INSULIN RELEASE FROM CULTURED B-CELLS.

David George Lambert. B.Sc(Hons), G.I. Biol.

Thesis submitted for the degree of Doctor of Philosophy.

The University of Aston in Birmingham.

September 1987.

The University of Aston in Birmingham.

David George Lambert 1987

Summary of thesis submitted for the degree of Doctor of Philosophy.

Insulin release from cultured B-cells.

Established RINm5F and IN111 R1 and newly available HIT-T15 and UMR 407/3 B-cell lines have been successfully maintained in vitro. With the exclusion of UMR 407/3 cells, all lines were continuously propagable. Doubling times and plating efficiencies for HIT-T15, RINm5F, IN111 R1 and UMR 407/3 cells were 20hours and 85%, 31hours and 76%, 24hours and 80% and 38hours and 94% respectively. All the cell lines were anchorage dependent, but only UMR 407/3 cells grew to confluence.

Only HIT-T15 and UMR 407/3 cells produced a true insulin response to glucose but glucose markedly increased the rate of D-[U^{14}C]glucose oxidation by all the cell lines. Glucose induced insulin release from HIT-T15 cells was biphasic with an exaggerated first phase. Insulin release from HIT-T15, RINm5F and IN111 R1 cells was stimulated by amino acids and sulphonylureas.

Glucagon stimulated insulin release from HIT-T15 and RINm5F cells while somatostatin and pancreatic polypeptide inhibited release. These observations suggest that net insulin release from the whole islet may be the result of significant paracrine interaction.

HIT-T15 and RINm5F cell insulin release was stimulated by forskolin and inhibited by imidazole. Ca^{2+} channel blockade and calmodulin inhibition suppressed insulin release from HIT-T15, RINm5F and IN111 R1 cells. In addition phorbol esters stimulated insulin release from RINm5F cells. These data implicate cAMP, Ca^{2+} and protein kinase-C in the regulation of insulin release from cultured B-cells.

Acetylcholine increased insulin release from HIT-T15 and RINm5F cells. Inhibition of the response by atropine confirmed the involvement of muscarinic receptors. HIT-T15 cell insulin release was also inhibited by adrenaline. These observations suggest a possible role for the autonomic nervous system in the modulation of insulin release.

Preliminary studies with a human insulinoma maintained in monolayer culture have demonstrated a limited life span of some seven weeks, a continuous low level of insulin release but no insulin response to glucose challenge.

KEY WORDS: Cultured B-cells, Human insulinoma, Insulin release, Glucose oxidation, Secretagogues, Inhibitors.

This thesis is dedicated to the four most important people in my life, to my father Frederick Norman and my mother Elizabeth for giving me such a wonderful start in life, to my wife Valerie for her constant support and encouragement and to my son Christopher David for making it all worthwhile.

"Your wife is the one who stands by you in all the trouble you would not have had if you had not married her"

Bernard Shaw.

Is this the situation between science and scientists ?

Acknowledgements.

Grateful thanks go to Dr R.F. Santerre of Eli Lilly Ltd, USA for providing samples of HIT-T15 cells, Dr S.J.H. Ashcroft from the John Radcliffe Infirmary, Oxford for providing samples of RINm5F cells, Dr S. Uchida of the University of Tokyo, Japan for providing samples of IN111 R1, Dr K.W. Ng from the Repatriation General Hospital, Australia for providing samples of UMR 407/3 cells and to Dr P.J. Hale, Dr M. Nattrass and Dr M. Baddeley from Birmingham General Hospital for providing the human insulinoma and for measuring C-peptide. Grateful thanks also go to Dr C.J. Bailey for helpful discussions and the loan of odd bits of apparatus.

My sincere thanks are extended to Mr Kevin Hughes for his help in setting up the perifusion system, writing the program for the area under the curve and for stimulating "chit-chat". Thanks mate !

My final thanks go to my supervisor Dr T.W. Atkins for giving me the opportunity to conduct research in such a stimulating area, for helpful guidance in experimental work and the preparation of this thesis. Cheers Terry !

Financial support from the University of Aston is gratefully acknowledged.

CONTENTS

List of tables plates and figures

CHAPTER 1.

1. GENERAL INTRODUCTION.

THE ORIGIN OF CULTURED INSULIN SECRETING TISSUES.

In 1909 Ross Harrison published a paper entitled "Observations on the live developing nerve fiber", in which he described the first reproducible attempts at tissue culture (1). His pioneering work is now generally accepted as the true beginning of tissue culture. Just four years after the discovery of insulin by Banting and Best in 1922 (2), Kapel made the first attempts at culturing foetal chick pancreas (3). The cells which grew from these pancreatic explants were almost certainly fibroblasts, as he stated that they resembled cultures of Rous sarcoma. Subsequent work of Murray and Bradley in 1935 involving the culture of human islet cell tumours (4) now represents one of the major directions for modern attempts to culture pancreatic islet tissue.

Why culture B-cells ?.

The problems hampering the study of insulin secretion *per se* are great; the islets of Langerhans are small, making up about 1% of the total pancreatic tissue, and are dispersed throughout and in close connection with the enzyme secreting acinar tissue (5). In addition the pancreatic islet is a heterogeneous mixture of at least four functionally different cell types: A, B, D and PP cells, secreting glucagon, insulin, somatostatin and pancreatic polypeptide respectively (6). Thus the preparation of large numbers of islets and the subsequent purification of B-cells is a costly and time consuming process. The establishment of a continuous representative cloned B-cell line could potentially overcome these problems and provide a useful tool enabling pure B-cell biochemistry and physiology to be more clearly defined.

The primary culture of isolated whole pancreas, pancreatic islets and islet cell monolayers.

Early work aimed at understanding the endocrine function of the pancreas involved investigation of the structure and function of the whole (endocrine plus exocrine) pancreas (7,8,9). Today organ culture of whole pancreas is a much used preparation, although emphasis is now on the production of potentially transplantable material (10,11,12). A recent study by Mandel et.al. using foetal pancreata obtained from women after hysterectomy and prostaglandin abortion has shown the foetal pancreatic explants could be maintained in vitro for up to 4 weeks and that the amount of insulin released correlated well with the gestational age of the foetus (13).

In the early 1960's the indroduction of mechanical microdissection by Hellerstrom (14) and collagenase digestion of pancreatic tissue by Lacy and Kostianovsky (15) for the isolation of the islets of Langerhans paved the way for the culture of islets and the subsequent production of islet cell monolayers. Isolated islets are relatively easy to maintain in static culture, their small dimensions limiting the amount of central necrosis seen in other larger pieces of tissue (16,17). Islets in static culture gradually settle to the bottom of culture vessels under unit gravity and attach to the substratum via fibroblast outgrowth on the second or third day of culture (18). Table 1.1 illustrates the effect of tissue culture on the physiology and biochemistry of the islets of Langerhans.

Table 1.1 Comparison of the characteristics of cultured and freshly isolated islets of Langerhans.

Islet/B-cell function	Effect of culture	Reference
Islet viability	Preserved	(18)
Insulin release	Reduced	(18)
Insulin content	Reduced	(18)
B-cell granulation	Reduced	(16)
Insulin biosynthesis	Reduced	(18,19)
Glucose oxidation	Reduced	(18)
Oxygen consumption	Reduced	(18)
DNA synthesis and B-cell replication	Increased	(20)

The biggest drawbacks associated with the use of isolated islets in experimental studies is their cell heterogeneity and their limited life span *in vitro*. In general islets isolated from adult animals have shorter life spans *in vitro* than foetal islets. Turcot-Lemay and colleagues were able to culture adult rat islets for 14 days (21) while Jonsson *et.al.* managed to maintain adult rat islets for almost 30 days in culture (22). Mandel and Georgiou have reported the maintenance of human foetal islets for periods of up to 40 days *in vitro* (23).

The next logical step from the culture of isolated intact islets was their disaggregation and maintenance as monolayers *in vitro*. Three approaches can be employed to dissociate primary tissues into single cells and small cell clusters for the initiation of

monolayer cultures. The three techniques involve either mechanical dissociation, enzymatic digestion or a combination of the two (24). The most commonly used technique for islet dissociation involves the use of trypsin/EDTA (25) although DNA-ase (26), collagenase and hyaluronidase have also been used (27). Table 1.2 illustrates some of the more commonly used methods for the enrichment and purification of B-cells.

Table 1.2 Commonly used methods for the enrichment and purification of islet B-cells.

Technique	Principle	Reference
Use of obese mouse islets	Islets contain increased numbers of B-cells	(28)
Use of thiomersal	Removes fibroblasts from primary cultures	(29)
Differential adhesion	Fibroblasts adhere more rapidly, endocrine cells may subsequently be poured off	(30)
Cysteine free medium	Inhibits fibroblast attachment	(31)
Differences in buoyant density	Ficoll gradient separates endocrine from acinar cells	(31)

Primary monolayer cultures of isolated islets are plagued by the rapid overgrowth of fibroblastoid cells (29), resulting in overcrowding, a rapid build up of toxic metabolites, nutrient depletion and subsequent impairment of B-cell function. Although a modest amount of DNA-synthesis and B-cell replication has been identified in islet cell monolayers, a process that can be increased by high glucose and suppresed by dexamethasone (32), islet cell monolayers have limited life spans in vitro. Such monolayers have been maintained for periods of up to 68 days (33). However disruption of the normal islet cell stochiometry and microenvironment especially cell-cell contact depresses the amounts of insulin released compared with intact islets (26). Although primary cultures of islet tissue exhibit the same pattern of glucose sensitivity observed with freshly isolated islets the limited life span and heterogeneous cell make up of this preparation are a major disadvantage.

The primary culture of islet cell tumours.

The culture of islet cell tumours could potentially provide a limitless supply of insulin secreting tissue for basic and clinical research. In this section the term 'insulinoma' will be used to describe the following confusing terminologies; insulin producing islet tumour, insulin producing insulinoma, insulin producing pancreatic tumour and islet cell adenoma. This simplified terminology stresses the fact that the main characteristic of these tumours is their ability to produce insulin, although not exclusively, while their appearance and origin is not defined (34).

Adult human insulinomas have proved difficult or impossible to maintain in long term culture. The first attempts were reported by Murray and Bradley in 1935, though hormone synthesis and release by these tumours was not demonstrated (4). Between 1973 and 1975 three papers by Chick et.al. (35), Yip et.al. (36) and Adcock et.al. (37) reported the maintenance of human insulinomas in culture for periods of up to 6months. These three reports all concluded that human insulinomas have limited lifespans in vitro, continue to secrete insulin in vitro, although this declines rapidly during the initial culture period, and do not respond to physiological concentrations of glucose. Some studies have reported a glucose stimulation of insulin release from insulinoma tissue, but these observations were obtained with freshly isolated tissue (38,39) or tissue that had been cultured for only a short period of time (40). In 1981 Akagi et.al. maintained 4 human insulinomas in culture for periods of up to 10 months (41). Insulin release declined rapidly and by 35 days in culture only 4.4% of the initial rate of insulin release could be demonstrated. The glucose sensitivity of the tumours was not measured (41). The only study to report significant growth of a human insulinoma in vitro was documented by Saxe et.al. in 1982. This tumour was maintained for over 40 days in culture and had a doubling time of 5 days (38). Although the fresh tissue did secrete insulin in response to glucose, the cultured tissue did not. Saxe and colleagues have also reported the successful cryopreservation of human insulinoma tissue, the procedure did not result in a significant reduction in the ability of the tissue to secrete insulin (38). The major disadvantages of using human insulinomas for experimental work is their rarity, finite lifespan and inconsistent response to glucose.

Rakieten and colleagues were first to report that the combined intraperitoneal administration of streptozotocin and nicotinamide to rats resulted in the induction of insulinomas (42). The induction time was lengthy and varied between 14 and 18 months with a success rate of about 64%. Kazumi et.al. also induced insulinomas by including picolinamide in the induction mixture. This addition did not reduce the lengthy induction time which remained on average around 12 months. The freshly isolated tissue released insulin in response to glucose and perifused insulinoma cells revealed both monophasic and biphasic insulin release profiles (43). Masiello et.al. were able to maintain streptozotocin/nicotinamide insulinoma cells in monolayer culture for some 15 weeks (44). The cultures variably secreted large amounts of insulin, glucagon and somatostatin and in common with human insulinomas the rate of insulin release declined rapidly within the first two weeks. No cell proliferation was observed (44). Streptozotocin/nicotinamide insulinomas maintained in vitro synthesise insulin (as determined by the incorporation of ^{3}H-leucine into insulin) and release insulin in response to high glucose challenge for the first two weeks in culture (45). The major disadvantages of using chemically induced insulinomas. in experimental studies are their heterogeneous cell composition and the extended period of time required for tumour induction.

Warren and colleagues in 1964 first reported the production of an X-ray induced insulinoma in parabiosed NEDH rats (46). In this study the right hand parabiont recieved a single supralethal dose of 1000rads of 250KV X-rays. The induction time was of the order of 12 months and the incidence of tumour production only 12% (46). This original tumour is still being passaged in vivo in the NEDH rat. Chick et.al. demonstrated ultrastructurally that the tumours were composed of B- and D-cells (47). On transplantation profound

hypoglycaemia was observed after 2 to 4 months which often lead to the death of the host animal. Tumour resection resulted in an amelioration of the hypoglycaemia. The transplanted X-ray induced tumours appeared to suppress normal islet function in the recipients, i.e., normal islets decreased in size and the amount of aldehyde-fuchsin staining in B-cells was markedly reduced compared with control non tumour bearing rats (47). Masiello et.al. (48) have shown that the X-ray induced insulinoma produced insulin and somatostatin confirming the work of Chick et.al. (47). In addtion it was shown that this tumour also produced glucagon and somatostatin (1: 3: 10, glucagon: somatostatin: insulin, in a molar ratio). These authors were able to culture this insulinoma as a monolayer, the cultured cells containing a small number of fibroblasts, formed small clusters a few hours after plating. The cultures were only maintained for a period of 5 to 7 days after which time, in contrast to fresh tumour cells, they released insulin in response to high glucose challenge (48).

In 1962 Kirkman reported the spontaneous development of well defined insulinomas in hamster pancreas which produced profound hypoglycaemia in the host animal (49). This insulinoma, passaged in vivo secreted substantial quantities of insulin and has been used to provide large quantities of tissue for basic research (50,51,52). Rae et.al. have been the only workers to attempt to culture the hamster insulinoma. After a total of 8 to 9 months culture on mitomycin treated fibroblasts as feeder cells the disaggregated insulinoma cells were capable of feeder cell independent growth. The cell line had an average doubling time of approximately 48 hours (53). The basic problem with these insulinoma cells is that they are incapable of synthesising and releasing insulin in vitro.

The initiation of IN111 R1, RINm5F, CR1, HIT-T15 and UMR 407/3 cell lines.

Uchida et.al. reported polyoncogenicity of BK virus infection in Syrian hamsters producing ventricular tumours, neuroblastomas, pineal tumours, peritoneal tumours, osteosarcomas and malignant insulinomas. The incidence of insulinoma production was 92% whether BK virus was injected intracranially, intraperitoneally or subcutaneously, and the insulinomas were generally malignant with hepatic metasteses. These insulinomas could be serially propagated in vivo with tumours of 1-3cm developing within periods of 2-5 weeks. Circulating insulin levels as high as 320uU/ml and circulating glucose levels as low as 0.4mmol/l were observed in tumour bearing hamsters. The freshly excised insulinoma could be cultured immediately after excision. Two distinct cell types could be cloned from this tumour, round cells that secreted insulin and designated IN111 R1 and epithelial cells which were not associated with insulin release designated IN111 E4 (54). The subsequent work of Kikkawa et.al. has shown that the IN111 R1 cell line produced glucagon and somatostatin in addition to insulin (193: 33: 1, insulin: glucagon: somatostatin in a molar ratio). The IN111 R1 cell line did not release insulin in response to glucose (55), and this might explain the infrequent use of this cell line in experimental diabetes research.

The RINr and RINm cell lines were developed from the X-ray induced insulinoma of the rat (46,47) by Gazdar et.al. in 1980 (56). Portions of the tumour were transplanted into rats and athymic nude mice to produce the RINr and RINm cell lines respectively. After numerous unsuccessful attempts to establish continuous cultures from the x-ray insulinoma maintained in rats, Gazdar et.al. elected to enhance the growth and attachment of these tumour cells in vitro

by growing the cells on feeder layers of a rat liver cell line BRL3A which had been exposed to a lethal dose of 6K rads of X-rays. After some 2 to 3 weeks the RINr cell line resulted but required further assistance from feeder layers of BRL3A cells for a period of some 3 months for optimal growth. After this time RINr cells could be subcultured onto plastic tissue culture ware without the use of further feeder layers. The RINr cell line was cloned by limiting dilution to yield five clones, RINr1 to RINr5, which have never been used for the study of insulin release (56).

The RINm cell line was established from the fourth nude mouse heterotransplant of the X-ray insulinoma. The cells grew spontaneously in vitro without the aid of feeder layers. RINm cells were also cloned by limiting dilution to yield five clones RINm1 to RINm5, one particular subclone RINm5F has been the subject of intense study over the last seven years. The characteristics of the parent RINr and RINm cell line are illustrated in Table 1.3.

Table 1.3 Characteristics of cultured RINr and RINm cell lines (56).

Characteristic	RINr	RINm
Rat X-ray tumour transplanted into	Rat	Nude mouse
Appearance	Epithelioid	Epithelioid
Doubling time	52 hours	60-80 hours
Split ratio	1:2 (every 7 days)	1:2 (every 7 days)
Modal chromosome number	40	40

Mc Evoy et.al. compared the growth characteristics of RINm5F cells cultured by groups in Uppsala, Chicago and New York (57). Cells cultured in New York had a greater plating efficiency, growth rate and releasd significantly more insulin than cells cultured in Uppsala and Chicago. These differences between groups presumably reflected differences in handling techniques and culture conditions (57). Praz et.al. demonstrated that RINm5F cells secreted glucagon and somatostatin in addition to insulin (38: 6: 1, Insulin: glucagon: somatostatin in a molar ratio) and subsequently confirmed the the original observations of Gazdar and his group (56) with the parent RINm cell line (58). RINm5F cells are generally accepted as being non-glucose sensitive (58). However, Giroix et.al. have demonstrated a slight but significant increase in insulin release in response to low glucose, 2.8mmol/l (59).

The CR1 cell line has been recently isolated from the X-ray insulinoma of the rat by Carrington et.al. (60). The derivation of the CR1 cell line was different to that reported for RINr/m cells (55) although it could be argued that the CR1 cell line merely represents another type of RIN cell. Carrington disaggregated the freshly excised tumour and maintained the isolated cells in vitro. About 90% of these cells died off in the initial culture period whilst the remaining 10% were maintained for between 0-49 days. These cells were subsequently reinjected into NEDH rats, maintained in vivo for 25-186 days, then excised and disaggregated as described above. Cells that eventually adapted to in vitro growth were cloned by limiting dilution and propagated as four cell lines designated CR1-G1, G5, D2 and D11. A further cell line CR1-10P was developed by direct culture of the initial tumour (60). These five cell lines variously released both insulin and glucagon but did not respond to glucose.

Niesor et.al. in 1979 first reported that infection of rat pancreatic islet cell monolayers with Simian virus 40 (SV40) resulted in in vitro transformation, although the cells were essentially immortalised they did not release insulin (61). Subsequently Santerre and co workers at Eli Lilly and Co Ltd developed the HIT cell line from SV40 virus transformed hamster islet cell monolayers (62). These workers initiated islet cell monolayers from the pancreata of 30 Syrian hamsters and maintained them in culture. The monolayers, after pretreatment with ethylmethanesulphonate (to induce unscheduled DNA synthesis), were transformed with SV40 virus for 1 hour at 37^{0}C. After several washings the cells were suspended in nutrient agar. Colonies of transformed cells, initially identified by the eighth week were isolated and established in monolayer culture, for subsequent B-cell cloning. The transformed cells possessed nuclear T-antigen (indicative of viral infection) but did not produce infectious virus particles. The resulting cell line was designated HIT (hamster insulin secreting tissue). Santerre reported the development of four HIT cell clones HIT-1B2, 5B5, 4C2 and T15, HIT-T15 being the main cell line quoted in his paper (62). HIT-T15 cells proliferate with an 'islet like' morphology, have a doubling time of 33-34 hours and a plating efficiency 75%. The HIT cell line has been reported to be glucose sensitive, secreting insulin in response to glucose concentrations of up to 10mmol/l. HIT-T15 cells appear to be homogeneous in their hormone secretory capacity, producing and secreting only insulin (62). The major disadvantage of this cell line appears to stem from the fact that the insulin content and subsequent insulin release declines rapidly with time in culture (and passage number). The HIT-T15 cell line remains to be extensively characterised.

In 1987 Ng and co workers described the generation and measured insulin release from a cloned precursor B-cell line derived from isolated 1 day old neonatal rat islets. These workers cultured freshly isolated neonatal rat islets for 48 hours and subsequently cultured these islets in double nutrient agar for 2 weeks. After this time individual, well defined islets were removed and cultured as monolayers. The epithelioid cells that subsequently grew from these islets were used as primary cell cultures. The latter were passaged 4 times over a period of 6 months and then cloned by limiting dilution on rat tail collagen. Eventually nine clones resulted, each one capable of growth without collagen. These clones were designated UMR 401 to 409. UMR 407 cells were cloned further to give 3 subclones UMR 407/1 to UMR 407/3. The UMR 407/3 cells in vitro have a doubling time of some 60 hours and release insulin in response to glucose concentrations of up to 16.7mmol/l. Ng et.al. also indicated that these cells had a low insulin content and a somewhat limited lifespan of around 27 passages (63).

The essential characteristics (hormones secreted, life span in vitro and sensitivity to glucose) of the presently available insulin secreting tissues are summarised in Table 1.4.

Table 1.4 General characteristics of the presently available insulin secreting tissues.

Tissue	Hormone(s) secreted	Life span *in vitro*	Glucose sensitivity
Primary islets, monolayers	I,G,S,PP	L	++(to 16.7mmol/l)
Insulinomas	I,G,S	L/UL	+/-
IN111 R1	I,G,S	UL	-
RINm5F	I,G,S	UL	-
CR1	I,G	UL	-
HIT-T15	I	UL	+(to 10mmol/l)
UMR 407/3	I	L	++(to 16.7mmol/l)

Key: I, Insulin
G, Glucagon
S, Somatostatin
PP, Pancreatic polypeptide
+, Glucose sensitive
-, Not glucose sensitive
L, Limited
UL, unlimited

Clearly the cell heterogeneity and limited life span of isolated islets and islet cell monolayers in vitro make them less than ideal models for the study of insulin release. Nevertheless the whole islet has been the experimental mainstay for the study of insulin release and the aetiology of diabetes mellitus since the early 1960's.

There are a number of alternatives to the conventional use of isolated islets/islet cell monolayers currently available. One such model, the insulinoma, is difficult to maintain long term in vitro and generally does not respond to physiological concentrations of glucose. However, in the last 8 years three cell lines have been developed from insulinoma tissue, the IN111 R1 (54), RINm5F (56), and CR1 (60) cell lines. The RINm5F cell line has been extensively characterised since its inception in 1980. Along with the other insulinoma cell lines it does not usually respond to glucose and variably produces glucagon and somatostatin in addition to insulin. These cell lines could be potentially useful tools for biochemical studies of insulin release where large amounts of tissue are required.

The present studies have been designed to characterise the insulin secretory properties of a newly derived B-cell line HIT-T15 (62). Preliminary studies with these cells by Santerre et.al. have indicated that this cell line produces only insulin and is glucose sensitive. A thorough characterisation may lead to the HIT-T15 cell line being adopted as an alternative to the conventional isolated islet and yield new information on the mechanism of insulin release and the aetiology of diabetes mellitus. The characterisation protocol employed can be reduced to four sections, wherever possible and practicable, parallel experiments will be carried out with RINm5F cells to provide a comparison.

In the first section attempts will be made to confirm the glucose sensitivity reported by Santerre et.al. (62) and to extend this work to encompass a range of nutrient secretogogues including D-glyceraldehyde, L-leucine and L-arginine and to include three sulphonylureas, glibenclamide, chlorpropamide and tolbutamide, used in the treatment of non insulin dependent diabetes mellitus.

Secondly, any possible paracrine role for glucagon, somatostatin and pancreatic polypeptide in the control of insulin release from the whole islet will be studied using cultured HIT-T15 and RINm5F cells The cloned B-cell is particularly suited for use in such studies.

Third, a basic evaluation will be made of the roles played by cAMP, calcium and inositol triphosphate. This could provide clues as to their involvement in the mechanism of insulin release from HIT-T15 and RINm5F cells.

The fourth section will examine the involvement of the autonomic nervous system in HIT-T15 and RINm5F cell insulin release using the parasympathetic and sympathetic neurotransmitters, acetyl choline and adrenaline.

Preliminary studies of insulin release from the infrequently used IN111 R1 cell line (54) and a clonal precursor B-cell line UMR 407/3 (63) developed in April 1987 will be carried out to evaluate their suitability for use in the study of insulin release. The in vitro maintenance of a human insulinoma has been effected in an attempt to develop a human insulin secreting cell line.

CHAPTER 2.

2. GENERAL MATERIALS AND METHODS

Sources of Chemicals.

Reagents of analytical grade and double distilled water were used throughout. The chemicals and their sources were as follows. N-2-hydroxyethyl-piperazine-N'-2-ethane sulphonic acid (HEPES), D-glucose, L-leucine, L-arginine, D-glyceraldehyde, colchicine, glucagon, somatostatin, pancreatic polypeptide, adrenaline, acetylcholine, hexamethonium, atropine, verapamil, diltiazem, nifedipine, trifluoperazine, TMB-8, D-myoinositol 1,4,5 triphosphate (IP_3), imidazole, 4-alpha phorbol-12,13-didecanoate (4PDD), 12-O-tetradecanoyl phorbol-13-acetate (TPA), dimethylsulphoxide (DMSO), glutathione (oxidized), powdered RPMI 1640 and Ham's F-12 were purchased from Sigma Chemical Co. Ltd, UK. Glibenclamide, chlorpropamide, and tolbutamide were purchased from Hoechst Pharmaceuticals Ltd, UK. Thiomersalate and selenous acid were purchased from BDH Chemicals Ltd, UK. Sodium metabisulphite and chloramine-T were from Hopkins and Williams UK. Porcine monocomponent insulin was purchased from Novo Research Institute, Bagsvaerd, Denmark. Worthington collagenase Class IV (145U/mg) was purchased from Cooper Biomedical Ltd, UK. Bovine serum albumin fraction V was obtained from Miles Laboratories Ltd, UK. Forskolin was purchased from Calbiochem Ltd, UK. Compounds M&B 13,753 and M&B 40,678 were kind gifts from Dr T. Brown of May and Baker, Dagenham UK. Human insulin standard RD 13 and binding reagent RD 12 were purchased from Wellcome Diagnostic Reagents Ltd, UK. Na[^{125}I] (IMS30,100mCi/ml) and D-[U-^{14}C]-glucose (278mCi/mmol) were obtained from Amersham International, UK. Foetal calf serum, horse serum penicillin/streptomycin (50x concentrate) and trypsin/EDTA were

purchased from Flow Laboratories Ltd, UK. All tissue culture plastic ware was purchased from either Costar, Cambridge, Mass, USA, Gibco, UK or Sterilin, UK. All other reagents were purchased from Fisons Scientific Apparatus, UK.

Preparation of stock solutions of glibenclamide, tolbutamide, nifedipine, forskolin, TPA and 4PDD.

These compounds were not readily soluble in Krebs buffer, pH 7.4 as a consequence they were first dissolved in a small volume of DMSO to which an appropriate volume of Krebs buffer was subsequently added to make up the stock solution. This stock solution was so designed such that 10 or 20 ul added to 1ml of incubation buffer gave the desired final concentration without altering the pH. The concentration of the desired stock was calculated using the following formula;

$$M_1 V_1 = M_2 V_2$$

Where: M_1 = desired final molarity (mmol/l)

M_2 = molarity of stock solution (mmol/l)

V_1 = volume of stock required (l)

V_2 = total incubation volume (l)

The final DMSO concentration never exceeded 0.4% and an appropriate amount was always added to each set of controls. It has been shown that low DMSO concentrations of this order have no significant effect on insulin secretion (64).

Maintenance of stock cell cultures

HIT-T15 cells.

Stock cultures of HIT-T15 cells were routinely maintained in Ham's F12 medium supplemented with 15% horse serum, 2.5% foetal calf serum, 0.1umol/l selenous acid, 10ug/ml glutathione, 100ug/ml streptomycin and 100IU/ml penicillin using 250 ml Costar tissue culture flasks.

RINm5F cells.

Stock cultures of RINm5F cells were routinely maintained in RPMI 1640 supplemented with 10% foetal calf serum, 100ug/ml streptomycin and 100IU/ml penicillin using 250ml Costar tissue culture flasks.

Media Preparation.

After initial studies using preprepared liquid medium (Flow Laboratories Ltd, UK.) economy dictated that subsequent work be carried out with powdered media. Powdered Ham's F12 and RPMI 1640 were prepared as follows, the contents of one bottle of powder (sufficient to make up 1L) was dissolved in 900ml of double distilled water at 15-20^{0}C. Any remaining powder in the bottle was removed by rinsing with 50ml of double distilled water. Whilst stirring 2.0g/l sodium bicarbonate was added to the RPMI 1640 and 1.162g/l was added to the Ham's F12. The pH was then adjusted to a level 0.25 units below the desired final pH of 7.4 with 1N HCl (the pH normally increases 0.2-0.3 units on filtering) The total volume of mixture was brought to 1000ml with the remaining distilled water.

Sterilization was achieved by membrane filtration using a 0.22um filter (Millipore , UK. Sterivex-GS incorporating a filling

bell) into 250ml tissue culture flasks. Batch sterility was monitored by incubation at 37^{0}C in a vented petri dish and by plating on blood agar. Any batches of medium showing bacterial contamination were discarded.

Cell passaging.

Adherent cells were brought into suspension for subsequent subculture or passage using a combination of trypsin and EDTA. Flasks to be passaged were rocked gently to free any loose or dead cells. The spent medium was removed and replaced with 9ml of trypsin/EDTA (0.05% w/v, 0.02%w/v). Each flask was incubated at room temperature for 1minute and the trypsin/EDTA quickly removed. A further 1ml was then added and each flask incubated for 6-10minutes to effect the suspension of cells. The action of trypsin was inhibited by the subsequent addition of 9ml of serum supplemented culture medium. The resulting cell suspension was centrifuged at 1,500 rpm (MSE Chillspin) for 3 minutes and the supernatant removed. The cell pellet was resuspended in 5ml of culture medium and enumerated using a haemocytometer. HIT-T15 and RINm5F cells were reseeded at 4 x 10^6 and 8 x 10^6 cells per 250ml Costar tissue culture flask containing 20ml of the appropriate culture medium.

HIT-T15 and RINm5F cells were routinely passaged once weekly and fed twice weekly. All cultures were maintained at 37^{0}C in 5% CO_2/ humidified air, using a temperature controlled CO_2 incubator. (Flow Laboratories, UK.) All experimental work was performed upon passages 47-67 for HIT-T15 cells and 85-105 for RINm5F cells.

Growth characteristics of cultured cells

General morphology.

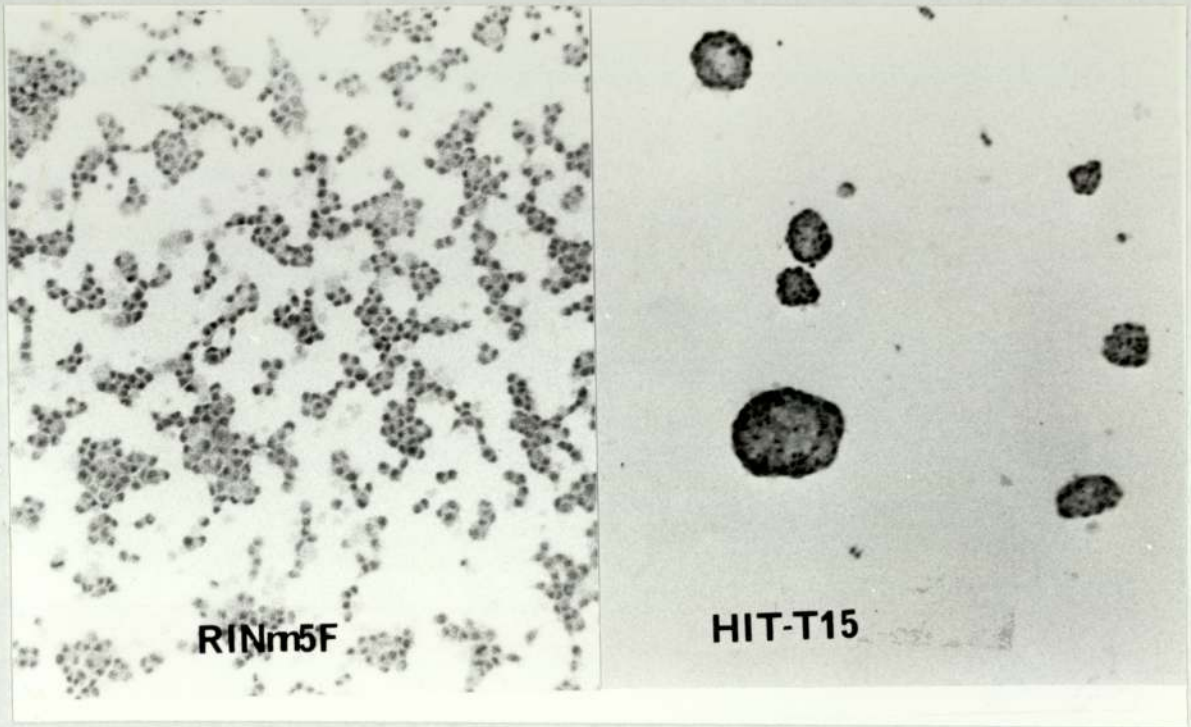
HIT-T15 cells.

Cultured HIT-T15 cells assumed an epithelioid appearance, tending to self-associate and form large multilayered islet-like structures rather than a confluent monolayer, Plate 1.1. Immediately prior to passaging large numbers of single cells could be observed in suspension, this may be due to overcrowding or medium depletion.

RINm5F cells.

Cultured RINm5F cells were also epithelioid in appearance and cytoplasmic processes could be seen extending from the free cell surfaces. The cultures did not appear multilayered nor did they reach confluence, Plate 1.1. When the monolayer reached >80% confluence lare numbers of cells became detached (again presumably due to overcrowding or nutrient depletion). These detached cells could not be replated.

Plate 1.1. 3 day cultures of HIT-T15 and RINm5F cells. The cells were cultured on glass coverslips and stained with giemsa. Magnification x100.



Plating efficiency.

To determine the plating efficiency of HIT-T15 and RINm5F cells (defined as the percentage of individual cells which give rise to colonies when inoculated into new culture vessels) 2-3x10^6 cells from freshly trypsinised stocks were seeded into Nunc vented petri dishes (60 x 15mm) and incubated for 24 hours. After this time any cells remaining in the supernatant were estimated using a haemocytometer. The plating efficiency P, was calculated using the equation below.

$$P\% = (T-S)/T \times 100$$

Where: T=initial inoculum

S=cells in the supernatant

The plating efficiencies of HIT-T15 and RINm5F cells were found to be 85.2 $\pm$ 1.9% (n=4) and 76.3 $\pm$ 3.8% (n=4) respectively. Values are means $\pm$ SEM.

Kinetics of cell growth.

Animal cells in culture display very much the same type of growth pattern as micro-organisms. In particular, they show the classical growth kinetics of bacteria (65). When cells are taken from a stationary culture there is an initial lag phase of some hours to days before growth commences, depending on the cell strain and growth conditions employed. Growth then proceeds steadily in an exponential fashion, this second phase is termed the log or exponential phase. At the end of the log phase the maximum population size is achieved and the cells enter the stationary phase (24). Stationary phase growth results from a combination of two factors, overcrowding which leads to a build up of toxic metabolites and nutrient depletion (65).

During the log phase growth the cell population increases according to the equation 1 below.

$$N = N_0 2^{kt}$$

Where: N_0 =initial inoculum

N =cell number at time,t

k =a regression constant

The term kt actually describes the generation number, defined as the number of generations involved in the increase by doubling at each cell generation, from N_0 to N. It follows that the mean generation or doubling time is represented by the inverse of k.

Rearranging equation -1 above for k,

$$\log N = \log N_0 + kt \log 2$$

$$\log N - \log N_0 = kt \log 2$$

since $1/\log 2 = 3.32$

it follows that,

$$3.32 (\log N - \log N_0) = kt$$

Therefore.

$$k = 3.32 (\log N - \log N_0) / t \quad \text{and doubling time} = 1/k$$

This calculation assumes that all the cells in the population divide and do so at the same rate. This can be assumed to be true for most cell cultures during log phase growth (24).

Growth curve for HIT-T15 cells.

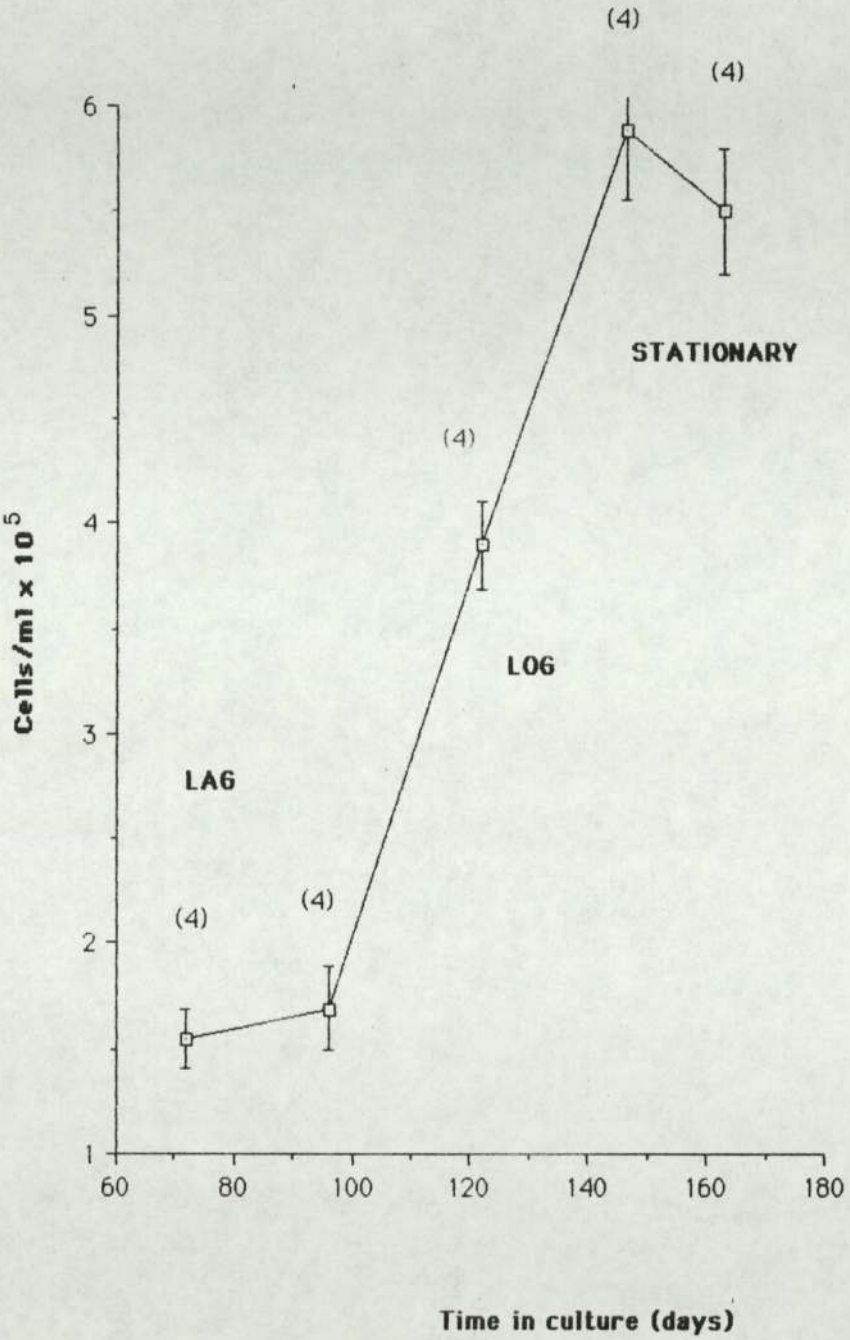
The growth of HIT-T15 cells was monitored over a seven day period by measuring the increase in cell number with time.

1×10^5 cells from freshly trypsinised stocks (stationary phase) were plated into 24 well Costar cluster plates containing 1ml of supplemented Ham's F-12 and maintained at 37^0C in 5% CO_2/air. Four wells were trypsinised and enumerated on days 2-7, any remaining cells were refed on days 3 and 6.

The growth curve for HIT-T15 cells illustrated in Figure 2.1 exhibits the classic three phases described earlier. Phase 1 the lag phase lasted approximately 72 hours. In this phase there is a build up of growth factors and essential metabolites by the initial innoculum, this process is referred to as conditioning (66) Phase 2 the log phase lasted between 100-140 hours, this phase was used to calculate the doubling time which was calculated by direct extrapolation from the graph and confirmed as 24 hours. The stationary phase >140 hours in HIT-T15 cells may be the result of overcrowding, in that, medium repletion on day 6 did not return the cells to log phase growth

Figure 2.1 Growth curve of HIT-T15 cells.

Values are means + SEM with n in parentheses.



Calculation of doubling times of HIT-T15 and RINm5F cells

The doubling times of HIT-T15 cells has also been estimated based on equation 1 page 35 The doubling time of HIT-T15 cells was found to be 28 hours. A crude estimate of RINm5F cell doubling time was performed based on the number of cell doublings over three days (72 hours). Using this method RINm5F cells were found to have a doubling time of 31 hours. The estimation of doubling time using equation 1 was the method of choice in so far as no subjective curve fitting methods were employed.

The doubling times observed in the present studies compared favourably with those quoted by Santerre et al (62) for HIT-T15 cells (33 hours) and Gazdar et al (56) for RINm-cells (40-80 hours, tending to fall in the later passages).

Cryopreservation of B-cell stocks

The preservation of cell cultures in the frozen state offers several advantages (67). This procedure enables multiple experiments to be performed upon the same cell stock. In addition parent and daughter cells can be compared and effective transportation of cell stocks can be carried out.

Effects of cryopreservation on biological systems.

When cells are cooled to below 0^0C three main changes occur. Ice crystals are formed, water is removed and the concentration of soluble materials in the cell increases. If cells are cooled sufficiently slowly they become dehydrated but do not freeze intracellularly. More rapid cooling however, produces intracellular ice but with smaller crystal size. Rapid thawing is required to prevent the conversion of these small crystals to larger crystals, which would occur if thawing was allowed to progress slowly. This phenomenon is called the recrystallisation of ice crystals (68). As a consequence of intracellular freezing the concentration of electrolytes in the remaining cell water increases. (69).

Cryoprotectants.

The two most commonly used cryoprotectants are glycerol and dimethylsulphoxide (DMSO). As the mechanisms of freezing damage are poorly understood it is not surprising that comprehensive explanations of the mechanism of cryoprotection are not yet available. The 'salt-buffer' hypothesis, advanced by Lovelock is the most commonly cited and fully elucidated concept of freezing injury and cryoprotection. Lovelock suggested that glycerol or DMSO exert cryoprotection by preventing excessive increases in intracellular electrolyte concentrations, i.e., they act as 'salt-buffers' (69,70,71).

Cryopreservation of isolated islets.

The interaction of cooling rate, warming rate and extent of cryoprotectant permeation have been studied during the cryopreservation of rat islets (72). Batches of islets were stored at -196^0C in liquid nitrogen after cooling at rates of 0.3, 3.0,10,30, 60,150 or $>1000^0$C/minute and then warmed at a rate of either 10

or 50^{0}C/minute. Survival was assessed by measuring the secretion of insulin during static incubation. Cooling rates extending over three orders of magnitude proved not to be the major criterion for survival. When islets were equilibrated with DMSO (and thawed at 50^{0}C/minute) a survival rate of >50% was achieved at all the cooling rates studied. Functional recovery was highly dependent on the cooling and warming rates, with peak survival occuring after slow cooling and rapid warming. Full permeation of the tissue with cryoprotectant offered maximal recovery of secretory function (72) Examples of the freezing rates used for the cryopreservation of islets from various animal species are shown in Table 2.1

Table 2.1. Freezing rates employed for the cryopreservation of islet tissue.

Animal	Freezing rate(^{0}C/minute)	Reference
mouse	5-25	(73,74)
dog	60	(75)
pig	1-30	(76,77)

In the present studies cultured HIT-T15 and RINm5F cells were frozen at 3.5^{0}C/minute in 10% DMSO and maintained at -196^{0}C in liquid nitrogen until required according to the following procedure.

Freezing down.

4-6 x 10^6 HIT-T15 or RINm5F cells from freshly trypsinised stocks (in log phase of growth) were suspended in 1ml of supplemented tissue culture medium containing 10% DMSO and placed in 1.2ml ampoulles (Nunc, Gibco Ltd, UK.) These ampou les were prechilled to 4^0C in a refridgerator for 20-30 minutes. The prechilling time was sufficient to allow complete equilibration of the DMSO a major determinant in the successful cryopreservation of islets (72). The chilled ampoulles were loaded onto a Handi Freeze 35HC freezing tray (Union Carbide, Indianapolis, USA.) set at position 3.5, which when placed into the neck tube of a Union Carbide 35HC liquid nitrogen freezer produced a freezing rate of approximately 3.5^{0}C/minute, Table 2.2. After 20 minutes, ampoulles maintained at about -65^{0}C were loaded onto canes and plunged into liquid nitrogen at -196^{0}C for storage.

Table 2.2 Performance characteristics of the Union Carbide handi freeze tray when inserted into a 35HC freezer neck tube. (Data supplied by the Union Carbide Corporation Ltd, USA)

Tray position number	Temperature after 20 minutes (^{0}C)	Calculated freezing rate(^{0}C/minute)
1	-160	8.2
2	-100	5.4
3	-80	4.2
4	-50	2.7
5	-40	2.2
6	-10	0.7

Thawing the frozen ampoules

When required ampoules at -196^{0}C were rapidly thawed in a shaking water bath at 37^{0}C. Thawing was complete in approximately 3 minutes (Thawing rate 80^{0}C/minute). The contents of the ampoulle were transferred to a 250 ml tissue culture flask containing 30 mls of medium, diluting the DMSO content to 0.3%. After 24 hours the medium was replaced and cell culture continued in the usual manner. Cells cryopreserved in this way maintain a post-freezing viability in excess of 70% as assessed by trypan blue dye exclusion.

Insulin radioimmunoassay.

Radioiodination of insulin.

Radioiodine is particularly convenient for the preparation of labelled protein tracers as it can be readily substituted into the tyrosine residues of proteins and peptides. Two convenient gamma emitting radioisotopes of iodine are widely available, ^{125}I and ^{131}I the former being the isotope of choice in that it has a longer half life (60 vs 8 days) and a greater counting efficiency.

For the production of labelled hormones of high specific activity without the use of large quantities of radioiodine, small quantities of protein need to be used. Methods developed as a consequence of this include the use of iodine monochloride (78), chemical oxidants (79), electrolytic (80) and enzymatic iodination (81).

The chloramine-T method of Hunter and Greenwood (82) first described for the labelling of human growth hormone is routinely used in our laboratory for the production of ^{125}I monoiodinated insulin of suitable purity and specific activity for use in insulin radioimmunoassays.

Chloramine-T radioiodination of insulin.

Chloramine-T the sodium salt of the N-monochloro derivative of p-toluene sulphonamide breaks down in solution to form hypochlorous acid (a mild oxidising agent). At pH 7.4 in the presence of chloramine-T, $Na^{125}I$ is oxidised to form the cationic iodine species, ^{125+}I. Tyrosine molecules in insulin at this pH will be only very slightly ionized due to the pK of the phenolic group of tyrosine being greater than 10. The iodination reaction proceeds through this small proportion of ionized groups, the iodine atom being substituted at the ortho-position to the hydroxyl group on the phenolic ring of tyrosine , 80% of the tyrosine being incorporated into tyrosine A14, the remaining 20% being incorporated into tyrosines B16 and B26 (83).

The amount of chloramine-T used in the reaction depends upon the amount of insulin to be iodinated and to the particular batch of $Na^{125}I$ used. Any excess chloramine-T remaining in the reaction is reduced by the addition of sodium metabisulphite (SMB) and free iodine is reduced to iodide. The quantity of SMB added is limited to twice that of chloramine-T. Albumin is also added to the reaction to act as a carrier for the labelled insulin. The concentration of chloramine-T was kept low and the reaction time was short (15-20seconds) to minimise the exposure of insulin to chloramine-T. The resulting reaction mixture was separated by gel filtration on Sephadex G50 fine.

Reagents for iodination

1. Phosphate buffers, 0.5 and 0.05 mol/l, Appendix A1 page 228
2. Column eluant, 0.5% BSA and 0.1% sodium azide (as a preservative) were dissolved in 0.05 mol/l phosphate buffer, pH 7.4.
3. Column primer, 2.5% BSA dissolved in 0.05 mol/l phosphate buffer, pH 7.4.
4. Porcine insulin (0.25 mg/ml). 0.25mg of porcine monocomponent insulin was dissolved in 1ml of 0.05 mol/l phosphate buffer, pH 7.4 and aliquoted into 25 microfuge tubes, which were stored frozen at -15^{0}C until required. A fresh tube of insulin was used for each iodination.
5. Chloramine-T (0.25 mg/ml). 25 mg of chloramine-T was added to an LP3 tube wrapped in foil to exclude light (84). Immediately prior to iodination the chloramine-T was diluted to 100ml with 0.05 mol/l phosphate buffer, pH 7.4.
6. Sodium metabisulphite (0.5 mg/ml). 50 mg of sodium metabisulphite was added to an LP3 tube wrapped in foil. Prior to iodination the sodium metabisulphite was dissolved in 100ml of 0.05 mol/l phosphate buffer, pH 7.4.
7. Na ^{125}I (1 mCi). 10uL of Na ^{125}I was supplied by Amersham International (UK) in a small reactivial made up in dilute sodium hydroxide, pH 7-11. 100uL of 0.5 mol/l phosphate buffer was added to the reactivial to bring the pH close to 7.4, the optimum required for the chloramine-T reaction (85).

Iodination protocol.

The iodination of porcine insulin was carried out two or three days after batch synthesis. Iodinations are carried out in the standard conical glass reactivial supplied. The quantities of insulin and chloramine-T used in the reaction depended upon the age of the Na ^{125}I. The 10uL aliquot of Na ^{125}I employed for the iodination of insulin had a projected activity of 1 mCi on the 'activity reference day' The 'activity reference day' is 15 days after batch synthesis, therefore on days 2 and 3 after synthesis (the iodination days) more than 1 mCi of Na^{125}I was present. On this basis the concentrations of the various reactants, especially insulin, were adjusted accordingly, Table 2.3

Table 2.3 Relative proportions of porcine insulin and chloramine-T (Chl-T) required in the reaction mixture for days 1-3 after Na^{125}I batch synthesis.

Days after synthesis	^{125}I (mCi)	Insulin (uL)	Chl-T (uL)	SMB (uL)	Phosphate buffer (uL)
1	1.176	11.76 (2.94ng)	23.52 (5.88ng)	20	200
2	1.162	11.62 (2.91ng)	23.24 (5.82ng)	20	200
3	1.149	11.49 (2.87ng)	22.98 (5.78ng)	20	200

SMB, sodium metabisulphite. Phosphate buffer 0.05 mol/l, pH 7.4 contained 2.5% BSA.

The following additions were made to the reactivial containing 1.162 mCi $Na^{125}I$ (day 2) in the following order.

1. 100uL 0.5 mol/l phosphate buffer to bring the pH to 7.4.
2. 11.62uL porcine insulin, (0.25 mg/ml)
3. 23.24uL chloramine-T, (0.25 mg/ml)

THEN AFTER 20 SECONDS REACTION TIME

4. 20uL sodium metabisulphite, (0.5 mg/ml)

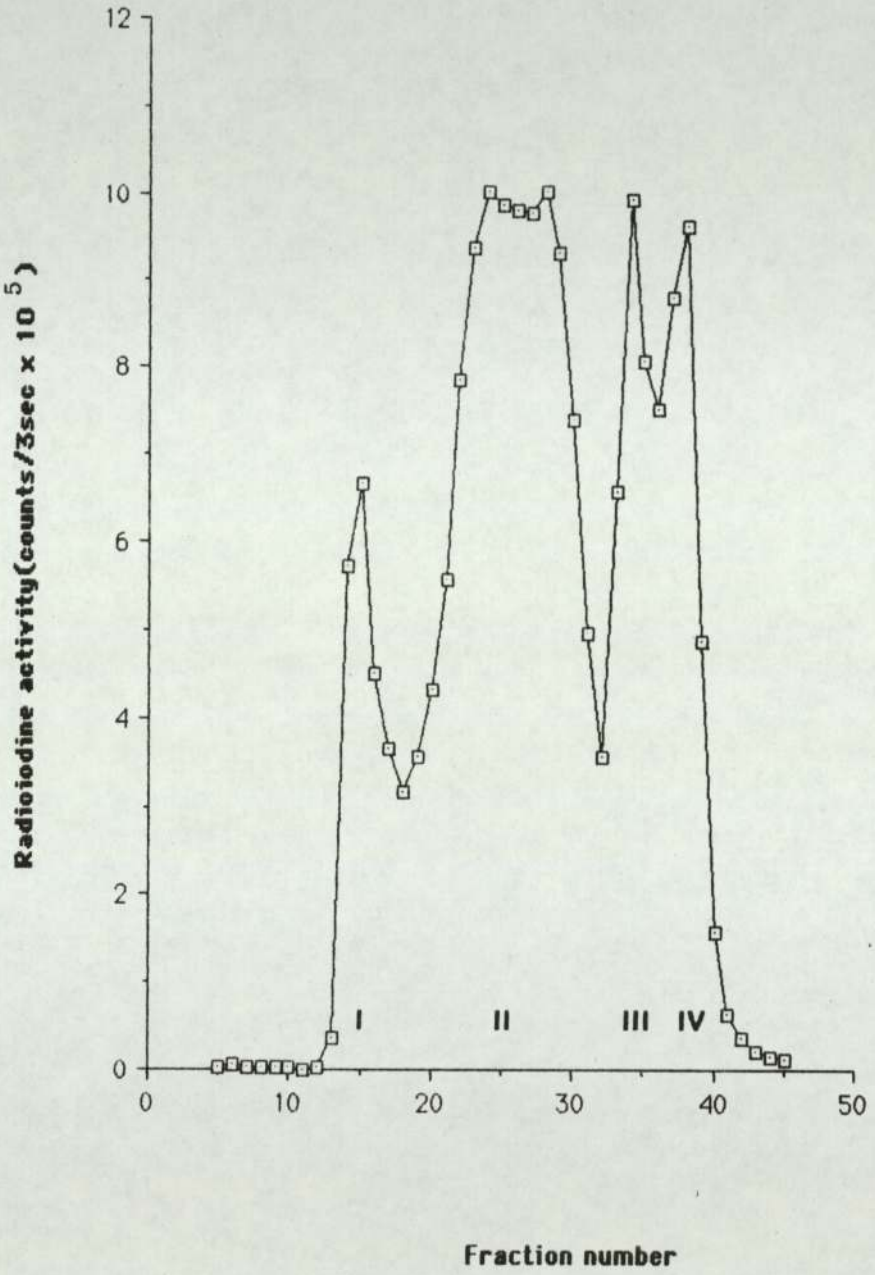
THEN AFTER 15 SECONDS STABILIZATION TIME

5. 200uL 0.05 mol/l phosphate buffer, pH 7.4 containing 2.5% BSA.

The labelled insulin was separated from unreacted iodine and damaged insulin fragments by gel filtration on Sephadex G-50 fine. The column was purged with 8ml of column primer to remove material remaining from the previous iodination and to prevent the adsorption of freshly generated labelled insulin onto the column surface itself. A 10uL aliquot was removed from the reactivial at the end of the reaction for the assessment of the percentage of ^{125}I incorporation into insulin. The remaining reaction mixture was then transferred to the column and eluted at a flow rate of approximately 1ml/minute. Sixty, one-minute fractions were collected in LP3 tubes on a Serva linear 2 fraction collector and each counted for 3 seconds using a gamma counter (ICN, Tracerlab), with a counting efficiency of 72%.

Four well defined peaks could be identified in the elution profile, Figure 2.2. Peak I (fractions 14-18) represented the high molecular weight fraction probably containing insulin aggregates (86) Peak II (fractions 19-30) contained monoiodinated insulin and

Figure 2.2 Typical elution profile of iodinated fractions serarated on sephadex G50 fine from the chloramine-T iodination of porcine insulin.



demonstrated maximum activity associated with insulin. Peak III (fractions 31-36) represented free unreacted ^{125}I mixed with small damaged insulin fragments. Peak IV (fractions 37-40) contained free unreacted ^{125}I.

Assessment of the specific activity and iodination damage of ^{125}I-insulin preparations.

A 10uL aliquot removed at the of the reaction to assess the percentage incorporation of ^{125}I into insulin and the specific activity of the preparation. 10uL aliquots were also removed from each fraction representing the apex of peak II to assess the percentage of iodination damage. 0.5ml of column eluant and 0.5ml of 10% trichloroacetic acid were added to each 10uL aliquot. The BSA present formed a visible precipitate which co-precipitated the undamaged intact ^{125}I-insulin. After counting the radioactivity of this sample followed by centrifugation (10minutes/2000rpm, MSE Mistral 4L) the supernatant was aspirated and the activity associated with the precipitate determined. The percentage of incorporation of ^{125}I into intact undamaged insulin (%I) or the percentage of undamaged insulin in peak II (%UD) was calculated as follows;

$$\%I \text{ or } \%UD = \frac{\text{Radioactive count of the precipitate}}{\text{Radioactive count of the total mixture}} \times 100$$

Since the total amount of ^{125}I (mCi) and insulin present (ng) in the reaction mixture and the percentage incorporation of ^{125}I into insulin (calculated above) were known the specific activity could be calculated using the following equation.

$$\text{Specific activity (uCi/ug)} = \frac{\text{\% incorporation x uCi of Iodine}}{\text{ug of porcine insulin}}$$

A typical iodination generated about 40 tubes each containing 50uL aliquots of ^{125}I-insulin (≈ 2-3 uCi/tube) with characteristics summarised in Table 2.4

Table 2.4 Percentage incorporation of ^{125}I into porcine insulin, percentage of undamaged insulin and the specific activity of ^{125}I-insulin.

Iodination characteristics	n	Mean ± SEM
% incorporation of ^{125}I into insulin	6	62.85 ± 3.76
% of undamaged insulin	6	94.35 ± 1.22
Specific activity (uCi/ug)	6	250.83 ± 14.99

n is the number of iodinations.

Repurification of ^{125}I-insulin.

Repurification of any remaining ^{125}I-insulin was carried out 1 month after synthesis when the iodination damage of the stored ^{125}I-insulin had increased by about 5%. Repurification was effected to preserve the activity for an additional month for use in radioimmunoassays. Repurification was carried out by gel filtration on Sephadex G50 fine as described previously page 46. Three peaks

were obtained in the elution profile, Figure 2.3. Tubes containing the maximum radioactivity associated with repurified monoiodoinsulin were identified in peak II (fractions 19-32). Peak I (fractions 12-18) represented aggregated iodination products of high molecular weight and peak III (fractions33-39) free unreacted iodine

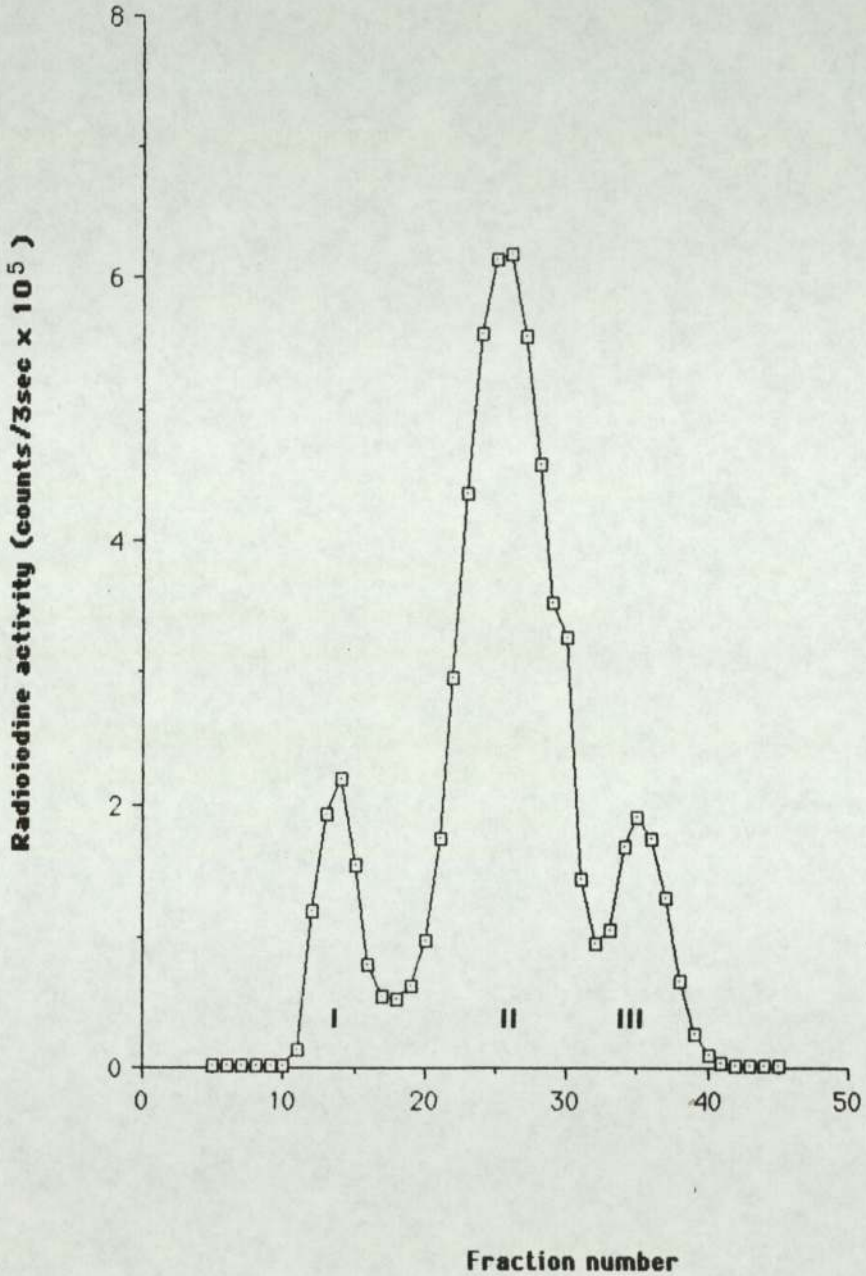
Four fractions from the apex of peak II were assessed for damage as described previously, page 48. Fractions from peak II containing less than 5-6% damage were pooled and distributed into 10-30 tubes (100-200uL and ≈2uCi/tube, specific activity remaining unchanged). The routine generation of ^{125}I-insulin using the chloramine-T method is an effective and economical means for the production of sufficient high quality ^{125}I-insulin for use in insulin radioimmunoassays over a period of approximately 2 months.

Principle of the radioimmunoassay for insulin.

Radioimmunoassay is dependent upon the competition between labelled and unlabelled antigen for binding sites on specific antibodies (87). Increasing amounts of unlabelled antigen in the sample produce a proportional decrease in the binding of labelled antigen to the antibody (88). Thus the level of radioactivity associated with the antibody-antigen complex is related to the concentration of unlabelled antigen in the original sample.

In the present work insulin was measured using a modification of the double antibody technique of Hales and Randle (89) where bound (to guinea pig anti porcine insulin precipitated with rabbit anti guinea pig globulin) and free ^{125}I labelled insulin were separated by centrifugation.

Figure 2.3 Typical elution profile of iodinated fractions separated on Sephadex G50 fine from the repurification of ^{125}I-insulin prepared by chloramine-T iodination.



Preparation of reagents for insulin radioimmunoassay

1. Diluent buffer. The following reagents were added to 1L of double distilled water: 6.2g sodium dihydrogen orthophosphate ($NaH_2PO_4 . 2H_2O$), 0.25g thiomersalate (as a preservative) and 5.1g of BSA. The pH was adjusted to 7.4.
2. Insulin binding reagent (RD 12). The lyophilised reagent consisted of guinea pig anti porcine insulin serum precipitated with rabbit anti guinea pig globulin. The reagent was reconstituted in 8ml of double distilled water on the day of the assay.
3. ^{125}I-iodinated insulin. ^{125}I-insulin was prepared to an average specific activity of 250 $\pm$ 15 (n=6) uCi/ug using the Chloramine-T method, page 43. The specific activity (of a 50-200uL aliquot) was reduced to 50 uCi/ug by the addition of a calculated amount of 'cold' unlabelled porcine insulin. The count rate was reduced with diluent buffer such that a 50uL aliquot used in the assay contained 9,500-11,000cpm
4. Insulin standards.The contents of 1 bottle of Wellcome human insulin standard RD13 (2.5-3.5 mIU) was diluted to 1mIU/ml with diluent buffer. 0.2ml aliquots of this solution were dispensed into LP3 tubes and stored at -15^{0}C. When required on the day of the assay one of these tubes was diluted with 0.8ml of diluent buffer to provide a top standard insulin concentration of 200uIU/ml. Five further serial dilutions were made from this stock standard tube to provide a range of standard insulin concentrations from 6.25-200uIU/ml. HIT-T15 and RINm5F cell insulin release was expressed in terms of human insulin equivalents. Since hamster insulin was not commercially available rat insulin standard (lot R8303081 NOVO) was also included in some assays.

Procedure for the radioimmunoassay of insulin.

Assay reagents were added to LP3 tubes in the order indicated in the reaction protocol summarised in Table 2.5 below.

Table 2.5 Protocol for the addition of reagents in the double antibody radioimmunoassay of insulin.

Tube description	Tube number	Initial reactants	Binding reagent	Iodinated insulin
Total counts	1-3	-	-	+
Blank	4-6	Buffer	Buffer	+
	7-9	6.25	+	+
Insulin	10-12	12.5	+	+
standards	13-15	25.0	+	+
(uIU/ml)	16-18	50.0	+	+
	19-21	100.0	+	+
	22-24	200.0	+	+
Unknown samples	24-n	+	+	+

All reactants were added in 50 uL aliquots.

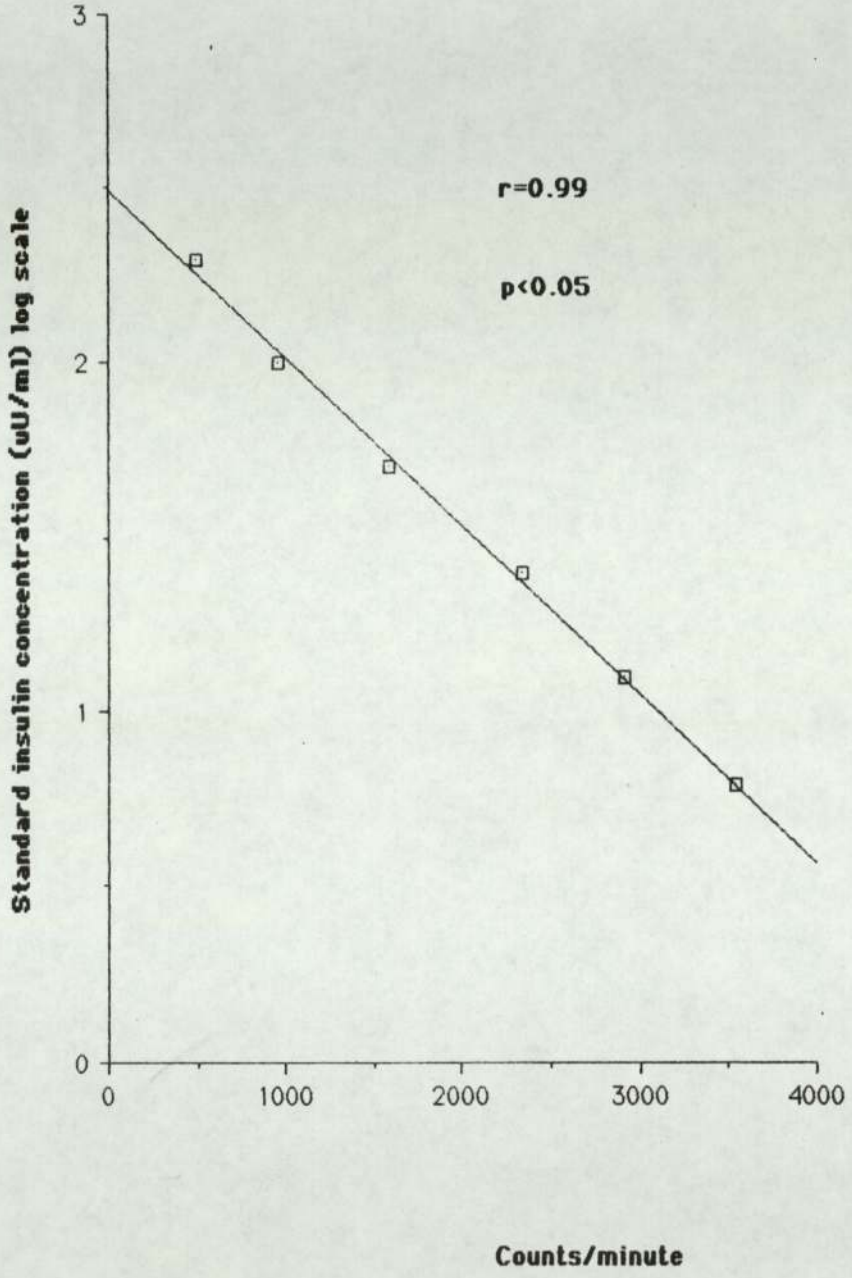
Insulin standards, total counts and blanks (containing buffer instead of standards and binding reagent) were assayed in triplicate. 50uL aliquots of insulin standards (human or rat) and unknown samples were transferred to LP3 tubes. A 50uL aliquot of insulin binding

reagent was added to all tubes (except totals and blanks). The contents of each tube was vortex mixed without frothing and incubated at 4^0C for 4 hours. A 50uL aliquot of ^{125}I-insulin (9,500-11,000 cpm,specific activity 50uCi/ug) was then added to all tubes. After gentle vortex mixing, all tubes were incubated for a further 16-18 hours at 4^0C. After this second incubation 0.5ml of diluent buffer was added to all tubes except the totals and the contents vortex mixed. Free and antibody bound ^{125}I-insulin were separated by centrifugation (1,500g Mistral MSE, coolspin) for 30 minutes at room temperature. The supernatant containing free labelled insulin was carefully decanted by inverting the tubes and the remaining drops of buffer aspirated from the rim of the inverted tube with the aid of a pasteur pipette connected to a vacuum pump. The tubes were then left at an angle of 30^0 for approximately 4 hours to dry at room temperature. The ^{125}I-activity associated with the precipitate was counted for 1 minute on a Compu-gamma gamma counter (LKB Instruments Ltd, Sweden), with a counting efficiency of 82%.

Computation of results.

The construction of standard curves and the computation of results was performed using an RIA package associated with the gamma counter. Log_{10} of the standard insulin concentration was plotted against the counts per minute in the bound fraction (spline plot) and the sample unknown insulin concentration was determined automatically by direct extrapolation from the graph (A copy of a typical printout is given in appendix, page 229). A typical standard curve is illustrated in Figure 2.4. Computation of the correlation coefficient and the minimal sensitivity was achieved using a BBC computer software package (appendix, page 231) In the present studies no significant differences could be detected between human

Figure 2.4 Typical standard curve for the double antibody radioimmunoassay of human insulin.



and rat standard curves, Figure 2.5. Thus insulin values stated in this thesis are expressed in terms of human insulin equivalents, (uU/ml). Quality control assessment of the data obtained in the last six assays using human insulin standards is shown in table 2.6. In the present studies the insulin assay had an intra- and interassay coefficient of variation of 4.83 and 9.49% respectively.

Table 2.6 Quality control assessment of data obtained in the last six insulin assays.

Parameter	Mean ± SD	CV %
Blank/total counts %□	5.62 ± 0.98 %	15.90
Minumum sensitivity	2.54 ± 0.11 uU/ml	4.30
Correlation coefficient	0.99 ± 0.01 ($p<0.05$)	1.02

CV %, correlation coefficient (SD/Mean x 100 %)

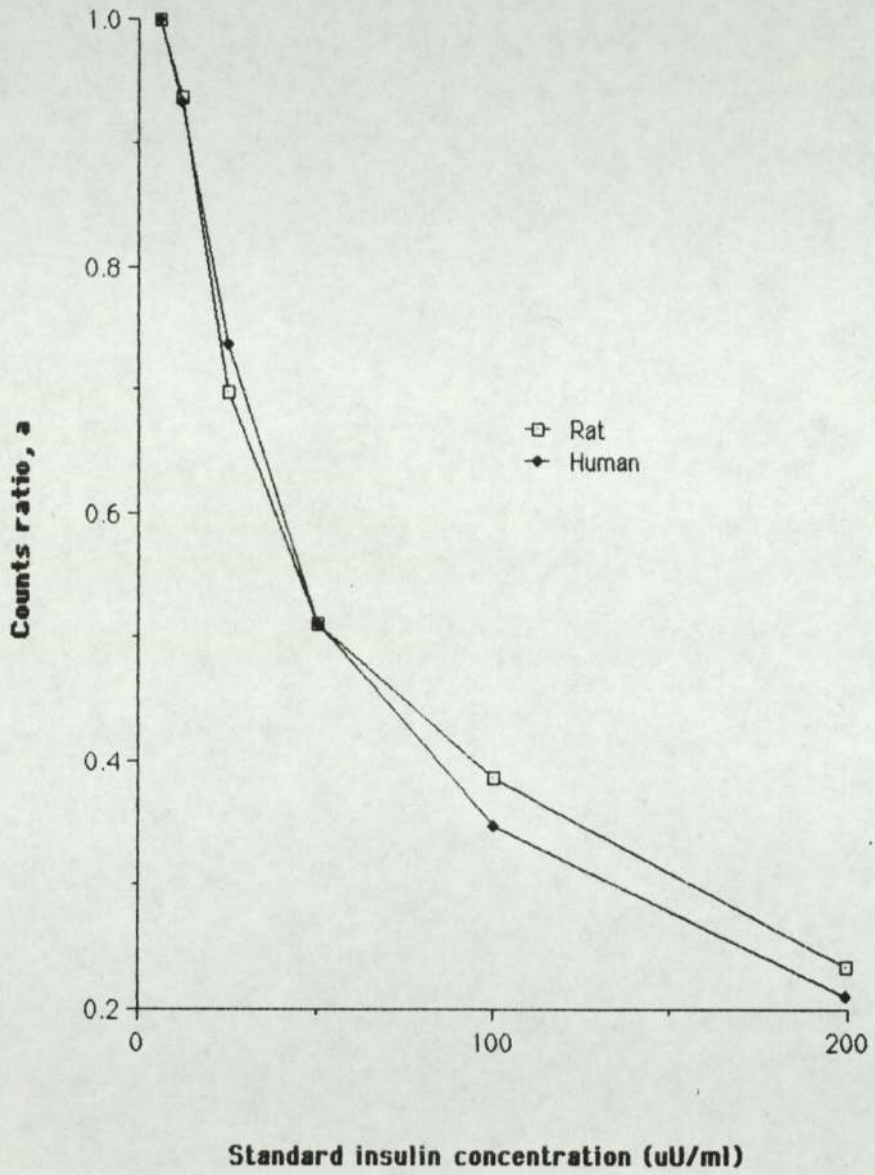
□ Blank/total counts is an estimate of non antibody specific ^{125}I binding i.e., to the assay tubes.

Criteria required for a satisfactory assay.

1. There must be good agreement between replicate values for all standards, blanks and total counts. The coefficient of variation should be around 5%. Where appropriate replicate sample count rates should be maintained around 5%.
2. The nonspecific binding (blank/total) should be between 5-7.5% of the total counts.

Figure 2.5 Comparison of standard curves constructed using rat and human insulin standards in the double antibody radioimmunoassay.

Data are means of 3 separate assays. For clarity SEM's are not shown but in all cases were <8 %, $p > 0.05$, no difference between standards at all concentrations.



a, counts in the bound fraction for each standard were expressed as a ratio of the 6.25uU/ml standard. This maneouvre reduces the effect of slightly differing amounts ot total label added in different assays.

C-peptide radioimmunoassay.

C-peptide determinations were kindly performed by Dr M. Nattrass of Birmingham General Hospital using a kit designed for the estimation of human C-peptide (Novo, Bagsvaerd, Denmark.) The protocol is a modification of that used by Heding (90) involving the use of synthetic human C-peptide as the standard and ^{125}I-synthetic Tyr-C-peptide as label. Bound and free radioactivity was separated by centrifugation. All samples were assayed in duplicate. Differences between duplicates was below 5% and the minimum sensitivity of the assay was 0.02pmol/ml.

Insulin release from statically incubated HIT-T15 and RINm5F cells.

Choice of incubation regimen.

The most frequently used incubation procedure used for isolated islets has involved preincubation for 30 minutes followed by a 60 minute test incubation using a basic Krebs bicarbonate buffer (91,92). Lindstrom and Sehlin have recently shown that the omission of the bicarbonate/CO_2 buffer system results in intracellular alkalinization and an impairment of insulin release (93).

In the present studies HIT-T15 and RINm5F cell insulin release was determined in monolayer culture. This regimen was chosen to enable normal contact between B-cells, which is believed to be important in the regulation of insulin release (25). In order to avoid cell detachment by repeated changes of media monolayers of cultured B-cells were incubated for 90 minutes in the presence and absence of test agents without prior preincubation.

HIT-T15 and RINm5F cell incubation protocol.

2×10^5 HIT-T15 or RINm5F cells from freshly trypsinised stocks were transferred to each well of a 24 well Costar cluster plate containing either 1ml of Ham's F-12 (HIT-T15) or RPMI 1640 (RINm5F). Plates were incubated at 37^0C in 5%CO_2/humidified air for 48 hours to allow adhesion and proliferation. Cultures were then washed in Krebs/bicarbonate/HEPES buffer, pH7.4 containing BSA, 0.2g/l (Appendix A2 page 228) and subsequently test incubated for 90 minutes in 1ml of fresh buffer, supplemented with the test agents at the appropriate concentrations. After incubation 0.2ml of the mildly agitated and centrifuged (1000rpm) supernatant was frozen at -15^0C for the subsequent estimation of insulin by double antibody radioimmunoassay.

In order to determine the mean number of HIT-T15 and RINm5F cells present in each well during secretory experiments, 4 wells in every cluster plate were trypsinised and the cell concentration confirmed using a haemocytometer. This value was used for both the quantification of insulin release and the extent of sample dilution for optimal radioimmunoassay. Control wells containing zero (basal) and 7.5mmol/l glucose for HIT-T15 and zero (basal) glucose for RINm5F cells were included in every batch experiment.

Insulin release was expressed as either uU insulin/ 10^5cells/ 90 minutes or as the percentage change from basal (absence of glucose).

Insulin release from perifused HIT-T15 and RINm5F cells.

The dynamic release of insulin in response to glucose, glibenclamide, colchicine and acetyl choline was monitored using a perifusion system (94).

5×10^5 HIT-T15 or RINm5F cells were seeded into individual multiwell chambers containing a 15mm round Thermanox coverslip (Miles Laboratories, Ltd, UK.) and incubated at 37^0C in 5% CO_2/humidified air for 48 hours to allow adhesion and proliferation. After this time coverslips carrying 3×10^5 cells (HIT-T15: $2.99 \pm 0.53 \times 10^5$, n=5; RINm5F: $3.27 \pm 0.33 \times 10^5$, n=3) were transferred to 25mm swinnex filter chambers (Millipore Ltd, UK.) filled with Krebs/HEPES buffer, pH 7.4 containing BSA, 0.2g/l.

Cells were preperifused for 30 minutes with basal Krebs/HEPES buffer (or with Buffer containing 1mmol/l colchicine in colchicine experiments) and then test perifused for a further 60 minutes with Krebs/HEPES buffer containing the test agents at the appropriate concentrations.

The perifusion system consisted of a Gilson minipuls 2 pump (Anachem Ltd, UK.) and an LKB 2212 helirac fraction collector fitted with a number 1 cassette (LKB Instruments, Bromma, Sweden.) 1ml fractions were collected throughout and the perifusion system had a dead volume of 2.5ml, which at a flow rate of 1ml/minute gave a respone time of 2.5 minutes. The rate of insulin release was expressed as uU insulin/minute/(coverslip = plate)

Measurement of the rate of glucose oxidation.

As the circulating concentration of glucose is the primary physiological stimulus for insulin release and biosynthesis (95) the measurement of glucose oxidation may yield evidence as to the control of these processes. The rate of glucose oxidation, the conversion of D-glucose to carbon dioxide and water, can be assessed by measuring either the rate of oxygen consumption (96) or glucose oxidation. Two methods are currently available for the measurement of glucose oxidation employing either the measurement $^{14}CO_2$ production from D-[^{14}C] glucose or $^{3}H_2O$ production from D-[^{3}H] glucose. (95) The stoichiometry of both of these reactions is illustrated below.

$^{14}CO_2$ production;

$$[^{14}C\text{-}C_5]H_{12}O_6 + 6O_2 \rightleftharpoons {}^{14}CO_2 + 5CO_2 + 6H_2O + \text{Energy}.$$

$^{3}H_2O$ production;

$$C_6[^{3}H\text{-}H_{11}]O_6 + 6O_2 \rightleftharpoons 6CO_2 + [^{3}H\text{-}HO] + 5H_2O + \text{Energy}.$$

In the present studies the rate of HIT-T15 and RINm5F cell glucose oxidation was estimated by determining the rate of $^{14}CO_2$ production from D-[U^{14}C] glucose using a modification of the method of Ashcroft et. al. (92,95)

Measurement of HIT-T15 and RINm5F cell glucose oxidation.

Batches of either 10^5 HIT-T15 or 10^5 RINm5F cells were incubated in small plastic reaction tubes (5 x 35mm, Luckhams Ltd, UK.) containing 100ul of Krebs/HEPES buffer, pH7.4 supplemented with BSA (0.2g/l). The incubation medium contained either glucose

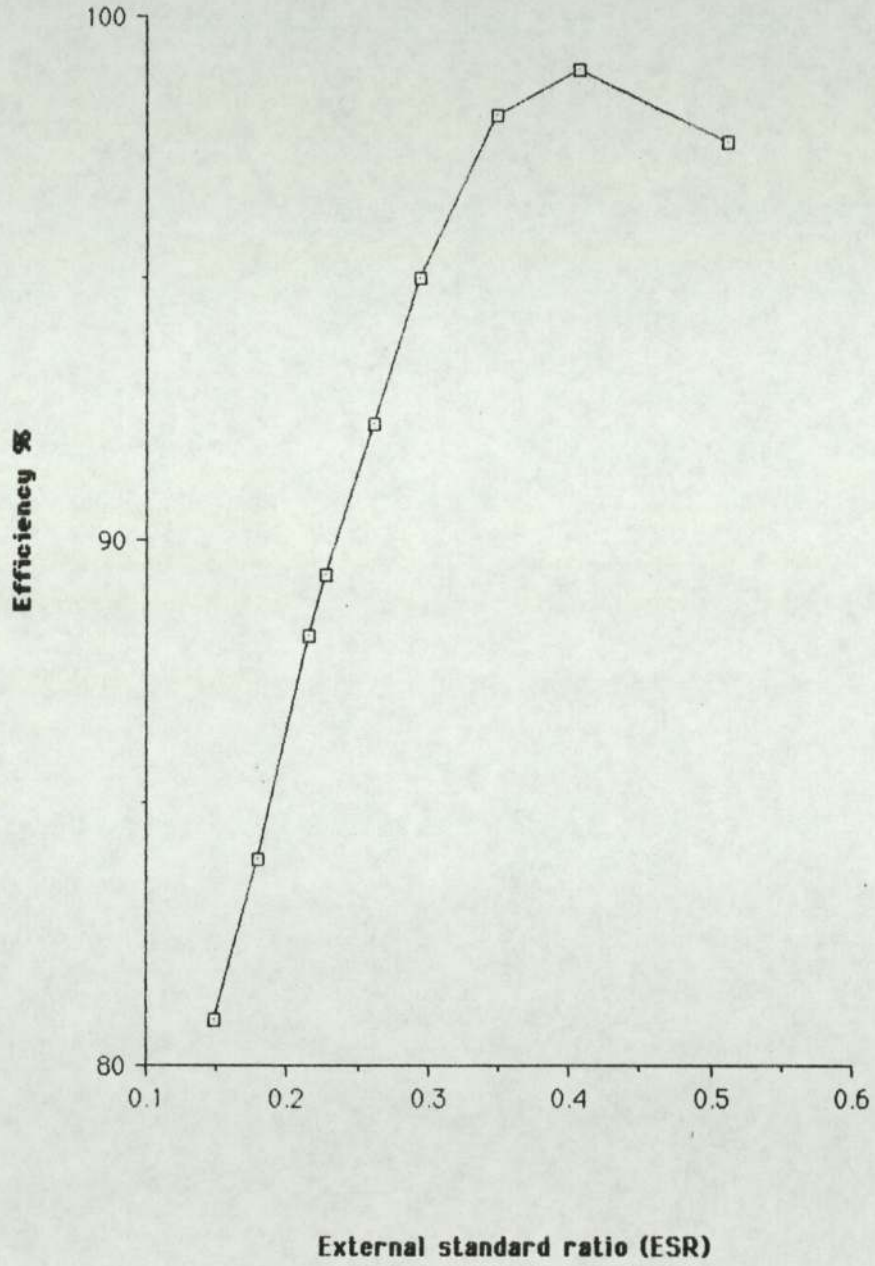
(2 or 7.5 mmol/l) alone or in combination with a variety of test agents at the appropriate concentrations and a tracer amount of D-[U^{14}C] glucose (4uCi/ml). The reaction tubes were placed inside glass scintillation vials and the atmosphere gassed with 95%O_2:5%CO_2. The scintillation vials were then sealed using rubber subaseal lids and incubated at 37^{0}C in a shaking water bath (100 cycles/minute) for 90 minutes. After incubation the vials were removed and chilled over ice. 1ml of NaOH (1mol/l) was injected through the subaseal into the outer compartment and B-cell metabolism stopped by the injection of 200ul of HCl (0.1mmol/l) into the reaction tube. The vials were then left at room temperature overnight to allow the absorption of $^{14}CO_2$ into the NaOH. Radioactivity trapped in the NaOH was measured by liquid scintillation counting. (Tricarb 2260, Packhard Instruments Ltd, UK.) using 10ml of NE260 scintillant (Nuclear Enterprises Ltd, UK). The counting efficiency was in excess of 97% estimated by reference to a preprepared quench curve Figure 2.6 (quenching was achieved artificially using chloroform).

Calculation of HIT-T15 and RINm5F cell glucose oxidation rates.

A blank consisting of 1ml of NaOH and 10ml of scintillant was included in each experiment. This value was automatically subtracted from the experimental values prior to multiplication by the counting efficiency. The rate of glucose oxidation expressed as umol/l of D-[U^{14}C] glucose oxidised per 10^5 cells per 90 minutes was computed using the following formula:

$$\text{D-[U}^{14}\text{C] glucose oxidation (umol)} = \frac{^{14}CO_2 \text{ formed (dpm)}}{\text{Specific activity of D-[U}^{14}\text{C]glucose (dpm/umol)}}$$

Figure 2.6 Chloroform quench curve for ^{14}C showing the relationship between counting efficiency and external standard ratio (ESR). All ESR values were in excess of 0.35 giving a counting efficiency of >97%.



Isolation of Lean mouse islets of Langerhans using collagenase digestion.

Pancreatic islets were isolated from from lean (HO) mice by a modification of the technique first described by Lacy and Kostianovsky (15). Mice were culled by cervical dislocation, positioned ventral side up and the viscera displayed using a mid ventral incision. The viscera were displaced to the left to reveal the full extent of the pancreas. The latter was distended with Krebs/HEPES buffer, pH7.4 using a syringe and a 24 gauge needle and removed from its points of contact with the stomach and duodenal loop with butterfly scissors. The excised pancreas was trimmed of fat and connective tissue, washed and cut into small pieces with round ended scissors. Pieces of pancreas derived from two mice were transferred to a conical flask (25ml) containing 8ml of Krebs/HEPES buffer and 40mg of Worthington collagenase. The contents were then vigorously shaken in a shaking water bath (120 cycles/minute) at 37^{0}C for 20-30 minutes. The digest was subsequently made up to 15 ml with ice cold Krebs/HEPES buffer to prevent any further digestion. The contents were then transferred to a centrifuge tube (15ml) and the contents allowed to sediment. This procedure was repeated to remove all traces of collagenase. The final sediment was resuspended in 10ml of Krebs/HEPES buffer and transferred to a glass petri dish. At this stage DNA-ase (200ug/digest) was added to prevent clumping of the debris. Islets free from acinar tissue could be picked out using a pasteur pipette under a dissecting microscope (magnification x 10). Approximately 150 clean islets could be harvested from the pancreata of two mice by this method.

Insulin release from statically incubated mouse islets of Langerhans.

Immediately after isolation islets were preincubated 'en masse' in Krebs/HEPES buffer, pH7.4 supplemented with BSA, 0.2g/l and glucose, 5.6mmol/l, at 37^{0}C in 5%CO_2/humidified air for 30 minutes to establish a steady state of insulin release. After this preincubation batches of 10 islets were transferred to each well of a Costar 24 well multitray containing 1ml of the appropriate test buffer and incubated for a further 90 minutes At the end of the incubation 100ul of buffer was removed and stored at -15^{0}C for the subsequent determination of insulin by double antibody radioimmunoassay.

Determination of the number of cells per lean mouse islet.

In order to make a quantitative comparison between islet data and data obtained using cultured B-cells six batches of 50-100 islets were disaggregated for 8 minutes with trypsin/EDTA (0.05%w/v : 0.02%w/v) to yield single cell suspensions. Enumeration of these cell suspensions using a haemocytometer indicated that lean (HO) mouse islets contained 4280 $\pm$ 547 cells/islet (mean values $\pm$ SEM, n=6)

Data presentation and statistical analysis.

Results are expressed throughout as means $\pm$ SEM with the number of determinations in parentheses. Student's t-test was used for statistical comparisons and the differences were considered significant if $p<0.05$. In perifusion experiments statistical significance of the differences between secretory profiles was computed using an analysis of the areas under the secretory profiles (Appendix A5, page 235).

CHAPTER 3.

3. THE EFFECTS OF GLUCOSE, AMINO ACIDS, SULPHONYLUREAS AND COLCHICONE UPON INSULIN RELEASE FROM HIT-T15 AND RINm5F CELLS.

INTRODUCTION.

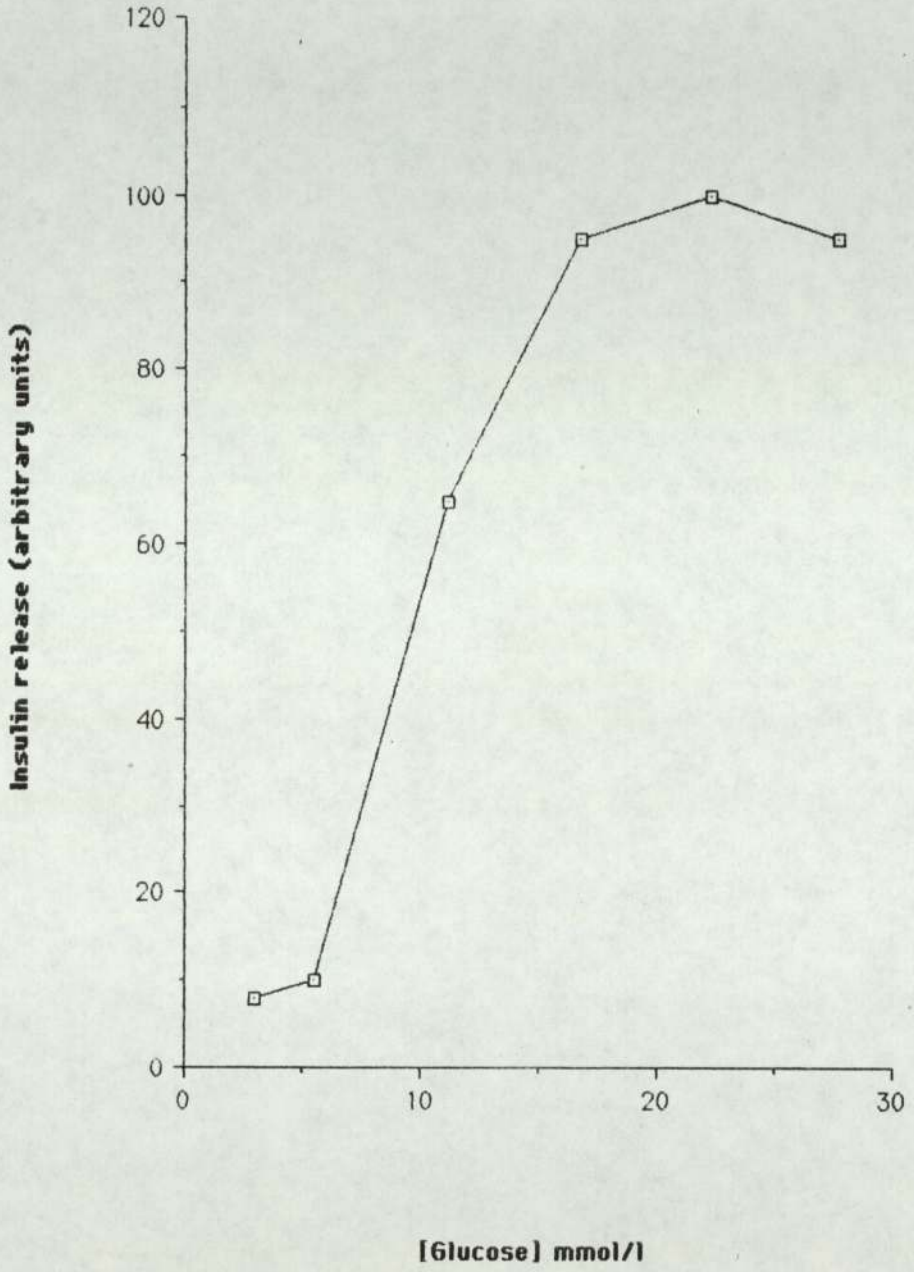
The effect of glucose on insulin release.

Insulin release from the islets of Langerhans in response to extracellular glucose is classically sigmoidal in nature, this relationship is illustrated in figure 3.1 (97). Glucose concentrations <3mmol/l fail to stimulate insulin release, the threshold being close to 4mmol/l. The most marked increase in insulin release occurs between 4 and 17mmol/l, with the secretory rate reaching a plateau at glucose concentrations in excess of 17mmol/l. Half maximum insulin secretory rates occur at glucose concentrations of 6-8mmol/l. (97,98).

Glucose recognition by the pancreatic B-cell.

The mechanism by which the pancreatic B-cell recognises glucose, the major physiological stimulus for insulin release, has remained largely unknown. Two main hypotheses have been advanced in an effort to explain this process. The first theory, the fuel, or substrate site hypothesis suggests that stimulus recognition is the consequence of glucose metabolism in the B-cell (99). The second hypothesis, the regulator site hypothesis, postulates that glucose combines with a specific membrane bound receptor on the B-cell leading to the initiation of insulin release (100).

Figure 3.1 Characteristics of glucose induced insulin release from isolated islets of Langerhans. (97)



The major experimental evidence supporting the fuel hypothesisis is based on the following observations. The insulinotrophic capacity of sugars correlates with their capacity to be metabolised in the B-cell, table 3.1 (98,101,102). Insulin release can be stimulated by provoking the production of endogenous glucose via glycogenolysis from B-cell glycogen stores (104). The metabolic inhibitor, mannoheptulose inhibits glucose oxidation and insulin release (105). Phloretin, a competitive inhibitor of glucose transport also inhibits insulin release (106).

In intact islets there are two components involved in the process of glucose utilization, a high Km component associated with with insulin release, reflecting glucokinase (EC 2.7.1.2) activity and a low Km component reflecting hexokinase (EC 2.7.1.1) activity (107). Figure 3.2 illustrates some of the early steps in the metabolism of glucose by pancreatic islets.

Figure 3.2 Schematic view of the early stages of glucose metabolism by the pancreatic B-cell. (Modified from Sener et al, ref 108). ⟶, indicates major pathway

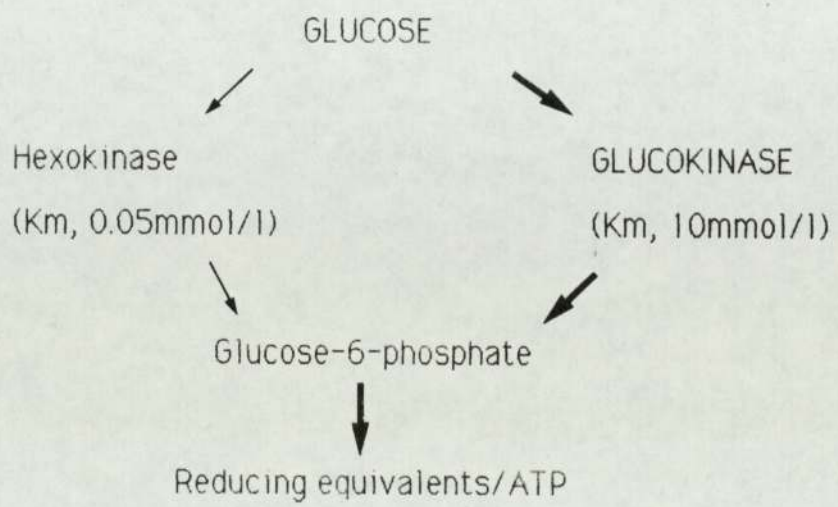


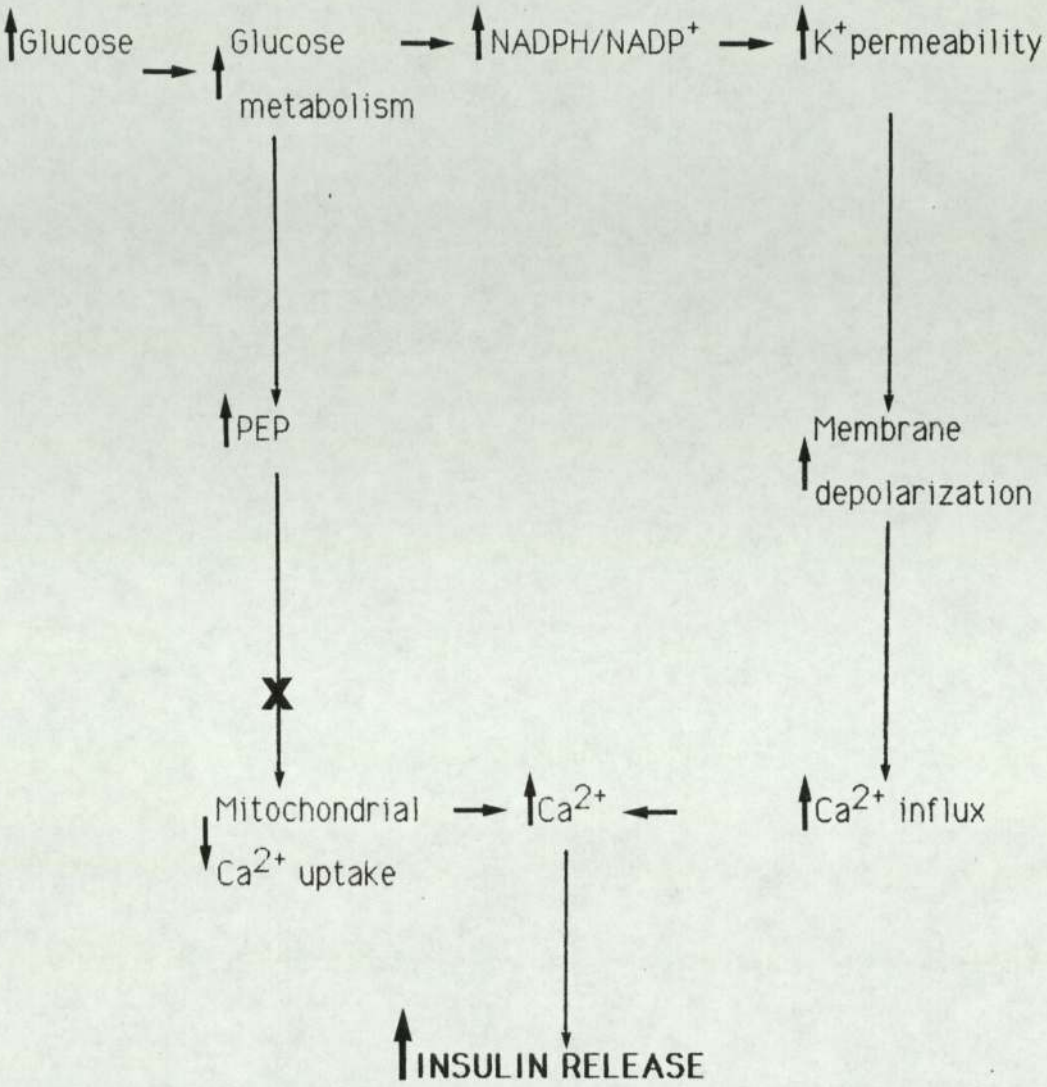
Table 3.1 The effect of various sugars upon islet metabolism and insulin release. (modified from Aschcroft, ref 103).

Sugar	Effect upon islet	
	metabolism	Insulin release
Glucose	+	+
N-acetylglucosamine	+	+
N-acetylmannosamine	-	-
Allose	-	-
Altrose	-	-
Arabinose	-	-
2-deoxyglucose	-	-
Fructose	+/-	+/-
Fucose	-	-
Galactose	-	-
L-Glucose	-	-
Galose	-	-
Idose	-	-
Mannose	+	+
3-methylglucose	-	-
Ribitol	-	-
Ribose	-	-
Sorbitol	-	-
Sucrose	-	-
Talose	-	-
Xylitol	-	-

All sugars are D-sterioisomers unless otherwise stated.

In islet homogenates hexokinase accounts for 80-90% of the total rate of glucose phosphorylation, in the presence of 8.3mmol/l glucose. Yet, in intact islets, the rate of glucose utilization in the presence of low glucose concentrations, sufficient to saturate hexokinase, represents only one third of that observed in the presence of 8.3mmol/l glucose. This difference can be accounted for by the fact that hexokinase is inhibited by glucose-6-phosphate (109). The high Km glucokinase may thus play a vital role in the rate of glucose phosphorylation by islets. It has been proposed that glucokinase may be the pancreatic B-cell glucoreceptor (110). However, Malaisse and Sener have suggested that the anomeric specificity of hexose utilization in pancreatic islets and the perturbation of glucose catabolism in islets from fasted rats illustrate the relevance of regulatory steps beyond glucose phosphorylation in the control of glucose metabolism and that it is unlikely that glucokinase is the pancreatic B-cell glucoreceptor (111). Ashcroft has proposed that an increase in glucose concentration leads, via an enhanced rate of glucose metabolism, to increased concentrations of phosphoenolpyruvate (PEP) and of reduced pyridine nucleotides. The latter influence membrane permeability to potassium leading to depolarization and consequent calcium influx into the B-cell. The resultant increase in intracellular calcium which is accentuated by the restraining effect of PEP on mitochondrial calcium uptake stimulates insulin release (103). Figure 3.3.

Figure 3.3 Proposed metabolic model for glucose stimulus insulin secretion coupling in the pancreatic B-cell. (103).



Key;

X inhibited

The putative glucoreceptor protein in the B-cell membrane has been defined by Ashcroft as 'the molecular species in the B-cell, that, by virtue of its ability to bind glucose, confers on the B-cell, sensitivity to changes in extracellular glucose concentrations' (112). It is thought that the binding of glucose to its receptor leads to a conformational change in the receptor which activates the secretory machinery via second messengers such as calcium, cAMP and inositol phospholipids. The major experimental evidence supporting this regulator site hypothesis can be summarized as follows. The alpha anomer of D-mannose is a more potent insulin secretagogue than the beta anomer, yet mannose-6-phosphate isomerase (EC 5.3.1.7) exerts a preferrential affinity for the beta anomer (113). Insulin release has been demonstrated from an accellular composite secreting system which was not accounted for by an increase in glucose metabolism. The system required ATP and calcium for the secretory system to function. (114). The diabetogenic effect of alloxan can be protected by glucose, alpha-D-glucose providing more protection than beta-D-glucose. (100,115,116) Also the diabetogenic effect of streptozotocin can be protected by 4,6-O-ethylidene glucose, a glucose transport inhibitor (117). Digitoxose, the sugar moiety of digitoxin inhibits glucose stimulated insulin release from islets without affecting either their glucose oxidation or oxygen consumption (92). Glucose saturated lentil lectin stimulates insulin release. The complex is not transported into B-cells and the lectin alone did not stimulate insulin release (118). Several attempts have been made to characterize the putative glucoreceptor. Price demonstrated that solubilized B-cell membranes of dog pancreatic islets, in contrast to membranes of acinar cells, include a component which forms complexes with the insulin secretagogues glucose, mannose and fructose. Galactose and 3-O-methylglucose, which are not insulin

secretagogues do not form complexes (119). Similar observations have been noted by Kobayashi et.al. these workers found that D-[^{14}C] glucose binding to rat islet cell membranes was inhibited by D-glucose but not by L-glucose. In addition D-galactose, 3-O-methylglucose, phloridzin and alloxan were found to inhibit the binding of D-[^{14}C] glucose (120). The major disadvantage of such studies is their inability to differentiate between the glucose transporter in the B-cell (121) and the putative glucoreceptor. It is possible however, that the glucoreceptor and transporter may be closely linked.

The effect of amino acids on insulin release.

In man, protein meals (122) or the intravenous infusion of amino acids (123) have been shown to enhance plasma insulin levels. Intraduodenal administration of a mixture of amino acids induces higher circulating insulin levels than intravenous administration (124), possibly suggesting a modification of amino acid induced insulin release by gastrointestinal hormones (125). A wide variety of amino acids have been shown to stimulate insulin release, Table 3.2. In the present studies L-arginine and L-leucine were used as representative amino acids.

The effect of L-arginine and L-leucine on insulin release.

Of the individual amino acids known to stimulate insulin release, L-arginine is the most potent (133) and its effect appears to be dependent upon the prevailing glucose concentration. Arginine is unable to stimulate insulin release in the absence of glucose (135). The insulinotrophic action of L-leucine has been demonstrated by many authors (126,134), and these studies express the view that leucine can stimulate insulin release in the absence of glucose. A maximum effect is observed at around 20mmol/l (136).

Table 3.2. The effect of amino acids on insulin release

Amino acid	Effect on insulin release	Reference
Arginine	+ 1*	(126)
Leucine	+ 3*	(126)
Alanine	+/-	(127,128)
Glutamate	-	(129)
Glycine	+/-	(130,131)
Histidine	+/o	(132,133)
Lysine	+ 2*	(133)
Methionine	+/o	(130,133)
Ornithine	+	(133)
Phenylalanine	+	(130)
Proline	+	(134)
Threonine	+	(122)
Tryptophan	+/o	(122,126)

+, stimulation, -, inhibition, o, no effect.

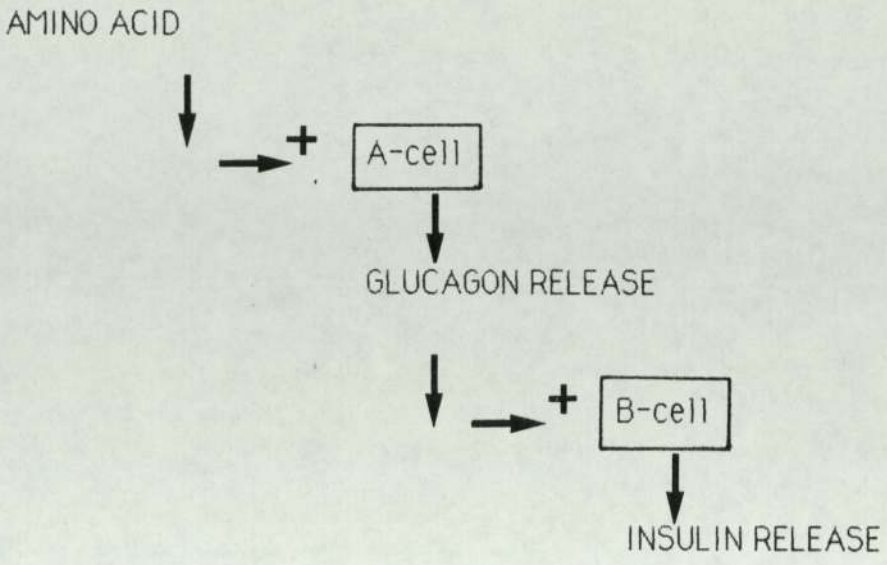
1^*, 2^*, 3^* denotes the degree of potency on insulin release.

Possible mechanisms for leucine and arginine induced insulin release.

Via amino acid induced glucagon release.

Milner suggested that amino acid induced insulin release may be the result of amino acid induced glucagon release (126), Figure 3.4. Certainly arginine has been shown to stimulate the release of glucagon from isolated pancreatic islets (137).

Figure 3.4 Possible mechanism for amino acid induced insulin release via amino acid induced glucagon release. (+, stimulation.)

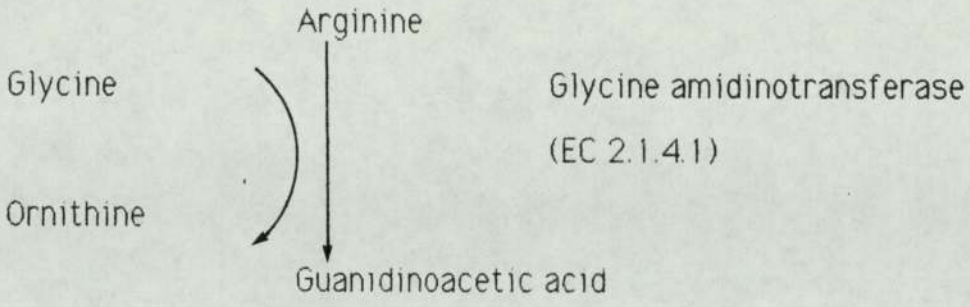


Arginine and leucine metabolism as a trigger for insulin release.

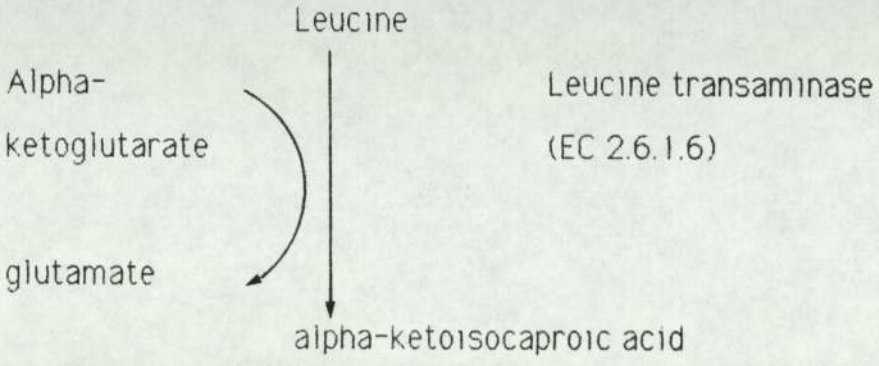
Arginine metabolism *per se* does not appear to be the trigger for insulin release, this is supported by the observation that the rate of arginine oxidation by the B-cell is extremely low (138). However, this observation does not rule out the possibility that an early metabolite of arginine may be the trigger for insulin rlease. Guanidinoacetic acid, the transamination product of arginine, Figure 3.5a does not stimulate insulin release from isolated rat islets (139). On the other hand, leucine metabolism appears to play a role in the capacity of the amino acid to stimulate insulin release. (140). Certainly, leucine is both transported into (141) and metabolized by (142) islet cells. Alpha-ketoisocaproate, the transamination product of leucine, Figure 3.5b, is a potent stimulator of insulin release (143). The most significant argument against leucine metabolism being involved in the insulinotrophic action of leucine is the fact that 2-aminobicyclo [2,2,1] heptane-2-carboxylate (BCH), a nonmetabolizable analogue of leucine stimulates insulin release (144).

Figure 3.5. Catabolism of a/ arginine to guanidinoacetic acid and b/ leucine to alpha-ketoisocaproic acid.

a/ Arginine



b/ Leucine



Possible intracellular mechanisms for arginine and leucine induced insulin release.

Calcium has been shown to be required for the stimulation of insulin release by amino acids (145). In the absence of extracellular calcium or in the presence of cobalt (146) or verapamil (147) the amino acids do not stimulate insulin release. Both leucine and arginine increase the net retention of calcium by isolated islets (140,145). Using rat islet cell homogenates, Howell and Montague have demonstrated that leucine and arginine have no effect on adenyl cyclase activity (148).

The effect of sulphonylureas on insulin release.

The development of sulphonylurea drugs was the result of investigations on the antibiotic properties of sulphonamide derivatives. The so called first generation sulphonylureas developed in the early 1950's and 1960's, were superceeded by a second generation of far more potent compounds (149). Table 3.3.

Table 3.3 Characteristics of first and second generation sulphonylureas. (from Lebovitz and Feinglos, 149)

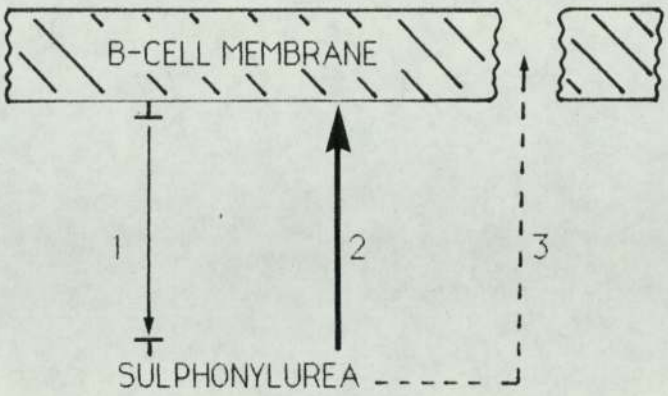
FIRST GENERATION COMPOUNDS (longer acting)	
Name	Effective dose range (mg)
Tolbutamide	500-3000
Chlorpropamide	100-500
Tolazamide	100-750
Acetohexamide	500-1500
SECOND GENERATION COMPOUNDS (more potent shorter acting)	
Name	Effective dose range (mg)
Glibenclamide	2.5-20
glipizide	2.5-40
Glibornuride	12.5-100

Although hypoglycaemic sulphonylureas may have several extrapancreatic effects which are beneficial to the diabetic (150,151), the stimulation of insulin release appears to be their essential property (152).

The binding of sulphonylurea to the B-cell.

In a recent study by Hellman et. al. six hypoglycaemic sulphonylureas (carbutamide, tolbutamide, chlorpropamide, glibornuride, glipizide and glibenclamide) were compared with regard to their ability to bind to B-cell rich pancreatic islets of obsese (ob/ob) mice (153). All of the sulphonylureas tested bound rapidly to the islets. There was no correlation between the insulinotrophic potency and binding characteristics. Glibenclamide was found to be exceptional among the sulphonylureas tested because it did not rapidly reach uptake equilibrium. Instead it accumulated progressively in large amounts within the B-cell. This accumulation of glibenclamide was resistent to washing with sulphonylurea free medium. The intracellular accumulation of glibenclamide could be inhibited by 4-acetamido-4'-isothiocyanate -stilbene-2, 2'-disulphonic acid (SITS), an inhibitor of anion channels in the B-cell membrane (154). It has therefore been suggested that glibenclamide accumulation after initial binding is via anion channels. The intracellular uptake of glibenclamide may explain why prolonged exposure to this agent results in functionally deficient B-cells (155). Since the insulin releasing action of these drugs appears to be due to an interaction with the B-cell membrane, it is important to consider the mechanism of sulphonylurea binding. Hellman has proposed a model to describe sulphonylurea binding to B-cells, Figure 3.6. The model pays particular attention to the fact that sulphonylureas at physiological pH, have a net negative charge, and that there is an increased binding of sulphonylureas with large non-polar groups. Thus sulphonylurea binding to B-cells can be assumed to be the product of hydrophobic interactions counteracted by electrostatic repulsion from fixed negative charges at the B-cell surface (156).

Figure 3.6 Putative model to explain sulphonylurea binding to the B-cell membrane (156).



1, negative charge repulsion

2, hydrophobic binding

3, anion channel permeation (glibenclamide)

The possible intracellular mechanisms of sulphonylurea induced insulin release.

Early work by Matthews and Dean reported that tolbutamide depolarises the B-cell membrane (157). Subsequent work demonstrated that both glibenclamide (158) and tolbutamide (159) evoked electrical depolarization of the B-cell membrane as well as spike activity in the presence of 3mmol/l glucose. In glucose free medium tolbutamide (<75umol/l) slightly depolarizes the B-cell membrane without inducing any spike activity (159). Studies on ion fluxes in isolated islets have been performed to elucidate the electrical and insulin secretory properties of sulphonylureas. Lebrun et.al. concluded that the electrical activity induced by tolbutamide could not be attributed to the inhibition of either Ca^{2+}-sensitive K^+ permeability or ouabain sensitive Na^+, K^+-ATP-ase (160).

The fact that sulphonylureas can raise the intracellular concentration of cAMP may explain their general potentiating action upon glucose stimulated insulin release (161). It would appear from the binding characteristics of sulphonylureas that the observed increase in the concentration of intracellular cAMP is not due to phosphodiesterase inhibition but rather to an activatiion of membrane bound adenylate cyclase.

The requirement for extracellular calcium in sulphonylurea induced insulin release is well established (162). Malaisse et. al. in 1971 showed that there is an almost perfect correlation between the rate of sulphonylurea induced insulin release and the radioactivity retained by islets after incubation with ^{45}Ca (163). The rapidly initiated sulphonylurea stimulated ^{45}Ca uptake, which decays with time, suggests that Ca^{2+} enters the B-cell through voltage dependent channels that are subject to progressive inactivation (164).

The present work sets out to characterise nutrient and sulphonylurea induced insulin release from HIT-T15 and RINm5F cells. Attempts have been made to answer the following questions. Is the glucose sensitivity of HIT-T15 cells observed by Santerre et.al. (62) reproducible and is it accompanied by an increase in the rate of glucose oxidation ?. How does the response of HIT-T15 cells compare with RINm5F cells ?. Secondly, are HIT-T15 cells capable of responding to glucose in a biphasic manner ?. Finally are HIT-T15 and RINm5F cells responsive to amino acids, L-leucine and L-arginine and sulphonylureas, tolbutamide, glibenclamide and chlorpropamide ?. Further elaboration of the dynamics of insulin release have been carried out using the first phase stimulator glibenclamide (165) and the second phase inhibitor colchicine (166).

RESULTS

The effect of glucose upon insulin release and the rate of glucose oxidation from cultured HIT-T15 and RINm5F cells.

Glucose stimulated insulin release from statically incubated HIT-T15 cells, maximum and half maximum stimulation occurring at 7.5 and 2.9 mmol/l respectively, Figure 3.7. RINm5F cells did not consistently respond to glucose challenge. In experiment I raising the glucose concentration from 0-7.5mmol/l produced a marginal (37%) but significant increase in insulin release, whilst this observation was not repeated in experiment II, Table 3.4.

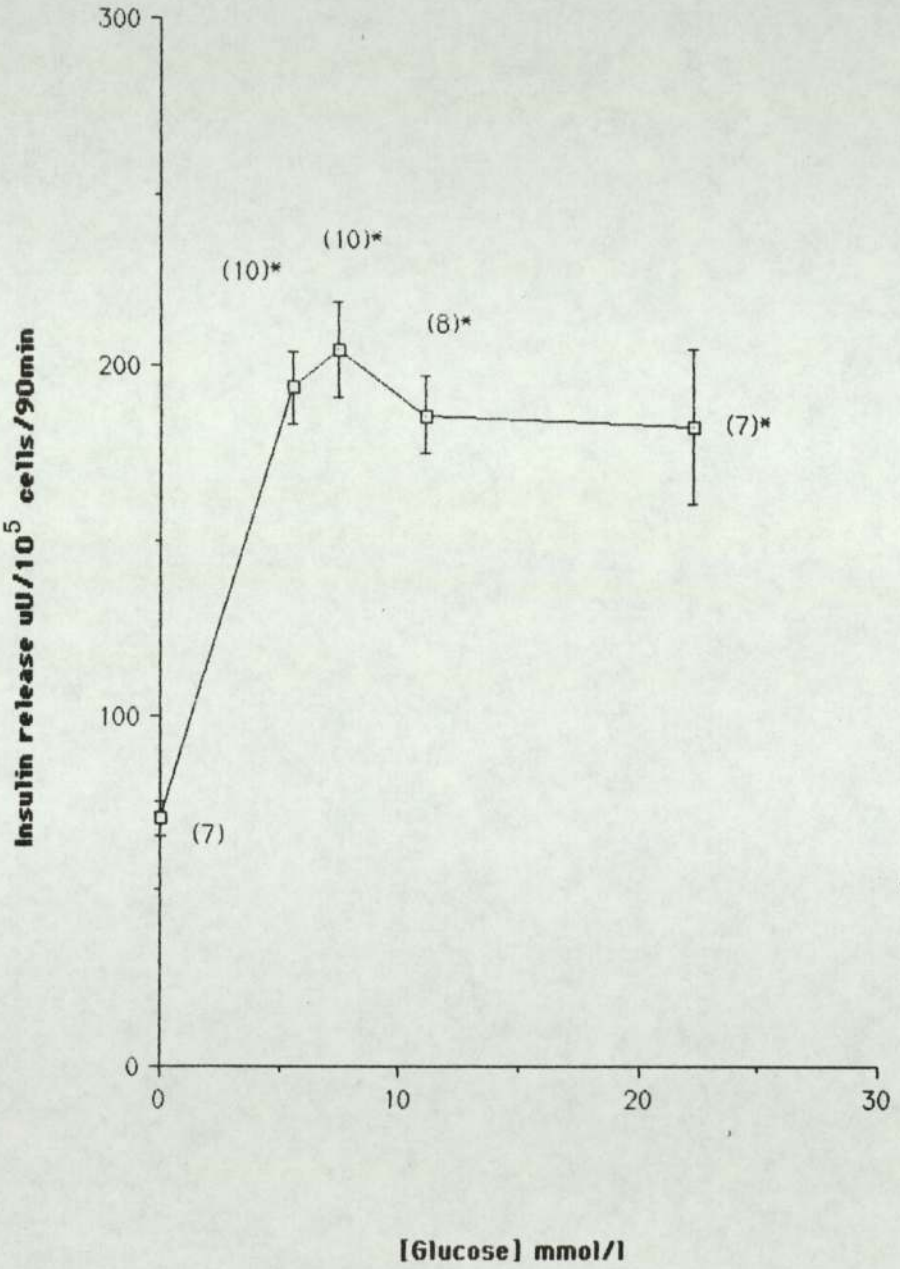
Table 3.4. The effect of glucose on insulin release from statically incubated RINm5F cells.

Experiment	[Glucose] mmol/l	n	Insulin release uU/10^5cells/90minutes
I	0	22	16.38 ± 0.70
	7.5	9	22.62 ± 2.86[a]
II	0	5	11.38 ± 1.33
	7.5	5	12.90 ± 1.50

a, $p<0.05$ significantly increased compared with 0 mmol/l glucose.

Figure 3.7 The effect of glucose on insulin release from statically incubated HIT-T15 cells. Values are means ± SEM with n in parentheses.

* p<0.05 significantly increased compared with basal (zero glucose)



When isolated islets were used, raising the glucose concentration from 0-16.7 mmol/l significantly stimulated insulin release, Table 3.5. When insulin release from islets is expressed as uU/10^5cells/90minutes (based on the observation that mouse islets contain 4280 cells/islet Chapter 2 page 65), islets release 5 fold more insulin than HIT-T15 cells and 100 fold more insulin than RINm5F cells in the absence of glucose.

Table 3.5. The effect of glucose upon insulin release from statically incubated mouse islets of Langerhans.

[Glucose] mmol/l	n	INSULIN RELEASE uU/islet/90min	INSULIN RELEASE uU/10^5cells/90min
0	4	47.16 ± 3.03	1101.82 ± 70.80
16.7	5	99.57 ± 16.82[a]	2326.41 ± 392.99[a]

a, $p<0.05$ significantly increased compared with 0mmol/l glucose

Raising the glucose concentration from 2-7.5 mmol/l caused a marked and significant increase in the rate of glucose oxidation by both HIT-T15 and RINm5F cells. This increase in glucose oxidation was accompanied by an increase in insulin release from HIT-T15 but not RINm5F cells, Table 3.6.The increase in the rate of glucose oxidation by RINm5F cells was more than double that shown by HIT-T15 cells.

Table 3.6. The effect of glucose on the rate of D-[U^{14}C] glucose oxidation and insulin release from HIT-T15 and RINm5F cells. (n is in parentheses)

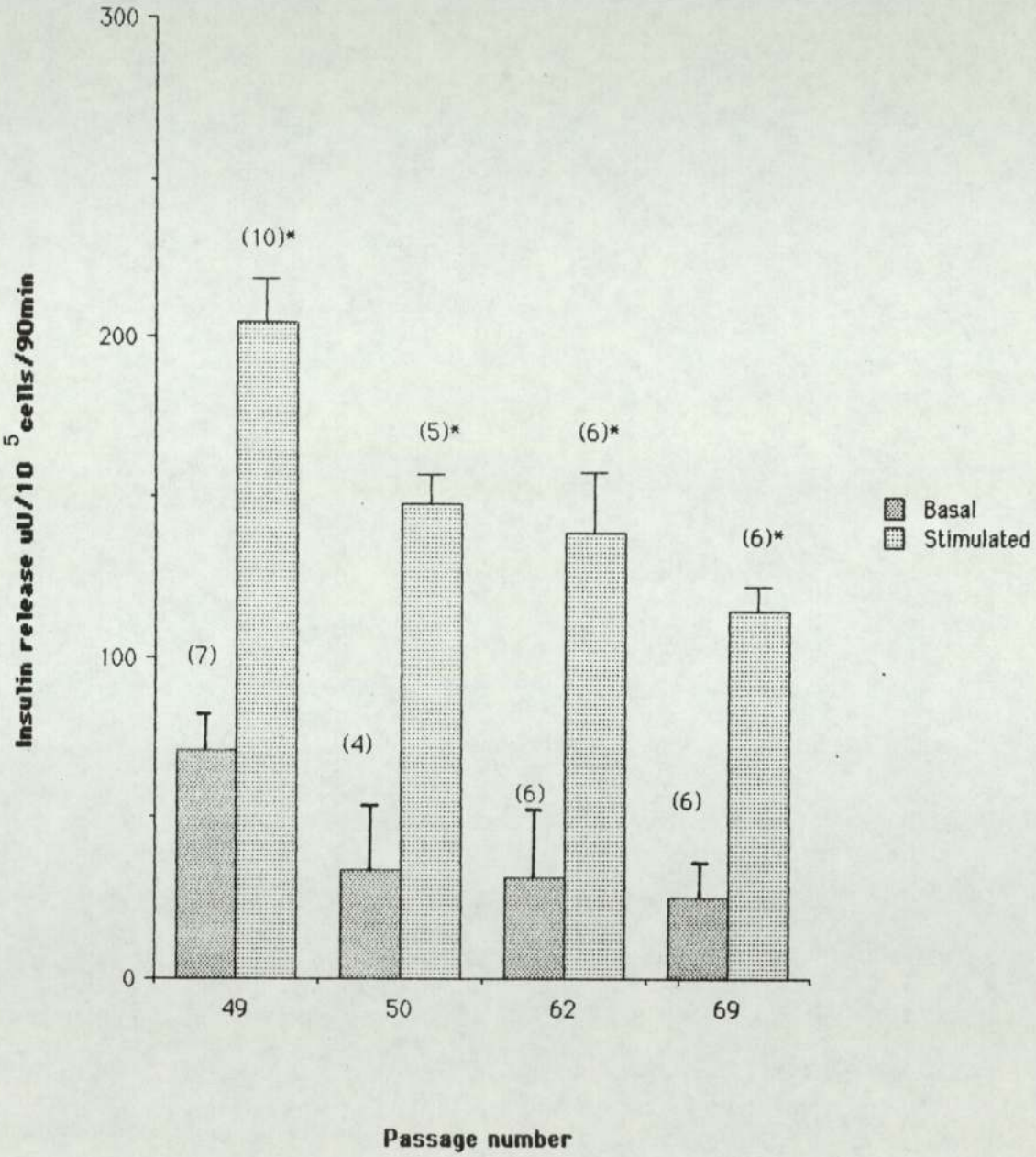
Cell line	[Glucose] mmol/l	Glucose oxidation umol/10^5cells/90min	Insulin release uU/10^5cells/90min
HIT-T15	2.0	7.14 ± 1.14 (12)	29.11 ± 7.42 (6)
	7.5	11.88 ± 2.53 (9)[a]	91.69 ± 15.40 (6)[a]
RINm5F	2.0	1.59 ± 0.06 (3)	20.27 ± 4.55 (3)
	7.5	26.97 ± 1.43 (3)[a]	21.83 ± 5.68 (3)

a, $p<0.05$ significantly increased compared with 2mmol/l glucose

Insulin release from cultured HIT-T15 cells is strongly affected by the passage effect, described by, Santerre et.al. (62) and Ashcroft et.al. (167) In the present studies this phenomenon was represented by a reduction in the basal (absence of glucose) and glucose (7.5mmol/l) stimulated insulin release with increasing passage number, Figure 3.8 Regression analysis of 7.5mmol/l glucose stimulated insulin release compared with passage number revealed a 3.25uU drop in insulin release per passage. No such reduction in basal (absence of glucose) insulin release was observed for RINm5F cells with increasing passage number. At passage 61, HIT-T15 cells had an insulin content of 283.4 uU/10^5cells. At passage 100, RINm5F cells had an insulin content of 184.4 uU/10^5cells. Basal insulin release from HIT-T15 cells at passage 61 and RINm5F cells at passage 100 accounted for some 11 and 8% of their insulin contents respectively

Figure 3.8 The effect of passage number on basal (absence of glucose) and glucose stimulated (7.5mmol/l) insulin release from statically incubated HIT-T15 cells. Values are means ± SEM with n in parentheses.

* p<0.05 significantly increased compared with basal.



Throughout this thesis data obtained from work performed using either a single or up to 3 consecutive passages were expressed as uU/10^5cells/90minutes. The results of experiments performed over more than 3 consecutive passages were expressed in terms of the percentage stimulation from basal. This maneouvre reduced the influence of the passage effect and is consistent with a similar manipulation of data carried out by Ashcroft et.al. (167)

The effect of D-glyceraldehyde upon insulin release from statically incubated HIT-T15 and RINm5F cells.

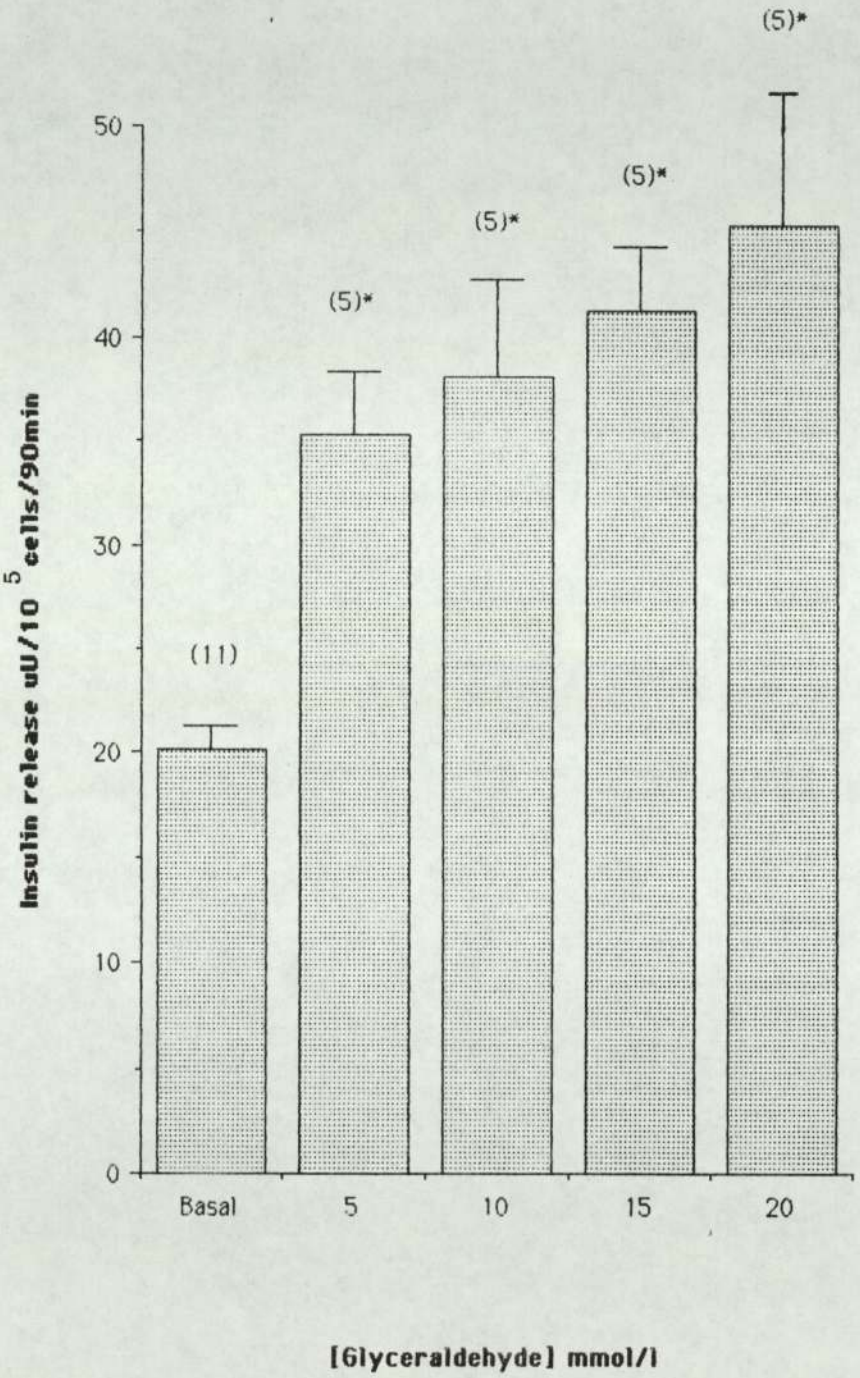
Glyceraldehyde stimulated insulin release from statically incubated RINm5F cells, maximum and half maximum stimulation occurring at 20 and 4.66 mmol/l respectively, Figure 3.9. Glyceraldehyde did not stimulate insulin release from statically incubated HIT-T15 cells, Table 3.7.

Table 3.7 The effect of D-glyceraldehyde upon insulin release from statically incubated HIT-T15 cells.

[D-glyceraldehyde] mmol/l	n	Insulin release uU/10^5cells/90minutes
0	6	39.16 ± 9.66
5	6	49.34 ± 0.77
10	5	51.91 ± 2.14
15	6	29.40 ± 1.01
20	6	42.21 ± 0.43

Figure 3.9 The effect of D-glyceraldehyde on insulin release from statically incubated RINm5F cells. Values are means ± SEM with n in parentheses.

* $p<0.05$ significantly increased compared with basal (absence of glyceraldehyde).



The effect of L-leucine and L-arginine upon insulin release from statically incubated HIT-T15 and RINm5F cells.

Both L-leucine and L-arginine, in the absence of glucose stimulated insulin release from statically incubated HIT-T15, Figure 3.10 and RINm5F cells, Figure 3.11. The maximum observed release of insulin from both cell lines occurred in response to an amino acid concentration of 15mmol/l. L-arginine and L-Leucine were not significantly different as regards their ability to stimulate the release of insulin from HIT-T15 cells. However with RINm5F cells, L-leucine was a significantly more potent insulin secretagogue than L-arginine ($p<0.05$) when used at concentrations of 15 and 20 mmol/l.

Insulin release from HIT-T15 cells in response to 7.5mmol/l glucose was significantly enhanced by the addition of either 15mmol/l L-arginine, or 15mmol/l L-leucine alone or by the two in combination, Table 3.8. This combination of amino acids in the absence of glucose produced a significantly greater release of insulin than either amino acid or 7.5mmol/l glucose alone.

Figure 3.10 The effect of L-leucine and L-arginine upon insulin release from statically incubated HIT-T15 cells. Values are means ± SEM with n in parentheses.

* $p<0.05$ significantly increased compared with basal (absence of amino acid).

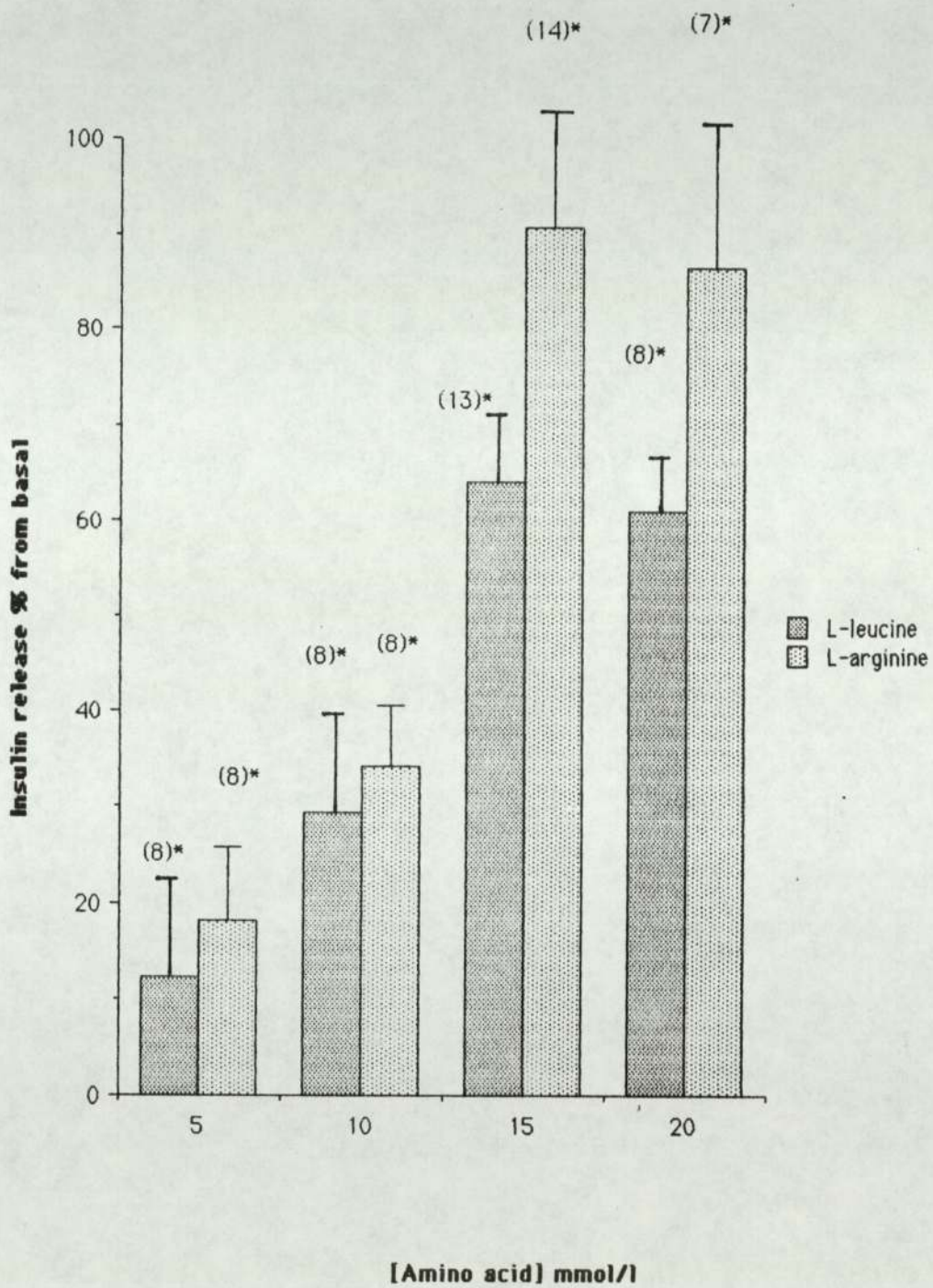


Figure 3.11 The effect of L-leucine and L-arginine upon insulin release from statically incubated RINm5F cells. Values are means ± SEM with n in parentheses.

* p<0.05 significantly increased compared with basal (absence of amino acid)

$ p<0.05 significantly increased compared with L-arginine.

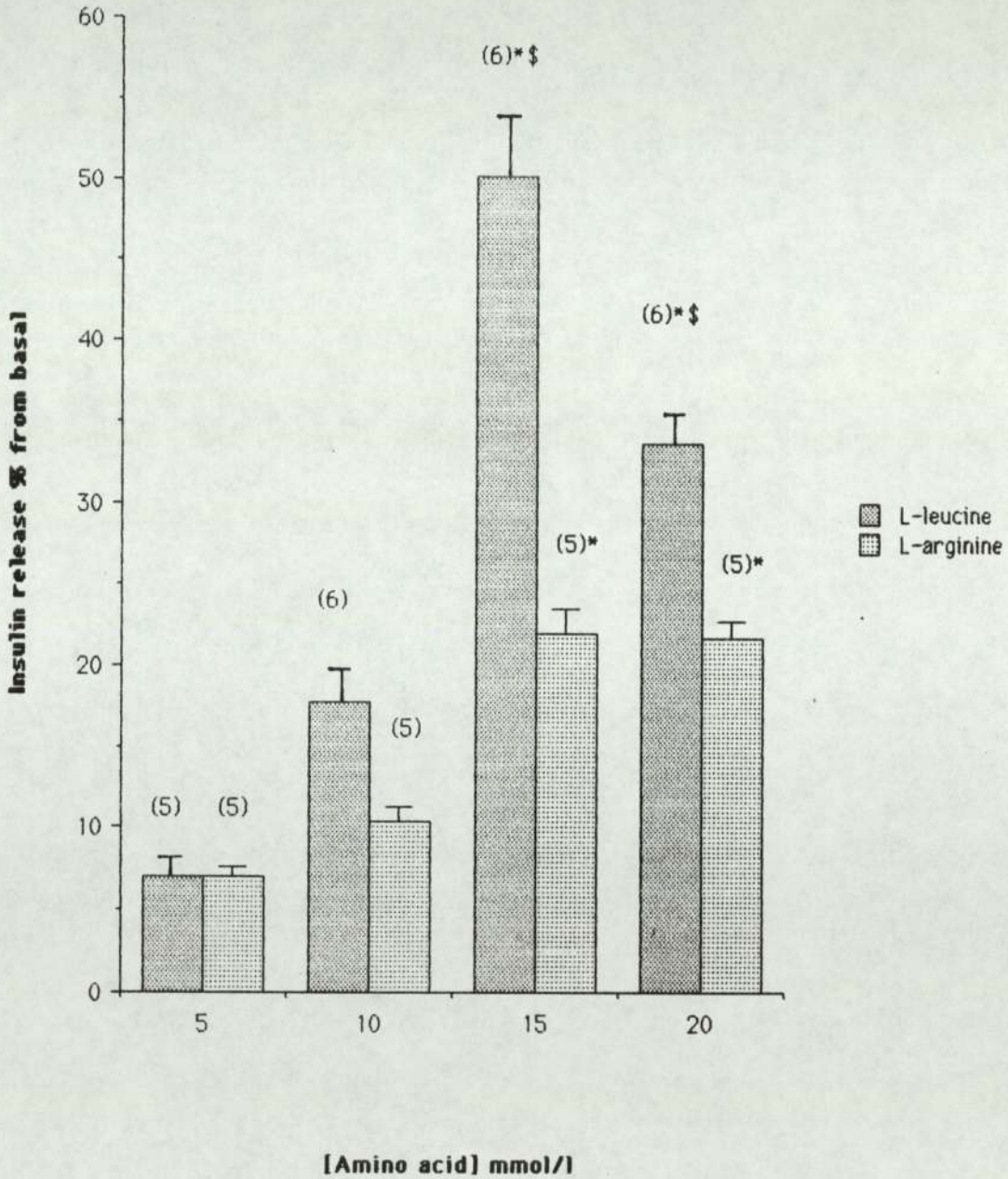


Table 3.8. The effect of glucose, L-arginine and L-leucine alone or in combination upon insulin release from statically incubated HIT-T15 cells.

Treatment	n	Insulin release % from basal
Glucose, 7.5mmol/l	37	118.81 ± 9.20
L-leucine, 15mmol/l	13	63.95 ± 12.91[c]
L-arginine, 15mmol/l	14	90.73 ± 13.44
L-leucine + L-arginine	5	170.52 ± 16.32[a,b]
Glucose + L-leucine	6	220.06 ± 48.51[a]
Glucose + L-arginine	6	303.92 ± 63.62[a]
Glucose + L-leucine + L-arginine	6	342.69 ± 38.45[a]

a, $p<0.05$ significantly increased compared with 7.5mmol/l glucose.

b, $p<0.05$ significantly increased compared with either amino acid alone.

c, $p<0.05$ significantly decreased compared with 7.5mmol/l glucose.

The effect of tolbutamide, glibenclamide and chlorpropamide upon insulin release and the rate of glucose oxidation by cultured HIT-T15 and RINm5F cells.

In the absence of glucose tolbutamide stimulated insulin release from HIT-T15 cells, maximum and half maximum stimulation occurring at 100 and 5umol/l respectively. The stimulation of insulin release was linear over the whole range of concentration used ($r=0.98$, $p<0.05$), Figure 3.12. This stimulation of insulin release from HIT-T15 cells by tolbutamide was not accompanied by an increase in the rate of D-[U^{14}C] glucose oxidation, Table 3.9.

Figure 3.12 The effect of tolbutamide upon insulin release from statically incubated HIT-T15 cells. Values are means ± SEM with n in parentheses.

* p<0.05 significantly increased compared with basal (absence of tolbutamide).

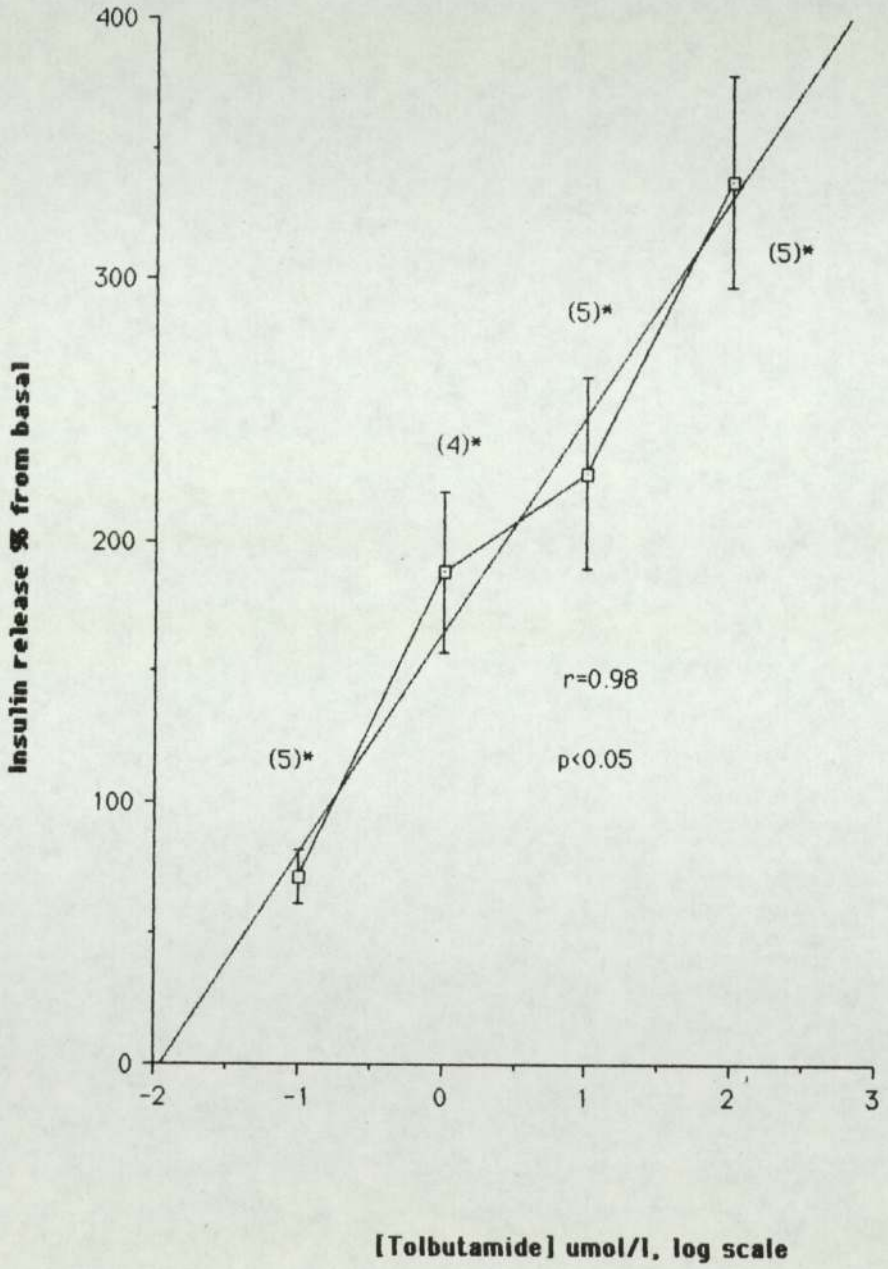


Table 3.9 The effect of tolbutamide upon the rate of D-[U^{14}C]glucose oxidation by HIT-T15 cells.

[Tolbutamide] umol/l	n	D-[U^{14}C] glucose oxidation umol/10^{5}cells/90minutes
0	12	7.14 + 1.14
0.1	4	4.48 + 1.28
1.0	5	5.34 + 1.32
10.0	4	5.23 + 1.93
100.0	6	7.75 + 1.91

The rate of D-[U^{14}C] glucose oxidation was measured at a glucose concentration of 2 mmol/l.

Glibenclamide and chlorpropamide at concentrations of 8umol/l (the concentration most commonly used for isolated islets, (92) stimulated the release of insulin from both RINm5F, Table 3.10 and HIT-T15 cells, Table 3.11.

Table 3.10. The effect of glibenclamide and chlorpropamide upon insulin release from statically incubated RINm5F cells.

Treatment	n	Insulin release, %from basal
Glibenclamide, 8umol/l	5	68.10 ± 7.09[a]
Chlorpropamide, 8umol/l	6	41.12 ± 1.79[a]

a, $p<0.05$ significantly increased compared with basal (zero glucose)

Table 3.11 The effect of glibenclamide and chlorpropamide upon insulin release from statically incubated HIT-T15 cells.

Treatment	n	Insulin release, %from basal
Glucose, 7.5mmol/l	37	118.80 ± 9.20[a]
Glibenclamide, 8umol/l	7	119.83 ± 18.35[a]
Chlorpropamide, 8umol/l	8	72.36 ± 17.13[b]
Glucose + glibenclamide	8	143.18 ± 25.03[a]
Glucose + chlorpropamide	6	246.04 ± 37.31[a,c]

a, $p<0.05$ significantly increased compared with basal (zero glucose)

b, $p<0.05$ significantly reduced compared with 7.5mmol/l glucose.

c, $p<0.05$ significantly increased compared with 7.5mmol/l glucose.

Glibenclamide was the more potent of the two sulphonylureas used for both cell lines ($p<0.05$, compared with 8umol/l chlorpropamide). Insulin released in response to both glibenclamide and chlorpropamide was greater from HIT-T15 cells than RINm5F cells. Of the two sulphonylureas used only chlorpropamide significantly potentiated 7.5mmol/l glucose stimulated insulin release from HIT-T15 cells.

Dynamics of insulin release from HIT-T15 cells in response to glucose, glibenclamide and colchicine.

Dynamic insulin release from perifused HIT-T15 cells in response to 7.5mmol/l glucose revealed a reproducible biphasic profile, Figure 3.13. The total area under the mean glucose stimulated profile was 620 $\pm$ 40 uU/hr/plate (n=5) compared with a total area of 345 $\pm$ 37 UU/hr/plate (n=5) obtained in the absence of glucose, $p<0.05$. Maximum rates of release during phase I (12.4 $\pm$ 1.8 uU/min/plate, n=5) and phase II (11.7 $\pm$ 1.7 uU/min/plate, n=5) occurred some 10 and 41 minutes respectively after increasing the perifusate glucose concentration to 7.5mmol/l.

Glibenclamide at 8umol/l specifically and significantly stimulated insulin release from HIT-T15 cells in the absence of glucose, Figure 3.14. The maximum mean rate of insulin release (12.8 $\pm$ 1.5 uU/min/plate, n=5) occurred 20minutes after adding the sulphonylurea. The total area under the mean glibenclamide stimulated phase I profile was 582 $\pm$ 30 uU/hr/plate (n=5) compared with a total area of 345 $\pm$ 37 uU/hr/plate (n=5) obtained in the absence of glucose, $p<0.05$.

Figure 3.13 The effect of glucose (7.5mmol/l) upon insulin release from perifused HIT-T15 cells. Values are means ± SEM with n in parentheses.

* p<0.05 significantly increased compared with basal (absence of glucose), when analysing the areas under the secretory profiles.

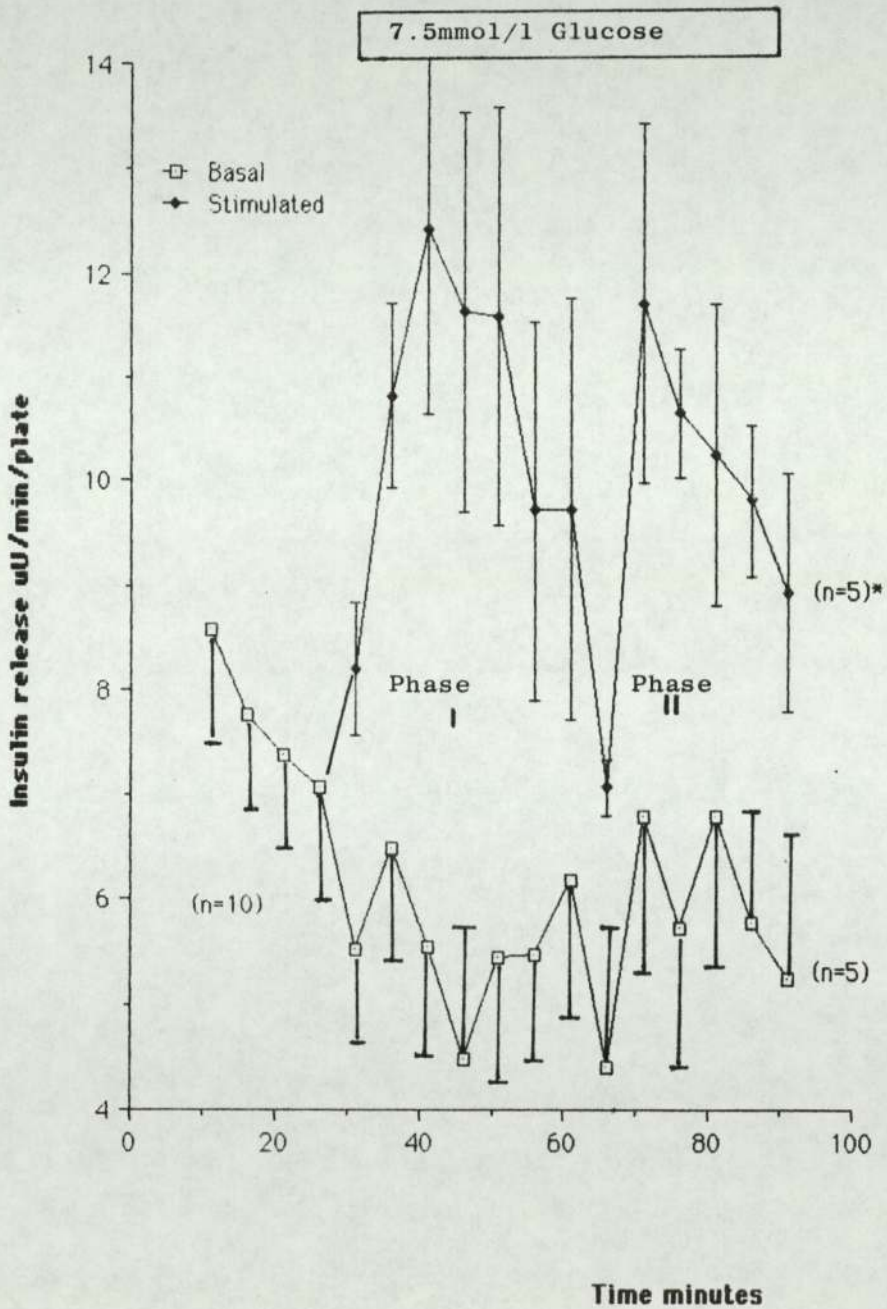
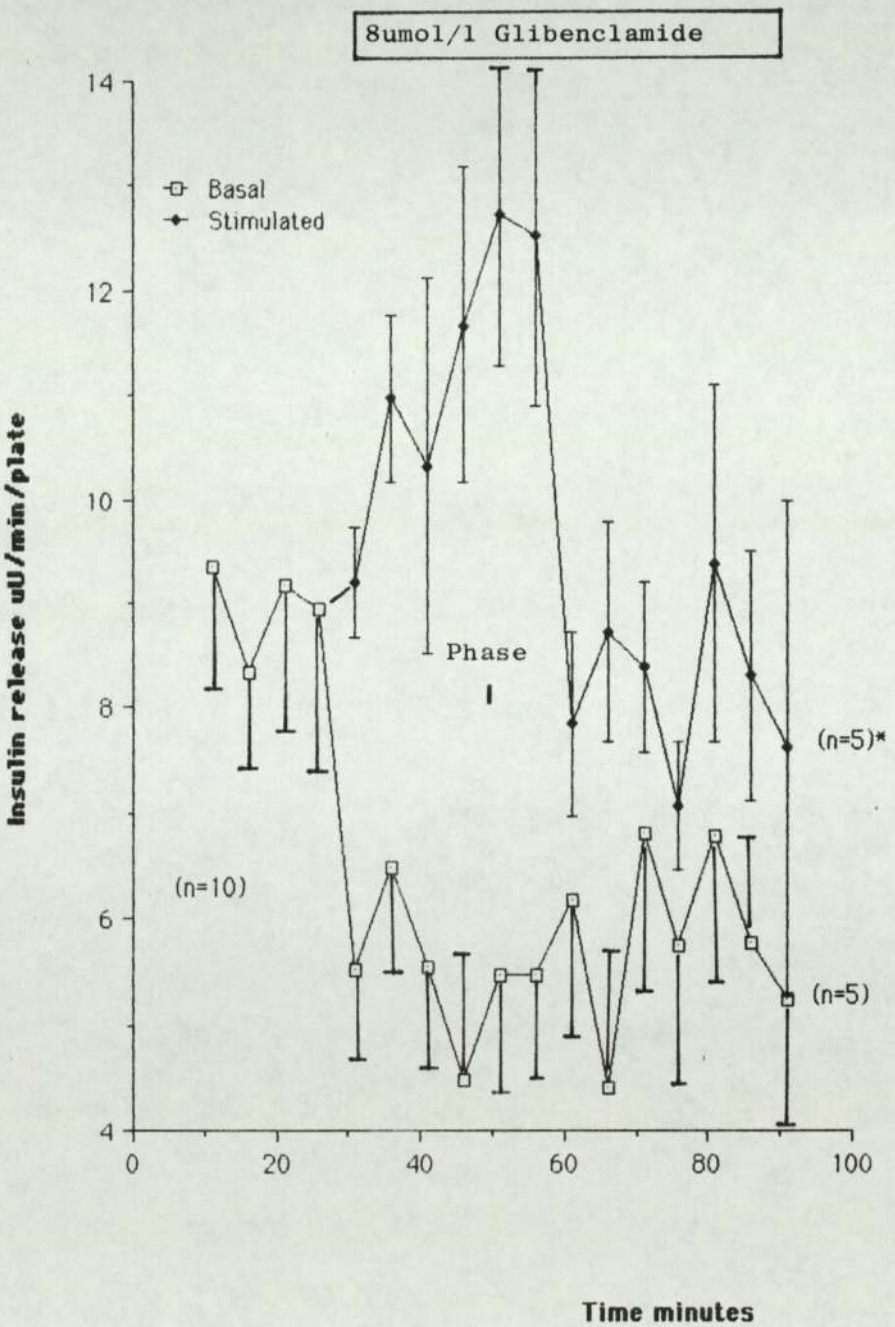


Figure 3.14 The effect of glibenclamide (8umol/l) upon insulin release from perifused HIT-T15 cells. Values are means ± SEM with n in parentheses.

* p<0.05 significantly increased compared with basal (absence of glucose or glibenclamide), when analysing the areas under the secretory profiles.



Pretreatment of HIT-T15 cells with 1mmol/l colchicine for 30 minutes, and subsequent exposure during perifusion eliminated the phase II insulin response to 7.5mmol/l glucose but did not significantly affect phase I insulin release, which still showed a healthy maximum mean rate of insulin release of 14.7 ± 2.1 uU/min/plate (n=6), Figure 3.15. The total area associated with the mean glucose stimulated phase I profile remaining after colchicine inhibition of phase II was 567 ± 44 uU/hr/plate (n=6) compared with a total area of 270 ± 87 uU/hr/plate (n=5) obtained for colchicine treated cells in the absence of glucose, $p<0.05$. Colchicine treatment did not significantly affect basal insulin release from HIT-T15 cells perifused in the absence of glucose, $p<0.05$.

Comparison of Insulin and C-peptide release from statically incubated HIT-T15 cells in response to glucose, arginine and chlorpropamide.

Glucose (7.5mmol/l), L-arginine (15mmol/l), and chlorpropamide, 8umol/l all stimulated the release of insulin and C-peptide from statically incubated HIT-T15 cells, Table 3.12. Values for C-peptide release were considerably lower than those for insulin. Raising the glucose concentration from 0 to 7.5mmol/l caused a 1.25 fold increase in insulin release but only a 0.77 fold increase in the release of C-peptide. This difference might have been due to a poor cross reactivity between hamster and human C-peptides in the radioimmunoassay.

Figure 3.15 The effect of colchicine (1mmol/l) upon glucose (7.5mmol/l) stimulated insulin release from perifused HIT-T15 cells. Values are means ± SEM with n in parentheses.

* $p<0.05$ significantly increased compared with basal (absence of glucose), when analysing the areas under the secretory profiles.

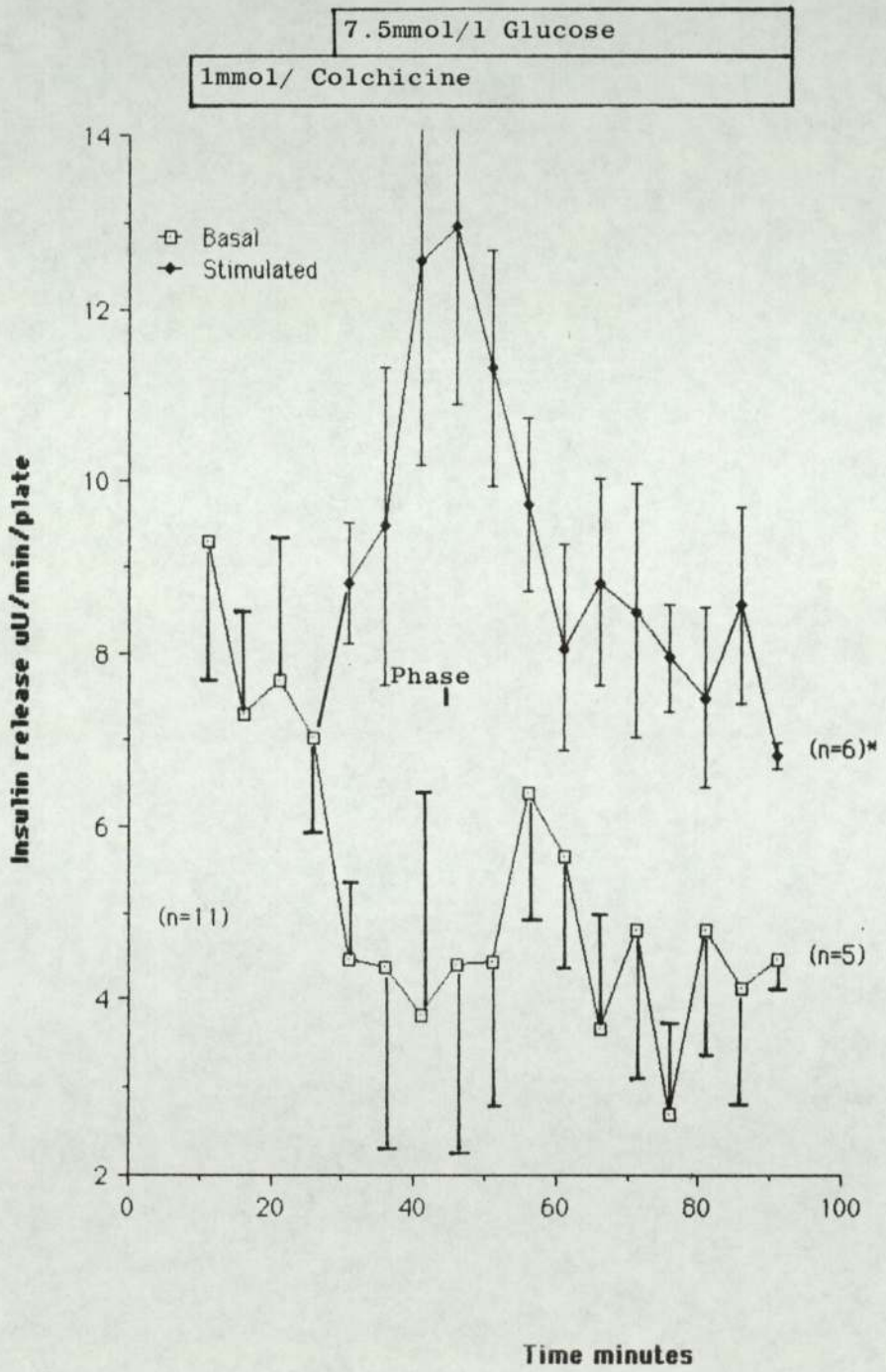


Table 3.12 The effect of glucose, L-arginine and chlorpropamide on the release of C-peptide and insulin from statically incubated HIT-T15 cells. (n is in parentheses).

Treatment	C-peptide release pmol/10^5cells/90min	Insulin release pmol/10^5cells/90min
Basal	0.022 ± 0.002 (5)	0.368 ± 0.035 (13)
Glucose, 7.5mmol/l	0.039 ± 0.004 (9)[a]	0.828 ± 0.127 (14)[a]
L-arginine, 15mmol/l	0.043 ± 0.002 (4)[a]	0.702 ± 0.103 (14)[a]
Chlorpropamide, 8umol/l	0.033 ± 0.001 (4)[a]	0.634 ± 0.150 (8)[a]

a, $p<0.05$ significantly increased compared with basal absence of glucose.

DISCUSSIONS.

The present studies confirm the modest glucose sensitivity of cultured HIT-T15 cells (62,167), maximum release occurring in response to 7.5mmol/l glucose. The stimulation of insulin release compares rather poorly with the maximum stimulation of insulin release from mouse islets of Langerhans which occurs at glucose concentrations in the region of 16.7-22.2mmol/l (98). Certainly glucose releases 30fold more insulin from intact rat islets than from single B-cells (168) and hamster islets release 46fold more insulin than HIT cell monolayers in response to 7.5mmol/l glucose (62).

Stimulation of the rate of HIT-T15 cell glucose oxidation by glucose has been reported by Ashcroft et.al. (167) Although the absolute rates of glucose oxidation were considerably higher in the present study. This increase may reflect differences in culture conditions and the passage number of cells used. A recent study by Meglasson et.al. (169) reported that glucose transport in HIT-T15 cells was slow and that intracellular and extracellular glucose concentrations are not equalised, even after 30 minutes. These authors suggested that the glucose transport step in HIT-T15 cells is a nonequilibrium reaction and contributes to the regulation of glucose metabolism in this cell line. It may be that in the presence of 7.5mmol/l glucose, the maximum stimulatory concentration for insulin release, that the glucose transporter is saturated. This possibility could also be used to account for the modest glucose sensitivity of HIT-T15.

The amount of insulin released by HIT-T15 cells in culture declined steadily with time in culture, this phenomenon, the passage effect, has previously been described by Santerre et.al. (62) and

Ashcroft et.al. (167) and is believed to be the result of a progressive reduction in the insulin secretory pool and total insulin content. Although the amount of insulin released did vary considerably with time in culture, in the present study glucose consistently produced at least a doubling in the basal insulin secretory rate.

The RINm5F cell line proved unreliable in its response to glucose. This is consistent with the previous observations of Praz et.al. (58) who described the glucose insensitivity of RINm5F cells. Groix et.al. has described a slight but significant secretory response to 2.8mmol/l glucose (59). The lack of a reproducible and physiologically meaningful insulin response to glucose may reflect abnormal glucose metabolism in these cells (170) or possibly the lack of specific membrane bound glucoreceptors (100). The glucose transport mechanism in RINm5F cells appears to be intact (169) and glucose has been shown to increase the cytosolic NADH/NAD^+ ratio, the rate of oxygen uptake and the ATP/ADP ratio (171). The vast excess of hexokinase 2 activity over glucokinase activity in RINm5F cells makes it highly unlikely that glucokinase could act as the rate limiting enzyme in these cells. Even assuming profound inhibition of hexokinase by glucose-6-phosphate, the contribution of glucokinase to the overall rate of glucose phosphorylation would remain small which is in contrast to the situation in islets. Under these circumstances, no sizeable change in the rate of glycolysis, resulting from a glucose concentration effect on the velocity of the glucokinase reaction, should be expected. Thus, genetic overexpression of hexokinase 2 may explain the unreliability of an insulin response to glucose in RINm5F cells (172).

The stimulation of insulin release from RINm5F cells by glyceraldehyde is consistent with that previously reported (58) and compares favourably with data obtained using isolated islets (173). In addition Ashcroft et.al. has shown that 10mmol/l glyceraldehyde stimulates insulin release from HIT-T15 cells (167). In the present work no such stimulation of insulin release could be demonstrated. This discrepancy is difficult to explain since the cells were of the same origin. Different culture regimens seem the most likely explanation, i.e, Ashcroft et.al. (167) use RPMI 1640, in the present study HIT-T15 cells were cultured in Ham's F12 medium.

Cultured HIT-T15 cells did release insulin in response to conventional concentrations of the amino acids L-leucine and L-arginine in the absence of glucose. The stimulation of HIT-T15 cell insulin release by 20mmol/l L-leucine has also been reported by Ashcroft et.al. (167), who in addition indicated that 2-ketoisocaproate, the transamination product of leucine, also stimulated insulin release, while L-glutamine and L-isoleucine were without effect.

RINm5F cells also release insulin in response to L-leucine and L-arginine. This finding is consistent with the work of Praz et.al. (58) who used 20mmol/l L-leucine and L-arginine, although their study involved the use of media containing glucose at 2.8mmol/l. RINm5F cells have also been shown to release insulin in response to both L-leucine methylester (174) and BCH (171). In contrast to glucose, BCH also reduced the rate of oxygen consumption and lowered the ATP/ADP ratio. These data suggest that L-leucine metabolism is not an essential requirement of the secretory response to L-leucine in RINm5F cells.

The present studies with HIT-T15 and RINm5F cells have indicated that insulin release in response to amino acids does not require the presence of glucagon, normally secreted from adjacent A-cells in the islets. It should be noted however that RINm5F cells have been shown to contain minute amounts of glucagon (58).

Glucose induced insulin release from HIT-T15 cells was potentiated by L-leucine and L-arginine alone or in combination. This observation is consistent with the provision in vivo of enhanced insulin release in response to a mixed meal. The insulinotrophic effect of L-leucine and L-arginine in combination was synergistic rather than additive. Similar synergism between L-leucine and L-arginine upon insulin release from isolated islets has been reported by Khalid and Rahman (175).

Glibenclamide and chlorpropamide stimulated insulin release from HIT-T15 and RINm5F cells, tolbutamide also stimulated insulin release fron HIT-T15 cells in the absence of glucose. These observations are consistent with the effects of these sulphonylureas on mouse islets (153) and the effects of tolbutamide and glibenclamide upon HIT-T15 cells (167). The posibility that tolbutamide might stimulate insulin release by interacting with glucose metabolism in the B-cell prompted the examination of the effect of this sulphonylurea upon the rate of ^{14}C glucose oxidation by HIT-T15 cells. In the past tolbutamide has been shown to stimulate (176), inhibit (177) or have no effect (177) on the rate of glucose oxidation by pancreatic islets. The present studies suggest that tolbutamide induced insulin release from HIT-T15 cells may result from the release of a readily available insulin secretory granule pool, possibly associated with the plasma membrane. The initiation of insulin release by tolbutamide does not require glucose metabolism

It is now well established that the release of insulin from the B-cell is biphasic in nature (111,178). In a recent study, the insulin response of perifused HIT-T15 cells was shown to be monophasic whether carried out in the presence of low or high glucose (94). A biphasic response could only be demonstrated using a combined stimulus of high glucose and 3-isobutyl-1-methylxanthine. In the present studies perifused HIT-T15 cells generated a reproducible biphasic insulin release in response to square wave glucose (7.5mmol/l) challenge. Similar observations have been reported by Ashcroft et.al. (167) in response to 10mmol/l glucose. Compared with the usual biphasic insulin release profiles of normal B-cells to glucose, the first phase of insulin release was pronounced and sustained whilst the second phase was reduced and of slower onset. This observation might reflect a difference in the size and distribution of secretory granule pools in HIT-T15 cells compared with normal B-cells (179). The pronounced first phase may be the product of rapid intracellular transport and emiocytosis of readily available insulin secretory granules associated with the microtubular-microfilamentous system of the HIT-T15 cells. The reduced second phase may well reflect either a decrease in the subsequent association of stored and newly formed B-granules with this system or a decrease in insulin biosynthesis per se in HIT-T15 cells. Certainly, ultrastructural studies have demonstrated modest numbers of secretory granules in HIT-T15 cells (62) which in appearance were characteristic of mature beta granules of normal hamster B-cells (180).The monophasic phase I stimulation of insulin release from HIT-T15 cells in response to glibenclamide, demonstrated in the present study is consistent with the presence of a substantial readily available insulin secretory granule pool which is not rapidly restored by subsequent insulin biosynthesis and granule margination.

Colchicine, an agent which inhibits microtubule polymerisation (181), did not affect the first phase of glucose induced insulin release from HIT-T15 cells, but did produce a significant inhibition of the second phase. The absence of an effect of colchicine on the first phase may be releated to the length of pretreatment and the concentration of colchicine used which were limited to 30 minutes and 1mmol/l respectively to prevent cell damage (182) and possible effects on cell metabolism (181). The inhibition of phase II of glucose induced insulin release by colchicine suggests a role for the microtubular system in the phasic mechanism of insulin release from HIT-T15 cells.

The results of the initial characterisation of the insulin secretory responses of cultured HIT-T15 and RINm5F cells have been summarised in Table 3.13

Table 3.13. Summary of the initial characterisation of the insulin secretory responses of cultured HIT-T15 and RINm5F cells.

INSULIN RELEASE IN STATIC CULTURE		
Secretagogue	Effect on insulin release from	
	HIT-T15	RINm5F
D-Glucose	+	+/-
D-Glyceraldehyde	-	+
L-Leucine	+	+
L-Arginine	+	+
Glibenclamide	+	+
Chlorpropamide	+	+
Tolbutamide	+	

INSULIN RELEASE FROM PERIFUSED HIT-T15 CELLS		
Secretagogue	Effect on phase	
	I	II
D-Glucose	+	+
Glibenclamide	+	
Colchicine		-

+, Stimulation, -, Inhibition.

CHAPTER 4.

4. PARACRINE MODIFICATION OF INSULIN RELEASE FROM CULTURED HIT-T15 AND RINm5F CELLS.

INTRODUCTION.

The close anatomic relationships between islet A,B,D and PP cells has led to the suposition that these islet cells might influence one another in a paracrine fashion (183). Paracrine regulation of insulin release can be simply defined as the direct effect of an agent(s) secreted by an islet cell into the islet interstitium without entry into the vascular space. Pipeleers put forward a possible paracrine model for islet cell interactions, in which he suggested that islet cells interact either by direct contact or via their released products (184).

The effect of insulin on the release of glucagon, somatostatin and pancreatic polypeptide.

The fact that insulin can suppress glucagon secretion *in vitro* and *in vivo* has been well documented (185,186). Suppression of circulating glucagon levels in man by 20-30% is readily achieved by raising the circulating insulin levels by some 30-200uU/ml during normoglycaemia (187,188). Studies with mammalian pancreas have failed to show any significant reproducible effect of exogenous insulin on pancreatic somatostatin secretion (189,190). In spite of this, specific insulin receptors have been identified on islet cells maintained in monolayer culture. These studies showed 50%, 30%, and 15% specific ^{125}I-insulin binding to B, A, and D cells respectively (191). These data also suggest an autocrine regulatory function for insulin upon insulin secretion (188).Interestingly C-peptide, the cleavage product of proinsulin has also been shown to inhibit insulin release (192). Insulin does not appear to influence the release of pancreatic polypeptide (189).

The effect of glucagon on the release of insulin, somatostatin and pancreatic polypeptide.

In contrast to the suppression of glucagon release by insulin, exogenous glucagon has been shown to stimulate insulin release *in vivo* (193). This stimulatory effect has been elegantly demonstrated *in vitro* by Trimble and Renold (194) who compared glucose induced insulin release from glucagon poor and glucagon rich islets of Langerhans form different regions of the same pancreas (194). Islets from the ventral primordium derived part of the pancreas contain more pancreatic polypeptide secreting cells and proportionately less glucagon secreting A-cells than islets from the dorsal primordium derived pancreas (195). The observation that glucose stimulated up to 50% more insulin release from dorsally situated islets than from ventrally situated islets, strongly suggests the possibility that glucagon exerts paracrine effects in islets and that endogenous glucagon amplifies glucose induced insulin release from islets (194). Exogenous glucagon has also been shown to stimulate somatostatin release from D-cells (196,197). Raised glucose concentrations potentiate glucagon induced stimulation of insulin release, as mentioned earlier, but do not potentiate the stimulation of somatostatin release. Glucose alone does, however, stimulate the release of somatostatin (198), this response being of the utmost importance in the the suppression of glucagon secretion (199). Glucagon has no effect on pancreatic polypeptide secretion (189).

The effect of somatostatin on the release of insulin, glucagon and pancreatic polypeptide.

Somatostatin rapidly inhibits the release of insulin (200), glucagon (201), and pancreatic polypeptide (202). Somatostatin appears to be equipotent as an inhibitor of glucagon and insulin release with weaker effects on the release of pancreatic polypeptide (202). Although there is evidence that the pancreas contains several molecular species of somatostatin (203) the major proportion of pancreatic islet somatostatin in the D-cell appears to be identical to hypothalamic somatostatin, SS14, containing 14 amino acids. The presence of a natural SS28 as a minor component of pancreatic D-cells has been reported by Benoit et. al. (204). SS28 is about 10-100 times more potent than SS14 as an inhibitor of arginine stimulated insulin release from the B-cell but equipotent with SS14 with respect to the arginine stimulated release of glucagon from A-cells (205) i.e., SS28 appears to be a selective inhibitor of insulin release from the B-cell.

The effect of pancreatic polypeptide on the release of insulin, glucagon and somatostatin.

Pancreatic polypeptide at 8ug/100g body weight inhibits insulin release from fed rats by some 46%. Pancreatic polypeptide at a concentration of 12ug/100g body weight also inhibited insulin release by 42% from fasted rats. Pancreatic polypeptide had no effect upon the release of glucagon (206). In the isolated perfused rat pancreas pancreatic polypeptide has also been shown to inhibit the release of somatostatin by between 40 and 60% (207).

The effect of gastric inhibitory polypeptide (GIP) on the release of insulin, glucagon, somatostatin and pancreatic polypeptide.

The major physiological role ascribed to GIP appears to be the potentiation of insulin release in hyperglycaemic situations (208,209). It has long been established that glucose taken orally causes a more marked elevation in serum insulin levels than glucose given intravenously (210). The term 'incretin' was first coined by La Barre in 1932 to designate the presumed agent(s) responsible for the stimulation of insulin release via the entero-insular axis (211).The glucose dependence of GIP stimulation of insulin release taken in concert with the observation that glucose stimulates GIP release (212) constitutes the main experimental evidence for the proposal that GIP is the 'incretin' described by La Barre (213,214). The major source of GIP appears to be localised in cells of the duodenum and jejunum (215). However, Smith et.al. have reported GIP immunoreactivity in A-cells of the islets of Langerhans (216). The effect of GIP on glucagon release from A-cells is far from clear. Conflicting reports have been presented to suggest both a stimulation of glucagon secretion (217) and no effect on secretion (208). There is no information on the effect of GIP on pancreatic polypeptide secretion.

Summary.

Aside from the anatomical juxtaposition of islet cells to one another suggestive of interaction between these cells information to date suggests that the hormones released by A, B, D and PP cells of the islet influence each others secretion in a specific and coordinated fashion, Table 4.1.

Table 4.1 Summary of the effects of insulin, glucagon, somatostatin, pancreatic polypeptide (PP) and GIP upon the secretory activity of islet cells.[modified from Montague (218)]

Hormone	Secretory activity			
	Insulin release	Glucagon release	Somatostatin release	PP release
Insulin	-(?)	-	NE	ND
Glucagon	+	ND	+	NE
Somatostatin	-	-	ND	-
PP	-	NE	-	ND
GIP	+	+(?)	ND	ND

Key: -, inhibits, +, stimulates, (?), speculative.
NE, no effect, ND, no data.

This section examines the effect of glucagon, somatostatin, pancreatic polypeptide and GIP upon insulin release from cultured HIT-T15 and RINm5F cells. Cultured cloned B-cells are particularly suited to the dissection of any possible potential paracrine control of insulin release presumed to occur in the islets of Langerhans, since exogenous hormone addition would act directly on the B-cell bypassing any indirect or combined effect on adjacent islet cells.

Materials and methods

The islet hormones, their sources and concentration ranges used in the present work are illustrated in Table 4.2.

Table 4.2 Sources of islet hormones and experimental concentration ranges used.

Islet hormone	Source	Concentration used
Glucagon	Bovine/Porcine mixture	0.01-2.5umol/l.
Somatostatin	Synthetic, human	10^{-10}-10^{-7}mol/l.
PP	Bovine	10-1000pg/ml.
GIP	Synthetic, porcine	1-100pg/ml.

Glucagon was tested upon basal insulin release from HIT-T15 and RINm5F cells. Stock solutions of glucagon contained 250KIU/ml trasylol. The effect of somatostatin and pancreatic polypeptide was evaluated upon 7.5 mmol/l glucose induced insulin release from HIT-T15 cells and because of the relative glucose insensitivity of RINm5F cells, upon 10mmol/l glyceraldehyde induced insulin release from these cells. The effect of GIP in the absence and presence of glucose was investigated on the release of insulin from HIT-T15 cells.

RESULTS

The effect of glucagon on insulin release from statically incubated HIT-T15 and RINm5F cells.

Glucagon stimulated insulin release from statically incubated HIT-T15, Figure 4.1 and RINm5F cells, Figure 4.2. Maximum stimulation occurred at 1umol/l glucagon for HIT-T15 cells and 2.5umol/l glucagon for RINm5F cells. Glucagon at a concentration of 1umol/l generated an insulin response from HIT-T15 cells equivalent to that generated by 7.5mmol/l glucose. Glucagon at concentrations of 1 and 2.5umol/l generated an insulin response from RINm5F cells equivalent to that generated by 10mmol/l glyceraldehyde. Values for the half maximum stimulation of insulin release from both cell lines were comparable, i.e., 0.28umol/l glucagon for HIT-T15 cells and 0.32umol/l glucagon for RINm5F cells. 1umol/l glucagon also potentiated glucose induced insulin release from HIT-T15 cells, Figure 4.3.

The effect of somatostatin on insulin release from statically incubated HIT-T15 and RINm5F cells.

Somatostatin inhibited 7.5mmol/l glucose induced insulin release from HIT-T15 cells, Figure 4.4 and 10mmol/l glyceraldehyde induced insulin release fron RINm5F cells, Figure, 4.5, maximum inhibition occurred at a somatostatin concentration of 10^{-7}mol/l. However, maximum statistically significant inhibition of insulin release occurred at somatostatin concentrations in excess of 10^{-10}mol/l for HIT-T15 and 10^{-8}mol/l for RINm5F cells. Somatostatin at concentrations of 10^{-10}, 10^{-8} and 10^{-7}mol/l reduced glucose stimulated insulin release from HIT-T15 cells to below basal values.

Figure 4.1. The effect of glucagon on insulin release from statically incubated HIT-T15 cells. Values are means ± SEM with n in parentheses

*, p<0.05 significantly increased compared with basal.

(basal secretion was 40.37 ± 2.11 uU/10^5cells/90minutes, n=14)

$, p>0.05 not significantly different from 1umol/l glucagon.

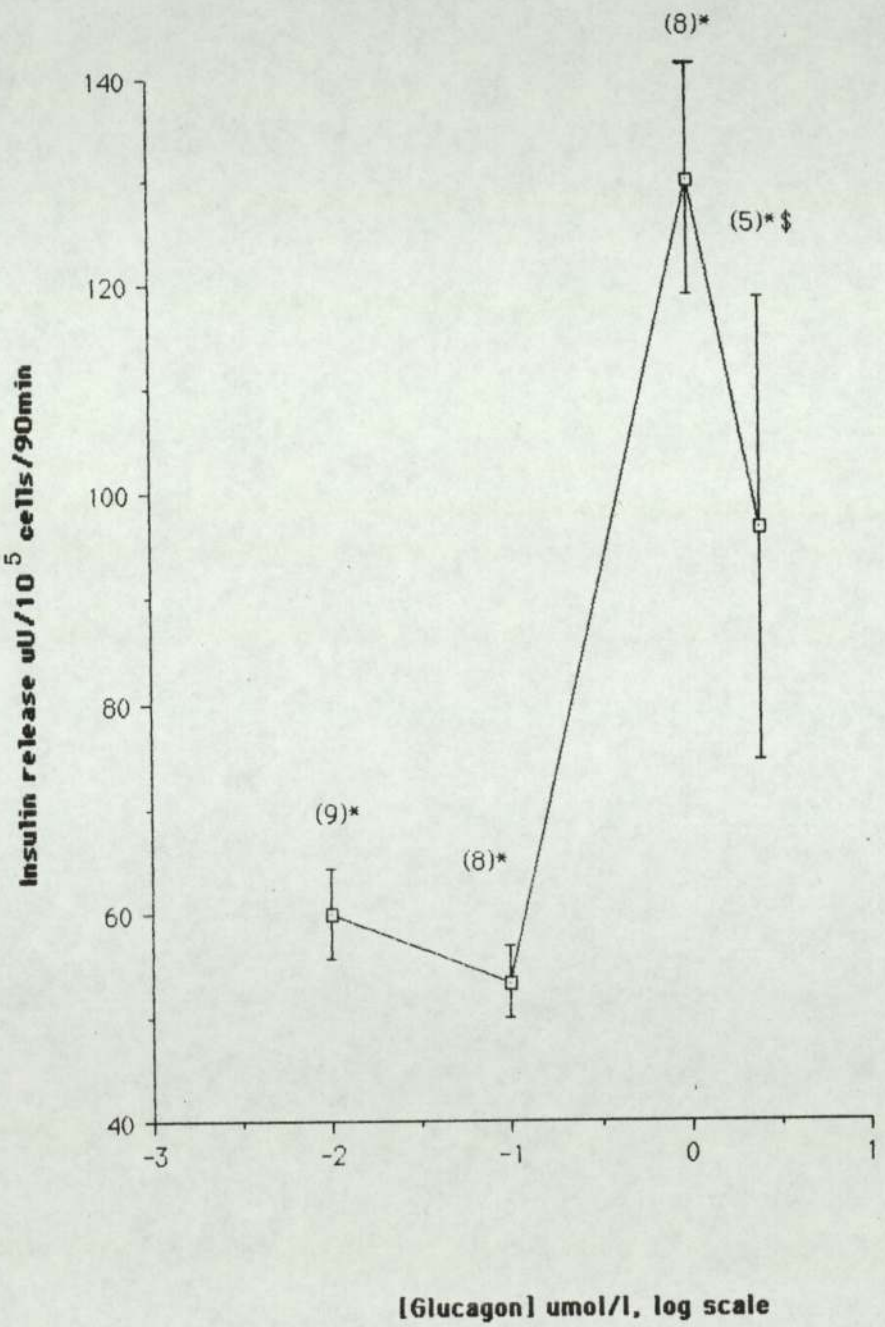


Figure 4.2. The effect of glucagon on insulin release from statically incubated RINm5F cells. Values are means ± SEM with n in parentheses.

*, $p<0.05$ significantly increased compared with basal.

(Basal secretion was 12.56 ± 0.53 uU/10^5cells/90minutes.)

$, $p>0.05$ not significantly different from 1umol/l glucagon.

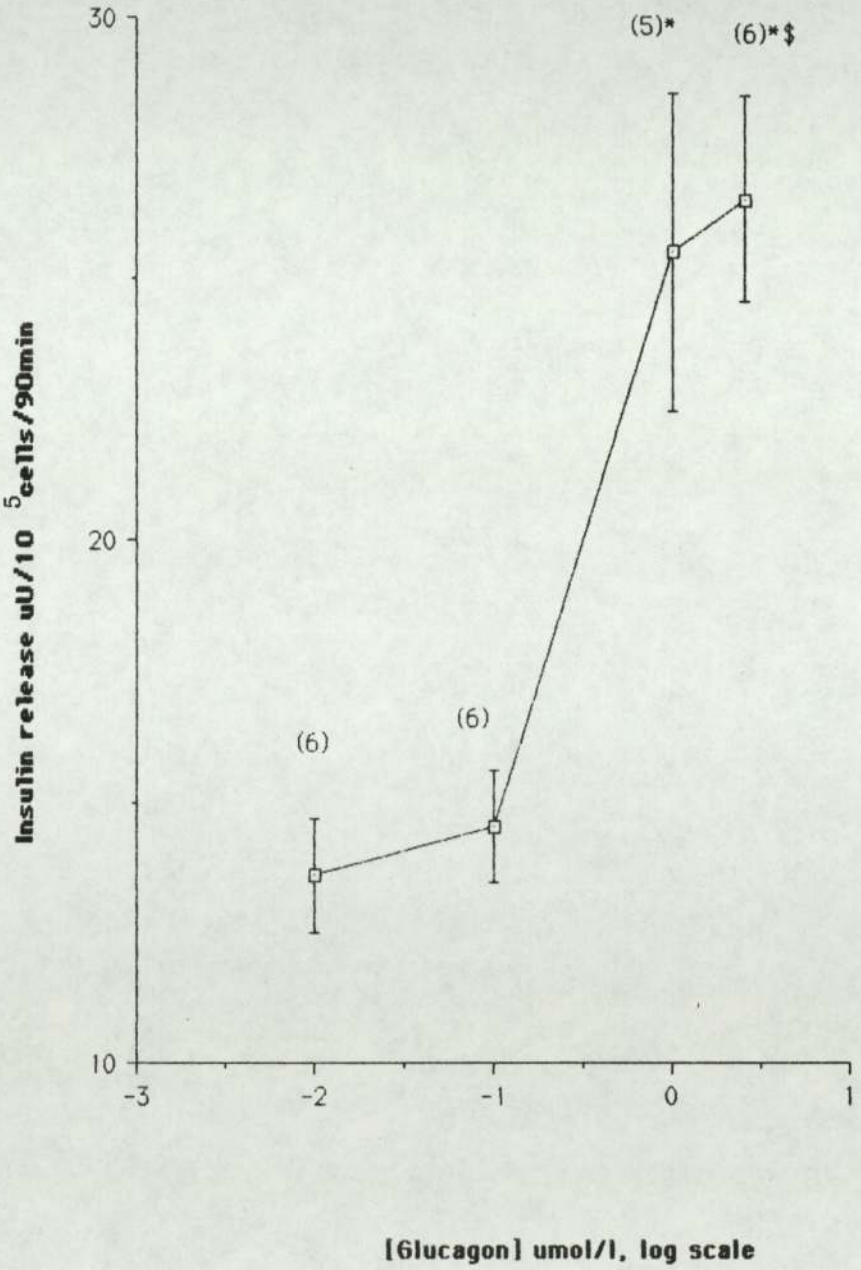


Figure 4.3. Potentiation of glucose (7.5mmol/l) induced insulin release from statically incubated HIT-T15 cells by glucagon (1umol/l). Values are means ± SEM with n in parentheses.

*, p<0.05 significantly increased compared with 7.5mmol/l glucose.

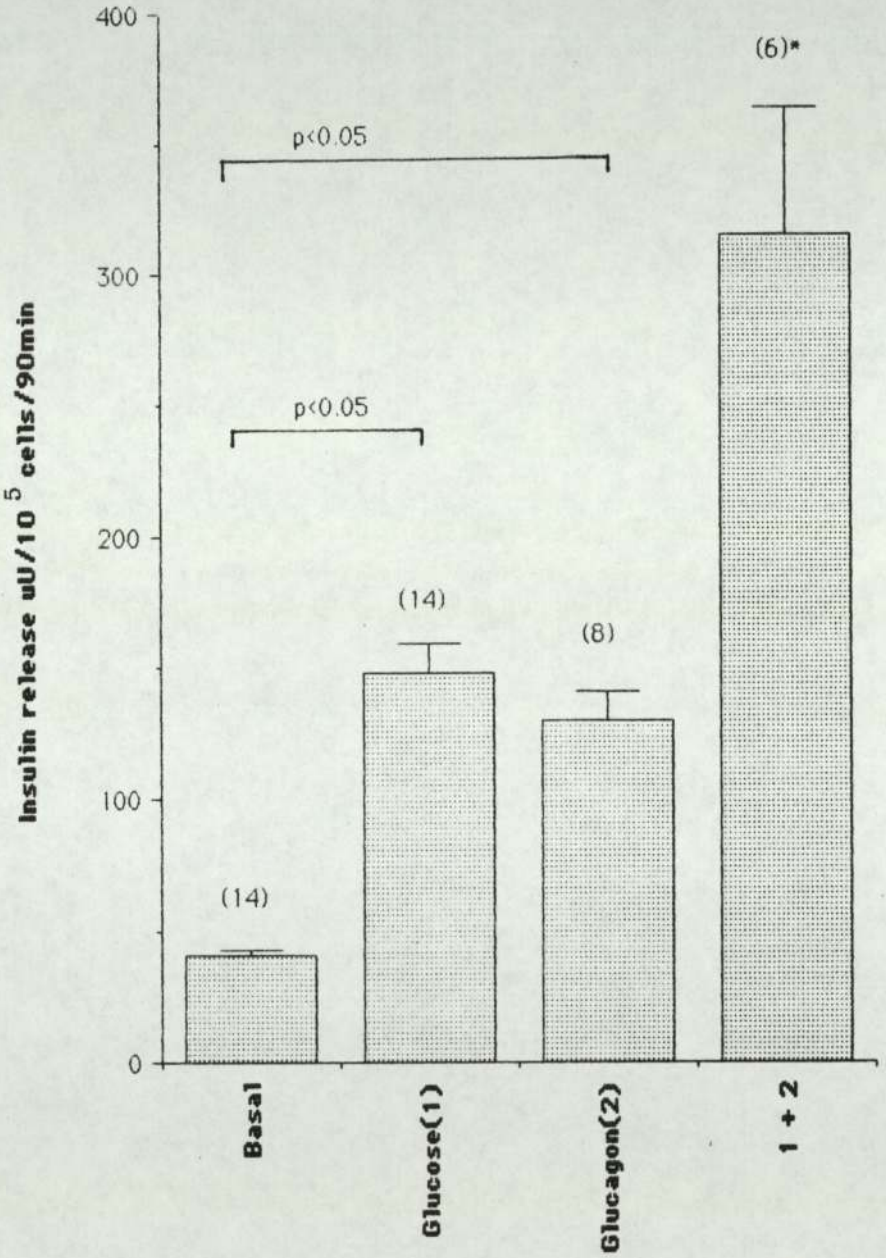


Figure 4.4. The effect of somatostatin on glucose (7.5mmol/l) induced insulin release from statically incubated HIT-T15 cells.

Values are mean ± SEM with n in parentheses.

* p<0.05 significantly reduced compared with 7.5mmol/l glucose.

$ p<0.05 significantly reduced compared with basal.

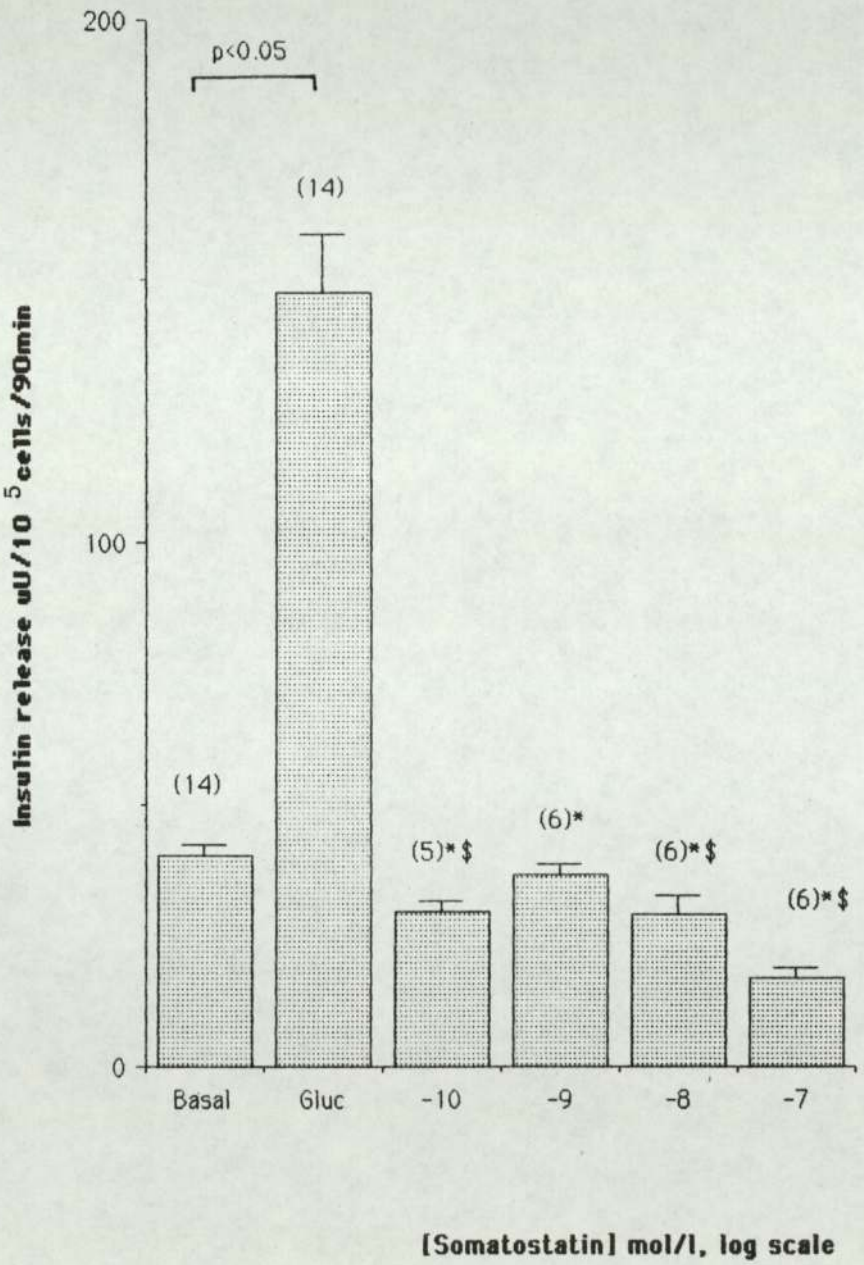
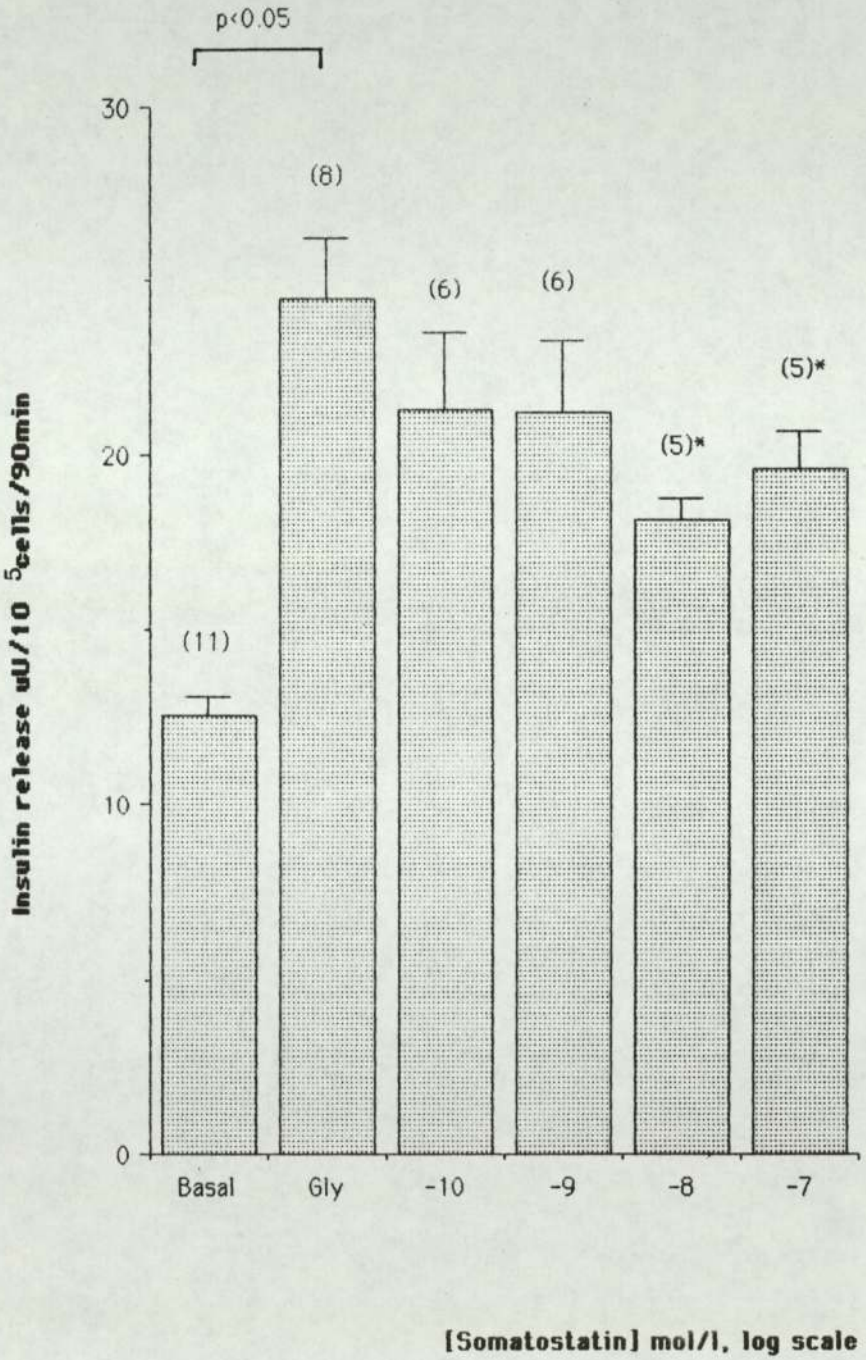


Figure 4.5. The effect of somatostatin on glyceraldehyde (10mmol/l) induced insulin release from statically incubated RINm5F cells.
Values are mean ± SEM with n in parentheses.
*, $p<0.05$ significantly reduced compared with 10mmol/l glyceraldehyde.



The effect of pancreatic polypeptide on insulin release from statically incubated HIT-T15 and RINm5F cells.

Pancreatic polypeptide inhibited 7.5mmol/l glucose induced insulin release from HIT-T15 cells, Figure 4.6 and 10mmol/l glyceraldehyde induced insulin release from RINm5F cells, Figure 4.7. Significant inhibition of 7.5mmol/l glucose stimulated insulin release from HIT-T15 cells was achieved with all pancreatic polypeptide concentrations in excess of 10pg/ml. Only 1000pg/ml pancreatic polypeptide was able to significantly inhibit 10mmol/l glyceraldehyde stimulated insulin release from RINm5F cells.

The effect of somatostatin and pancreatic polypeptide in combination on insulin release from statically incubated HIT-T15 and RINm5F cells.

The combination of 10^{-7}mol/l somatostatin and 1000pg/ml pancreatic polypeptide significantly inhibited 7.5mmol/l glucose induced insulin release from HIT-T15 cells to below basal values (70% reduction in basal secretion). The combination of somatostatin and pancreatic polypeptide also inhibited 10mmol/l glyceraldehyde induced insulin release from RINm5F cells the effect being less marked than that observed for HIT-T15 cells, Table 4.3.

Figure 4.6. The effect of pancreatic polypeptide on glucose (7.5mmol/l) induced insulin release from statically incubated HIT-T15 cells. Values are mean ± SEM with n in parentheses.

*, $p<0.05$ significantly reduced compared with 7.5mmol/l glucose.

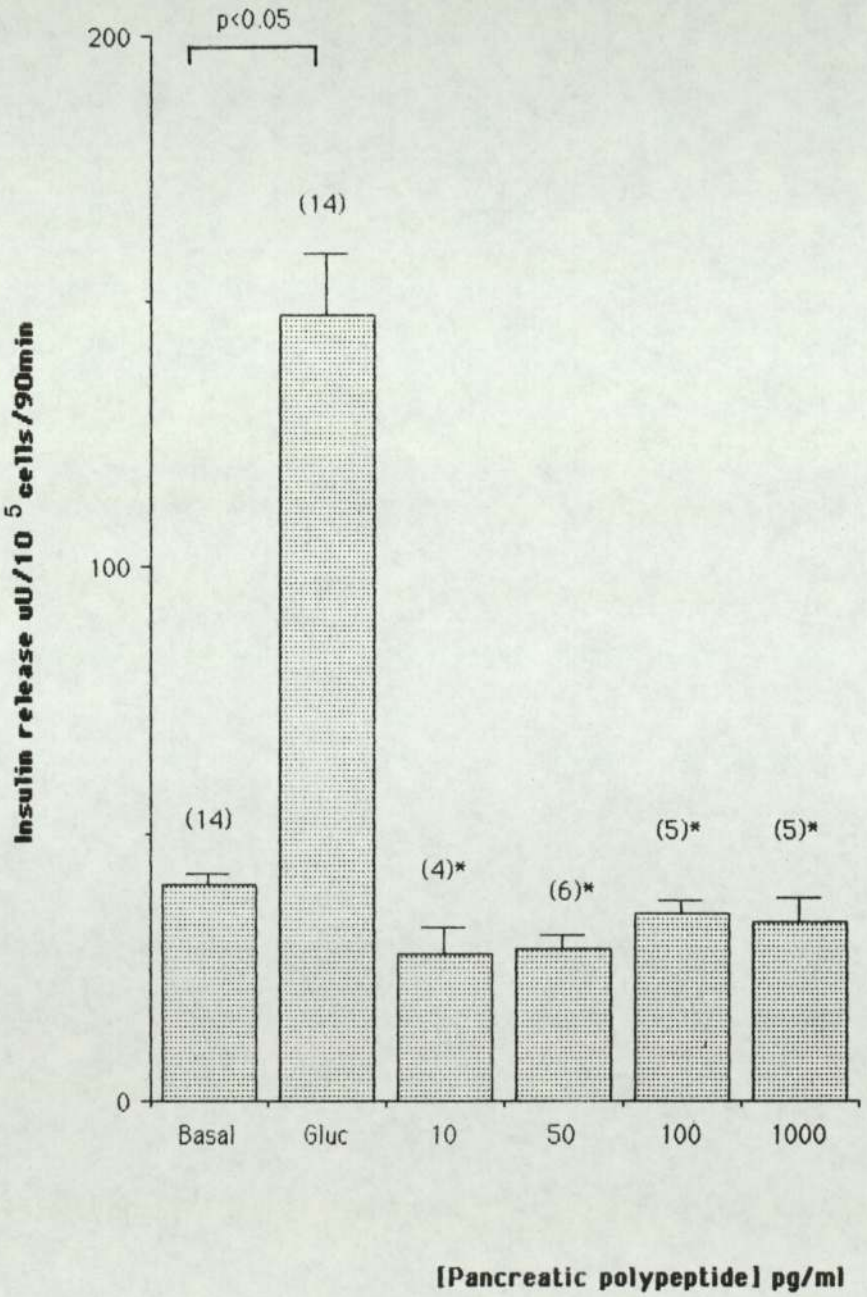


Figure 47. The effect of pancreatic polypeptide on glyceraldehyde (10mmol/l) induced insulin release from statically incubated RINm5F cells. Values are means ± SEM with n in parentheses.
*, $p<0.05$ significantly reduced compared with 10mmol/l glyceraldehyde.

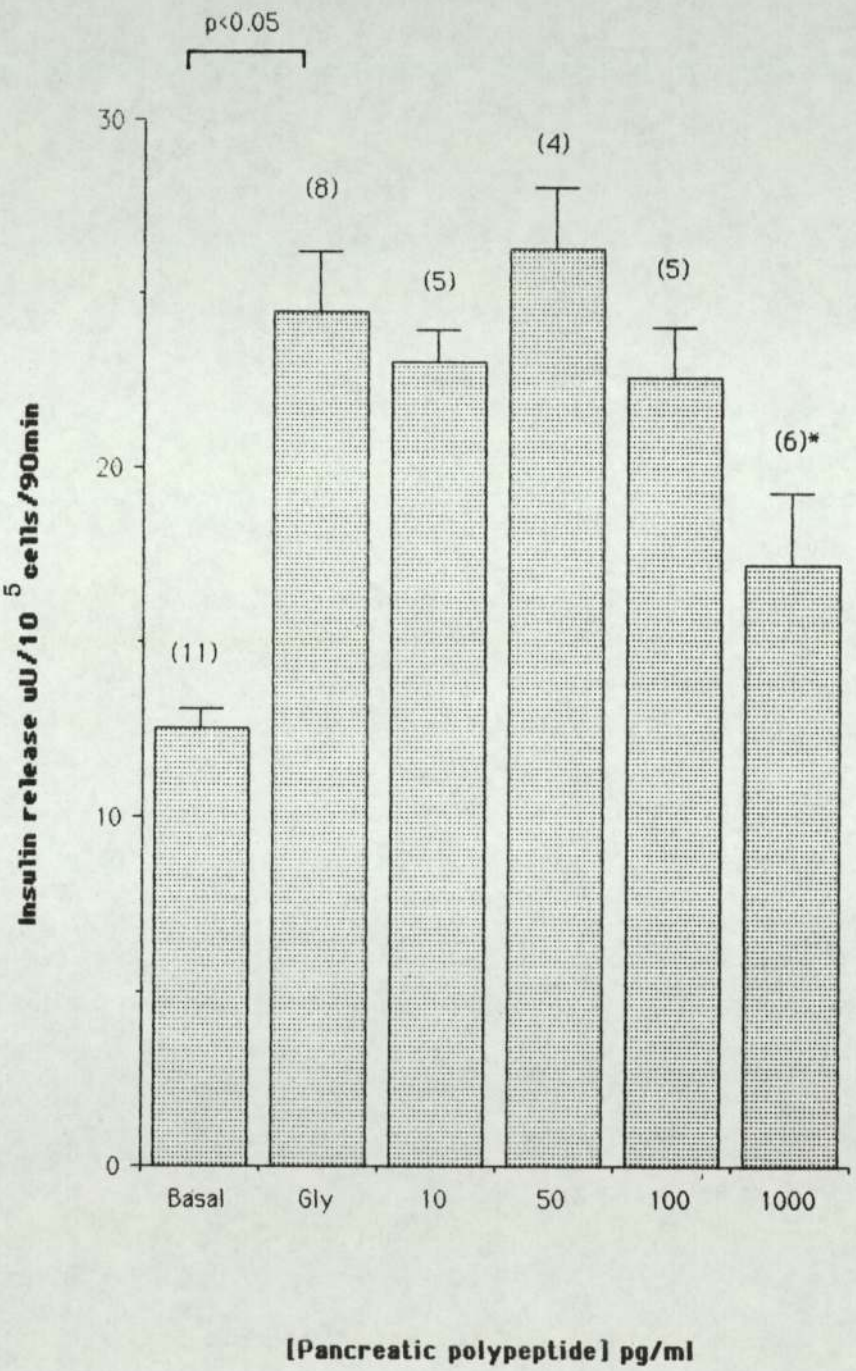


Table 4.3 The effect of somatostatin and pancreatic polypeptide (PP) in combination upon glucose induced insulin release from HIT-T15 cells and glyceraldehyde induced insulin release from RINm5F cells.

Treatment	Insulin release (uU/10^5cells/90minutes)			
	n	HIT-T15	n	RINm5F
Basal	14	40.37 ± 2.11	11	12.56 ± 0.53
Glucose,7.5mmol/l or Glyceraldehyde, 10mmol/l	14	147.52 ± 11.16[a]	8	24.50 ± 1.78[a]
+ Somatostatin 10^{-7}mol/l and PP 1000pg/ml	6	11.91 ± 2.31[b,c]	4	19.09 ± 0.73[d]

a, $p<0.05$ significantly increased compared with basal.

b, $p<0.05$ significantly reduced compared with 7.5mmol/l glucose.

c, $p<0.05$ significantly reduced compared with basal.

d, $p<0.05$ significantly reduced compared with glyceraldehyde, 10mmol/l.

The effect of somatostatin and pancreatic polypeptide alone and in combination on glucagon induced insulin release from statically incubated HIT-T15 and RINm5F cells.

Somatostatin 10^{-7}mol/l and pancreatic polypeptide 1000pg/ml alone or in combination significantly inhibited glucagon induced insulin release from HIT-T15 cells. The effect in combination was additive, i.e., somatostatin and pancreatic polypeptide alone inhibited insulin release by 36 and 35% respectively giving a total

additive inhibition of 71% inhibition. The actual observed effect of this combination was a 69% reduction in glucagon stimulated insulin release. Somatostatin but not pancreatic polypeptide inhibited glucagon induced insulin release from RINm5F cells. The effect of these hormones in combination produced a significantly greater inhibition of glucagon stimulated insulin release than somatostatin alone, Table 4.4.

Table 4.4 The effect of somatostatin and pancreatic polypeptide (PP) alone and in combination upon glucagon induced insulin release from HIT-T15 and RINm5F cells.

Treatment		Insulin release (% from basal)		
	n	HIT-T15	n	RINm5F
Glucagon, 1umol/l	14	194.21 ± 16.16[a]	5	103.02 ± 12.30[a]
+ Somatostatin, 10^{-7}mol/l	5	125.03 ± 27.11[b]	5	66.51 ± 6.72[b]
+ PP, 1000pg/ml	5	128.47 ± 11.99[b]	5	87.70 ± 12.10
+ somatostatin and PP	5	60.97 ± 8.73[b]	5	27.71 ± 1.98[b]

a, $p<0.05$ significantly increased compared with basal (absence of glucose).

b, $p<0.05$ significantly reduced compared with 1umol/l glucagon.

The effect of GIP on insulin release from statically incubated HIT-T15 cells.

GIP stimulated insulin release from statically incubated HIT-T15 cells, figure 4.8, maximum and half maximum stimulation of insulin release occurring at 100 and 9.4ng/ml respectively. A fresh ampoulle of HIT-T15 cells confirmed the stimulation of insulin release in the absence of glucose (GIP, 100ng/ml produced a 75.34 ± 9.32% stimulation from basal, n=4 this value did not differ from that shown in Figure 4.7). The effect of GIP on glucose induced insulin release was additive rather than synergistic. GIP did, however, potentiate 15mmol/l arginine induced insulin release, Table 4.5

Table 4.5 Potentiation of arginine induced insulin release from HIT-T15 cells by GIP.

Treatment	n	Insulin release (%from basal)
Glucose, 7.5mmol/l	6	346.82 ± 24.66[a]
GIP, 100ng/ml	6	121.14 ± 27.41[a]
Glucose + GIP	6	423.04 ± 77.64[a]
Arginine, 15mmol/l	6	77.97 ± 6.00[a]
Arginine + GIP	6	400.18 ± 43.00[a,b]

a, $p<0.05$ significantly increased compared with basal.

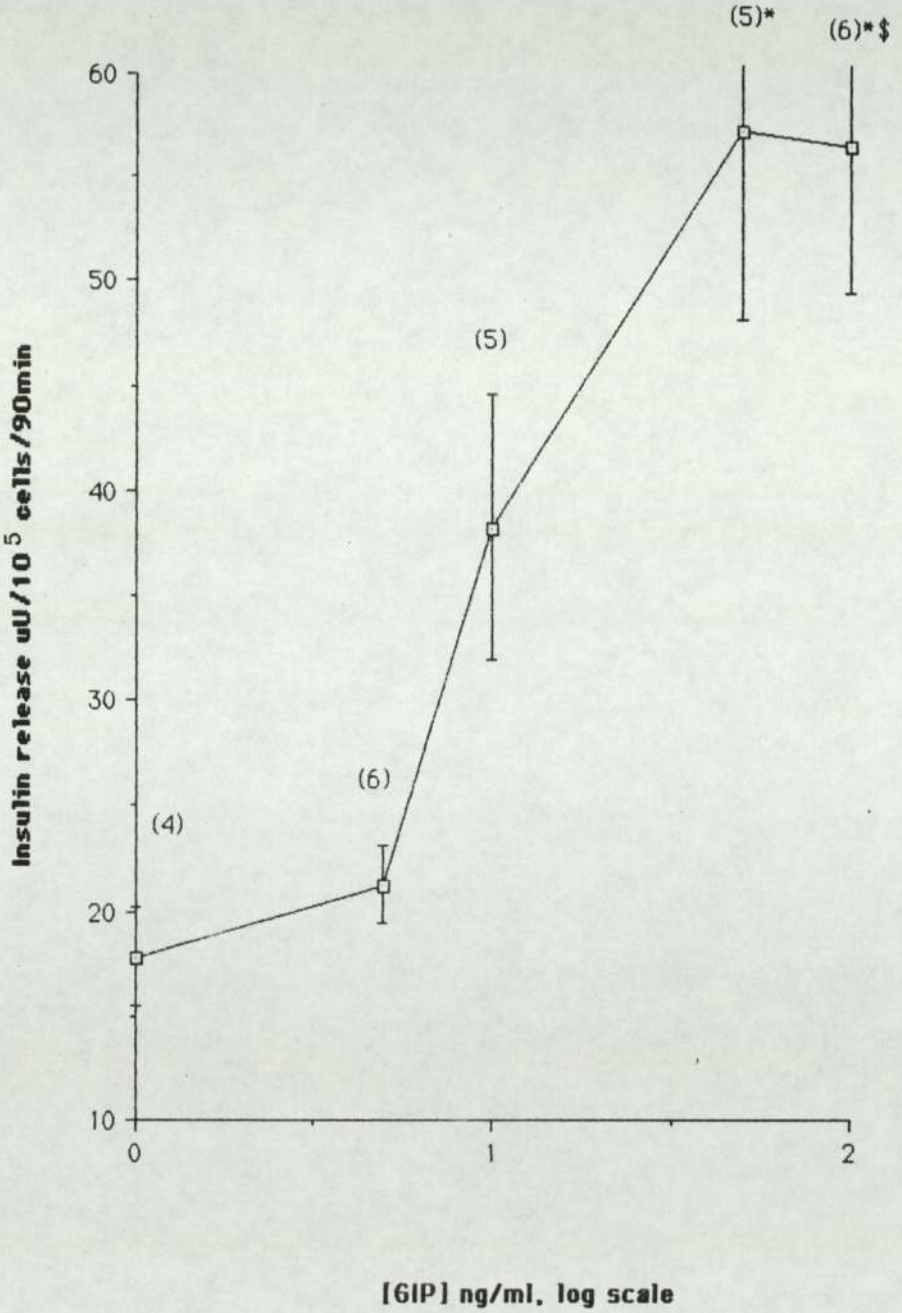
b, $p<0.05$ significantly increased compared with arginine or GIP alone.

Figure 4.8; The effect of GIP on insulin release from statically incubated HIT-T15 cells. Values are means ± SEM with n in parentheses.

*, p<0.05 significantly increased compared with basal.

(Basal secretion was 25.50 + 4.56 uU/10^5cells/90minutes, n=6)

$, p>0.05 not significantly different from 50ng/ml GIP



DISCUSSION

The stimulation of HIT-T15 and RINm5F cell insulin release in the present work by glucagon was consistent with the stimulatory effect of this hormone previously observed with isolated islets (193,194), HIT cells (62,167) and RINm5F cells (219). The concentration of glucagon required for the half maximum stimulation of insulin release from HIT-T15 and RINm5F cells was similar i.e., 0.28umol/l and 0.32umol/l respectively. This value for RINm5F cells compares favourably with the previous value obtained by Korman et.al. of 0.5umol/l also using RINm5F cells. It was also reported by these authors that glucagon stimulated the production of cAMP and that the dose-response relationships for cAMP formation and insulin release were superimposible. The glucagon receptor on RINm5F cells does not appear to be coupled to adenylate cyclase (219). The potentiation of glucose induced insulin release by glucagon observed with HIT-T15 cells is consistent with the situation in vivo (167). A possible paracrine effect for glucagon on insulin release should be considered when working with isolated islets. For example, it has been sugested that certain amino acids might stimulate insulin release via amino acid induced glucagon release. However previous work with HIT-T15 cells (Chapter 3, p 82) has indicated that glucagon secreting A-cells are not a necessary prerequisite for insulin release. The recent work of Fujimoto et.al. using antisomatostatin and antiglucagon serum has indicated that, although exogenous glucagon stimulates insulin release the effect of endogenously released glucagon from adjacent A-cells upon islet B-cells may be a restraining one which is probably mediated through an effect upon D-cells and their release of somatostatin (220).

Somatostatin inhibited both glucose and glyceraldehyde induced insulin release from HIT-T15 and RINm5F cells respectively. Santerre et.al. have demonstrated that 10^{-8}mol/l somatostatin inhibits insulin release from 96 hour cultures of HIT-T15 cells by some 80% (62). In the present studies 10^{-8}mol/l somatostatin also inhibited glucose stimulated insulin release from HIT-T15 cells by exactly 80%. Similar effects have been noted with 2 day rat islet cultures (221), and somatostatin has also been shown to inhibit bombesin stimulated insulin release from HIT-T15 cells (222). The somatostatin inhibition of glyceraldehyde induced insulin release from RINm5F cells is not as marked as the hormone inhibitory effect on glucose stimulated insulin release from HIT-T15 cells. This discrepancy could be due to the presence of either reduced numbers of somatostatin receptors or a reduction in existing receptor affinity on the RINm5F cell membrane, or due to low somatostatin concentrations used in the present studies. Reduced receptor number seems a more likely candidate since Pipeleers has calculated the somatostatin concentration in the islet venous capillary to be of the order 10^{-10}mol/l.

The inhibition of glucose induced insulin release from HIT-T15 cells and glyceraldehyde induced insulin release fron RINm5F cells by pancreatic polypeptide follows a similar pattern to the inhibition shown by somatostatin. Glucose induced insulin release from HIT-T15 cells was more sensitive to the inhibitory effects of this peptide than glyceraldehyde induced insulin release from RINm5F cells. Physiologically glucose has been shown to have a weak inhibitory effect upon pancreatic polypeptide secretion (199), thus coinhibiting any inhibitory effect of pancreatic polypeptide upon insulin release. The reduced effect of pancreatic polypeptide upon glyceraldehyde induced insulin release from RINm5F cells may be a

result of reduced receptor numbers or a reduction in existing receptor affinity for pancreatic polypeptide. The inhibitory effect of somatostatin and pancreatic polypeptide in combination on insulin release from HIT-T15 and RINm5F cells does not appear to be additive, Table 4.6.

Table 4.6 Percentage inhibition of insulin release from HIT-T15 and RINm5F cells by somatostatin and pancreatic polypeptide (PP) in combination.

Treatment	% inhibition of glucose or glyceraldehyde stimulated insulin release	
	HIT-T15	RINm5F
Somatostatin, 10^{-7}mol/l	80	20
PP, 1000pg/ml	77	29
Theoretical combined effect	157	49
Observed combined effect	92	22

This discrepancy between theoretical and observed effects might result if each individual concentration of somatostatin and pancreatic polypeptide employed produced a near maximal inhibition of insulin release.

Glucagon stimulated insulin release from HIT-T15 cells was inhibited by both somatostatin and pancreatic polypeptide and by the two in combination. Somatostatin and pancreatic polypeptide were more effective inhibitors of glucose and glyceraldehyde induced insulin release than of glucagon induced insulin release from HIT-T15 and RINm5F cells. In contrast to glucose, the inhibitory effect of somatostatin and pancreatic polypeptide in combination on glucagon induced insulin release was additive. Somatostatin alone and in combination with pancreatic polypeptide inhibited glucagon induced insulin release from RINm5F cells. Pancreatic polypeptide alone, however, did not reduce glucagon stimulated insulin release. These data for RINm5F cells suggest that glucagon induced insulin release is not affected by pancreatic polypeptide and this may be the result of receptor deficiency.

The data presented above suggest the involvement of a significant islet cell paracrine interaction in the net release of insulin from the pancreatic islet.

The stimulation of insulin release from isolated islets by GIP has been shown to be dependent on the presence of glucose concentrations in excess of 5mmol/l (208,209). However the present study demonstrates that GIP will stimulate insulin release from HIT-T15 cells in the absence of glucose. It might be suggested that the observed stimulation of insulin release in the absence of glucose from HIT-T15 cells is the result of a breakdown in the normal integrity of the intra-islet paracrine microenvironment. Since GIP has been shown to stimulate the release of both insulin and glucagon (209), the elevated glucagon levels might then stimulate the release of somatostatin which would inhibit insulin release. Glucose on the other hand inhibits glucagon secretion from

A-cells (199) and stimulates somatostatin secretion from D-cells (197). Clearly the effect of GIP on net insulin release from isolated islets is the result of a coordinated release of pancreatic hormones. In the present studies GIP is able to interact directly with cultured HIT-T15 cells to stimulate insulin release in the absence of glucose. The absence of a potentiating effect of GIP upon glucose induced insulin release from HIT-T15 cells may reflect the reduced glucose sensitivity of this cell line when compared with the glucose sensitivity of isolated islets. The potentiation of glucose induced insulin release by GIP may be a pharmacological rather than physiologiacal phenomenon. Sarson et.al. have recently demonstrated in man that the infusion of 10 or 20g iv glucose (which produced blood glucose levels of 4.7 and 9.6 mmol/l respectively) and GIP at 0.5pmol/Kg/min (which produced circulating levels of 0.2ng/ml) failed to augment insulin release compared with glucose infusion alone. However, at supraphysiological levels of glucose and GIP (15.5 mmol/l and 0.5ng/ml) there was a significant augmentation of insulin release. These data were taken to indicate that the augmentation of glucose induced insulin release by GIP may be a pharmacological rather than physiological phenomenon (223).

Although GIP did not potentiate glucose stimulated insulin release from HIT-T15 cells it did potentiate arginine induced insulin release from these cells. In a series of hyperglycaemic clamp studies in man, the release of endogenous GIP stimulated by oral glucose was found to exert an additive effect upon arginine induced insulin release. This observation may provide evidence of a common mechanism for arginine and GIP induced insulin release (224).

CHAPTER 5.

5. STUDIES ON THE INTRACELLULAR MECHANISM OF INSULIN RELEASE FROM HIT-T15 AND RINm5F CELLS.

INTRODUCTION.

The involvement of cAMP in the mechanism of insulin release.

The idea that cAMP might act in the pancreatic B-cell as a second messenger was first suggested by Samols et.al. (225), who reported that glucagon, an agent known to increase the intracellular concentration of cAMP, stimulated insulin release. When the concentration of cAMP in the B-cell is increased, the the rate of insulin release in response to glucose is enhanced. In the presence of nonstimulatory concentrations of glucose, cAMP levels can be raised without stimulating insulin release. As the concentration of glucose is increased however, the ability of cAMP to enhance the response to glucose is unmasked, i.e., the effect of cAMP is greatest in the presence of high glucose and least in the presence of low glucose concentrations. (226-229). In the isolated islet as in other tissues, the intracellular concentration of cAMP is expressed as a balance between its rate of synthesis via adenylate cyclase and its rate of breakdown via cAMP dependent phosphodiesterase enzymes.

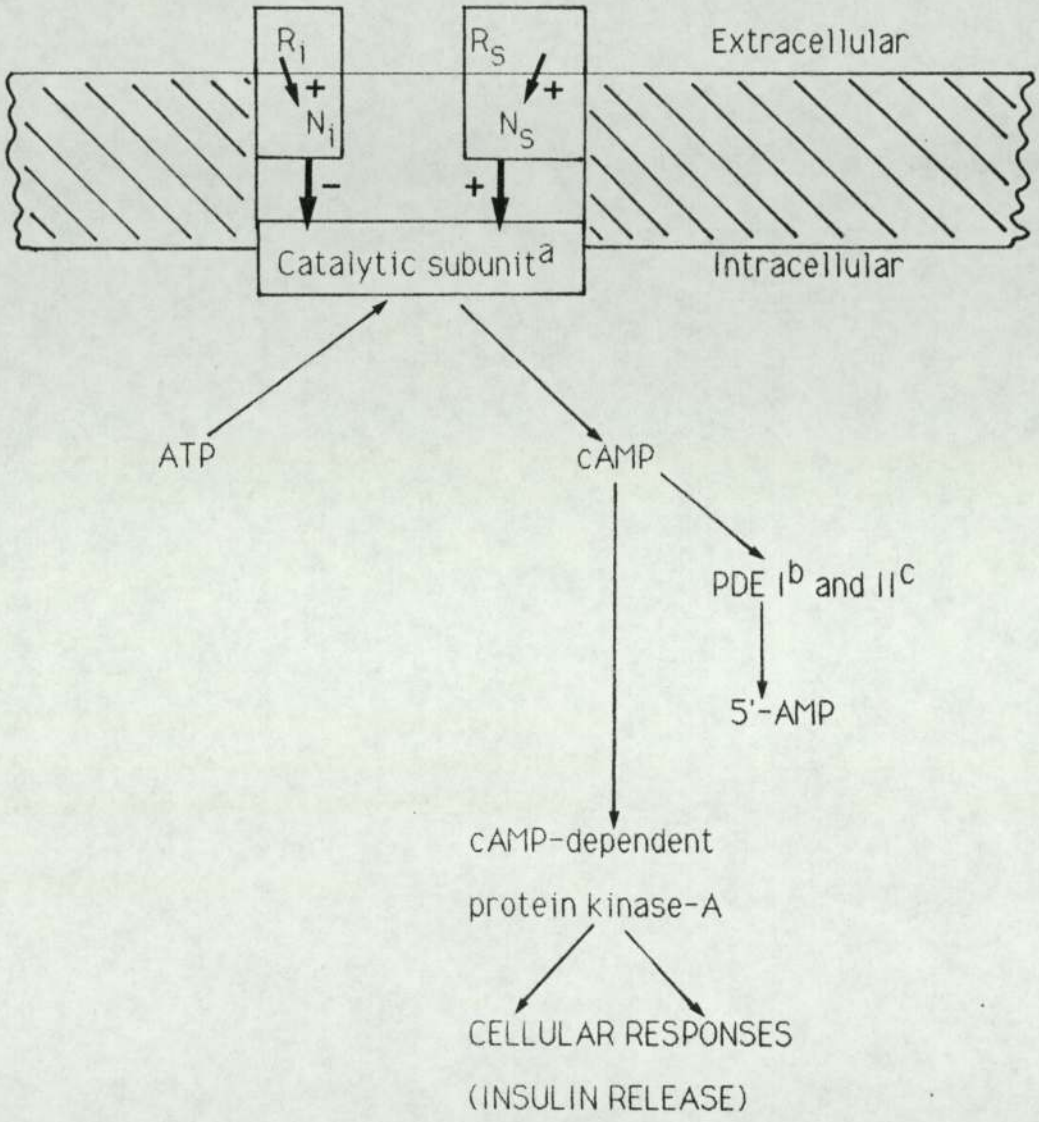
Adenylate cyclase is localised in the B-cell exclusively and almost uniformly in the plasma membrane (230). The enzyme converts ATP to cAMP with a Km for ATP of 0.07mmol/l (231). Excluding hormone receptor proteins adenylate cyclase consists of two functionally distinct subunits; the catalytic subunit which converts ATP to cAMP and two guanine nucleotide binding proteins (N_i inhibitory and N_s stimulatory), which are the required mediators of receptor input (232). Most investigators have failed to detect any direct effect of glucose upon adenylate cyclase activity (148,233).

cAMP dependent phosphodiesterase, the enzyme that catalyses the breakdown of cAMP to 5'-AMP is located in the islet cytosol. There appears to be two phosphodiesterase enzyme systems in islets referred to here as phosphodiesterase I and phosphodiesterase II. They differ in their Km values for cAMP, PDE I and PDE II have Km values of 2-9 and 30umol/l respectively (234,235). The low Km enzyme (PDE I) is believed to be largely in control of cAMP breakdown, this assumption being based on the fact that intracellular cAMP levels have been found to be of the order of 6umol/l, close to the Km of PDE I (179). The integration of adenylate cylase and cAMP phosphodiesterase activities in the control of intracellular cAMP levels is illustrated in Figure 5.1.

cAMP responsive target systems in the B-cell.

cAMP dependent protein kinase A activity has been identified in islet homogenates by many authors (236,237). It is generally believed but not unequivocally proven that cAMP exerts its insulinotrophic action via the activation cAMP dependent protein kinase A. The bulk of evidence suggests that cAMP induced insulin release is not the result of nutrient catabolism in islet cells (238). It has been suggested that cAMP dependent protein kinase A may stimulate microtubule polymerisation and subsequently insulin release (239). The majority of evidence also implicates an interaction between cAMP and calcium fluxes in the stimulation of insulin release. Incubation of islets in the presence of dibutryl cAMP (which is able to penetrate the cell membrane) induces a modest calcium uptake into B-cells (240). Brisson et.al. have proposed that cAMP acts on the secretory sequence by causing the intracellular redistribution of calcium to favour cytosolic accumulation rather than net uptake (241).

Figure 5.1 Integration of adenylate cyclase and cAMP phosphodiesterase activities in the control of intracellular cAMP levels.



Key; R_i & R_S inhibitory and stimulatory receptors.

N_i & N_S inhibitory and stimulatory guanine nucleotide binding proteins.

a, Km for ATP 0.07mmol/l

b, Km for cAMP 2-9umol/l

c, Km for cAMP 30umol/l

+, stimulation −, inhibition

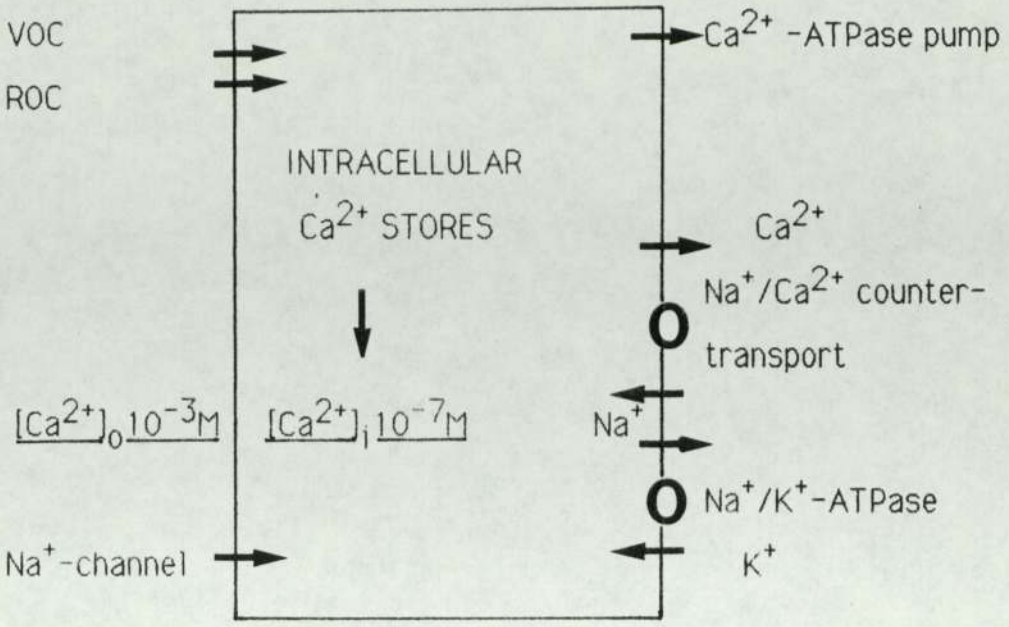
The involvement of calcium in the mechanism of insulin release.

Sustained insulin release is evoked by glucose only in the presence of sufficient amounts of extracellular calcium (Ca^{2+}). The threshold concentration *in vitro* is approximately 0.1mmol/l. Maximal effects of glucose occur with Ca^{2+} concentrations of the order 1-5mmol/l (242,243,244). Very high Ca^{2+} concentrations inhibit insulin release (243).

Calcium handling by the B-cell.

At the B-cell plasma membrane two processes are involved: a Ca^{2+} pump which derives its energy from ATP hydrolysis and a Na^{+}/Ca^{2+} countertransport, driven by an inward Na^{+} gradient (245,246). The Ca^{2+}-ATPase enzyme has a high affinity for Ca^{2+}, is activated by Ca^{2+}-calmodulin and is magnesium dependent (247). Na^{+}/Ca^{2+} countertransport, although not directly energy dependent, relies upon the maintenance of a steep inward Na^{+} gradient. This steep inward Na^{+} gradient is dependent upon the activity of the Na^{+}/K^{+}-ATPase. The inhibition of this enzyme in the B-cell leads to a decrease in Ca^{2+} efflux (248). Various intracellular organelles such as mitochondria, the endoplasmic reticulum and secretory granules also participate in the overall buffering of intracellular Ca^{2+}. These intracellular organelles have been termed intracellular Ca^{2+} stores (249). Stimulators of insulin release may also raise intracellular Ca^{2+} levels by the opening of receptor mediated (250) or voltage dependent Ca^{2+} channels (251). A summary of the various mechanisms for Ca^{2+} handling in the B-cell is illustrated in Figure 5.2.

Figure 5.2 Summary of the various mechanisms for calcium handling in the B-cell.(252)



Key; VOC voltage operated Ca^{2+} channel

ROC receptor operated Ca^{2+} channel

The involvement of calmodulin in the mechanism of insulin release.

Calmodulin was first described by Cheung in 1970 as an activator of brain cyclic nucleotide phosphodiesterase (253). Nine years later Sugden et.al. (254) and Valverde et.al. (255) identified and quantified the amount of calmodulin in islets. These reports estimated the calmodulin content of rat islets at between 0.11-0.13 pmol/islet. The important features of the calmodulin molecule is its ability to bind Ca^{2+} (4 x Ca^{2+}/mol with high affinity), with a dissociation constant in the micromolar range, and to undergo significant conformational changes as a result of Ca^{2+} binding. This conformational change enables the Ca^{2+}-calmodulin complex to bind to and activate cellular enzyme systems (256). Figure 5.3 illustrates the possible mechanisms involved in the activation of calmodulin and the subsequent binding of calmodulin with inactive receptor proteins (257).

Calcium and calmodulin responsive target systems.

Ca^{2+}-calmodulin has been shown to activate both adenylate cyclase (255) and cAMP-phosphodiesterase (258) in islet tissue. The activation of adenylate cyclase by calmodulin might explain how glucose increases intracellular cAMP levels independently of the direct activation of adenylate cyclase. Cheung has suggested that adenylate cyclase and cAMP-phosphodiesterase are activated sequentially in the brain. In this hypothesis the passage of Ca^{2+} through the membrane would activate membrane bound calmodulin which in turn would activate adenylate cyclase and generate cAMP. The subsequent rise in intracellular Ca^{2+} would activate cellular calmodulin which in turn would activate cAMP-phosphodiesterase converting cAMP to 5'-AMP, and terminating the cAMP signal (257), Figure 5.4. The existence of such a regulatory system in pancreatic B-cells remains to be determined.

Figure 5.3 Mechanism by which calmodulin mediates the biological actions of calcium ions (257)

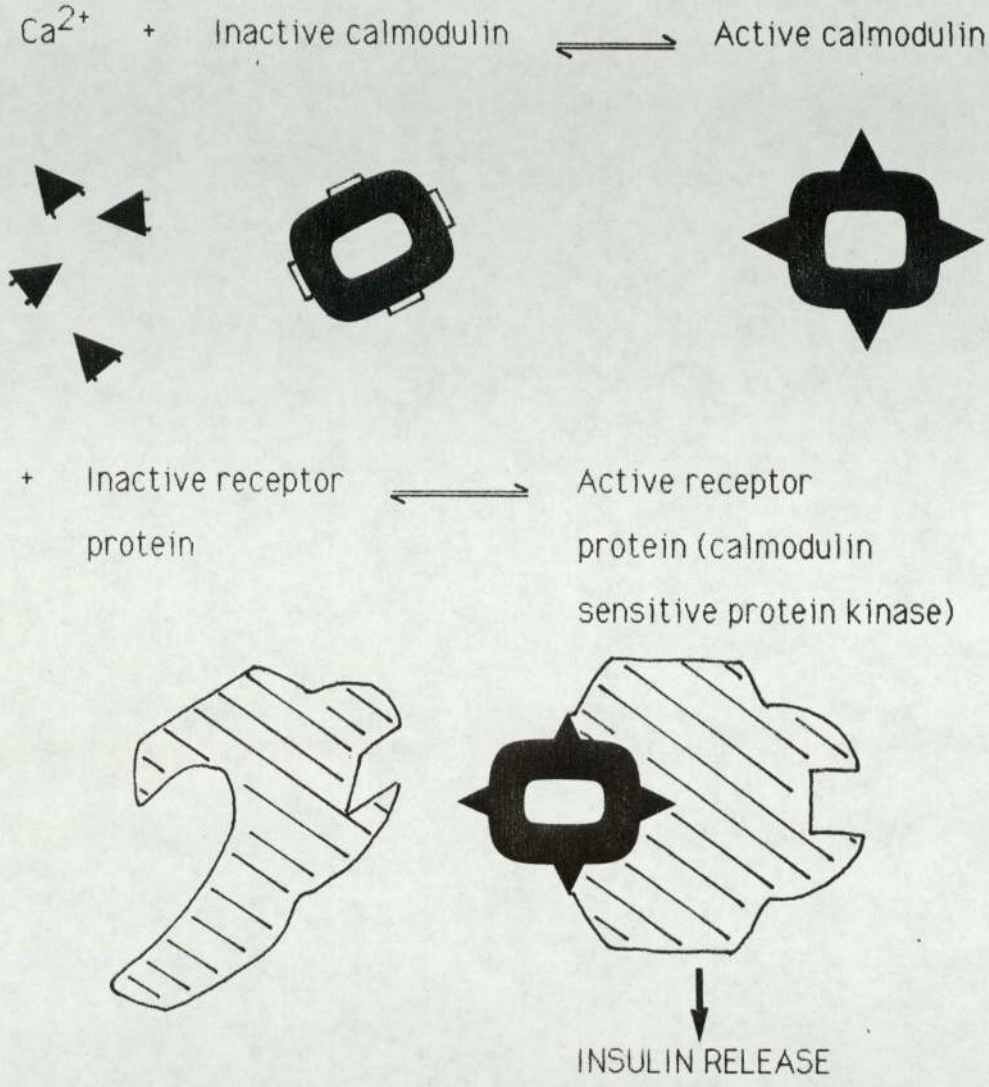
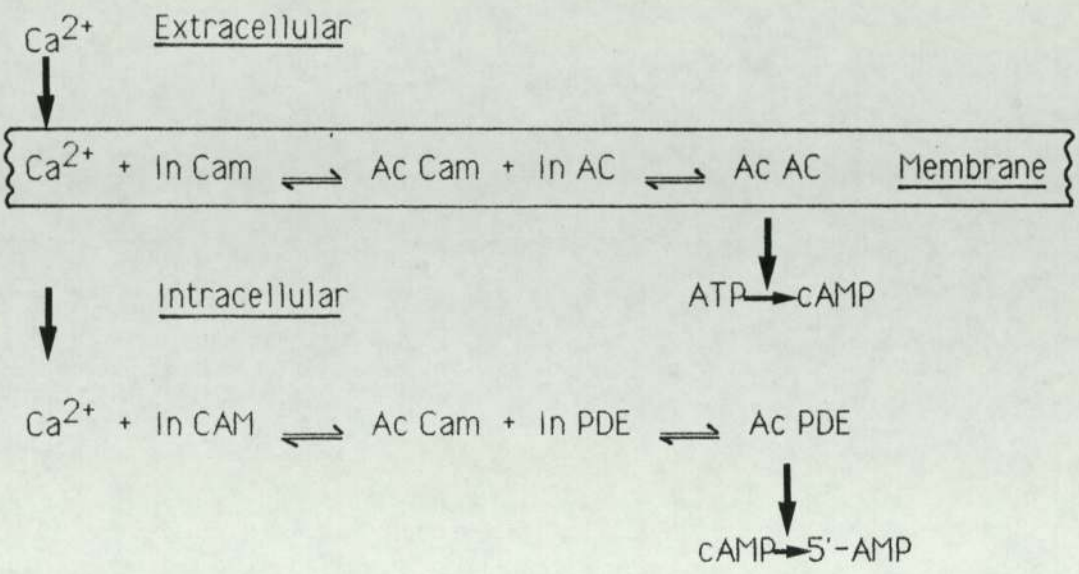


Figure 5.4 Proposed sequential activation of adenylate cyclase and cAMP dependent phosphodiesterase in the brain (257).



Key; In, inactive

Ac, active

Cam, calmodulin

PDE, phosphodiesterase

Calmodulin has been shown to inhibit the Ca^{2+}-ATPase in the B-cell plasma membrane (which indirectly controls the Na^{+}/Ca^{2+} countertransport, Figure 5.2), this inhibition results in an increase in intracellular Ca^{2+} levels (259). The inhibition of this enzyme may be important in the maintenance of high intracellular Ca^{2+} levels required for the stimulation of insulin release.

A calmodulin sensitive protein kinase has been isolated from rat islet homogenates (260). This calmodulin sensitive kinase has been shown to carry out two major functions. Firstly it phosphorylates the alpha and beta subunits on tubulin (261) and secondly it phosphorylates myosin, permitting interaction with actin which allows the microfilament to contract (262). Calmodulin has also been shown to increase the binding of secretory granules to inverted plasma membrane vesicles (263).

The involvement of phosphoinositides in the mechanism of insulin release.

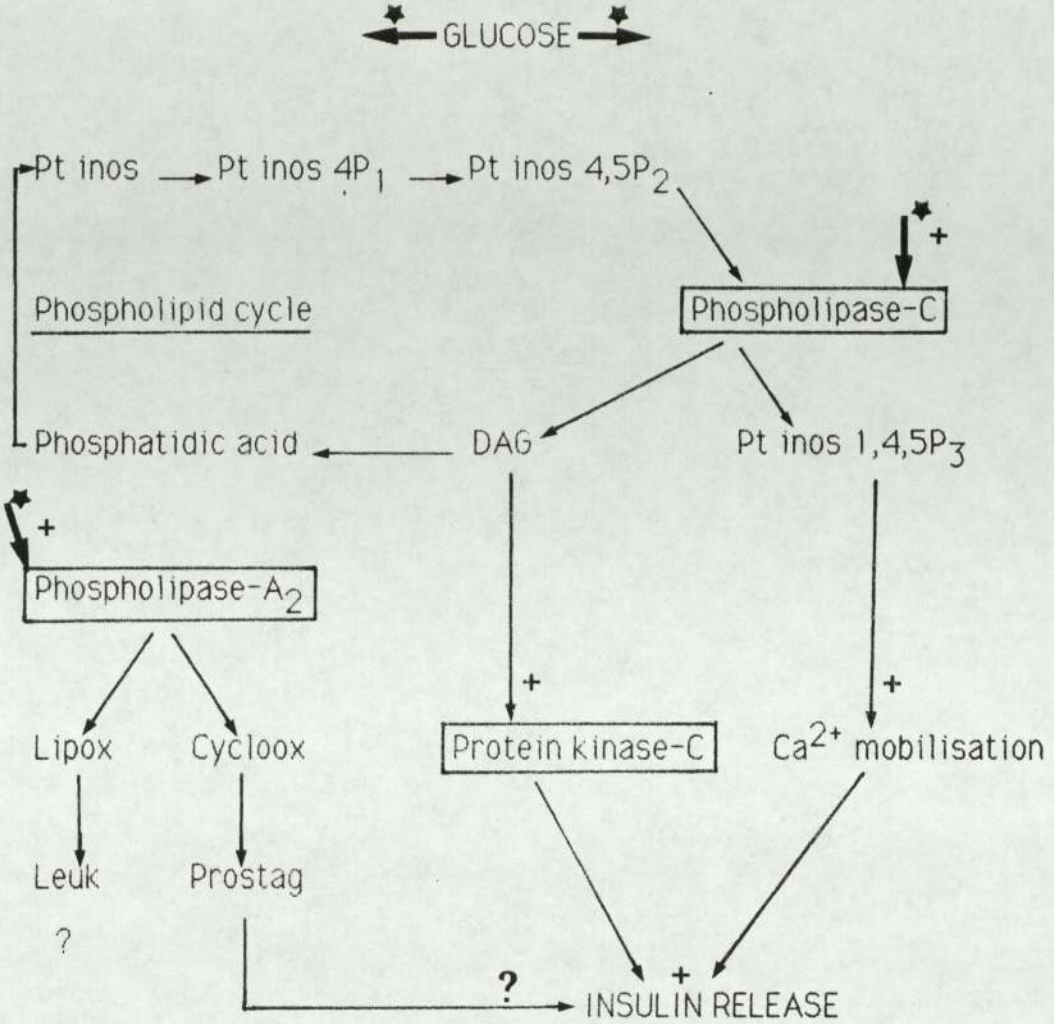
Hokin and Hokin were first to report an increase in phospholipid turnover in pancreatic exocrine tissue in response to acetyl choline (264). This increase in phospholipid turnover, particularly of the inositol containing phospholipids was called the phosphatidylinositol or PI effect (265). Subsequent work by Fex and Lernmark (266) and Freinkel et.al. (267) showed that glucose stimulated pancreatic islets exhibited an increased ^{32}P-labelling of phospholipids when preincubated in ^{32}P-containing medium. The consequence of stimulated phospholipid turnover appears to be threefold. Glucose activation of membrane bound phospholipase-C (268) increases the intracellular concentrations of D-myoinositol 1,4,5 triphosphate (IP_3), diacylglycerol and phosphatidic acid (269). Diacylglycerol activation of Ca^{2+} and phospholipid dependent

protein kinase-C (270,271) and IP_3 induced Ca^{2+} mobilisation from intracellular stores (271,272), stimulate the insulin secretory machinery in a synergistic fashion. Laychock has shown that glucose also stimulates phospohlipase-A_2 (273), causing an increase in the intracellular concentration of arachidonic acid. Arachidonic acid can be converted via the lipoxygenase pathway to leukotrienes and by the cyclo-oxygenase pathway to prostaglandins (265). Data obtained from studies investigating the effect of arachidonic acid metabolites on insulin release is contradictory. For example, prostaglandins have been shown to both stimulate (274) and inhibit (275) insulin release. Figure 5.5 summarises the interrelationships between phospholipid metabolism and the stimulation of insulin release.

The involvement of the microtubular-microfilamentous system in the mechanism of insulin release.

Lacy et.al. first suggested that glucose stimulated insulin release might involve the microtubular-microfilamentous system, these observations were based on the fact that colchicine, a potent inhibitor of microtubule formation, inhibited insulin release (276). These observations were later confirmed using vinblastine, deuterium oxide (277), nocodazole (278) and taxol (278), agents that also disrupt microtubule function. The ultrastructural studies of Orci et.al. (6) and Dean (279) have shown association between microtubules and insulin secretory granules in the B-cell. Evidence for the involvement of microfilaments in the mechanism of insulin release comes from studies using cytochalasin B which causes hypercontraction of microfilaments and a stimulation of insulin release (280)

Figure 5.5 The relationship between phospholipid turnover and glucose stimulated insulin release. (270)



Key, Pt inos, phosphatidyl inositol

DAG, diacylglycerol

Lipox, lipoxygenase pathway

Leuk, leukotrienes

Cycloox, cyclo-oxygenase pathway

Prostag, prostaglandins

+, stimulation

?, effect uncertain

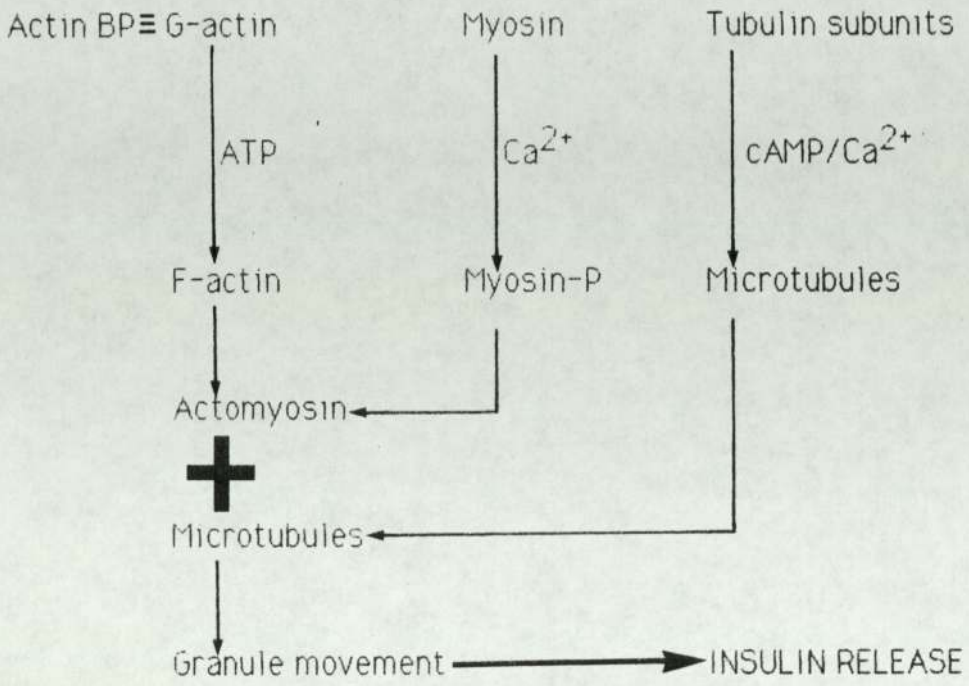
The three major proteins associated with the microtubular microfilamentous system in islets are tubulin (281), actin (282) (globular and filamentous) and myosin (283). Two microtubule associated proteins (Maps I and II) have also been identified in other tissues (284,285). The sites and presumed modes of action of these proteins are illustr ated in table 5.1

Table 5.1 proteins associated with microtubule/microfilament system.

Protein	Proposed role
Tubulin	polymerises to form microtubules.
Maps I	believed to mediate Ca^{2+} induced tubulin polymerisation.
Maps II	believed to mediate cAMP induced tubulin polymerisation.
Actin (globular)	polymerises to form microfilaments (filamentous actin).
Myosin	ATPase activity enhanced after phosphorylation and by association with actin (actomyosin).

A summary of the proposed role played by microtubules and microfilaments in the process of insulin release is shown in figure 5.6. Globular actin, released from actin binding proteins (e.g. actinogelin [286], gelsolin [287], fragmin [288] and villin [289]) is polymerised to form filamentous actin (290). The latter interacts with phosphorylated myosin (that may be located in the B-granule

Figure 5.6 Mechanism of the interaction between microtubules and microfilaments in glucose stimulated insulin release. Glucose generates increased Ca^{2+}, ATP and cAMP (291).



Key; BP, binding proteins

G, globular

F, filamentous

P, phosphorylated

membrane) to form the activated actomyosin complex (292). Microtubules are assembled in a Ca^{2+} (261) and cAMP (281) dependent process from tubulin subunits. The interaction of actomyosin and microtubules results in granule movement and insulin release by exocytosis.

In the present work the possible roles played by cAMP, Ca^{2+}, IP_3 and protein kinase C in the mechanism of insulin release from HIT-T15 and RINm5F cells have been evaluated by examining the effect of forskolin, a powerful stimulator of adenylate cyclase (293) and imidazole, a presumed cAMP phosphodiesterase activator (294) upon insulin release. The effect of raising and lowering extracellular Ca^{2+} concentrations, in conjunction with studies using Ca^{2+} antagonists (verapamil, diltiazem, nifedipine, TFP, M&B 13753, TMB-8 and M&B 40678) and the phosphoinositide IP_3 (that may enhance Ca^{2+} release from intracellular stores [272]) have been used to establish a role for Ca^{2+} in the mechanism of insulin release from HIT-T15 and RINm5F cells. The phorbol esters TPA and 4PDD have been used as probes to directly stimulate the Ca^{2+} and phospholipid dependent protein kinase C and elucidate any possible role for this kinase in RINm5F cell insulin release.

RESULTS

The effect of forskolin and imidazole on insulin release from statically incubated HIT-T15 and RINm5F cells.

10umol/l forskolin stimulated insulin release in the absence of glucose from statically incubated HIT-T15 and RINm5F cells. HIT-T15 cells produced significantly ($p<0.05$) more insulin than RINm5F cells in response to this agent. 7.5mmol/l glucose induced insulin release fron HIT-T15 cells and 10mmol/l glyceraldehyde induced insulin release from RINm5F cells was significantly suppressed by 0.1mmol/l imidazole, Table 5.2. In view of the general glucose insensitivity of RINm5F cells, glyceraldehyde at a concentration of 10mmol/l was chosen as a suitable insulin secretagogue (this concentration consistently produces a near doubling in basal insulin release).

The effect of extracellular calcium and calcium antagonists on insulin release from statically incubated HIT-T15 and RINm5F cells.

In the absence of glucose, calcium stimulated insulin release from HIT-T15 cells, maximum and half maximum stimulation occurred at 2.04 and 1.26mmol/l calcium respectively, Figure 5.7. The ommission of calcium from the incubation buffer completely abolished glucose induced insulin release, the response was reduced to below basal values. The combined effect of glucose and 2.04mmol/l calcium on insulin release was additive rather than synergistic. Insulin release in response to either 2.04mmol/l calcium or 2.04mmol/l calcium in combination with 7.5mmol/l glucose was suppressed by 15umol/l verapamil, Table 5.3.

Figure 5.7. The effect of extracellular calcium upon insulin release from statically incubated HIT-T15 cells.

Values are means ± SEM with n in parentheses.

a, calcium concentration in regular Krebs buffer (0.68mmol/l)

*, $p<0.05$ significantly increased compared with 0.68mmol/l Ca^{2+}.

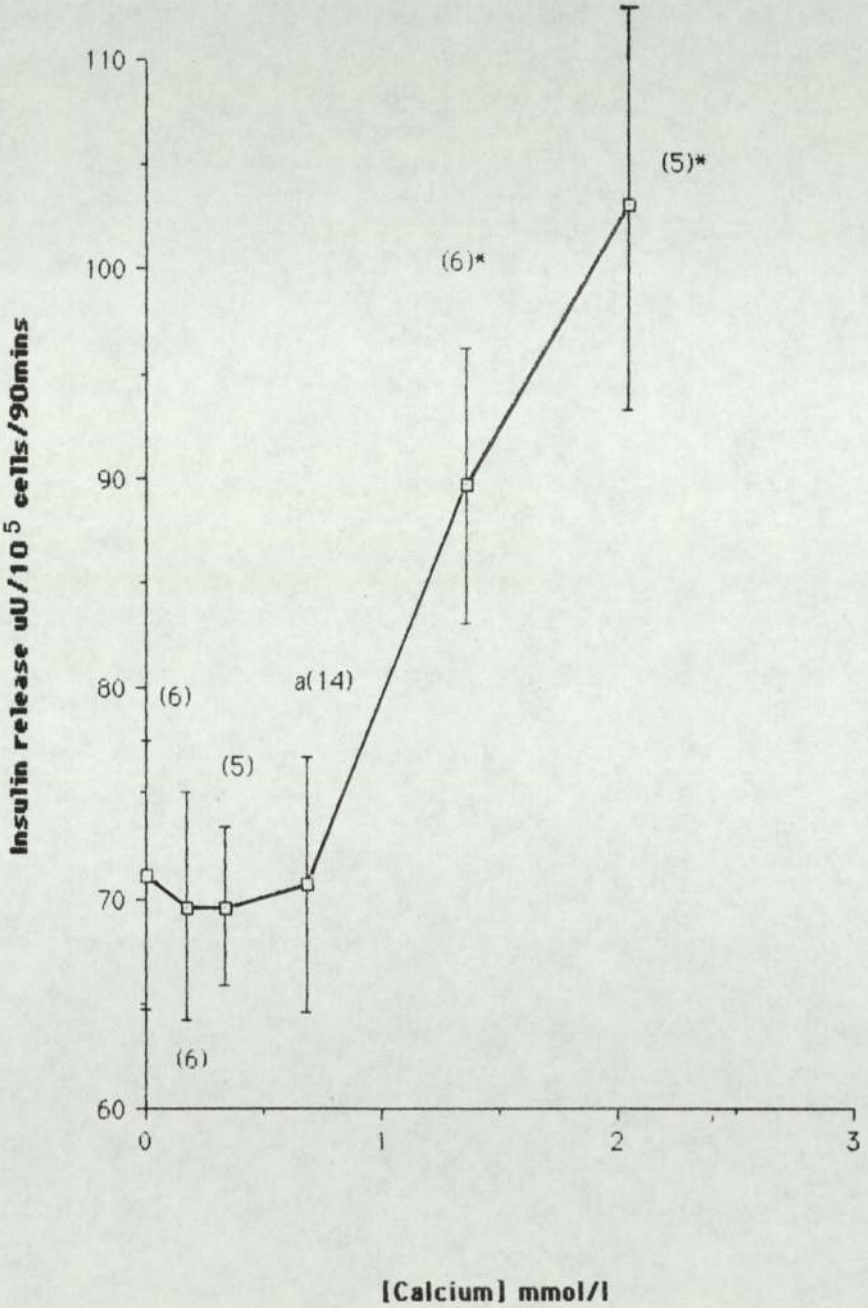


Table 5.2 The effect of forskolin on basal and imidazole on glucoase and glyceraldehyde induced insulin release from HIT-T15 and RINm5F cells.(n is in parentheses)

Treatment	Insulin release % from basal	
	HIT-T15	RINm5F
Forskolin, 10umol/l	94.55 ± 20.25[a](10)	53.10 ± 8.08[a](5)
Glucose, 7.5mmol/l	169.54 ± 10.65[a](48)	-
+ imidazole, 0.1mmol/l	46.71 ± 23.93[b](5)	-
Glyceraldehyde, 10mmol/l	-	90.31 ± 8.53[a](6)
+ imidazole, 0.1mmol/l	-	66.30 ± 4.06[c](5)

a, $p<0.05$ significantly increased compared with basal.

b, $p<0.05$ significantly reduced compared with glucose.

c, $p<0.05$ significantly reduced compared with glyceraldehyde.

Table 5.3 Insulin release from statically incubated HIT-T15 cells is calcium dependent and inhibited by verapamil.

Treatment	n	Insulin release % from basal
Glucose, 7.5mmol/l	48	169.54 ± 10.65[a]
Glucose in Ca^{2+} free medium	5	-17.21 ± 7.81[b]
Ca^{2+}, 2.04mmol/l	6	45.88 ± 4.36[a,e]
Ca^{2+} + glucose	5	171.90 ± 6.06[a]
Ca^{2+} + verapamil, 15umol/l	5	7.53 ± 7.93[c]
Ca^{2+} + verapamil + glucose	7	28.84 ± 4.25[a,d]

a, $p<0.05$ significantly increased compared with basal.

b, $p<0.05$ significantly reduced compared with basal.

c, $p<0.05$ significantly reduced compared with 2.04mmol/l Ca^{2+}.

d, $p<0.05$ significantly reduced compared with 7.5mmol/l glucose and 2.04mmol/l Ca^{2+}.

e, $p<0.05$ significantly reduced compared with 7.5mmol/l glucose.

Glucose induced insulin release from HIT-T15 cells and glyceraldehyde induced insulin release from RINm5F cells was significantly inhibited by calcium channel blockade, using 15umol/l verapamil, diltiazem and nifedipine and calmodulin antagonism, using 15umol/l TFP and M&B 13,753 (a new May and Baker calmodulin inhibitor). The effect of Ca^{2+} sequestration blockade, using 15umol/l TMB-8 and M&B 40,678 (a new May and Baker sequestration blocker) upon glucose stimulated insulin release from HIT-T15 cells was contradictory, in that TMB-8 but not M&B 40,678 inhibited glucose stimulated insulin release, Tables 5.4 and 5.5.

Table 5.4 The effect of calcium antagonists upon glucose induced insulin release from statically incubated HIT-T15 cells.

Treatment	n	Insulin release % from basal
Glucose, 7.5mmol/l	48	169.54 ± 10.65[a]
+ verapamil, 15umol/l	5	46.70 ± 15.27[b]
+ diltiazem, 15umol/l	5	38.82 ± 12.73[b]
+ nifedipine, 15umol/l	5	46.81 ± 24.74[b]
+ TFP, 15umol/l	6	39.88 ± 8.29[b]
+ M&B 13,753, 15umol/l	5	52.17 ± 9.91[b]
+ TMB-8, 15umol/l	5	54.97 ± 17.07[b]
+ M&B 40,678, 15umol/l	5	144.48 ± 15.17

a, $p<0.05$ significantly increased compared with basal.

b, $p<0.05$ significantly reduced compared with 7.5mmol/l glucose.

Table 5.5 the effect of calcium antagonists on glyceraldehyde induced insulin release from statically incubated RINm5F cells.

Treatment	n	Insulin release % from basal
Glyceraldehyde, 10mmol/l	6	90.30 ± 8.33[a]
+ verapamil, 15umol/l	5	41.20 ± 3.43[b]
+ diltiazem, 15umol/l	6	17.52 ± 1.33[b]
+ nifedipine, 15umol/l	5	49.72 ± 4.66[b]
+ TFP, 15umol/l	5	63.31 ± 1.06[b]
+ M&B 13,753, 15umol/l	5	37.53 ± 2.46[b]

a, $p<0.05$ significantly increased compared with basal.

b, $p<0.05$ significantly reduced compared with 10mmol/l glyceraldehyde

The effect of D-myoinositol 1,4,5 triphosphate (IP_3) on insulin release from statically incubated HIT-T15 cells.

2.5umol/l IP_3 , the concentration Biden et.al., found sufficient to mobilise intracellular calcium in RINm5F cells, failed to stimulate insulin release from HIT-T15 cells in the present studies, Table 5.6.

Table 5.6 The effect of IP_3 on insulin release from statically incubated HIT-T15 cells.

Treatment	n	Insulin release % from basal
Glucose, 7.5mmol/l	48	169.54 + 10.65[a]
IP_3, 2.5umol/l	19	-26.77 + 9.00

a, $p<0.05$ significantly increased compared with basal.

The effect of 12-O-tetradecanoylphorbol-13-acetate (TPA) and 4-alpha-phorbol-12,13-didecanoate (4PDD), tumour promoting phorbol esters, on insulin release from statically incubated RINm5F cells.

Both TPA and 4PDD, in the absence of glucose, stimulated insulin release from statically incubated RINm5F cells, maximum insulin release occurring at about 10^{-5}mol/l for both phorbol esters. TPA was significantly ($p<0.05$) more potent than 4PDD at concentrations of 10^{-7} and 10^{-8}mol/l. Half maximum stimulation of insulin release occurred at concentrations of 8nmol/l and 0.6umol/l for TPA and 4PDD respectively, representing a near 100-fold difference, Figure 5.8. The insulin response to 10^{-7}mol/l TPA and 10^{-6}mol/l 4PDD was not significantly inhibited by either imidazole or TFP. Imidazole did, however, significantly enhance ($p<0.05$) the response to TPA, Table 5.7.

Figure 5.8. the effect of TPA and 4PDD upon insulin release from statically incubated RINm5F cells.

Values are means ± SEM with n in parentheses.

*, $p<0.05$ significantly increased compared with basal.

(Basal secretion was 23.46 ± 1.85 uU/10^5cells/90mins [n=15]).

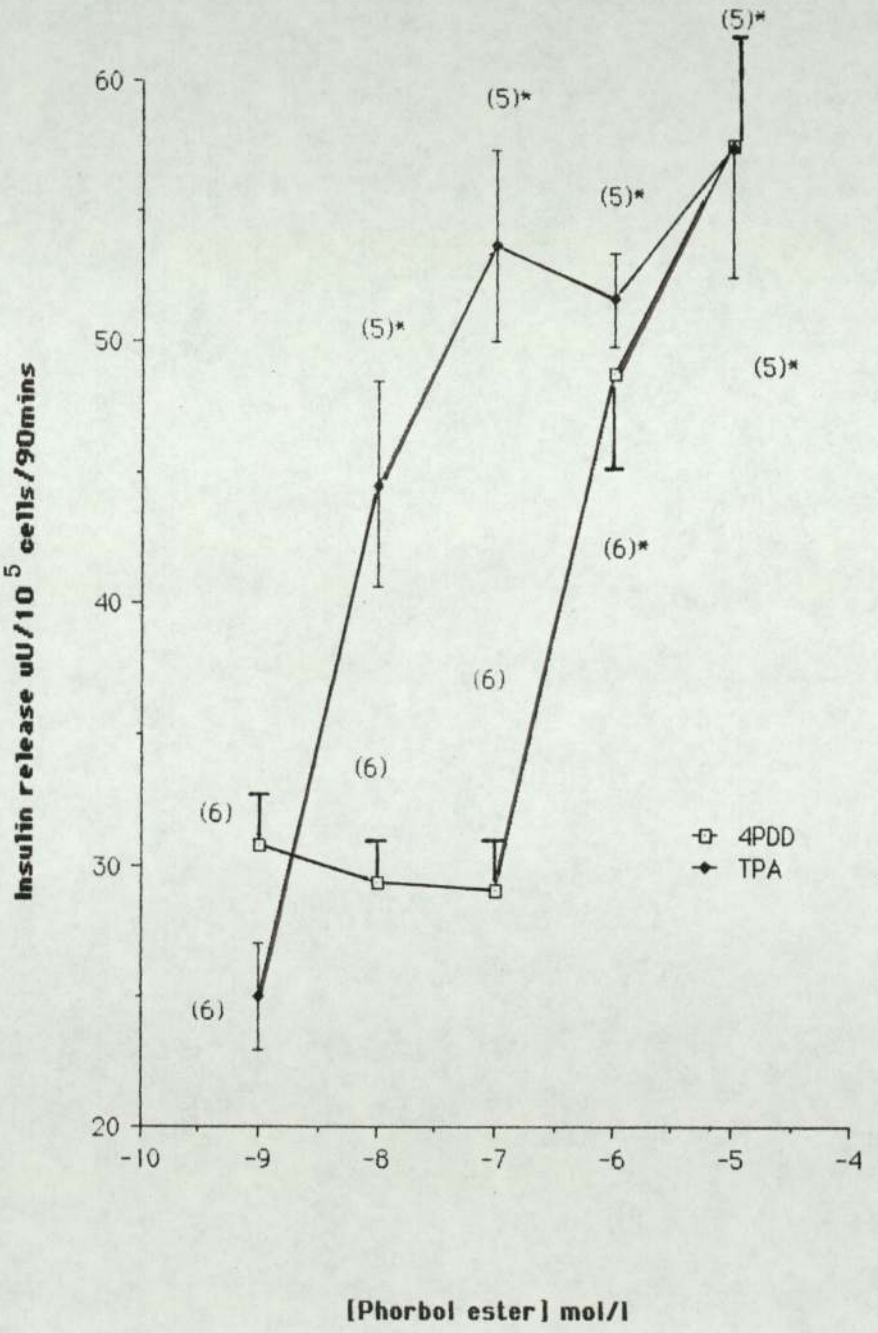


Table 5.7 The effect of imidazole and TFP on TPA and 4PDD induced insulin release from statically incubated RINm5F cells.

Treatment	n	Insulin release % from basal
TPA, 10^{-7}mol/l	5	131.30 ± 8.49[a]
+ imidazole, 0.1mmol/l	6	184.75 ± 14.60[a,b]
+ TFP, 15umol/l	5	132.24 ± 24.73[a]
4PDD, 10^{-6}mol/l	5	119.50 ± 20.03[a]
+ imidazole, 0.1mmol/l	4	74.21 ± 5.08[a]
+ TFP, 15umol/l	5	115.23 ± 9.52[a]

a, $p<0.05$ significantly increased compared with basal.

b, $p<0.05$ significantly increased compared with 10^{-7}mol/l TPA.

DISCUSSIONS

The stimulation of HIT-T15 and RINm5F cell insulin release by forskolin, a widely known and powerful stimulant of adenylate cyclase (293), suggests a role for the adenylate cyclase system in the mechanism of insulin release from these cells. The stimulation of cAMP production by forskolin in adipocytes has been shown to be via a direct activation of the adenylate cyclase catalytic subunit rather than the stimulation of cAMP-phosphodiesterase (295). Yamamoto et.al. have shown that forskolin stimulates insulin release and cAMP generation dose-dependently in isolated islets, with maximum stimulation of secretion occurring at 10umol/l forskolin (296) Ashcroft et.al. have demonstrated that 10umol/l forskolin stimulates insulin release and cAMP formation in HIT-T15 cells both in the absence and presence of glucose. In addition forskolin potentiated glucose stimulated insulin release (167). Forskolin has also been reported to stimulate the release of insulin from RINm5F cells (297).

Imidazole has been shown to stimulate cAMP dependent phosphodiesterases in a variety of tissues including heart (294), adipose tissue (298), parathyroid (299) and ileum (300). Imidazole is most effective at high concentrations of the order of 0.1mmol/l and its activation of cAMP dependent phosphodiesterase is Mn^{2+} dependent (301). The specificity of imidazole has been called into question since it has also been shown to inhibit cGMP dependent phosphodiesterase (302). However any resultant increase in cGMP would additionally activate cAMP phosphodiesterase (303). The net effect of imidazole is to reduce the intracellular concentration of cAMP. The existence of a cGMP activated cAMP dependent phosphodiesterase in islet tissue remains to be determined. In the

present studies 0.1mmol/l imidazole inhibited glucose induced insulin release from HIT-T15 cells and glyceraldehyde induced insulin release from RINm5F cells further supporting a role for cAMP in the regulation of insulin release from these cells.

The stimulation of insulin release from HIT-T15 cells by high extracellular Ca^{2+} in the absence of glucose, and its inhibition by the Ca^{2+} channel blocker verapamil suggests that Ca^{2+} may enter the B-cell by passive diffusion along a steep electrochemical gradient (304), possibly through the B-cell Ca^{2+} channel. The resultant increase in intracellular Ca^{2+} would trigger the insulin release mechanism. The omission of Ca^{2+} from the incubation medium did not inhibit basal insulin release but did significantly inhibit 7.5mmol/l glucose induced insulin release from HIT-T15 cells confirming earlier observations by Ashcroft et.al. (167)

The involvement of Ca^{2+} entry, via membranal Ca^{2+} channels, in the mechanism of glucose stimulated insulin release from HIT-T15 cells and glyceraldehyde induced insulin release from RINm5F cells was evaluated using three Ca^{2+} channel blockers, verapamil, diltiazem and nifedipine. These channel blockers inhibited glucose and glyceraldehyde stimulated insulin release from both HIT-T15 and RINm5F cells respectively, suggesting Ca^{2+} entry is an essential step in the initiation of the secretory sequence in these cells. Verapamil has previously been shown to inhibit glucose stimulated insulin release from islets (305) and HIT-T15 cells (306). In addition to the specific inhibition of Ca^{2+} channels, Triggle has shown that verapamil also inhibits fast Na^{+} currents, and muscarinic, adrenergic and opiate receptor binding in various muscle tissue (307). Similar non specific actions of the other Ca^{2+} channel blockers, diltiazem and nifedipine should not be ignored.

Calmodulin inhibitors, TFP and M&B 13,753 also significantly inhibited glucose stimulated insulin release from HIT-T15 cells and glyceraldehyde induced insulin release from RINm5F cells. M&B 13,753 is a phenoxypropanolamine derivative recently develped by May and Baker and described as a specific calmodulin inhibitor (Dr T. Brown. May and Baker. Personal communication) Recently it has been suggested that it may also be a beta adrenergic receptor antagonist (308). The calmodulin inhibitor TFP has been widely used in secretory studies using isolated islets (309,310). Fleischer et.al. using a Syrian hamster insulinoma has shown that TFP may also act as a Ca^{2+} channel blocker. In this study TFP at <10umol/l was reported to block the entry of ^{45}Ca while >10umol/l TFP appeared to enhance ^{45}Ca entry into insulinoma cells (311).

The Ca^{2+} sequestration blocker TMB-8 (a trimethoxybenzoate derivative which has been shown to reduce Ca^{2+} availibility in skeletal and smooth muscles by stabilising Ca^{2+} binding to cellular stores [312]) but not M&B 40,678 (a trimethoxybenzoate derivitave and putative Ca^{2+} sequestration blocker from May and Baker, Dr T. Brown, Personal communication) inhibited glucose stimulated insulin release from HIT-T15 cells. Any role a sequestration blocker such as TMB-8 might play in the mechanism of insulin release needs to be viewed with extreme caution. Especially in view of the potentiating effect of TMB-8 upon IBMX stimulated insulin release from isolated islets, recently reported by Weidenkeller and Sharp (313).

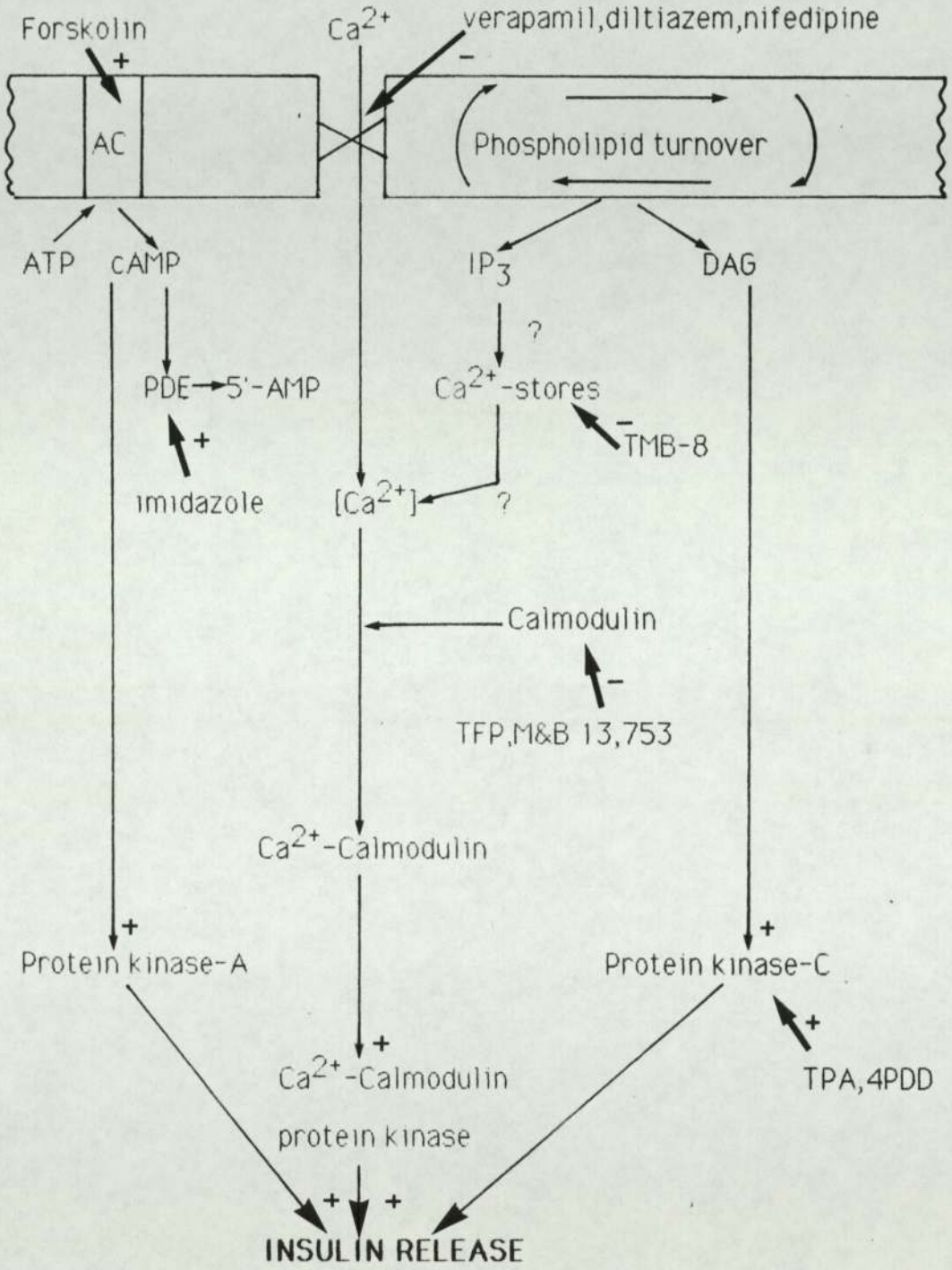
The incubation of HIT-T15 cells with 2.5umol/l IP_3 did not stimulate insulin release. Biden et.al. demonstrated that 2.5umol/l IP_3 was sufficient to mobilise intracellular calcium in digitonin permeabilised RINm5F cells, where 50% of the maximum response was observed within 15 seconds (272). Any possible role for IP_3 induced Ca^{2+} mobilisation in the mechanism of insulin release from intact cultured B-cells will require further study.

Castagna et.al. has shown that TPA, phorbol-12,13-didecanoate, -dibutyrate and -dibenzoate but not 4PDD or phorbol stimulate rat brain protein kinase-C (314). Virji et.al. was the first to report that TPA stimulated insulin release from isolated pancreatic islets. These workers showed that the stimulation of insulin release was dependent upon substimulatory concentrations of glucose (315). Subsequent work by Malaisse et.al. demonstrated that TPA could stimulate insulin release in the absence of glucose (316). In the present studies TPA and the structurally related 4PDD both stimulated insulin release in the absence of glucose from RINm5F cells, suggesting a role for protein kinase-C in the mechanism of insulin release from these cells. TPA has also been shown to stimulate insulin release from the X-ray induced insulinoma of the rat, the parent tumour of the RINm5F cell line (317). The half maximum value for TPA stimulated insulin release from RINm5F cells of 8nmol/l determined in the present studies compares favourably with that recently quoted by Arcoleo and Weinstein of 4nmol/l for purified protein kinase-C (318). Since imidazole and TFP had no effect on TPA and 4PDD induced insulin release in the present studies it can be inferred that these phorbol esters are probably acting via protein kinase-C to stimulate insulin release, rather than on cAMP dependent protein kinase-A or calmodulin

dependent protein kinase. The enhancement of the RINm5F cell insulin response to TPA by imidazole might well be the result of a reduction in protein kinase-A activity. The possibility that 4PDD may influence other parts of the insulin secretory process in RINm5F cells can not be excluded.

The effects of cAMP, Ca^{2+} and inositol containing phospholipids upon insulin release should be viewed as an integrated system. Hill et.al. have shown that an increase in adenylate cyclase activity in HIT-T15 cells induced by forskolin increases the levels of cAMP without altering the concentration of cytosolic free Ca^{2+} (319). Yet increasing the intracellular Ca^{2+} concentration in islets with the Ca^{2+} ionophore A23187 increases the intracellular cAMP levels (320). Certainly Ca^{2+} and calmodulin can activate adenylate cyclase in isolated islets (321,322). Stimulated phospholipid turnover could conceivably increase the intracellular Ca^{2+} concentration via IP_3 induced Ca^{2+} release from intracellular stores. The resultant co-ordinated increase in cAMP, Ca^{2+} and phospholipid levels would activate their respective protein kinases to produce the finely tuned insulin response to a spectrum of insulin secretagogues. A possible model representing such an integrated system for the control of insulin release from cultured HIT-T15 and RINm5F cells is illustrated in Figure 5.9

Figure 5.9 Putative mechanism for the integrative roles played by cAMP, calcium/calmodulin, phospholipids and protein kinases in the mechanism of insulin release from HIT-T15 and RINm5F cells.



Key; **X**, block

AC, adenylate cyclase

PDE, phosphodiesterase

DAG, diacylglycerol

+, stimulation

−, inhibition

?, uncertain

CHAPTER 6.

6. CHOLINERGIC AND ADRENERGIC MODULATION OF INSULIN RELEASE FROM HIT-T15 AND RINm5F CELLS.

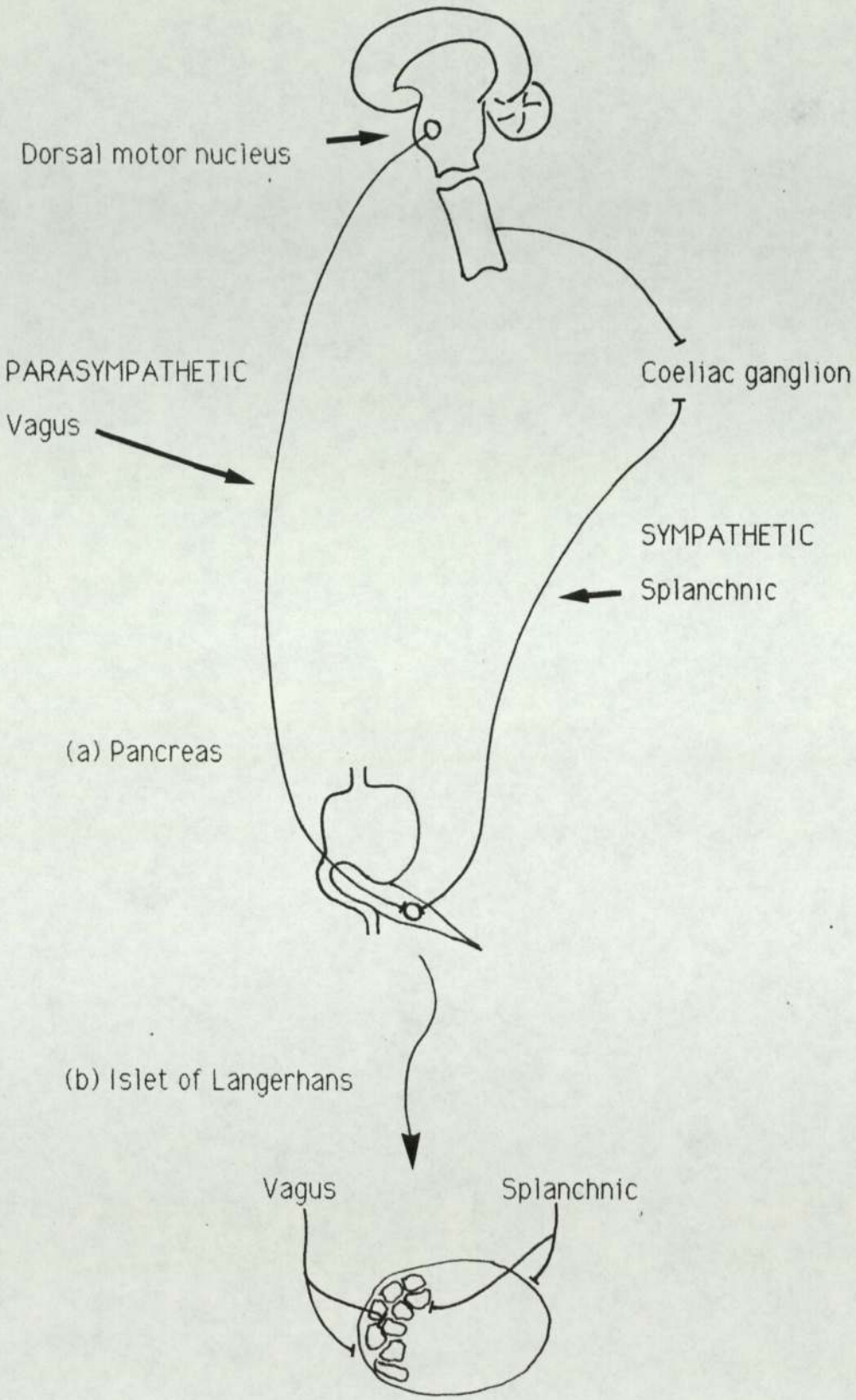
INTRODUCTION.

Innervation of the pancreas and the islets of Langerhans.

The early experiments of Clark in 1925 (323) and La Barre in 1927 (324) suggested that vagal nerve stimulation provoked insulin release. Subsequently in 1958 Coupland (325) demonstrated an anatomic relationship between the autonomic nervous system and both the endocrine and exocrine pancreas.

Preganglionic, parasympathetic cholinergic nerve fibers which innervate the pancreas originate in the dorsal motor nucleus of the vagus. These nerve fibers travel in the vagus and terminate in ganglia either within the exocrine pancreas or at the islet borders or directly at the surfaces of islet cells (326). Sympathetic nerve fibers from the splanchnic nerve traverse the coeliac ganglion and enter the pancreas. These fibers are closely associated with the blood vessels supplying the islets and also impinge upon. the surfaces of individual islet cells (326). The general layout of the autonomic innervation to the pancreas and the islets of Langerhans is illustrated in Figure 6.1.

Figure 6.1 Autonomic innervation of (a) the pancreas and (b) the islets of Langerhans.



Cholinergic modulation of islet hormone secretion.

Stimulation of the vagus nerve has been shown to stimulate insulin, glucagon and pancreatic polypeptide secretion in dogs, pigs and calves. The role of vagal nerve stimulation in the release of somatostatin is not clear cut, in that a stimulation of somatostatin release has been observed in the dog whilst inhibition has been observed in the pig, (327-332), Table 6.1.

Table 6.1 The effect of vagal nerve stimulation on the release of insulin, glucagon, somatostatin and pancreatic polypeptide.

Species	Effect of vagal nerve stimulation on the release of			
	Insulin	glucagon	somatostatin	PP
Dog	+(327)	+(327)	+(330)	+(332)
Pig	+(328)	+(328)	-(331)	+(332)
Calf	+(329)	+(329)	NM	+(329)

PP, pancreatic polypeptide.

+, stimulation, -, inhibition, NM, not measured.

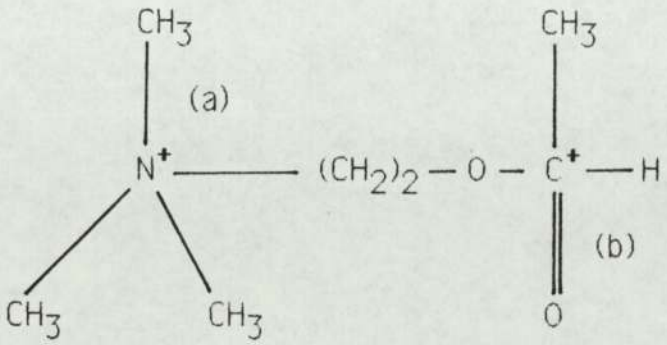
Insulin and glucagon release in response to vagal nerve stimulation is inhibited by atropine in dogs and calves (327,329) suggesting muscarinic receptor stimulation. Vagal induced somatostatin release in the dog is only partly blocked by atropine (327), suggesting some non- muscarinic actions of acetly choline. In the pig (332), dog (327) and calf (329) vagal induced pancreatic polypeptide secretion is also inhibited by atropine. Direct

stimulation by the cholinergic agents acetylcholine (333), carbamyl choline (333), methacholine (334) and carbachol (335) causes a marked increase in insulin release both in vivo and in vitro. Intrapancreatic infusion of acetyl choline also stimulates the release of glucagon, somatostatin and pancreatic polypeptide (336).

The cholinergic receptor.

Early studies of the biological actions of acetylcholine and its congeners on the clam heart (Venus mercinaria) has lead to the current understanding of cholinergic receptor structure (337). The binding of acetyl choline (whose structure is illustrated below in Figure 6.2,) to the cholinergic receptor is believed to occur at two distinct sites, the cationic nitrogen species binds electrostatically with an anionic cavity in the receptor (a) and the aldehyde group may either hydrogen bond or interact with electron donor groups at the receptor surface (b) [338].

Figure 6.2 Structure of acetylcholine.



The main antimuscarinic agent, atropine, an alkaloid of the deadly nightshade (Atropa belladonna) exhibits sufficient similarity with acetylcholine, i.e., it contains a cationic nitrogen group and an aldehydic group separated by a carbon chain, to interact directly with the cholinergic receptor blocking any subsequent binding and actions of acetylcholine. Islet cells clearly possess muscarinic receptors since L-[N-methyl-^{3}H] scopolamine, a specific muscarinic agonist, will bind to isolated islets (339,340) and to islet A-and B-cells (341). The binding of L-[N-methyl-^{3}H] scopolamine to isolated islets is inhibited by the cholinergic agonist carbamylcholine (340). Acetylcholine may also interact with nicotinic receptors (342), however, no such receptors have been identified on pancreatic B-cells. Nicotinic receptors do occur in the intrapancreatic ganglia where nicotinic actions of acetylcholine may stimulate insulin release (343).

Adrenergic modulation of islet hormone secretion.

Stimulation of the splanchnic nerve (or mixed pancreatic nerve after atropine pretreatment) inhibits insulin release in dogs (344), and calves (345) and glucagon release in dogs (344), calves (345) and pigs (328). Splanchnic nerve stimulation inhibits somatostatin and pancreatic polypeptide release in the dog (346), but produces an increase in pancreatic polypeptide secretion in the calf (347). The addition of exogenous adrenaline has been shown to inhibit insulin release from pieces of rat pancreas (333), isolated rat islets (348) and the perfused rat pancreas (349). Adrenaline also inhibits somatostatin release from isolated islets (350). In contrast to splanchnic nerve stimulation, exogenous adrenaline stimulates glucagon secretion from the perfused pancreas (351).

Adrenoreceptors and insulin release.

Ahlquist first described the existence of two types of adrenoreceptors, which he designated alpha and beta (352). Lands et.al. showed that two subclasses of beta receptors exist, $beta_1$ and $beta_2$ (353). Subsequent work by Drew et.al. and Hoffman and Lefkowitz demonstrated the existence of $alpha_1$ and $alpha_2$ subclasses of the classical alpha-adrenoreceptor (354,355). On the pancreatic B-cell adrenaline exerts its inhibitory action upon insulin release via direct activation of alpha-adrenoreceptors, this mode of action is suggested by the following evidence. Adrenaline induced inhibition of insulin release is abolished by alpha receptor blockade with phenoxybenzamine and phentolamine but not by beta receptor blockade with propranolol or nethalide (333). The alpha adrenergic agonist clonidine inhibits insulin release, and the inhibition is abolished by phentolamine (356). Nakaki et.al. have demonstrated that adrenaline acts specifically via $alpha_2$ adrenoreceptors on the basis that yohimbine, an $alpha_2$ receptor antagonist abolished adrenaline induced inhibition of insulin release, while prazosin, an $alpha_1$ receptor antagonist was ineffective (357). Malaisse and Moratinos have confirmed that the pancreatic B-cell is virtually devoid of $alpha_1$ adrenoreceptors (358). A physiological role for beta receptor activation in the regulation of insulin release is difficult to ascribe, certainly the binding of the beta adrenergic ligands [^{3}H] dihydroalprenolol and [^{125}I] cyanoiodopindolol would suggest that pancreatic islets possess beta adrenoreceptors (91). Any stimulation of insulin release by beta receptor activation is only possible when simultaneous alpha blockade is imposed (91,359), indicating the relative efficacies of the two receptor populations.

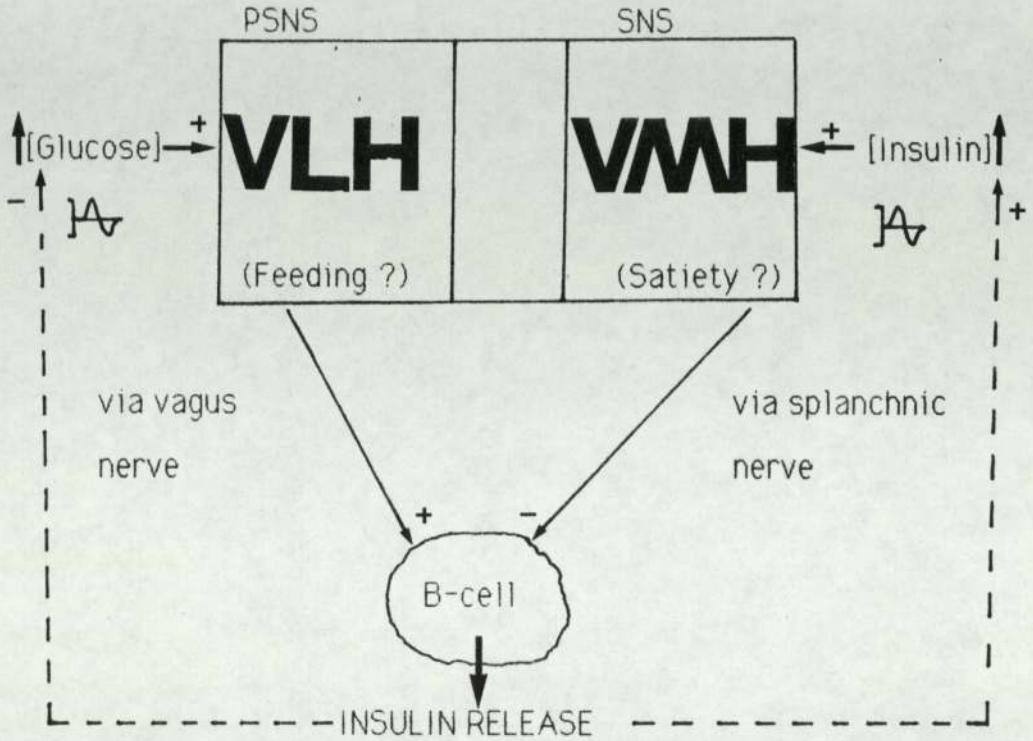
Central nervous system regulation of insulin release.

In 1849 Claude Bernard demonstrated that puncturing the floor of the fourth ventricle in dogs resulted in hyperglycaemia and glycosuria (360). This study provided the first evidence for central nervous system (CNS) control of glucose homeostasis. There is evidence for the presence of glucose sensitive (361,362) and insulin sensitive (363,364) areas within the hypothalamus. Intravenous glucose infusion increases electrical activity in the ventromedial hypothalamus (VMH) and decreases electrical activity in the ventrolateral hypothalamus (VLH), while insulin infusion has the opposite effects (365). Frohman and Bernardis have showed that electrical stimulation of the VMH results in hyperglycaemia, increased glucagon and decreased insulin levels (366). It has been suggested that the VLH and the VMH may be the parasympathetic and sympathetic centres involved in the control of glucose homeostasis respectively (367). Possible interrelationships which might exist between the VLH and the VMH in the control of insulin release are illustrated in Figure 6.3.

The role of neuropeptides in the control of insulin release.

Many peptides have been identified in pancreatic nerves, where they presumably act as as neurally controlled regulators of pancreatic islet function. Ahren et.al. have suggested five criteria for the identification of neuropeptides active in the endocrine pancreas (368), Table 6.2.

Figure 6.3 Interrelationships between the ventromedial (VMH) and ventrolateral (VLH) hypothalamus in the control of insulin release.



Key; PSNS, parasympathetic nervous system.

SNS, sympathetic nervous system.

+, stimulation.

-, inhibition.

Table 6.2 Criteria for the identification of pancreatic neuropeptides (368).

Criterion 1	Pancreatic localisation
Criterion 2	Pancreatic release during neural stimulation
Criterion 3	Ruling out classical neurotransmitters
Criterion 4	Effects on islet hormone secretion
Criterion 5	Neural effects inhibited by specific neuropeptide antagonists.

Ahren et.al. has recently described seven neuropeptides; vasoactive intestinal polypeptide (VIP), cholecystokinin (CCK), gastrin releasing peptide (GRP), galanin, neuropeptide-Y (NPY), calcitonin gene-related peptide (CGRP), substance-P and enkephalin (368). These presumptive neuropeptides satisfy criteria 1,2 or 4 as illustrated in Table 6.3 and these three criteria represent the mainstay of neuropeptide research to date. In order to rule out classical neurotransmitters (criterion 3) any change in islet hormone secretion during autonomic nervous system blockade will need to be monitored and fulfillment of criterion 5 will depend upon the subsequent development of specific neurpoeptide antagonists. In general terms, galanin, NPY, and CGRP are thought to be inhibitory and VIP, CCK, and GRP are thougnt to be stimulatory neuropeptides. The physiological significance of these opposing actions remains to be determined.

Table 6.3 Identification of the presumptive neuropeptides which satisfy the criteria 1, 2 and 4 proposed by Ahren et.al.(368) in Table 6.2.

Peptide	Criterion		
	1/ Pancreatic localisation	2/ Release during neural stimulation	4/ Effect on insulin release
VIP	+(369)	+(376)	+(378)
CCK	+(370)		+(370)
GRP	+(371)	+(377)	+(379)
Galanin	+(372)		-(372)
NPY	+(373)		-(373)
CGRP	+(374)		-(374)
Substance-P	+(375)		+/-(380,335)
Enkephalin	+(375)		+/-(381)

Examination of the literature, therefore, provides four possible levels for the neural control of insulin release involving the CNS, cholinergic, adrenergic and peptidergic axes. The present work examines two of these axes, cholinergic and adrenergic. The effects of acetylcholine the PSNS neurotransmitter have been studied upon the static and dynamic release of insulin from HIT-T15 and RINm5F cells together with studies using the muscarinic blocker, atropine and the nicotinic blocker hexamethonium (to delineate muscarinic and nicotinic effects). Any involvement of Ca^{2+} entry and cAMP metabolism have been evaluated using verapamil and imidazole. The effect of adrenaline, the SNS neurotransmitter has been examined on basal and 7.5 mmol/l glucose stimulated insulin release and 7.5mmol/l glucose stimulated glucose oxidation by HIT-T15 cells.

RESULTS

The effect of acetylcholine on insulin and C-peptide release from statically incubated HIT-T15 and RINm5F cells.

In the absence of glucose acetylcholine stimulated insulin release from statically incubated HIT-T15, Figure 6.4 and RINm5F cells, Figure 6.5. Maximum and half maximum stimulation occurred at 1000 and 8.9umol/l for HIT-T15 and 100 and 4.2umol/l for RINm5F cells. Acetylcholine also stimulated the release of C-peptide from HIT-T15 cells, maximum and half maximum stimulation occurring at 1000 and 19.9umol/l respectively, Figure 6.6. C-peptide values from HIT-T15 cells were considerably lower than the corresponding insulin values, possibly due to the poor crossreactivity of hamster and human C-peptides in the C-peptide radioimmunoassay. Although the release of C-peptide and insulin were not equimolar, there was a strong positive correlation between their respective release rates ($p<0.05$), Figure 6.7.

The effect of acetylcholine on glucose stimulated insulin release from statically incubated HIT-T15 and RINm5F cells.

Raising the glucose concentration to 7.5mmol/l significantly stimulated the release of insulin from HIT-T15 cells. Interestingly, this batch of RINm5F cells (passage 93) also released insulin in response to 7.5mmol/l glucose. The insulin response of RINm5F cells was some six fold lower than the corresponding insulin response of HIT-T15 cells. 1mmol/l acetylcholine generated a stimulation of insulin release that was equivalent in magnitude to that generated by 7.5mmol/l glucose from both cell lines. In addition 1mmol/l acetylcholine potentiated 7.5mmol/l glucose induced insulin release from HIT-T15 but not RINm5F cells, Table 6.4.

Figure 6.4 The effect of acetylcholine upon insulin release from statically incubated HIT-T15 cells.

Values are means ± SEM with n in parentheses.

*, $p<0.05$ significantly increased compared with basal.

(Basal release was 54.53 ± 5.29uU/10^5cells/90mins [n=13])

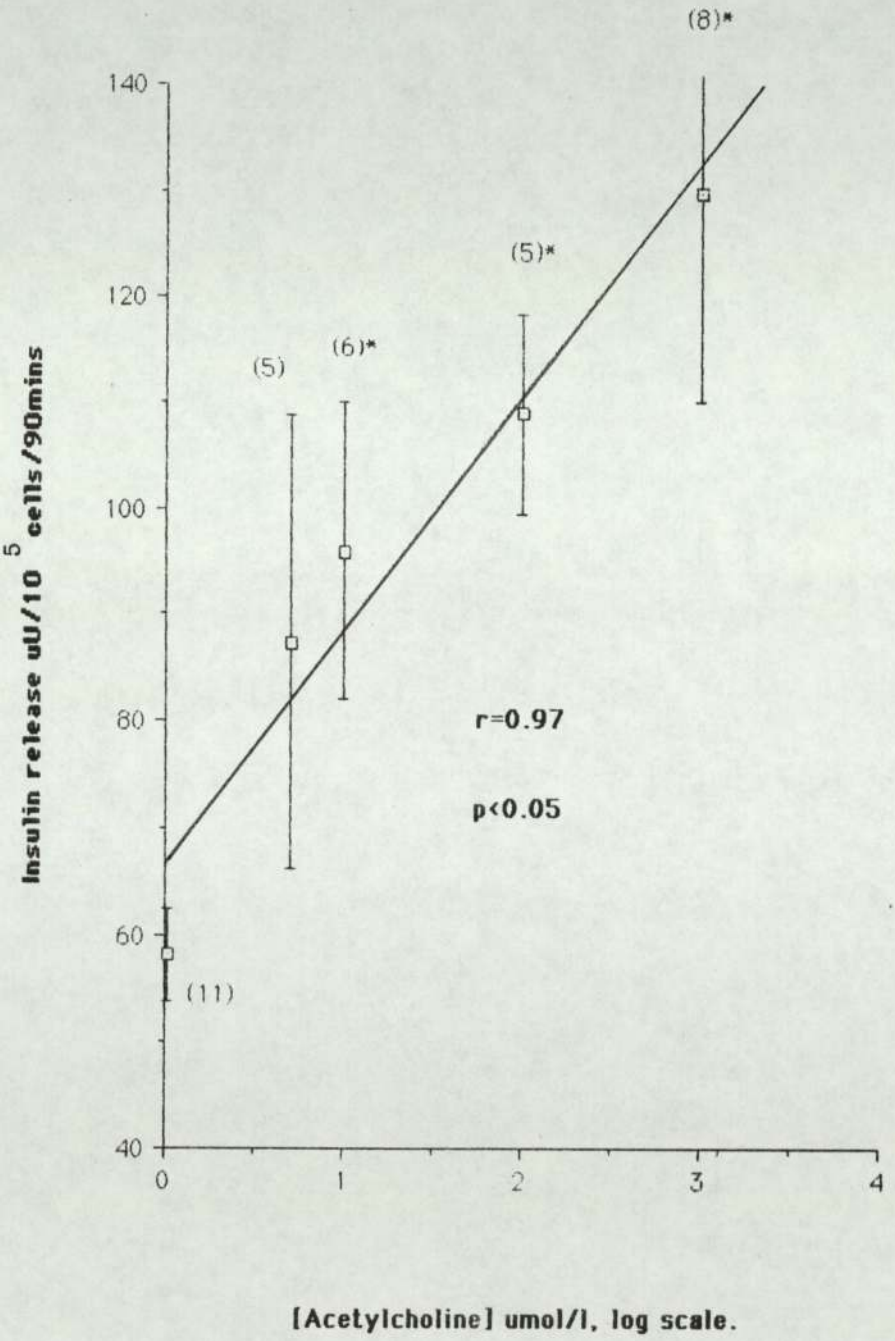


Figure 6.5 The effect of acetylcholine upon insulin release from statically incubated RINm5F cells.

Values are means ± SEM with n in parentheses

*, $p<0.05$ significantly increased compared with basal.

(Basal release was 16.38 ± 0.70uU/10^5cells/90mins [n=22])

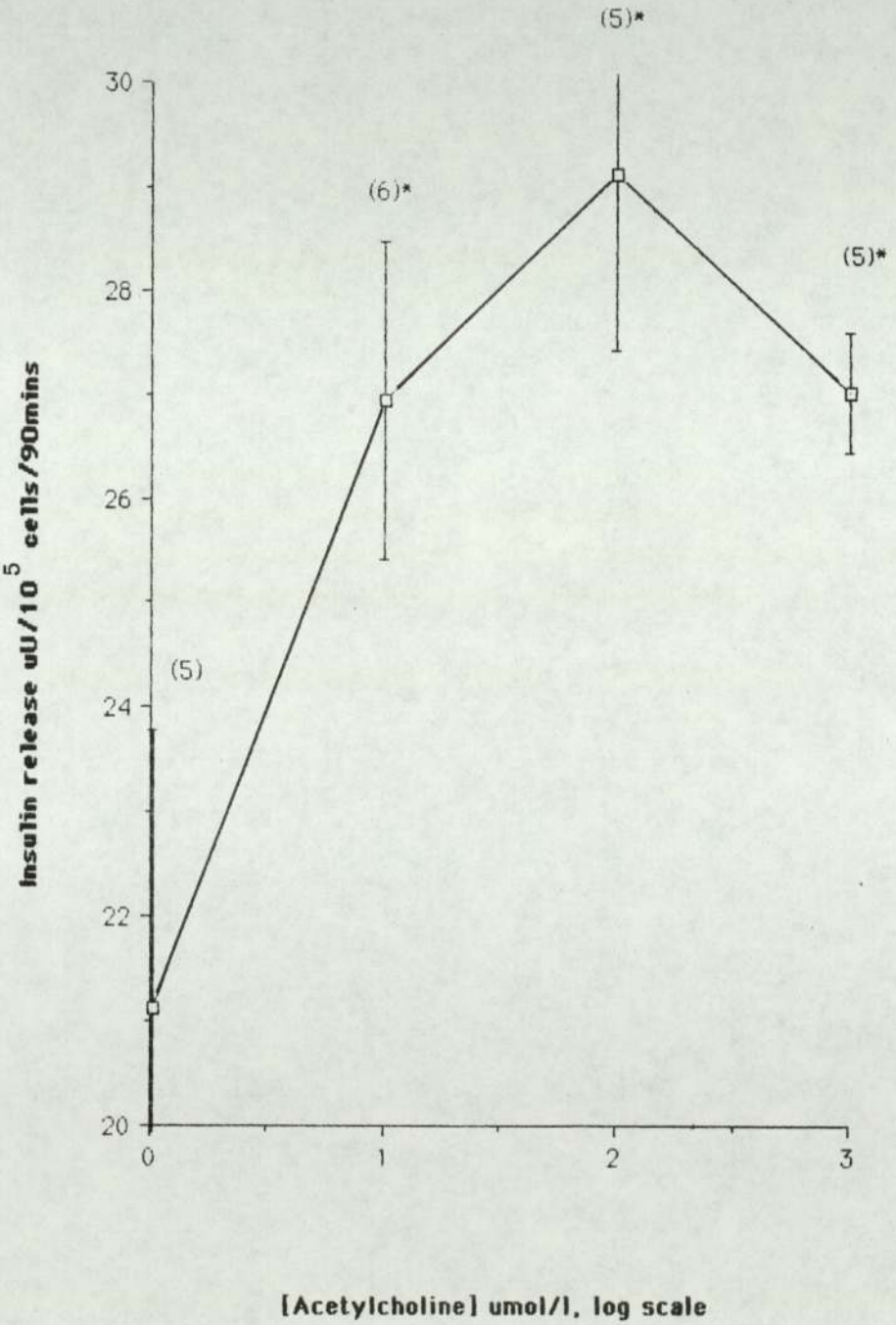


Figure 6.6 The effect of acetylcholine upon C-peptide release from statically incubated HIT-T15 cells.

Values are means ± SEM with n in parentheses.

*, $p<0.05$ significantly increased compared with basal.

(Basal release was 0.022 ± 0.002pmol/10^5cells/90mins [n=5])

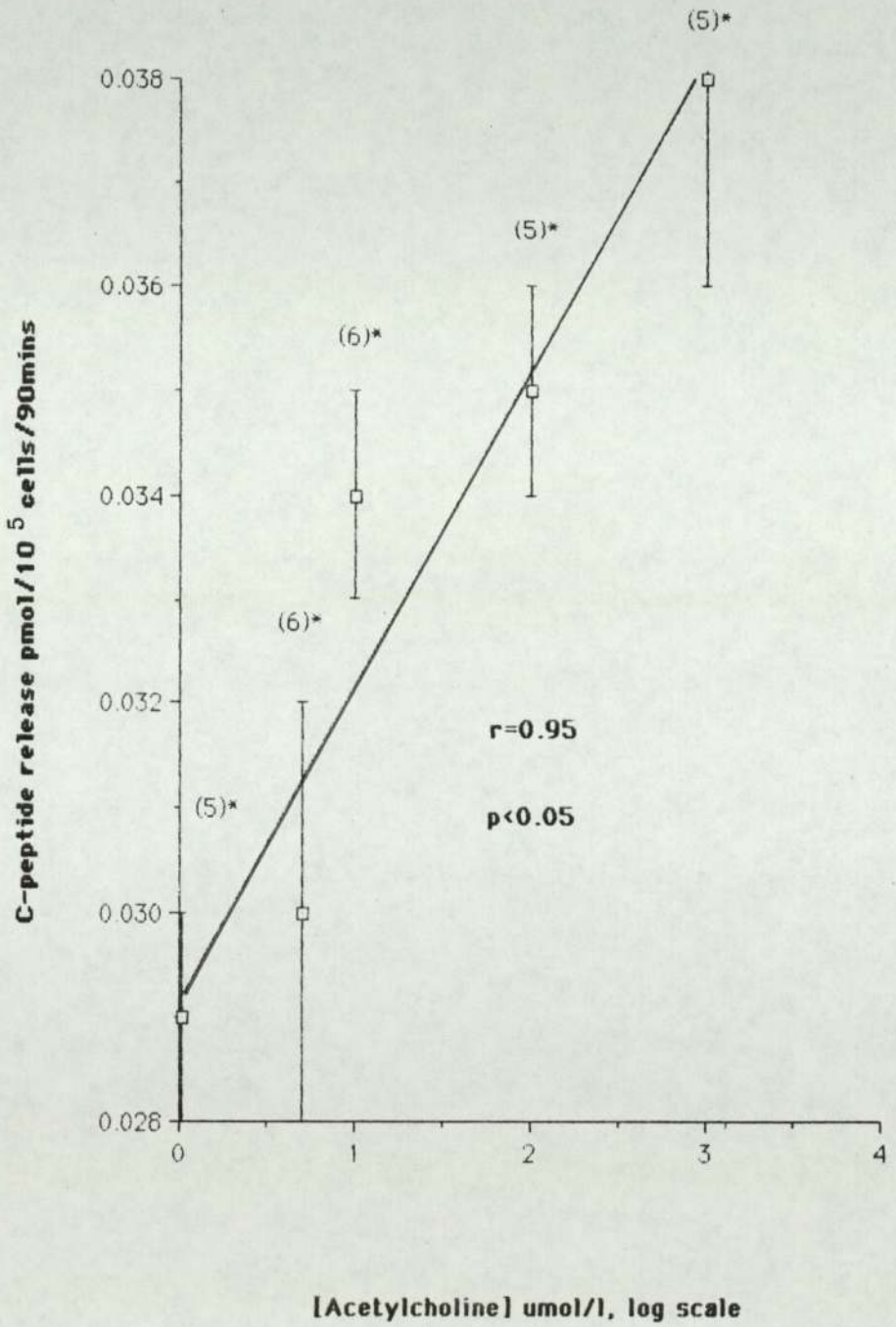


Figure 6.7 Positive correlation between C-peptide and insulin release from statically incubated HIT-T15 cells challenged with acetylcholine, the concentrations of which are given in parentheses. Data are mean values.

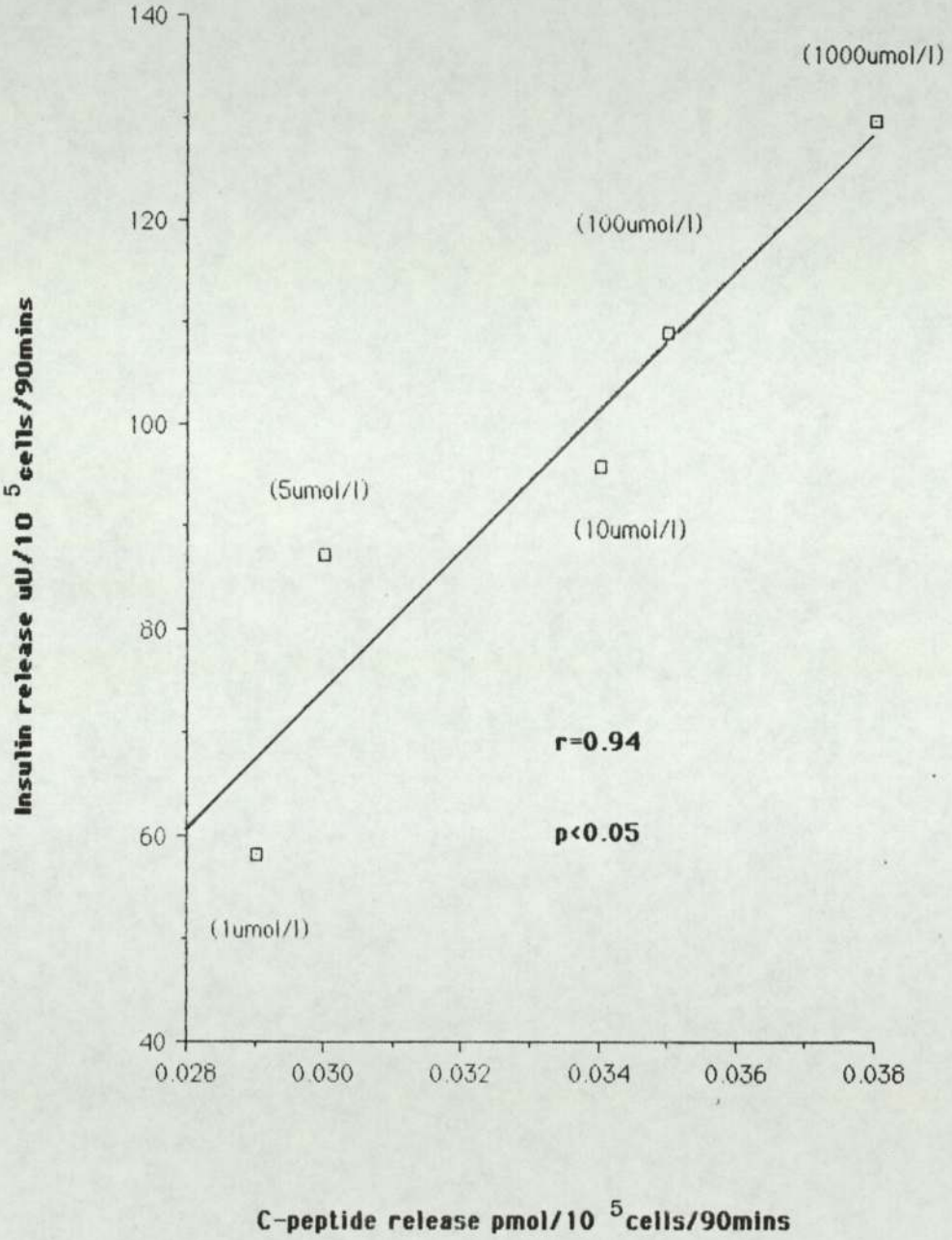


Table 6.4 The effect of acetylcholine on glucose stimulated insulin release from statically incubated HIT-T15 and RINm5F cells.

Treatment	Insulin release uU/10^5cells/90mins			
	n	HIT-T15	n	RINm5F
Basal	13	54.53 ± 5.29	22	16.38 ± 0.70
Glucose, 7.5mmol/l	14	124.80 ± 12.20[a]	9	22.62 ± 2.86[a]
Acetylcholine, 1mmol/l	8	129.70 ± 19.77[a]	5	27.00 ± 0.59[a]
Glucose + Acetylcholine	6	175.76 ± 16.62[b]	5	30.76 ± 5.10

a, $p<0.05$ significantly increased compared with basal.

b, $p<0.05$ significantly increased compared with 7.5mmol/l glucose.

The effect of atropine, hexamethonium, verapamil and imidazole upon acetylcholine induced insulin release from statically incubated HIT-T15 and RINm5F cells.

Acetylcholine, 1mmol/l, induced insulin release from HIT-T15 and RINm5F cells was inhibited by some 32.7 and 33.1% respectively using 1mmol/l atropine, a muscarinic receptor antagonist. The nicotinic receptor antagonist, hexamethonium, at a concentration of 1mmol/l had no effect upon acetylcholine induced insulin release from either cell line. Acetylcholine induced insulin release from HIT-T15 and RINm5F cells was also inhibited by the calcium channel blocker verapamil (15umol/l) and the cAMP-dependent phosphodiesterase activator imidazole (0.1mmol/l), Table 6.5.

Table 6.5 The effect of atropine, hexamethonium, verapamil and imidazole upon acetylcholine induced insulin release from statically incubated HIT-T15 and RINm5F cells.

Treatment	Insulin release uU/10^5cells/90mins			
	n	HIT-T15	n	RINm5F
Acetylcholine, 1mmol/l	8	129.70 ± 19.77[a]	5	27.00 ± 0.59[a]
+Atropine, 1mmol/l	6	86.82 ± 10.88[b]	6	18.06 ± 0.77[b]
+Hexamethonium, 1mmol/l	6	121.26 ± 4.04	5	25.73 ± 2.73
+Verapamil, 15umol/l	6	88.88 ± 3.72[b]	4	20.06 ± 2.03[b]
+Imidazole, 0.1mmol/l	6	77.92 ± 5.11[b]	5	18.31 ± 1.65[b]

a, $p<0.05$ significantly increased compared with basal.

b, $p<0.05$ significantly reduced compared with 1mmol/l acetylcholine

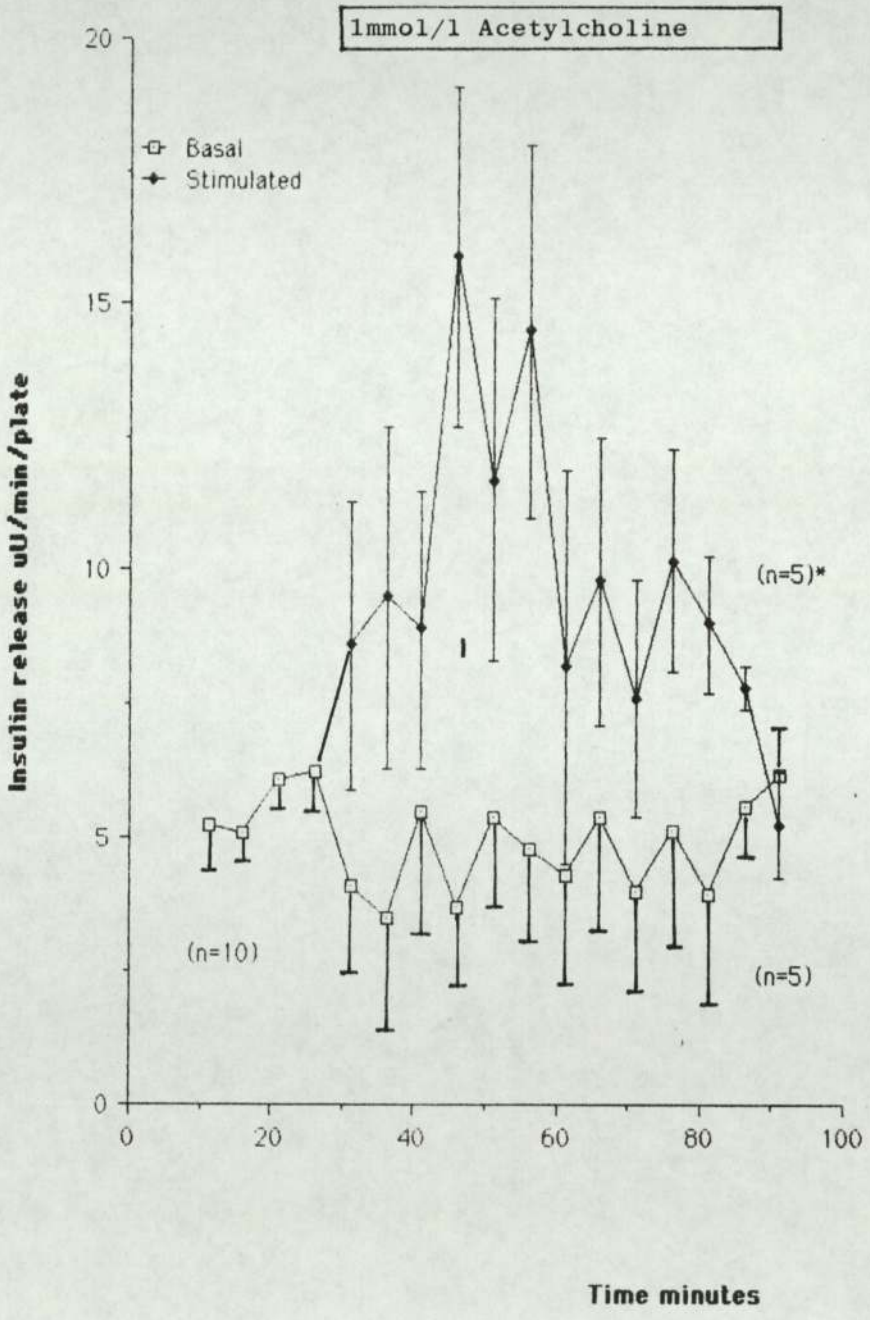
Dynamics of acetylcholine stimulated insulin release from perifused HIT-T15 and RINm5F cells.

The dynamic release of insulin from perifused HIT-T15 cells in response to 1mmol/l acetylcholine produced a monophasic release pattern which reached a maximum of 15.9 ± 3.2 uU/min/plate 15 minutes after the addition of 1mmol/l acetylcholine. The total area under the mean acetylcholine stimulated profile was 584.5 ± 61.1 uU/60mins/plate (n=5) compared with a total area of 281.8 + 17.1 uU/60mins/plate (n=5) obtained in the absence of glucose, $p<0.05$, Figure 6.8.

Figure 6.8 The effect of acetylcholine (1mmol/l) upon insulin release from perifused HIT-T15 cells.

Values are means ± SEM with n in parentheses.

*, $p<0.05$ significantly increased compared with basal (absence of acetylcholine), when analysing the areas under the secretory profiles.



Perifusion of RINm5F cells revealed a more modest (but of the same order of magnitude) multiphasic pattern of insulin release. The total area under the insulin release profile in response to 1mmol/l acetyl choline ammounted to 372.9 ± 20.5 uU/60mins/plate (n=4) and was significantly increased, $p<0.05$ compared with the total area of 264.3 ± 33.4 uU/60mins/plate (n=4) obtained in the absence of acetylcholine, Figure 6.9

The effect of adrenaline upon basal and 7.5mmol/l glucose stimulated insulin release from statically incubated HIT-T15 cells.

Adrenaline at concentrations up to 100umol/l caused a marginal but non-significant reduction in basal insulin release, Table 6.6.

Table 6.6 The effect of adrenaline upon basal insulin release from statically incubated HIT-T15 cells.

[Adrenaline] umol/l	n	Insulin release % from basal
1	4	-19.80 ± 7.38
10	4	-21.24 ± 4.46
100	4	-23.44 ± 6.75
1000	4	+45.93 ± 21.52

On the other hand, insulin release from HIT-T15 cells in response to 7.5mmol/l glucose was significantly reduced by adrenaline at all concentrations ranging from 1-1000umol/l, Figure 6.10.

Figure 6.9 The effect of acetylcholine (1mmol/l) upon insulin release from perifused RINm5F cells.

Values are means ± SEM with n in parentheses.

*, $p<0.05$ significantly increased compared with basal (absence of acetylcholine), when analysing the areas under the secretory profiles.

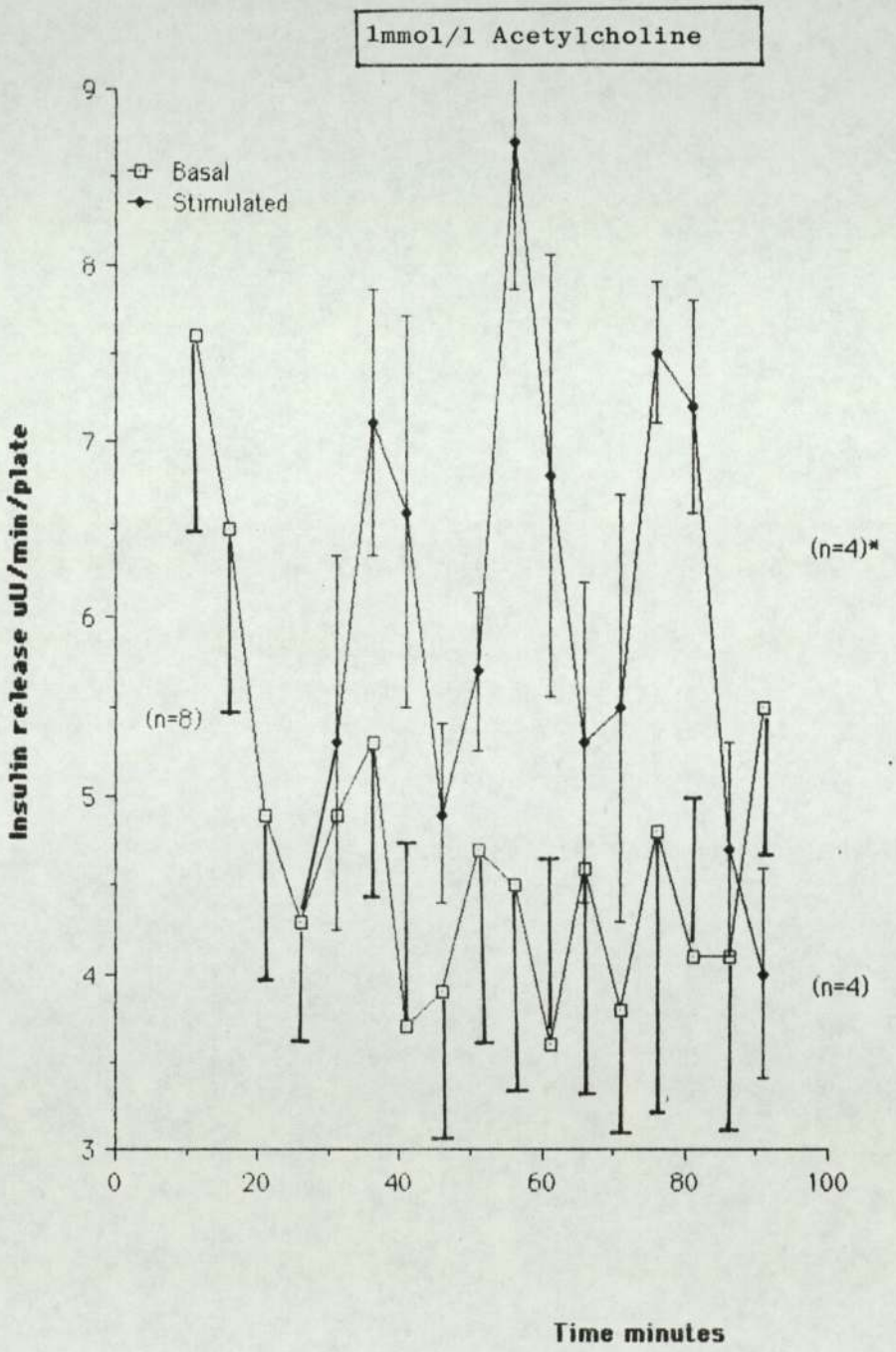
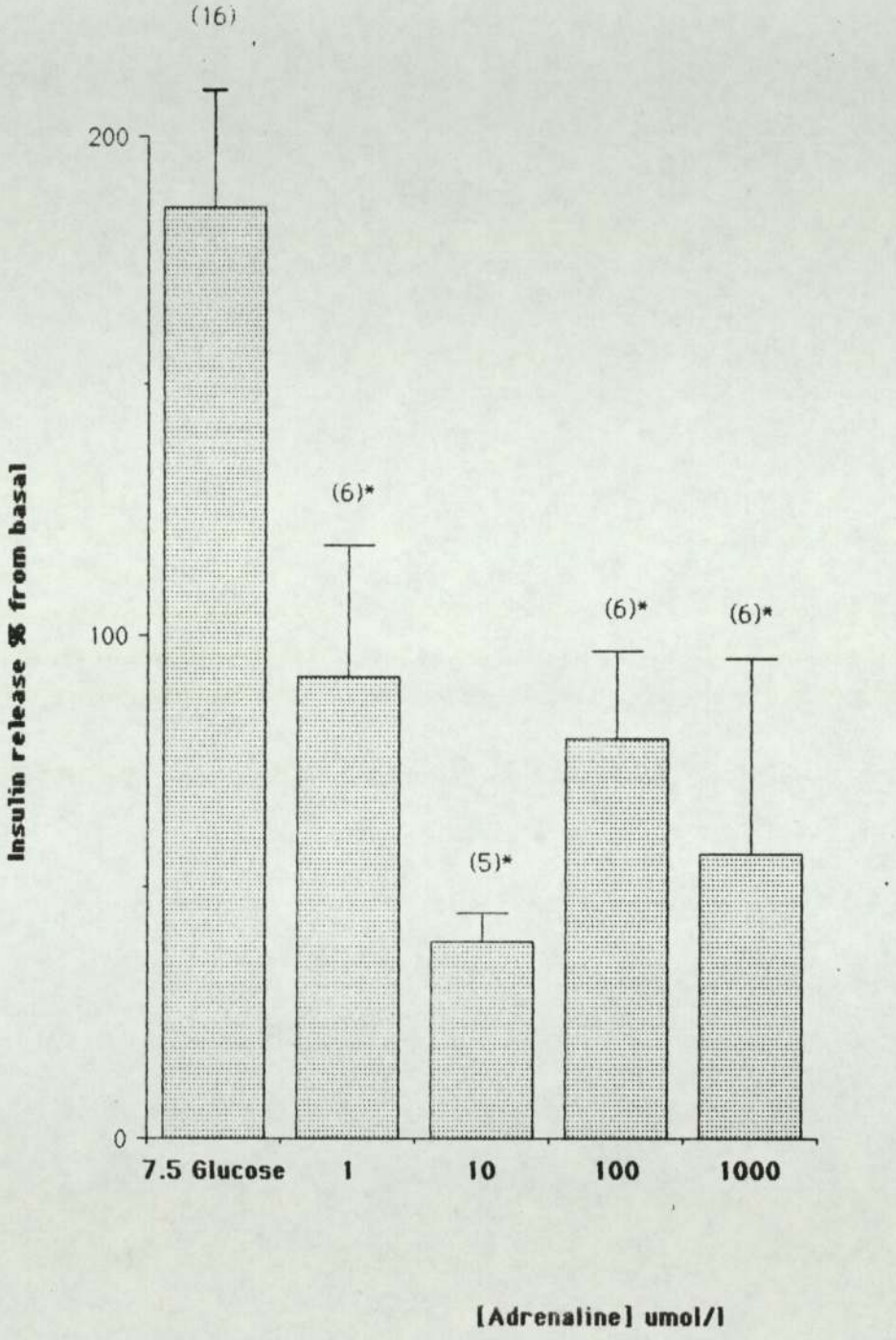


Figure 6.10 The effect of adrenaline upon glucose (7.5mmol/l) induced insulin release from statically incubated HIT-T15 cells.

Values are means ± SEM with n in parentheses.

*, $p<0.05$ significantly reduced compared with 7.5mmol/l glucose.



The effect of adrenaline upon the rate of D-[U^{14}C] glucose oxidation by HIT-T15 cells.

Raising the glucose concentration from 2 to 7.5mmol/l produced a marked and significant ($p<0.05$) increase in the rate of D-[U^{14}C] glucose oxidation. The rate of glucose oxidation in the presence of 7.5mmol/l glucose was significantly ($p<0.05$) inhibited (50.8%) by adrenaline at a concentration of 1mmol/l, Table 6.7.

Table 6.7 The effect of adrenaline on the rate of D-[U^{14}C]glucose oxidation by HIT-T15 cells.

Treatment	n	Rate of glucose oxidation umol/10^5cells/90mins
Glucose, 2mmol/l	11	4.81 ± 0.45
Glucose, 7.5mmol/l	5	17.74 ± 1.76[a]
Glucose, 7.5mmol/l + Adrenaline, 1mmol/l	9	9.02 ± 1.80[b]

a, $p<0.05$ significantly increased compared with 2mmol/l glucose.

b, $p<0.05$ significantly reduced compared with 7.5mmol/l glucose.

DISCUSSIONS

The stimulation of insulin release from cultured HIT-T15 and RINm5F cells by acetylcholine is consistent with the observed effects of this agent on isolated islets (382). However, previous studies using islets have suggested that cholinergic agents do not stimulate insulin release in the absence of glucose (333,382). The glucose requirement for acetylcholine stimulated net insulin release from islets may be a feature of the paracrine interaction between islet cells (Chapter 4, page 116), glucose being a potent stimulator of somatostatin release, and an inhibitor of glucagon and pancreatic polypeptide release (199). This paracrine interaction is not imposed on cultured HIT-T15 and RINm5F cells. A potentiating effect of acetylcholine upon glucose stimulated insulin release from HIT-T15 cells *in vitro* has been reported by Ashcroft *et.al.* (167). The potentiating effect of acetylcholine upon insulin release *in vivo* is believed to be the result of a combination of vagal activity, initiated by the sight, smell and anticipation of food and a postprandial elevation in blood glucose (323). This combined effect makes rapid and efficient use of absorbed nutrients.

Since acetylcholine has been shown to stimulate glucagon release (327,336) it could be argued that acetylcholine stimulates insulin release via the release of glucagon from adjacent A-cells in the islet. In the present work (Chapter 4, page 116) glucagon was found to be a potent stimulator of insulin release from both HIT-T15 and RINm5F cells. In these studies acetylcholine stimulated insulin release in the absence of glucagon secreting A-cells, although RINm5F cells have been shown to secrete minute amounts of

glucagon (58). This observation would suggest that glucagon is not a necessary prerequisite for the acetylcholine stimulation of insulin release from B-cells, and confirms our previous findings for L-arginine (Chapter 3, page 82) and GIP (Chapter 4, page 116).

The monophasic pattern of insulin release from HIT-T15 cells observed in response to acetylcholine (in the absence of glucose) was consistent with the observations of Kimura et.al. using the perfused rat pancreas (383). This sustained monophasic release pattern confirmed the presence of a substantial readily available insulin secretory granule pool in HIT-T15 cells. The much reduced multiphasic insulin release profile observed with RINm5F cells could concievably suggest a failure of the normal synthesis and margination of B-cell granules, although ultrastructural studies would be required to confirm this.

Acetylcholine mediated insulin release from both HIT-T15 and RINm5F cells clearly involves muscarinic receptor activation. This was confirmed by the inhibitory effect of atropine, a muscarinic receptor antagonist and the absence of an effect for hexamethonium, a nicotinic receptor antagonist on acetylcholine stimulated insulin release.

The stimulation of insulin release from HIT-T15 and RINm5F cells by acetyl choline appears to be dependent upon calcium uptake, in so far as verapamil, a calcium channel blocker (305,306) inhibited insulin release. This observation is consistent with the calcium dependency of acetylcholine induced insulin release reported by Wollheim and Biden using RINm5F cells (384). The inhibitory effect of imidazole, a cAMP dependent phosphodiesterase activator (298-300) upon acetyl choline induced insulin release

from both HIT-T15 and RINm5F cells is more difficult to explain. Especially since acetylcholine does not appear to increase the intracellular concentrations of cAMP in isolated islets of Langerhans (385). In addiyion acetylcholine does not increase the intracellular concentration of cGMP (385). It is conceivable that imidazole may interfere with phospholipid turnover in the B-cell membrane and there is much evidence to suggest that acetylcholine induced insulin release is mediated by increased phospholipid turnover (386). Occupation of muscarinic receptors has been shown to result in the activation of phospholipase-C (387) and to increase the influx of extracellular calcium. (388). Increased turnover of phospholipids (386) ultimately leads to an increase in the concentrations of diacylglycerol and IP_3 (269,386). Increased concentrations of diacylglycerol would activate protein kinase-C and stimulate insulin release (386,389). The increase in IP_3 induced calcium mobilisation brought about by cholinergic agents may not be sufficient to stimulate insulin release, since acetyl choline induced insulin release is dependent upon extracellular calcium (382).

Adrenaline has been reported to inhibit 10mmol/l glucose induced insulin release from HIT-T15 cells [result of a single experiment] (167) and 10mmol/l alanine induced insulin release from RINm5F cells (390). RINm5F cells have been shown to possess both $alpha_1$ and $alpha_2$-adrenoreceptors (390), and the usual cellular response to the activation of $alpha_2$ receptors is the inhibition of plasma membrane adenylate cyclase and a decrease in the intracellular concentration of cAMP (391). Certainly using isolated islets, $alpha_2$ receptor activation with clonidine inhibited adenylate cyclase and the production of cAMP (392). Adrenaline also

inhibits potassium induced insulin release from RINm5F cells a process which presumably involves the hyperpolarisation of the B-cell membrane (58). Adrenergic inhibition of insulin release has also been shown to alter calcium fluxes in the B-cell, certainly adrenaline inhibited glucose induced calcium entry (393) and reduced calcium retention (394).

The results of the present studies suggests that the adrenergic inhibition of glucose stimulated insulin release from HIT-T15 cells may be the result of an inhibition of glucose oxidation, in so far as adrenaline inhibited $^{14}CO_2$ production from D-[U^{14}C] glucose. This inhibition of glucose oxidation would reduce the capacity to manufacture reducing equivalents and ATP to fire essential parts of the secretory machinery, such as the B-cell Na^+/K^+-ATPase and the Ca^{2+}-ATPase, but gives no information as to how adrenoreceptor occupancy is linked to the inhibition of glucose metabolism.

CHAPTER 7.

7. INSULIN RELEASE FROM CULTURED IN111 R1 AND UMR 407/3 CELLS AND A HUMAN ISLET CELL ADENOMA.

INTRODUCTION.

The IN111 R1 cell line and its derivation.

The induction of insulinomas by BK virus (395) infection in Syrian hamsters has been well documented. Uchida et.al. demonstrated that after intracranial injection of BK virus, 47% of animals developed malignant pancreatic insulinomas (396). Corallini et.al. reported that intracranial or intraperitoneal injection of BK virus resulted in the production of pancreatic islet cell tumours, however, the success rate of 12% (397) was lower than that reported by Uchida et.al. (396). These tumours were shown histologically to contain insulin, C-peptide, glucagon, calcitonin and gastrin immunoreactivity (397).

In an attempt to confirm their earlier findings Uchida et.al. repeated the BK virus infection studies. Intraperitoneal, intracranial or subcutaneous injection of BK virus resulted in the production of malignant insulinomas (usually with hepatic metasteses) in up to 92% of animals. One of these tumours IN111 was serially transplanted in vivo. After 2 to 5 weeks, tumours measuring 1 to 3 cm in diameter could be identified. These tumours were associated with blood glucose levels as low as 0.4mmol/l and serum insulin levels as high as 320uU/ml (54).

The IN111 tumour maintained in vitro was composed of two morphologically different cell types, round and epithelial cells. These cell types were subsequently cloned to yield IN111 R1 and IN111 E4 cell lines. Insulin release and the development of

insulinomas in vivo was only associated with the round IN111 R1 cells (54). During continuous passage, the characteristic round cells may subsequently be replaced with epithelial cells which no longer produce insulin and instead switch to the production of glucagon (Dr S. Watanabe, Department of Enteroviruses, NIH, Tokyo, Japan, Personal communication). Despite the potential for insulin production and the ability to be continuously passaged this cell line has been little used and is seldom quoted in the literature.

The UMR 407/3 cell line and its derivation.

In 1987 Ng et.al. described the production and insulin release from from a cloned precursor beta cell line derived from isolated 1 day old neonatal rat islets (63). Freshly isolated islets were cultured for 2 days as loose monolayers, after which time they were gently removed with a pasteur pipette and subsequently cultured in double nutrient agar for 2 weeks. After this time the remaining individual islets were picked out and cultured as monolayers. Cells that subsequently grew from these islets were used as the primary cell cultures. These cells were passaged 4 times over the subsequent 6 months and then cloned by limiting dilution on rat tail collagen. Eventually nine clones resulted, each one capable of growth without collagen. These clones were designated UMR 401 to 409, UMR 407 was further cloned to give three subclones UMR 407/1 to UMR 407/3. (63). The ability of these cells to form colonies in soft agar and their limited life span (up to 27 passages) suggested that these cells were likely to be progenitor or precursor B-cells (398,399). UMR 407/3 cells characteristically grow to a confluent monolayer and can be maintained for up to 27 passages after which time the cells begin to crenate and detach, such features are characteristic of the development of in vitro senescence(63).

The culture of human insulinomas

In the past adult insulinomas have proven difficult or impossible to maintain long term *in vitro* (35-41). This difficulty is accentuated by the low frequency of occurrence. Creutzfeldt *et.al.* reported the biochemical and histological findings of some 30 insulinomas collected over a seven year period from six university medical schools (34). The previous attempts to culture human insulinomas (reviewed in Chapter 1, page 10) based on the length of culture period, continued insulin release *in vitro* and glucose sensitivity have been summarised in Table 7.1.

Table 7.1 Summary of the previous attempts to culture human insulinoma tissue.

Study	Maximum culture period	Insulin release % of day 1 value	Glucose sensitivity
Murray *et.al.*(4)	5-6weeks	ND	ND
Chick *et.al.*(35)	2months	26.8% by 34d	-
Yip *et.al.*(36)	>2months	6.3% by 20d	-
Adcock *et.al.*(37)	>7months	5.0% by 50d	-
Akagi *et.al.*(41)	>10months	4.4% by 35d	ND
Saxe *et.al.*(38)	>40days	By 30d IRI had increased by 75% of 10d value	-(+)*

ND, not determined

(+)*, fresh but not cultured tissue was glucose sensitive up to 16.7mmol/l

The present work describes the initial characterisation of insulin release from INIII RI cell line, in terms of glucose sensitivity (of both insulin release and glucose oxidation), and responsiveness to a range of insulin secretagogues (glyceraldehyde, L-arginine, glibenclamide and chlorpropamide) and inhibitors (adrenaline, TFP and verapamil). In addition initial studies confirming the glucose sensitivity of the newly derived UMR 407/3 cell line together with the effect of tolbutamide on insulin release from these cells and their rate of glucose oxidation have also been included. Attempts have been made to culture a human islet cell adenoma in vitro. The tumour, one of ten identified following partial pancreatectomy was obtained at surgery from Birmingham General Hospital. A preliminary evaluation of the culture characteristics, insulin release and the capacity for cryopreservation are presented.

MATERIALS AND METHODS

Maintenance of stock cultures of IN111 R1 and UMR 407/3 cells.

Stock cultures of IN111 R1 and UMR 407/3 cells were routinely maintained in RPMI 1640 supplemented with 10% foetal calf serum, 100ug/ml streptomycin and 100IU/ml penicillin at 37^{0}C in 5% CO_2/humidified air using 250ml Costar tissue culture flasks.

Stock cultures were passaged once weekly and fed twice weekly. IN111 R1 and UMR 407/3 cells were reseeded at 4×10^6 and 2×10^6 cells per flask respectively. All experimental work was performed with passages 27 to 30 for IN111 R1 cells and passages 10 to 14 for UMR 407/3 cells. Aliquots of IN111 R1 at passage 27 and UMR 407/3 cells at passage 10 were frozen in liquid nitrogen (Chapter 2, page 38) for future use.

Insulin release from statically incubated IN111 R1 and UMR 407/3 cells

Either 2×10^5 IN111 R1 or 4×10^5 UMR 407/3 cells from freshly trypsinised stocks were transferred to each well of a 24 well Costar cluster plate containing 1ml of supplemented RPMI 1640 medium. Plates were incubated for 48 hours to allow adhesion and proliferation. After this time UMR 407/3 cells formed a confluent monolayer. Cultures of IN111 R1 and UMR 407/3 cells were then washed in Krebs/bicarbonate/HEPES buffer, pH 7.4 containing BSA, 0.2g/l (Appendix, page 228) and subsequently test incubated for 90minutes with the test agents at the appropriate concentrations. After incubation 0.2ml of the mildly agitated and centrifuged (1000rpm) supernatant was frozen at -15^{0}C for the

subsequent estimation of insulin by double antibody radioimmunoassay. The number of cells present in each well during secretory experiments was determined as described previously (Chapter 2, page 38). Insulin release was expressed as uU/10^5cells/90minutes.

Measurement of the rate of glucose oxidation by IN111 R1 and UMR 407/3 cells.

Batches of either 10^5 IN111 R1 or 10^5 UMR 407/3 cells were incubated in small plastic reaction tubes containing 100uL of Krebs/bicarbonate/HEPES buffer, pH 7.4 supplemented with BSA, 0.2g/l. The incubation medium contained either glucose (5.6 or 16.7 mmol/l) alone or in combination with a variety of test agents at the appropriate concentrations and a tracer amount of D-[U^{14}C] glucose (4uCi/ml). The incubation protocol and the calculation of the rates of glucose oxidation have been described previously (Chapter 2, page 61).

Origin and maintenance of the human insulinoma.

Clinical background.

A 21 year old caucasian male presented with an 18month history of intermittent altered consciuosness, particularly after exercise or missing meals. Diagnosis of insulinoma was confirmed by the finding of a 24hourfastedblood glucose of 1.5mmol/l with a simultaneous serum insulin concentration of49.9mU/l. Serum calcium was elevated at 2.8-3.0mmol/l with a parathyroid hormone concentration of 0.35ng/ml (normal value <0.2ng/ml), consistent with primary hyperparathyroidism and multiple endocrine adenomatosis type 1. Histology following partial pancreatectomy revealed multiple adenomata of the islets of Langerhans. This tumour is referred to hereafter as 'insulinoma'.

Insulinoma disaggregation and culture protocol.

A cross sectional segment of an excised 0.7cm diameter insulinoma was transported from the operating theatre to the laboratory and maintained overnight at 37^0C in an atmosphere of 5%CO_2/humidified air using RPMI 1640 supplemented with 10% foetal calf serum, 100ug/ml streptomycin and 100IU/ml penicillin. The following morning the insulinoma was disaggregated by vigorous shaking in 10 ml of fresh supplemented RPMI 1640 until only the fibrous capsule remained. The resultant cell suspension and any debris from the overnight culture period were pooled, centrifuged (1000rpm/3minutes) and resuspended in supplemented RPMI 1640. Prior to attempts at extended culture the cell yield, viability (trypan blue exclusion), ability to release insulin in response to glucose and insulin content were determined using a small number of cells ~7×10^5 in total.

All of the isolated cells were plated into a 250ml Costar tissue culture flask containing 20ml of supplemented RPMI 1640. Spent culture medium was replaced every two days, at which time insulin released into the medium was determined by double antibody radioimmunoassay and expressed as uUinsulin/flask/2days. Cultures were passaged at a split ratio of 1:1 on days 7 and 24 and at a ratio of 1:2 on day 34 into 50ml tissue culture flasks. On day 24 the detached cells were frozen in liquid nitrogen (Chapter 2, page 38) for a period of 1 month over the Christmas break until required.

Measurement of insulin release from human insulinoma cells in response to glucose.

10^5 freshly disaggregated insulinoma cells were incubated for 1 hour in LP3 tubes (Luckhams Ltd, UK.) containing 1ml of Krebs/bicarbonate/HEPES buffer, pH 7.4 supplemented with BSA, 0.2g/l and either 5.6 or 16.7 mmol/l glucose. After incubation the tubes were centrifuged (1000rpm) and 0.2ml of the supernatant was removed and stored at -15^0C for the subsequent estimation of insulin by double antibody radioimmunoassay. Insulin release was expressed as uU/10^5cells/hour.

RESULTS

Characteristics of IN111 R1 cells in culture.

Cultures of IN111 R1 cells were mainly composed of round cells, although a very small number of epithelial cells could be detected. The round cells tended to self associate to form diffuse islet like structures (comparable with HIT-T15 cell cultures) never reaching confluence. The IN111 R1 cells had a plating efficiency of 80.1 ± 3.4 % (n=3) and a doubling time of 24 hours (estimated based on the number of cell doublings over 48 hours). This figure compared favourably with the 30 hour doubling time obtained by Kikkawa et.al. for these cells (55).

The effect of glucose on insulin release and the rate of glucose oxidation by cultured IN111 R1 cells.

Raising the glucose concentration from 0 to 16.7mmol/l failed to significantly stimulate insulin release from statically incubated IN111 R1 cells, Figure 7.1. Raising the glucose concentration from 5.6 to 16.7mmol/l did, however, significantly increase the rate of IN111 R1 cell glucose oxidation, Table 7.2.

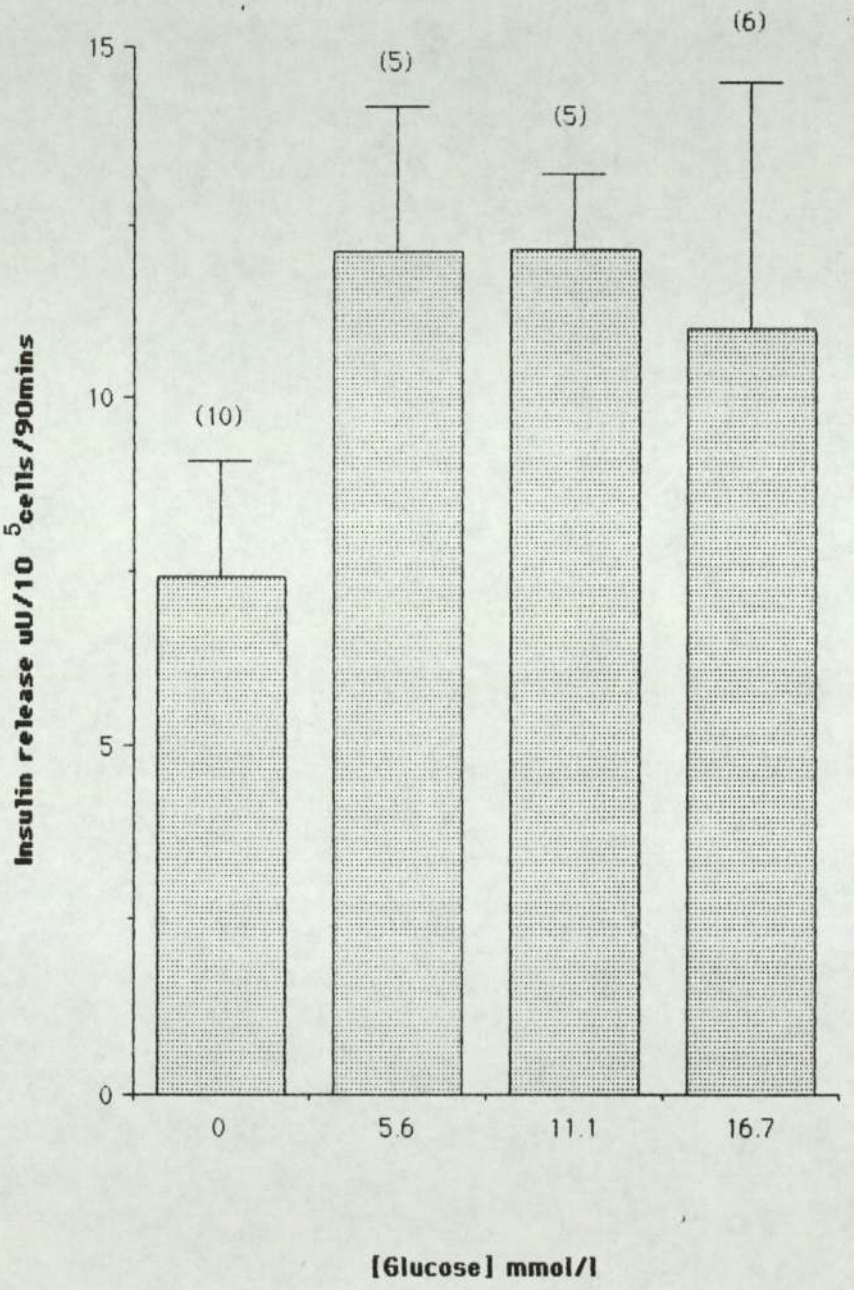
Table 7.2 The effect of glucose on the rate of glucose oxidation and insulin release from cultured IN 111 R1 cells.

[Glucose] mmol/l	n	Glucose oxidation umol/10^5cells/90min	n	Insulin release uU/10^5cells/90min
5.6	6	75.56 ± 6.11	5	12.12 ± 2.06
16.7	6	222.79 ± 12.78[a]	6	10.99 ± 3.53

a, $p<0.05$ significantly increased compared with 5.6mmol/l glucose.

Figure 7.1 The effect of glucose on insulin release from statically incubated IN111 R1 cells.

Values are means ± SEM with n in parentheses.



At passage 29 IN111 R1 cells had an insulin content of 159.2uU/10^5cells, insulin release in response to 5.6mmol/l glucose from this passage accounted for some 7.6% of the total insulin content per 90mins.

The effect of glyceraldehyde, L-arginine, glibenclamide and chlorpropamide upon insulin release from statically incubated IN111 R1 cells.

In the absence of glucose, 10mmol/l D-glyceraldehyde, 15mmol/l L-arginine, and 8umol/l glibenclamide and chlorpropamide significantly stimulated insulin release, Table 7.3. At the concentrations used in the present work glibenclamide was the most potent insulin secretagogue followed by L-arginine, chlorpropamide and glyceraldehyde.

Table 7.3 The effect of glyceraldehyde, L-arginine, glibenclamide and chlorpropamide on insulin release from statically incubated IN111 R1 cells.

Treatment	n	Insulin release uU/10^5cells/90mins
Basal (no glucose)	10	7.42 ± 1.66
Glyceraldehyde, 10mmol/l	5	12.74 ± 1.66[a]
L-arginine, 15mmol/l	5	15.19 ± 2.97[a]
Glibenclamide, 8umol/l	6	17.21 ± 3.80[a]
Chlorpropamide, 8umol/l	6	13.87 ± 1.69[a]

a, $p<0.05$ significantly increased compared with basal.

The effect of adrenaline, TFP, and verapamil upon glyceraldehyde induced insulin release from statically incubated IN111 R1 cells.

Since IN111 R1 cells do not release insulin in response to glucose glyceraldehyde was chosen as a suitable secretagogue for the subsequent use of adrenaline, verapamil and TFP. Adrenaline at a concentration of 1mmol/l, the calcium channel blocker verapamil and the calmodulin antagonist TFP, at 15umol/l, all inhibited 10mmol/l glyceraldehyde induced insulin release from statically incubated IN111 R1 cells, Figure 7.2.

Characteristics of UMR 407/3 cells in culture.

UMR 407/3 cells were epithelioid in appearance and grew to form a confluent monolayer with a plating efficiency of 94 ± 1% (n=4) and a doubling time of 38 hours (based on the number of cell doublings over 120 hours) This value was considerably lower than the value of 60 hours previously recorded by Ng et.al. (63). However, these workers maintained their cells in alpha modified MEM rather than RPMI 1640, the higher glucose concentration in RPMI (11.1mmol/l) compared with alpha modified MEM (5.6mmol/l) may have caused the increased the growth rate.

The effect of glucose on insulin release and the rate of glucose oxidation by cultured UMR 407/3 cells.

Raising the glucose concentration from 5.6 to 16.7 mmol/l significantly stimulated the rates of insulin release and glucose oxidation by cultured UMR 407/3 cells, Table 7.4. At passage 12 UMR 407/3 cells had an insulin content of 0.28uU/10^5cells and the amount of insulin released in response to 5.6mmol/l glucose accounted for 314% of the insulin content per 90mins.

Figure 7.2 The effect of adrenaline (1mmol/l), TFP (15umol/l) and verapamil (15umol/l) upon glyceraldehyde (10mmol/l) induced insulin release from statically incubated IN111 R1 cells.

Values are means ± SEM with n in parentheses.

*, p<0.05 significantly reduced compared with 10mmol/l glyceraldehyde.

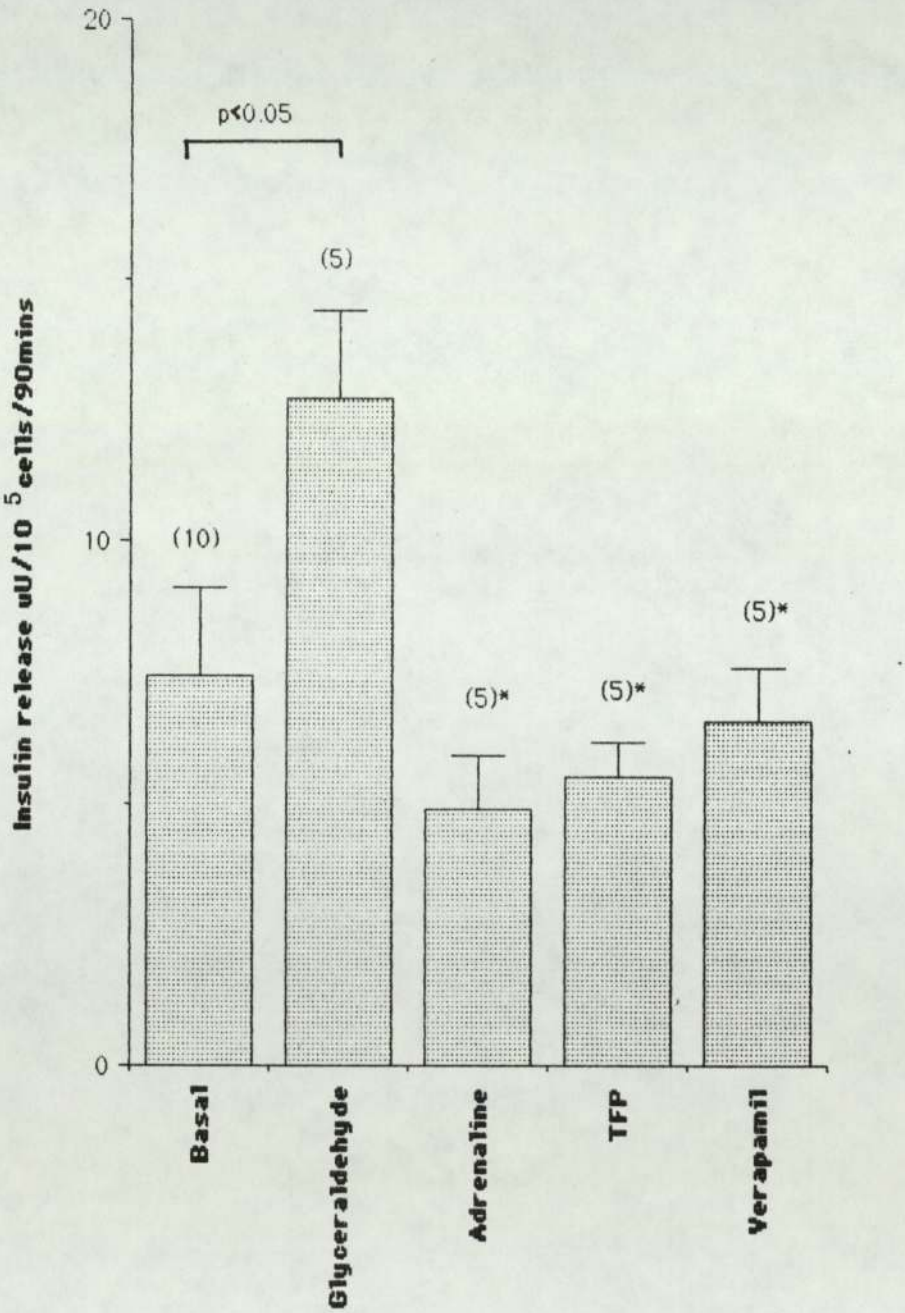


Table 7.4 The effect of glucose on insulin release and the rate of glucose oxidation by cultured UMR 407/3 cells.

[Glucose] mmol/l	n	Glucose oxidation umol/10^5cells/90mins	n	Insulin release uU/10^5cells/90mins
5.6	7	16.01 ± 3.19	5	0.88 ± 0.12
16.7	7	63.99 ± 14.74[a]	5	1.49 ± 0.22[a]

a, $p<0.05$ significantly increased compared with 5.6 mmol/l glucose.

The effect of tolbutamide on the rates of insulin release and glucose oxidation by cultured UMR 407/3 cells.

In the absence of glucose, tolbutamide stimulated insulin release from UMR 407/3 cells, maximum and half maximum stimulation occurring at concentrations of 10 and 2 umol/l tolbutamide respectively, Figure 7.3. The stimulation of insulin release from UMR 407/3 cells by tolbutamide was not accompanied by an increase in the rate of D-[U^{14}C] glucose oxidation, Table 7.5.

Figure 7.3 The effect of tolbutamide on insulin release from statically incubated UMR 407/3 cells.

Values are means ± SEM with n in parentheses.

*, $p<0.05$ significantly increased compared with basal.

(Basal release was 0.88 ± 0.12uU/10^5cells/90mins, n=5)

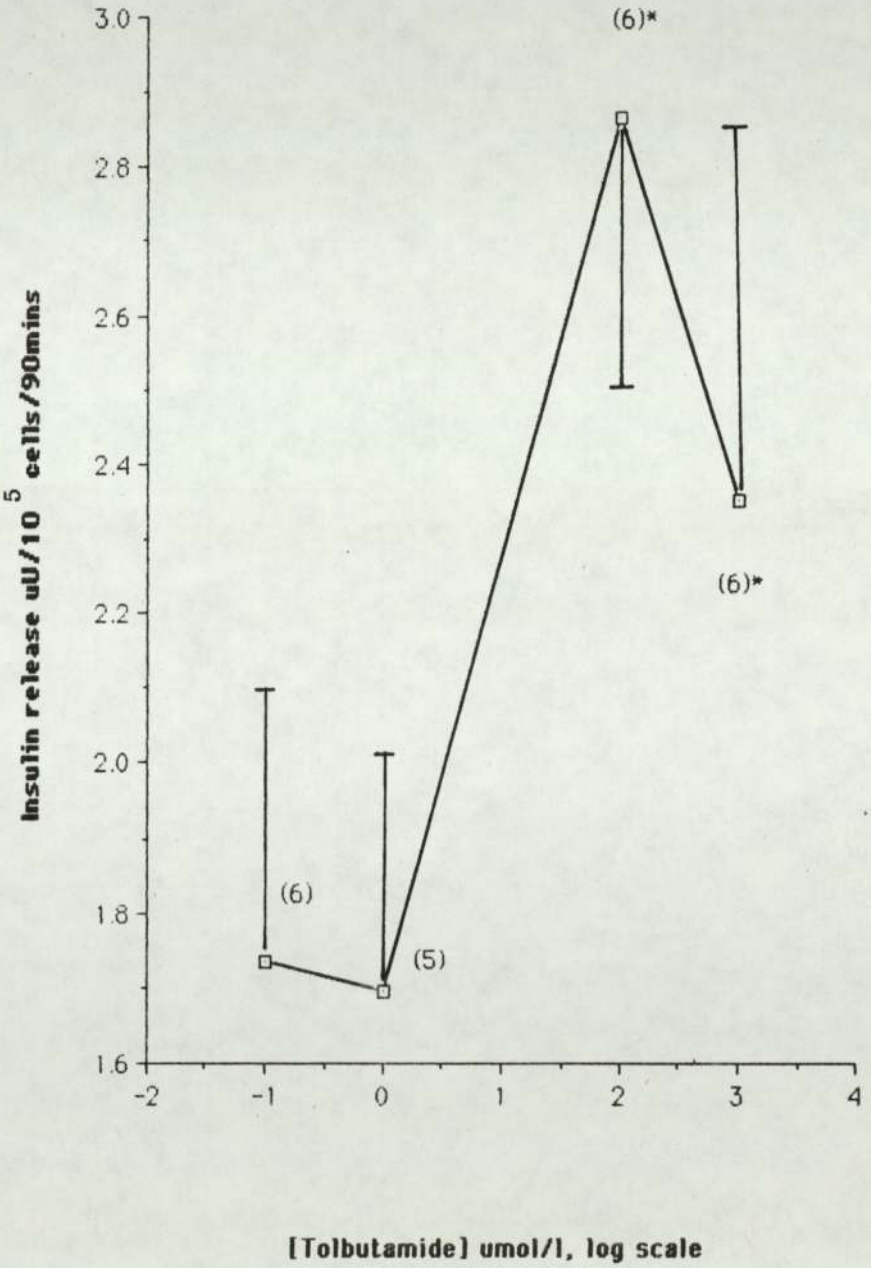


Table 7.5 The effect of tolbutamide on the rate of glucose oxidation by UMR 407/3 cells. (the incubation buffer contained 5.6mmol/l glucose as a substrate)

[Tolbutamide] umol/l	n	Glucose oxidation umol/10^5cells/90mins
0	7	16.01 ± 3.19
0.1	7	19.04 ± 5.60
1.0	7	16.98 ± 3.31
10.0	7	13.50 ± 3.57
100.0	7	13.70 ± 3.58

Characteristics of the disaggregated human insulinoma.

The disaggregated cross sectional segment of insulinoma yielded a total of 35 x 10^6 cells with a viability of 64.3% (assessed by trypan blue exclusion) and a plating efficiency of 66.5%. The gross morphology of an adjacent insulinoma removed from the same pancreas is shown in plates 7.1 to 7.4.

The effect of glucose on insulin release from freshly disaggregated and statically incubated human insulinoma cell suspensions.

Raising the glucose concentration from 5.6 to 16.7 mmol/l failed to stimulate insulin release from statically incubated human insulinoma cells. Based on an insulin content of 22.9uU/10^5cells, insulin release in response to 5.6mmol/l glucose accounted for 15.6% of the insulin content per hour, Table 7.6.

Plate 7.1 Haematoxylin/Eosin sections of a human insulinoma excised from a 21 year old male caucasian at partial pancreatectomy.(magnification X 6.25)

Key: a, Insulinoma tissue

b, Fibrous capsule

c, Surrounding area of normal pancreatic exocrine tissue

d, Small insulinoma beginning to develop.

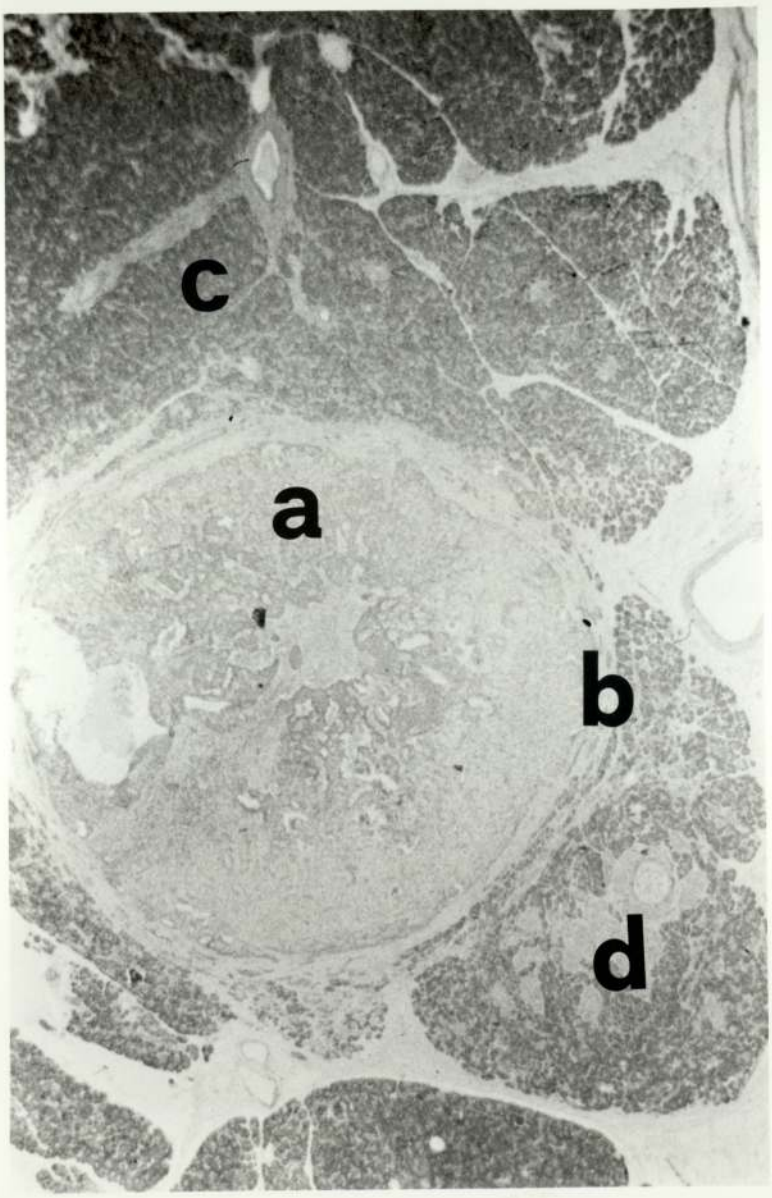


Plate 7.2. Haematoxylin/Eosin section of a human insulinoma excised from a 21 year old male caucasian at partial pancreatectomy showing the boundary between the insulinoma and the normal pancreatic tissue (magnification X10)

Key: a, Insulinoma tissue

b, Fibrous capsule

c, Normal pancreatic exocrine tissue

d, Normal pancreatic islet

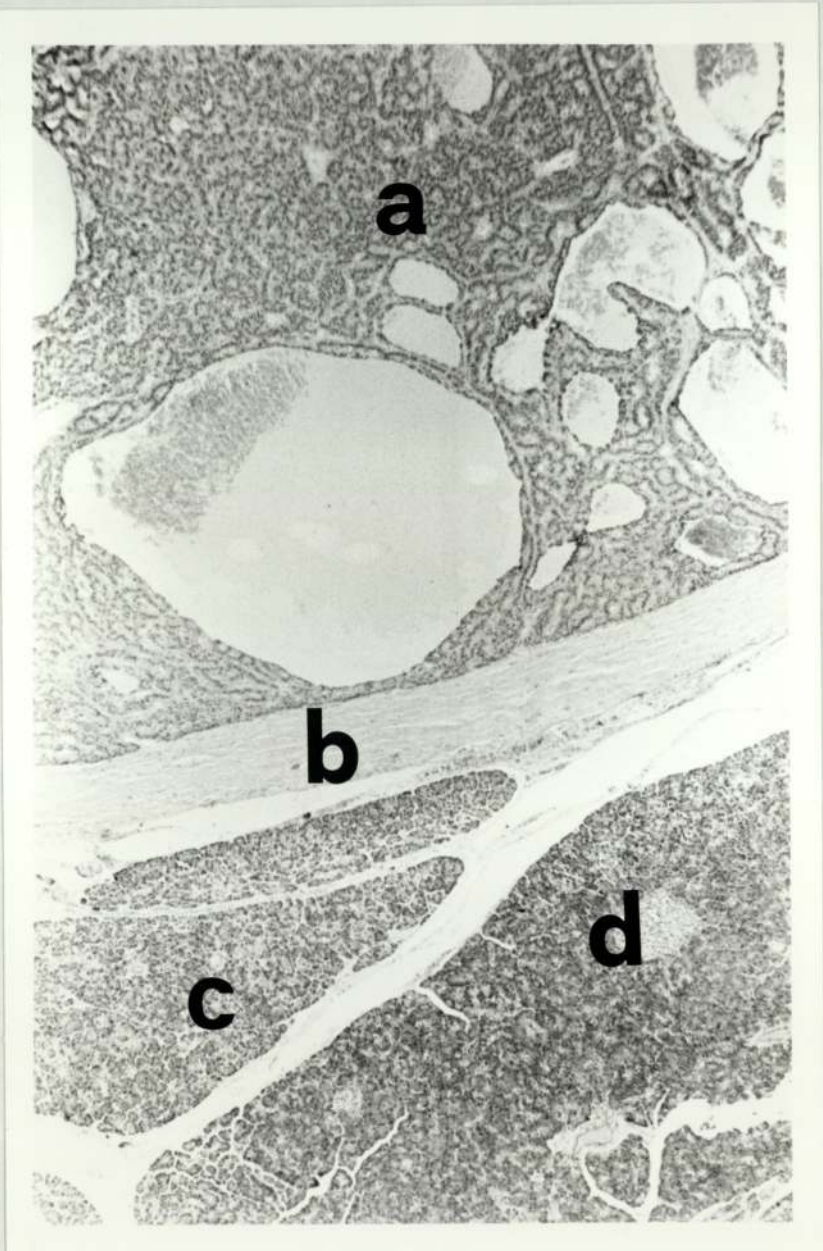


Plate 7.3 Haematoxylin/eosin section of a human insulinoma excised from a 21 year old male caucasian at partial pancreatectomy showing the boundary between the insulinoma and the pancreatic exocrine tissue.(magnification X25)

Key: a, Insulinoma tissue

b, Fibrous capsule

c, normal pancreatic exocrine tissue

d, normal pancreatic islet

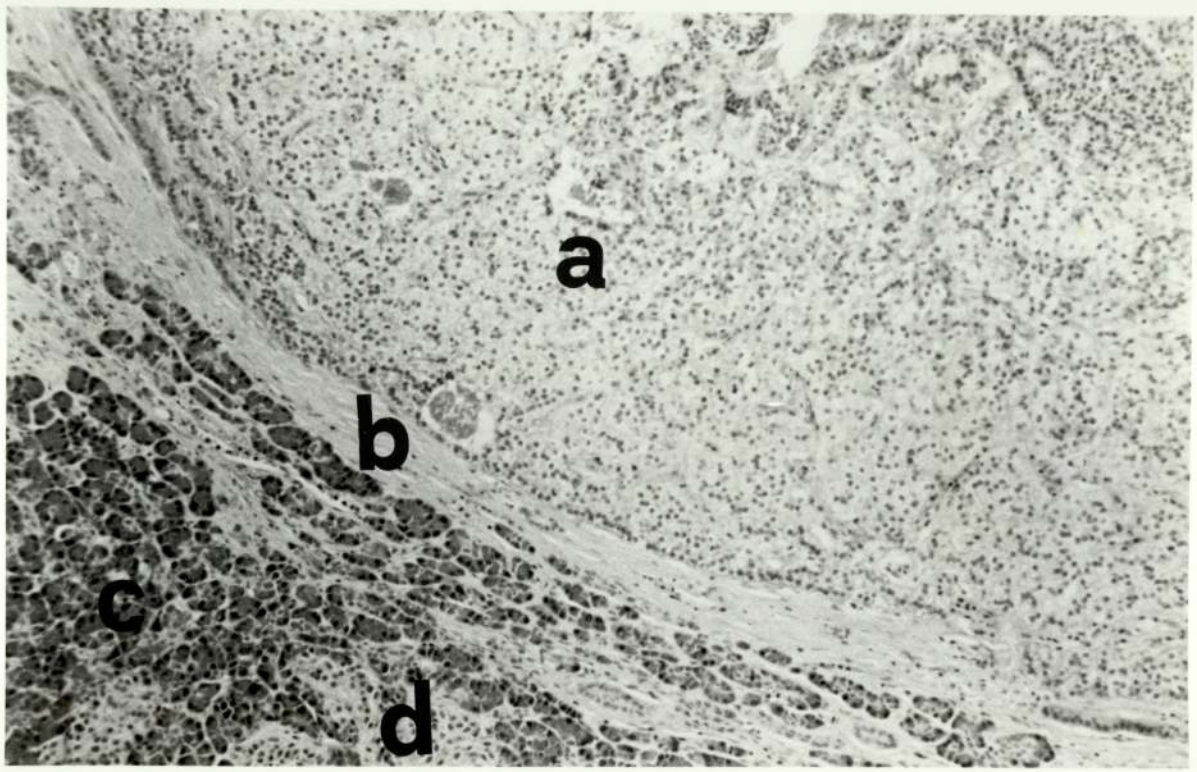


Plate 7.4 High magnification haematoxylin/eosin sections of a human insulinoma excised from a 21 year old male caucasian at partial pancreatectomy showing chords of insulinoma cells. (magnification X100)

The cells in this plate were shown immunohistochemically to be mainly insulin producing cells with some scattered glucagon and somatostatin producing cells in the minority.

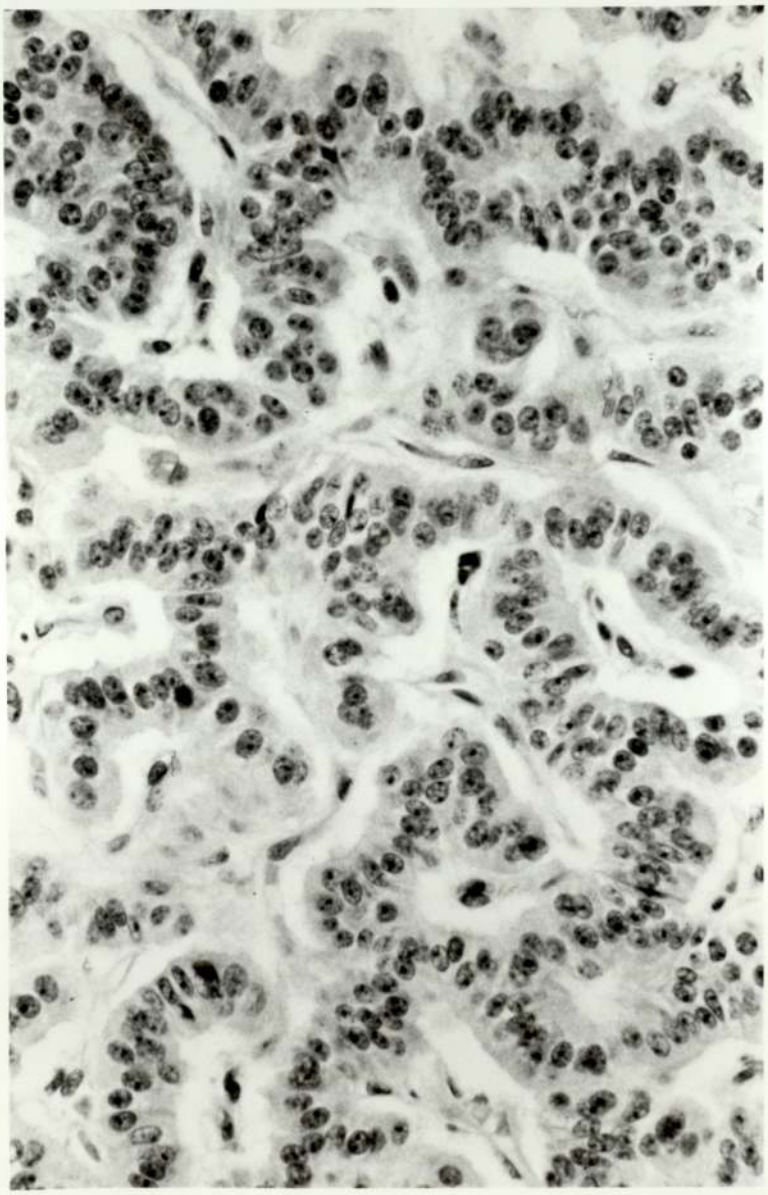


Table 7.6 The effect of glucose on insulin release from freshly disaggregated and statically incubated human insulinoma cells.

[Glucose] mmol/l	n	Insulin release uU/10^5cells/hour	Insulin release % of contents/hour
5.6	3	3.58 ± 0.40	15.63 ± 1.75
16.7	3	4.71 ± 0.31	20.57 ± 1.35

Monolayer culture of cells from a human insulinoma.

During the first week of culture cell numbers declined rapidly from 35 x 10^6 to 1.1 x 10^6cells/flask and insulin released into the medium declined from 6.3 to 0.03mU/flask/2days, Figure 7.4. During the subsequent six weeks the cells secreted on average 0.077±0.023 mU/flask/2days, n=6 (range 0.033-0.159 mU/flask/2days). The cells which were epithelioid in appearence and grew as scattered monolayer colonies did not visibly increase over the entire culture period. Cells passaged on day 24, Figure 7.4, and frozen for a period of 1 month retained their morphology and continued to release insulin when thawed and returned to culture. The cells were passaged a third time on day 34 at a split ratio of 1:2, Figure 7.4. The quantity of insulin accumulated in the medium by these two cell cultures is shown in Figure 7.5. The amount of insulin released into the medium increased steadily over the seven day period. After a total of seven weeks in culture the insulinoma cells began to crenate and detach, indicating the development of *in vitro* senescence.

Figure 7.4. The decline in insulin release and cell number (inset) with time for cultured human insulinoma cells.

Arrows indicate where the cultures were passaged and the split ratios employed.

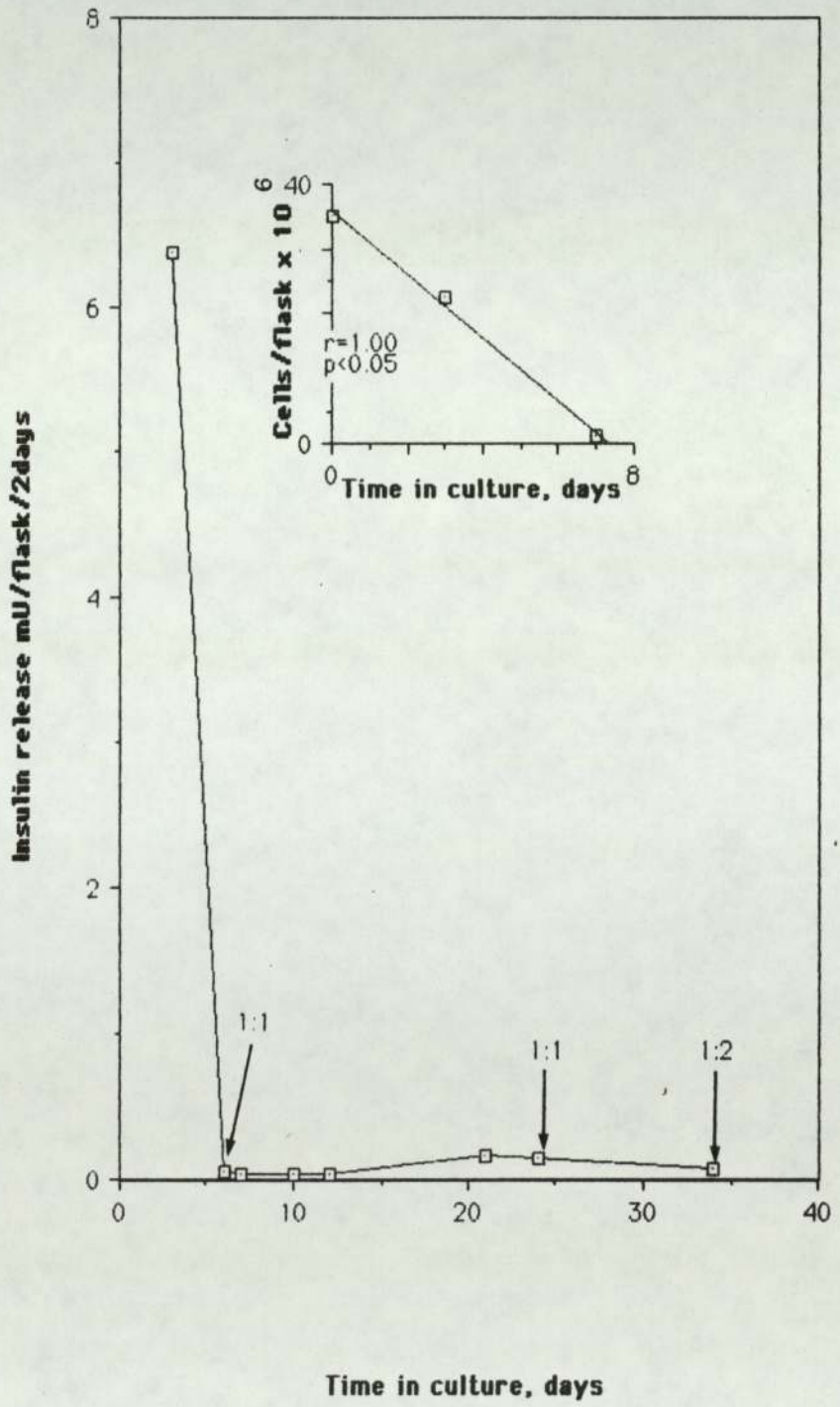
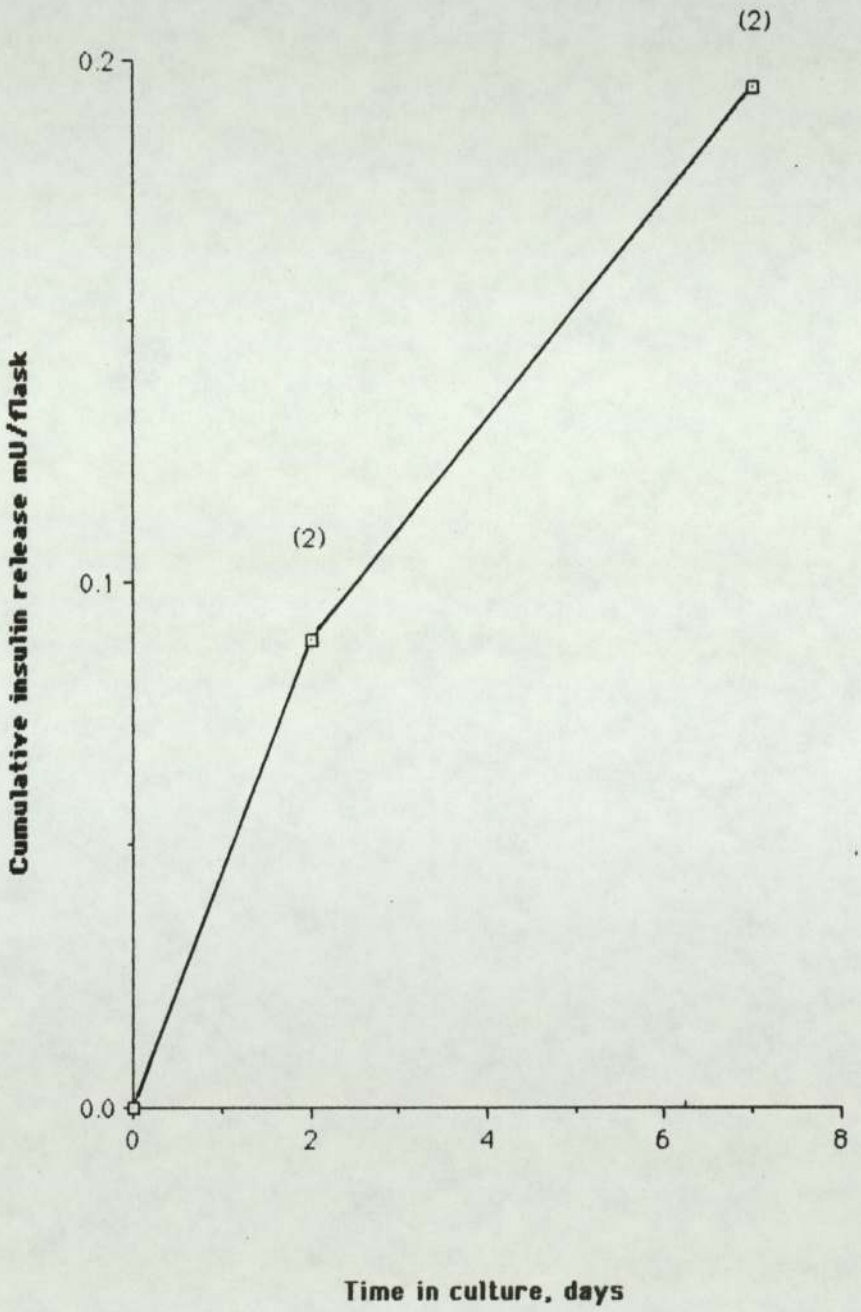


Figure 7.5. Accumulation of insulin in the medium of cultured human insulinoma cells over one week.

Insulin release at day 7 was calculated as the sum of the insulin released over days 0-2 + days 2-7.

Values are means of two separate cultures.



DISCUSSIONS.

The present study confirms the lack of glucose sensitivity of IN111 R1 cells first described by Kikkawa et.al. (55). However, in the present work glucose did cause a marked and significant increase in the rate of glucose oxidation by these cells. The insensitivity of the insulin release mechanism to glucose may be the result of the absence of steriospecific membrane bound glucoreceptors (100) or abnormal glucose metabolism by these cells.

IN111 R1 cells did release insulin in response to commonly used concentrations of D-glyceraldehyde, the amino acid L-arginine and the sulphonylureas glibenclamide and chlorpropamide. The shorter acting glibenclamide was a more effective insulin secretagogue than the longer acting chlorpropamide. The stimulation of insulin release by L-arginine in the absence of glucose further supports the suggestion that amino acids can interact directly with the pancreatic B-cell to release insulin and not act via the subsequent release of glucagon from adjacent A-cells. (126,137). It must be noted, however, that IN111 R1 cells have been reported to contain small amounts of glucagon and somatostatin [193:33:1 in a molar ratio for insulin: glucagon: somatostatin] (55).

Glyceraldehyde induced insulin release from IN111 R1 cells was siginificantly inhibited by the sympathetic nervous system (SNS) neurotransmitter, adrenaline, indicating the possible existence of adrenoreceptors at the level of the B-cell membrane, and a role for the SNS in the regulation of insulin release. The

calcium channel blocker, verapamil (305), and the calmodulin antagonist, TFP (309) also significantly inhibited glyceraldehyde induced insulin release suggesting that calcium entry and calmodulin activation may play a part in the mechanism of glyceraldehyde induced insulin release from IN111 R1 cells.

Glucose stimulated insulin release could be demonstrated with UMR 407/3 cells confirming the original report of Ng et.al. (63). The stimulation of insulin release by glucose from these cells is accompanied by an increase in the rate of glucose oxidation. The absolute amount of insulin released by UMR 407/3 cells is minute compared with that release by isolated islets and cultured HIT-T15 and RINm5F cells (Chapter 3, page 82). Ng et.al. have suggested that the low insulin content, rate of insulin release and rate of insulin biosynthesis (~5% of isolated islets) is due to the fact that UMR 407/3 cells are precursor B-cells i.e., not terminally differentiated (63). In the present study insulin release was well in excess of the cellular insulin content. This has also been reported for cultures of CR1 cells by Carrington et.al. (60), and may result from a combination of the low insulin biosynthetic rate and a possible defect in the storage of insulin secretory granules. Insulin release from UMR 407/3 cells decreases with time in culture and by passage 27 these cells no longer secrete detectable levels of insulin in response to glucose (63).

Insulin release from UMR 407/3 cells could also be elicited by tolbutamide, the observed stimulation did not involve an increase in the rate of glucose oxidation by these cells. This observation is consistent with the lack of dependence on glucose oxidation for insulin release from HIT-T15 cells (Chapter 3, page 82). The fact that tolbutamide did not influence the rate of B-cell glucose

oxidation comfirms the earlier work of Kawazu et.al. (177), but is at varience with observations made by Panten et.al. who reported that tolbutamide increases the rate of oxygen consumption by isolated islets (400).

The present work on disaggergated cultured human insulinoma cells confirms the general insensitivity of this tissue to glucose and the presence of a low insulin content (37,41). However, in some studies fresh insulinoma tissue has shown modest responsiveness to glucose while other studies have failed to demonstrate glucose sensitivity (38,39). The unresponsiveness of cultured insulinoma tissue to glucose may represent in vitro dedifferentiation. Human insulinomas release insulin in vitro but this capacity rapidly declines (37,41). In the present study after 7 days only 1.2% of the amount of insulin initially released at day 3 was released. This rapid decline in insulin release is probably due to the detachment and death of large numbers of cells unable to adapt to the in vitro environment and culture. Insulinomas in general tend to have a finite life span in culture. The only evidence of significant cell proliferation in vitro is that reported by Saxe et.al. who reported a doubling time of 5 days (38). The generalised lack of proliferation in these insulinomas may reflect their benign nature. The ability to proliferate in vitro could be used by the clinician to establish the possibility of malignancy in vivo. Insulinoma tissue can be cryopreserved in the usual way and fresh and cryopreserved tissue behave identically in terms of general morphology and the capacity to release insulin.

CHAPTER 8.

8. GENERAL DISCUSSIONS

In the past studies directed towards understanding the precise mechanisms involved in the regulation of insulin release have been hampered by certain fundamental problems. Isolated islets and islet cell monolayers are laborious to prepare and in order to obtain sufficient tissue for biochemical studies large numbers of experimental animals are generally required. Islets and islet cell monolayers maintained *in vitro* have limited lifespans of the order 40 days for islets (23) and 70 days for islet cell monolayers (33). Islet cell monolayers still contain the constituent cells present in the islet (6) and are invariably contaminated with and subsequently overgrown by fibroblasts (29). Tissue culture medium from islet cell monolayers contains high concentrations of insulin, glucagon, somatostatin and pancreatic polypeptide.

Besides the X-ray induced insulinoma of the rat (46), chemically induced insulinomas (42) and spontaneous hamster insulinomas (49), there are a number of insulin secreting cell lines currently appearing that could potentially provide large numbers of B-cells quickly and cheaply for use in biochemical studies of the mechanism of insulin biosynthesis and release. These cell lines include RINm5F (56), IN111 R1 (54), HIT-T15 (62) and UMR 407/3 (63). The RINm5F cell line has been well characterised and although it does not reliably release insulin in response to glucose it is widely used in studies of the mechanism of insulin release (58).

In the present studies the growth characteristics of these cultured cell lines was found to be strikingly similar. UMR 407/3 cells grew to confluence but had the slowest growth rate, with a doubling time of 38 hours. The IN111 R1 cell line demonstrated the fastest growth rate, with a doubling time of only 24 hours and like the RINm5F and HIT-T15, cell lines did not grow to confluence. The plating efficiencies of all the cell lines exceeded 76%, but the human insulinoma (a primary culture) had a reduced plating efficiency of only 67%.

The insulin contents of the cultured cells varied considerably, taking into account the fact that differing passages of the various cell lines were used, HIT-T15 cells (passage 61) demonstrated the greatest insulin content of 283.4uU/10^5cells whilst UMR 407/3 cells (passage 12) had the lowest insulin content of only 0.3uU/10^5cells. Despite these differences in insulin content all the cell lines retained the capacity to release insulin <u>in vitro</u>.

In the present studies, HIT-T15 and RINm5F cells (and to a lesser extent IN111 R1 and UMR 407/3 cells) proved capable of releasing insulin in response to a variety of insulin secretagogues. In addition insulin release from these cells could be reduced by well established inhibitors of insulin release. The generalised effects of the various secretagogues and inhibitors used in the present work have been summarised in Table 8.1.

The greatest variability in insulin release from these cell lines was that generated in response to glucose. Whilst an elevation in the glucose concentration stimulated the rate of glucose oxidation by all the cell lines, glucose did not increase the rate of insulin release from all cell lines. HIT-T15 and UMR 407/3 cells did release

Table 8.1 The effects of secretagogues and inhibitors upon insulin release from cultured HIT-T15, RINm5F, IN111 R1 and UMR 407/3 cells.

Secretagogue/Inhibitor	Effect on insulin release from HIT-T15	RINm5F	IN111 R1	UMR 407/3
a/ Nutrients.				
D-glucose	+	+/-	-	+
D-glyceraldehyde	-	+	+	
L-leucine	+	+		
L-arginine	+	+	+	
b/ Sulphonylureas.				
Glibenclamide	+	+	+	
Chlorpropamide	+	+	+	
Tolbutamide	+			+
c/ Pancreatic hormones.				
Glucagon	+	+		
Somatostatin	-	-		
Pancreatic polypeptide	-	-		
GIP	+			
d/ Pharmacological agents.				
Forskolin	+	+		
Imidazole	-	-		
Calcium	+			
Verapamil	-	-	-	
Diltiazem	-	-		
Nifedipine	-	-		
TFP	-	-	-	
M&B 13,753	-	-		
TMB-8	-			
M&B 40,678	NE			
IP3	NE			
TPA		+		
4PDD		+		
e/ Neurotransmitters.				
Acetlycholine	+	+		
Adrenaline	-		-	

+, stimulates, -, inhibits, NE, no effect

insulin in response to glucose [confirming the previous observation of Santerre et.al (62) and Ng et.al. (63)]. The glucose response of HIT-T15 cells was biphasic in nature with an exaggerated first phase. Human insulinoma cells and IN111 R1 cells did not release insulin in response to glucose. RINm5F cells proved unpredictable in their responsiveness to glucose, in one study glucose did cause a slight but significant increase in the rate of insulin release. The absence of an insulin response to glucose by RINm5F cells, IN111 R1 cells and human insulinoma cells might be attributed to abnormal glucose metabolism in these cells or the absence of membrane bound glucoreceptors. A recent study by Ashcroft et.al have suggested that the glucose transporter in HIT-T15 cells is the glucose sensor. This finding was based on the nonequilibrium between extracellular and intracellular glucose (confirming the earlier work of Meglasson et.al. [169]) and the inhibitory effect of phloridzin and phloretin (glucose transport inhibitors) upon insulin release. It was concluded that glucose transport into HIT-T15 cells is the rate limiting step and therefore the glucose transporter is the glucose sensor in these cells (401). Studies to identify the glucose sensor in UMR 407/3 cells remain to be carried out. D-glyceraldehyde markedly stimulated insulin release from RINm5F and IN111 R1 cells, but not HIT-T15 cells. This effect of glyceraldehyde may implicate glucose metabolism in the stimulation of insulin release and that an early defect in the conversion of glucose to glyceraldehyde may be manifest in RINm5F and IN111 R1 cells.

Glucose and glyceraldehyde induced insulin release from HIT-T15 and RINm5F cells respectively was inhibited by the calcium channel blockers verapamil, diltiazem and nifedipine and the calmodulin inhibitor trifluoperazine. These data implicate calcium entry through specific membranal calcium channels and the

subsequent activation of a calcium sensitive regulator protein calmodulin in the process of insulin release from these cells. A role for intracellularly sequestered calcium in the mechanism of insulin release proved difficult to demonstrate, since two apparently specific sequestration blockers exerted opposite effects on insulin release from HIT-T15 cells. TMB-8 inhibited insulin release while M&B 40,678 was without effect. The sequestration agonist IP_3 which is believed to facilitate calcium release from intracellular stores (269) failed to stimulate insulin release from HIT-T15 cells at a concentration of 2.5umol/l. Yet Biden et.al. have demonstrated that this concentration of IP_3 is sufficient to mobilise intracellular calcium in cultured RINm5F cells, although insulin release was not measured (272). These observations suggest that IP_3 induced calcium mobilisation alone may be insufficient to trigger the release mechanism and may only act as a positive modulator of insulin release in the presence of secretagogues which increase the turnover of inositol phospholipids.

Studies using isolated islets over the years have confirmed that glucose induced insulin release involves an increased turnover of cAMP (226-229). In the present work the stimulation of insulin release by forskolin and inhibition by imidazole confirmed a role for cAMP in the process of insulin release and the integrity of adenylate cyclase and cAMP phosphodiesterase in cultured HIT-T15 and RINm5F cells. Since no direct effect of glucose on B-cell adenylate cyclase has yet been demonstrated (148, 233) it may be that the observed increase in B-cell cAMP levels observed after glucose stimulation of isolated islets (226-229) may be due to an activation of adenylate cyclase by the calci-calmodulin complex. (255).

The combined actions of elevated cAMP, calcium and inositol containing phospholipids could stimulate insulin release via the activation of protein kinase-A, calcium calmodulin dependent protein kinase and protein kinase-C (237). Certainly the stimulation of insulin release from RINm5F cells by the phorbol ester TPA in the present work indirectly demonstrates a role for protein kinase-C in the initiation of insulin release from these cells.

Not only does insulin release appear to be the product of a co-ordinated series of intracellular events, net release from the islet involves a co-ordinated paracrine intraislet interaction involving the hormonal products of A, B, D and PP cells (183,184). Cultured HIT-T15 and RINm5F cells were found to be particularly suitable systems for unravelling the paracrine interactions between islet cells. Insulin release from HIT-T15 and RINm5F cells was stimulated by glucagon while glucose and glyceraldehyde induced insulin release from these cells was inhibited by both somatostatin and pancreatic polypeptide. In addition glucagon induced insulin release from both cell lines was inhibited by somatostatin and pancreatic polypeptide. These studies strongly suggest that net insulin release from the whole islet may be the result of a significant paracrine interaction. An interaction that appears to be essential for the normal in vivo function and integrity of the islet (184). A paracrine involvement had been suggested for the effects of L-arginine, gastric inhibitory polypeptide and acetylcholine on insulin release since all three agents stimulate the release of glucagon (137,209,327). The present studies indicate that a paracrine effect of glucagon (i.e., glucagon stimulation of insulin release) does not occur in this instance. However the stimulation of insulin release by these three agents was thought to depend on the presence of glucose, no such glucose dependence could be

demonstrated in these studies. The direct stimulation of insulin release from HIT-T15 cells by GIP would appear to confirm the observations of Sarson et.al. (223) and may result from a pharmacological rather than physiological phenomenon. This is supported by the fact that GIP concentrations of less than 1ng/ml have been reported in human serum after oral glucose loading. In the present studies significant insulin release from HIT-T15 cells could only be produced by GIP at concentrations in excess of 50ng/ml. The precise mechanisms involved in the paracrine regulation of insulin release from the islet remain to be determined but may involve a sequential activation of A, B, D and PP cells.

There is a great deal of evidence to support the existence of a neural control for insulin release in vivo. Stimulation of the vagus (327-332) or splanchnic nerves (344,345) initiates a stimulation and inhibition of insulin release respectively. As with conventional islets (333), cultured HIT-T15 and RINm5F cells also respond to cholinergic stimulation with acetylcholine. The stimulation of insulin release by acetylcholine is mediated via muscarinic receptors, based on the inhibitory effect of atropine but not hexamethonium. The insulin response of HIT-T15 and RINm5F cells to acetylcholine appears to be calcium dependent since it was inhibited by verapamil. The inhibition of acetylcholine induced insulin release by imidazole may be a phenomenon exclusive to cultured B-cells and confirmation of a role for cAMP in acetylcholine induced insulin release would require the measurement of cAMP turnover in these cells. Both insulin release and glucose oxidation by HIT-T15 cells was inhibited by adrenaline, indirectly suggesting the presence of adrenoreceptors at the level of the plasma membrane.

All of the cultured cells used in the present studies were of animal origin, recently however, the opportunity arose to evaluate the insulin response of human B-cells obtained from a human insulinoma. The insulinoma was removed during surgery at Birmingham General Hospital, and maintained in monolayer culture for some seven weeks. Continued insulin release was observed duing this period. However the cells did not proliferate and were unresponsive to glucose challenge. These observations are entirely consistent with the reports of Chick et.al. (35), Yip and Schimmer (36), Adcock et.al. (37) and Akagi et.al. (41). Reports of the successful culture of human insulinoma cells are few and far between but in 1982 Boyd and co workers managed to immortalise a human insulinoma by hybridising the insulinoma cells with LMTK$^-$C11D, an anchorage dependent murine fibroblastoid cell line. The cocultures were hybridised with polyethylene glycol (PEG 1000). One of the resulting hybrid clones 1-1C-8h secreted proinsulin like material for some seven months in vitro Immunoreactive insulin release from the hybrid cells was minimal (402). This technique successfully immortalised the insulinoma cells but it also apparently interfered with the normal biosynthesis of insulin. Insulin release in response to glucose by this hybrid clone was not determined.

The studies presented in this thesis involving cultured B-cells confirm the glucose sensitivity of HIT-T15 and UMR 407/3 cells and indicate their usefulness in the study of insulin biosynthesis and release, and go some way to providing legitimate B-cell models for use in unravelling the aetiology of diabetes mellitus.

APPENDIX.

APPENDIX

A1. Preparation of phosphate buffers for radioiodination.

1/ 0.5mol/l: 73g Na_2HPO_4 combined with 14.04g NaH_2PO_4 $2H_2O$ and made up to 1 litre with 2 x distilled water, pH adjusted to 7.4 with 1mol/l NaOH.

2/ 0.05mol/l: 7.3g Na_2HPO_4 combined with 1.404g NaH_2PO_4 . $2H_2O$ and made up to 1 litre with 2 x distilled water, pH adjusted to 7.4 with 1mol/l NaOH.

A2. Basic Krebs/bicarbonate/HEPES incubation buffer.

The incubation medium used throughout this thesis was Krebs/bicarbonate/HEPES with the following composition, molarity (mmol/l) is shown in parentheses.

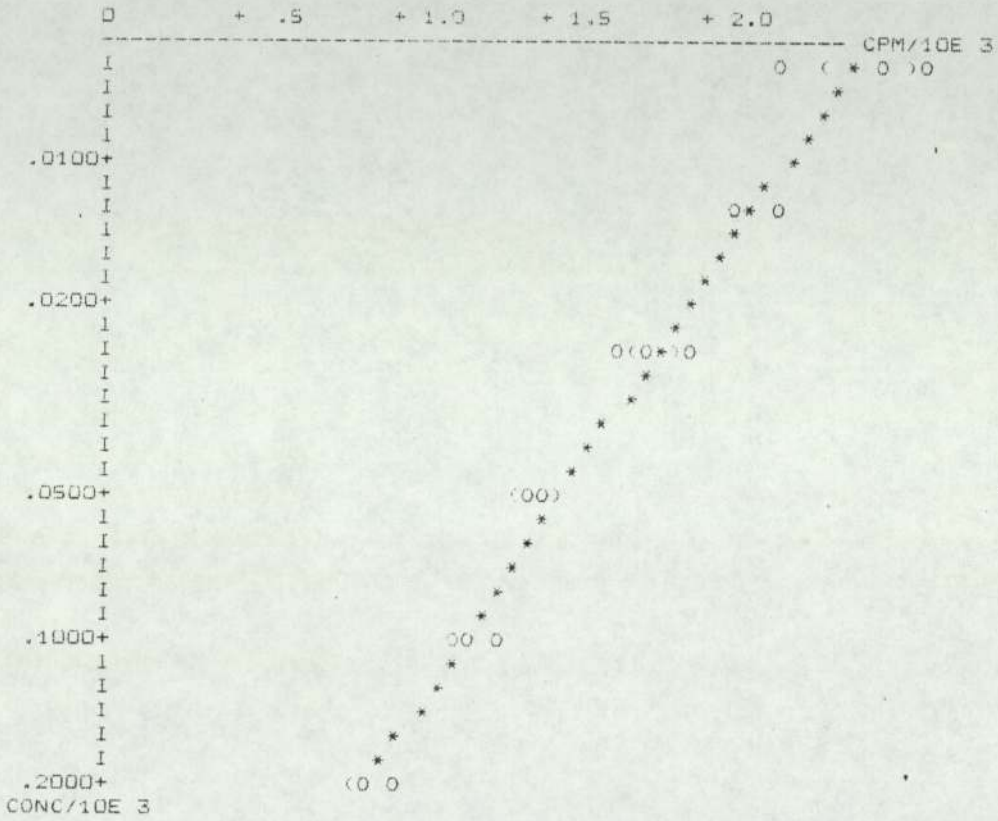
NaH_2PO_4 (0.2), $NaHCO_3$ (20), Na_2HPO_4 (1.8), KCl (4.5), $MgSO_4$ (1.2), and $CaCl_2$ (2.56). After gassing the buffer for 20-30 minutes with 95% O_2 : 5% CO_2, HEPES (16) was added and the pH adjusted to 7.4 with either 2mol/l NaOH or 2mol/l HCl.

A.3. Typical gamma counter printout for the double antibody radioimmunoassay of insulin.

```
 1 LISTING          Y
 2 DUAL LABEL       N
 3 STD TIME         60
 4 UNK TIME         45
 5 COUNTS           900000
 6 WINDOW           035-110
 7 METHOD           6
 8 UNK BLAN         0
 9 UNK REPL         2
10 CONC.FACTORS     N
11 CURVE EDIT       Y
12 FREE FRACTION    N
13 PRINT            -1-2-4-6-7-8-9-10-11-12

14 CODING           POS-CODE
                    001-TOA
                    004-BLA
                    007-6.25
                    010-12.5
                    013-25
                    016-50
                    019-100
                    022-200
                    025-UNKS
```

POS	CODE1	CTIME	COUNTS1	COR.CPM1	ERR1%	RATIO1	S.E1%
001	TOA	60	9227	9293.3	1.0		
002	TOA	60	9558	9627.6	1.0		
003	TOA	60	10043	10117.4	1.0		
	MEAN			9679.4	0.6		2.5
004	BLA	60	534	536.6	4.3		
005	BLA	60	609	611.9	4.1		
006	BLA	60	518	520.5	4.4		
	MEAN			556.3	2.5		5.1
007	6.25	60	2152	2163.3	2.2		
008	6.25	60	2481	2494.3	2.0		
009	6.25	60	2625	2639.1	2.0		
	MEAN			2432.2	1.2	1.0000	5.8
010	12.5	60	2125	2136.2	2.2	0.8783	
011	12.5	60	2126	2137.2	2.2	0.8787	
012	12.5	60	2002	2012.5	2.2	0.8274	
	MEAN			2095.3	1.3	0.8615	2.0
013	25	60	1718	1726.8	2.4	0.7100	
014	25	60	1857	1866.6	2.3	0.7674	
015	25	60	1628	1636.3	2.5	0.6728	
	MEAN			1743.2	1.4	0.7167	3.8
016	50	60	1340	1346.8	2.7	0.5537	
017	50	60	1367	1373.9	2.7	0.5649	
018	50	60	1394	1401.1	2,7	0.5760	
	MEAN			1373.9	1.6	0.5649	1.1
019	100	60	1213	1219.1	2.9	0.5012	
020	100	60	1114	1119.5	3.0	0.4603	
021	100	60	1131	1136.6	3.0	0.4673	
	MEAN			1158.4	1.7	0.4763	2.7
022	200	60	821	825.0	3.5	0.3392	
023	200	60	822	826.0	3.5	0.3396	
024	200	60	886	890.3	3.4	0.3661	
	MEAN			847.1	2.0	0.3483	2.6


0 + .5 + 1.0 + 1.5 + 2.0
CPM/10E 3
.0100+
.0200+
.0500+
.1000+
.2000+
CONC/10E 3

A4 BBC microcomputer programme used to calculate unknown insulin concentrations and estimate the correlation coefficient of standard curves obtained in the double antibody radioimmunoassay of insulin.

```
>LIST
  10REM** Original program devised by **
  20REM** S.G. Howitt **
  30REM** Adapted for BBC microcomputer by **
  40REM** K. Hughes **
  50REM DATA
  60DATA "Mouse","ng/ml",5,.5,1,2.5,5,10
  70CLS
  80DATA "Human","uU/ml",6,6.25,12.5,25,50,100,200
  90REPEAT
 100PRINT:PRINT:INPUT "DO YOU WANT PRINTOUT ",PR$
 110CLS
 120UNTIL PR$="Y" OR PR$="YES" OR PR$="NO" OR PR$="N"
 130IF PR$="Y" OR PR$="YES" THEN 140 ELSE 190
 140CLS:PRINT:PRINT:PRINT "EXPERIMENTAL TITLE ETC. FOR PRINTOUT "
 150PRINT
 160INPUT TI$:PROCPRINT1
 170REM
 180REM
 190REPEAT
 200CLS
 210PRINT:PRINT:PRINT:PRINT:INPUT "STANDARDS : Mouse or Human.  (M or H) ",MHB$
 220IF MHB$="M" THEN 250
 230IF MHB$="H" THEN 290
 240UNTIL MHB$="M" OR MHB$="H"
 250GOSUB 390
 260LET INM=IN
 270LET SLM=SL
 280IF MHB$="M" THEN 800
 290RESTORE
 300READ R$,R$,R%
 310FOR J%=1 TO R%
 320READ R
 330NEXT J%
 340GOSUB 390
 350LET INH=IN
 360LET SLH=SL
 370GOTO800
 380REM
 390READ Sp$,Un$
 400CLS
 410PRINT: PRINT Sp$
 420PRINT "Stds."
 430PRINT
 440PRINT Un$;TAB(8);"cpm (if value unknown enter '0')"
 450IF PR$="N" OR PR$="NO" THEN 470
 460PROCPRINT2
 470X=0:Q=0:Y=0:S=0:W=0:N%=0
 480READ StN%
 490FOR J%=1 TO StN%
 500READ StCo
 510PRINT TAB(0);StCo;TAB(6);" ";
 520INPUT StCpm
 530IF StCpm <> 0 THEN 560
 540IF PR$="N" OR PR$="NO" THEN 650
 550PROCPRINT3:GOTO650
 560IF PR$="N" OR PR$="NO" THEN 580
 570PROCPRINT4
 580LET StCo = LOG(StCo)
 590LET X=X+StCpm
 600Q=Q+StCpm^2
```

```
  610Y=Y+StCo
  620S=S+StCo^2
  630W=W+StCo*StCpm
  640N%=N%+1
  650NEXT J%
  660SL=(W-X*Y/N%)/(Q-X^2/N%)
  670IN=(Y-SL*X)/N%
  680CoCo=(W-X*Y/N%)/SQR((Q-X^2/N%)*(S-Y^2/N%))
  690CLS
  700PRINT:PRINT "Best line : "
  710PRINT TAB(6);"Intercept (cpm=0) = ";IN
  720PRINT TAB(18);"Slope = ";SL
  730PRINT"Correlation coefficient = ";CoCo
  740IF PR$="N" OR PR$="NO" THEN 760
  750PROCPRINT5
  760RETURN
  770REM
  780REM
  790REM
  800PRINT:PRINT "Input sample name, or  END' to stop     program or 'DIL' to ch
ange dilution     factor or  SUBH' to insert subheading   for printout followed
by cpm."
  810IF PR$="N" OR PR$="NO" THEN 820
  820PRINT:PRINT "'*' indicates result outside linear     region of curve and wi
ll be incorrectly calculated by this program"
  830REM
  840IF MHB$="B" THEN 960
  850PRINT:PRINT TAB(38),"Insulin concn."
  860PRINT TAB(38),"ref. ";Sp$;" standard"
  870IF PR$="N" OR PR$="NO" THEN 890
  880PROCPRINT6
  890IF MHB$="H" THEN 930
  900PRINT "Sample","  cpm","   ng/ml","  uU/ml"
  910IF PR$="N" OR PR$="NO" THEN 1030
  920PROCPRINT7:GOTO1030
  930PRINT "Sample","  cpm","        uU/ml"
  940IF PR$="N" THEN 1030
  950PROCPRINT8:GOTO1030
  960PRINT:PRINT TAB(38),"Insulin concentration    Insulin cocn."
  970PRINT "Sample","cpm"," ng/ml","uU/ml"," uU/ml"
  980IF PR$="N" OR PR$="NO" THEN 1030
  990PROCPRINT9
 1000REM
 1010REM
 1020REM
 1030PRINT:INPUT "Dilution factor ",DF :PRINT
 1040LET MX$="*":LET HX$="*"
 1050INPUT Sam$;
 1060IF Sam$ <> "SUBH" THEN 1080
 1070PRINT:INPUT TI$:PROCPRINT10:GOTO 1050
 1080IF Sam$ = "END" THEN 1330
 1090IF Sam$ = "DIL" THEN 1030
 1100PRINT" "," ";:INPUT Cpm:PRINT:PRINT Sam$;TAB(12);Cpm;
 1110IF MHB$="H" THEN 1210
 1120LET CoM=INT(EXP((INM+SLM*Cpm)*LN(10))*10+.5)/10
 1130IF CoM < .5 THEN 1160
 1140IF CoM > 12.8 THEN 1160
 1150LET MX$=" "
 1160IF MHB$="B" THEN 1210
 1170PRINT TAB(20);MX$;TAB(21)DF*CoM;TAB(30);MX$;TAB(31);25*DF*CoM
 1180IF PR$="N" OR PR$="NO" THEN 1200
 1190PROCPRINT11
 1200IF MHB$="M" THEN 1040
 1210LET CoH=INT(EXP((INH+SLH*Cpm)*LN(10))+.5)
 1220 IF CoH < 12.5 THEN 1250
 1230 IF CoH > 320 THEN 1250
```

```
1240 LET HX$=" "
1250IF MHB$="B" THEN 730
1260PRINT TAB(25);HX$;TAB(27);DF*CoH
1270IF PR$="N" OR PR$="NO" THEN1040
1280PROCPRINT12:GOTO1040
1290PRINT FMBT$,MX$,DF*CoM,MX$,25*CoM,Hx$,DF*CoH
1300IF PR$="N" OR PR$="NO" THEN 1040
1310PROCPRINT(13)
1320GOTO 1040
1330IF PR$="N" OR PR$="NO" THEN END
1340 END
1350DEF PROCPRINT1
1360*FX6
1370VDU2
1380PRINT TI$
1390PRINT:PRINT:
1400VDU3
1410ENDPROC
1420DEF PROCPRINT2
1430VDU2
1440VDU21
1450PRINT Sp$
1460PRINT "Standards"
1470PRINT TAB(9);Un$;TAB(22);"cpm"
1480VDU6
1490VDU3
1500ENDPROC
1510DEF PROCPRINT3
1520VDU2
1530VDU21
1540PRINT StCo
1550VDU6
1560VDU3
1570ENDPROC
1580DEF PROCPRINT4
1590VDU2
1600VDU21
1610PRINT TAB(9);StCo;TAB(22);StCpm
1620VDU6
1630VDU3
1640ENDPROC
1650DEF PROCPRINT5
1660VDU2
1670VDU21
1680PRINT:PRINT:
1690PRINT"Computed best line : Intercept (cpm=0) = ";IN
1700PRINT TAB(33);"Slope = ";SL
1710PRINT TAB(15);"Correlation coefficient = ";CoCo
1720VDU6
1730VDU3
1740ENDPROC
1750DEF PROCPRINT6
1760VDU2
1770VDU21
1780PRINT:PRINT:
1790PRINT TAB(39);"Insulin concentration"
1800PRINT TAB(26)"Dilution";TAB(39)"ref.  ";Sp$;" standard"
1810VDU6
1820VDU3
1830ENDPROC
1840DEF PROCPRINT7
1850VDU2
1860VDU21
1870PRINT"SAMPLE";TAB(13);"cpm";TAB(27);"factor";TAB(44);"ng/ml";TAB(54);"uU/ml
"
```

```
1880PRINT
1890VDU6
1900VDU3
1910ENDPROC
1920DEF PROCPRINT8
1930VDU2
1940VDU21
1950PRINT"Sample";TAB(13);"cpm";TAB(27)"factor";TAB(48);"uU/ml"
1960PRINT
1970VDU6
1980VDU3
1990ENDPROC
2000DEF PROCPRINT9
2010VDU2
2020VDU21
2030PRINT
2040PRINT
2050PRINT
2060PRINT
2070VDU6
2080VDU3
2090ENDPROC
2100DEF PROCPRINT10
2110VDU2
2120VDU21
2130PRINT:PRINT"  ";T1$ :
2140VDU6
2150VDU3
2160ENDPROC
2170DEF PROCPRINT11
2180VDU2
2190VDU21
2200PRINT  Sam$;TAB(13);Cpm;TAB(29);DF;TAB(42);MX$;TAB(44);DF*CoM;TAB(52);MX$;T
AB(54);25*DF*CoM
2210VDU6
2220VDU3
2230ENDPROC
2240DEF PROCPRINT12
2250VDU2
2260VDU21
2270PRINT Sam$;TAB(13);Cpm;TAB(29);DF;TAB(43);HX$;TAB(49);DF*CoH
2280VDU6
2290VDU3
2300ENDPROC
```

A5. BBC microcomputer programme used to calculate the area under the curve for data obtained during perifusion of HIT-T15 and RINm5F cells.

```
>LIST
   10CLS:PRINT:PRINT:
   20PRINT CHR$(141);"THIS PROGRAM WAS WRITTEN BY K.HUGHES"
   30PRINT CHR$(141);"THIS PROGRAM WAS WRITTEN BY K.HUGHES"
   40PRINT CHR$(141);TAB(12)"NOVEMBER 1985"
   50PRINT CHR$(141);TAB(12)"NOVEMBER 1985"
   60INPUT TAB(7,20)"PRESS RETURN WHEN READY ";A$:IFA$=INKEY$(-74)  THEN 70 ELSE
 10
   70DIM A(100):DIM BX(100)
   80CLS:PROCstimulated:PROCbasal:PROCtest:PROCprint1:
   90INPUT"       DO YOU WANT TO PRINT                          OUT OF THESE RESULTS ";
Y$
  100IF Y$="YES" OR Y$="Y" THEN PROCprint2 ELSE 110
  110PRINT:PRINT:
  120INPUT"DO YOU WANT TO ENTER MORE DATA ";D$:IF D$="YES" OR D$="Y" THEN 80 ELS
E PRINT:PRINT:PRINT"END OF THE PROGRAM"
  130PRINT:PRINT:END
  140DEF PROCstimulated
  150CLS
  160PRINT:PRINT:
  170INPUT"INPUT NUMBER OF STIMULUS EXPT'S ";N
  180IF N<2 THEN PROCerror1
  190PRINT:PRINT:
  200A=0:B=0
  210FOR I=1 TO N
  220AREA=0
  230PRINT "      TIME (MINS)";TAB(20);"INSULIN (uU/min)"
  240FOR T=31 TO 91 STEP5
  250PRINT T;
  260INPUT TAB(20)"INPUT VALUE ";BX(T)
  270IF T=31 THEN NEXT T
  280AREA=AREA+((BX(T-5)+BX(T)))*2.5
  290NEXT T
  300CLS
  310 PRINT "DATA FOR EXPERIMENT ";I;" NOW BEING STORED!"
  320PRINT:PRINT:
  330LET A(I)=AREA
  340A=A+A(I)
  350B=B+A(I)^2
  360NEXT I
  370C=A/N
  380V=(B-(C*A))/(N-1)
  390D=SQR(V)
  400FORX=1TO1000
  410NEXT X
  420ENDPROC
  430DEF PROCbasal
  440CLS
  450PRINT:PRINT:
  460INPUT"INPUT NUMBER OF BASALS ";N1
  470IF N1<2 THEN PROCerror2
  480PRINT:PRINT:
  490A=0:B=0
  500FOR I=1 TO N1
  510AREA=0
  520PRINT "      TIME (MINS)";TAB(20);"INSULIN (uU/min)"
  530FOR T=31 TO 91 STEP5
  540PRINT T;
  550INPUT TAB(20)"INPUT VALUE ";BX(T)
  560IF T=31 THEN NEXT T
  570AREA=AREA+((BX(T-5)+BX(T)))*2.5
```

```
580NEXT I
590CLS
600 PRINT "DATA FOR EXPERIMENT ";I;" NOW BEING STORED!"
610PRINT:PRINT:
620LET A(I)=AREA
630A=A+A(I)
640B=B+A(I)^2
650NEXT I
660C1=A/N1
670V=(B-(C1*A))/(N1-1)
680D1=SQR(V)
690FORX=1TO1000
700NEXT X
710ENDPROC
720DEF PROCtest
730T=(C-C1)/SQR(D^2/N+D1^2/N1)
740ENDPROC
750DEF PROCprint1
760CLS
770PRINT:PRINT:
780PRINT"MEAN FOR STIMULATED  = ";C
790PRINT
800PRINT"S.D. FOR STIMULATED  = ";D
810PRINT
820PRINT"NO. OF STIMULATED    = ";N
830PRINT
840PRINT"MEAN FOR BASAL       = ";C1
850PRINT
860PRINT"S.D. FOR BASAL       = ";D1
870PRINT
880PRINT"NO. OF BASAL         = ";N1
890PRINT
900PRINT"RESULT OF T TEST     = ";T
910PRINT
920PRINT"DEGREES OF FREEDOM   = ";(N+N1)-2
930PRINT:PRINT:
940ENDPROC
950DEF PROCprint2
960CLS
970PRINT:PRINT:
980INPUT"ENTER EXPERIMENTAL TITLE ";EX$
990VDU2
1000*FX6
1010VDU21
1020PRINT:PRINT
1030PRINT TAB(20);EX$
1040PRINT:PRINT:
1050PRINT TAB(15);"MEAN FOR BASAL      = ";C1
1060PRINT TAB(15);"S.D. FOR BASAL      = ";D1
1070PRINT TAB(15);"NUMBER OF BASAL     = ";N1
1080PRINT:PRINT
1090PRINT TAB(15);"MEAN FOR STIMULUS   = ";C
1100PRINT TAB(15);"S.D. FOR STIMULUS   = ";D
1110PRINT TAB(15);"NUMBER OF STIMULUS  = ";N
1120PRINT:PRINT
1130PRINT TAB(15);"RESULT OF T.TEST    = ";T
1140PRINT TAB(15);"DEGREES OF FREEDOM  = ";(N+N1)-2
1150VDU6
1160VDU3
1170ENDPROC
1180DEF PROCerror1
1190PRINT:PRINT
1200VDU7
1210PRINT"YOU MUST ENTER AT LEAST TWO EXPERIMENTAL RESULTS AT A TIME. THE COMPU
TER CAN'T  DIVIDE BY ZERO !":PRINT:PRINT
1220INPUT"PRESS RETURN WHEN READY ";X$:IF X$=INKEY$(-74) THEN CLS: GOTO 160
```

```
1230ENDPROC
1240DEF PROCerror2
1250PRINT:PRINT
1260VDU7
1270PRINT"YOU MUST ENTER AT LEAST TWO BASAL        RESULTS AT A TIME. THE COMPUT
ER CAN'T   DIVIDE BY ZERO !":PRINT:PRINT
1280INPUT"PRESS RETURN WHEN READY ";X$:IF X$=INKEY$(-74) THEN CLS:GOTO 450
1290ENDPROC
```

A6. Publications arising from this thesis.

Regular papers.

1/Lambert, D.G., Hughes, K. and Atkins, T.W (1986) Insulin release from a cloned B-cell line (HIT-T15). The effects of glucose, amino acids, sulphonylureas and colchicine. Biochem. Biophys. Res. Commun. 140(2): 616-625.

2/Lambert, D.G., Hughes, K. and Atkins, T.W (1986) 12-0-tetradecanoylphorbol-13-acetate and 4 alpha phorbol-12, 13 didecanoate stimulate insulin secretion from a cloned B-cell line RINm5F. IRCS. Med. Sci 14: 922-923.

3/Lambert, D.G., Douglas, J.P. and Atkins, T.W (1986) C-peptide parallels insulin release from a cloned B-cell line HIT-T15. IRCS. Med. Sci. 14: 1137-1138.

4/Lambert, D.G. and Atkins, T.W (1987) Modification of insulin release from cloned B-cell lines HIT-T15 and RINm5F by agents capable of altering the turnover of intracellular cAMP. Med. Sci. Res. 15: 139-140.

5/Lambert, D.G. and Atkins, T.W (1987) Nutrient induced insulin release from an insulinoma derived B-cell line. Acta. Diabetologica. Latina. In Press.

Reviews and articles.

1/Atkins,T.W. and Lambert, D.G (1986) Diabetes research with cultured cells. Balance 94: 15-16

2/Lambert, D.G. and Atkins, T.W (1987) Cultured insulin secreting tissues. Med. Sci. Res. 15: 279-281.

Published conference abstracts. (**P**=poster, **O**=oral)

1/Lambert, D.G. and Atkins, T.W. (1985) Potentiation of glucose induced insulin release from a cloned B-cell line (HIT-T15) by arginine and leucine. Diabetic. Med. 2(6): 515A **P**

2/Lambert, D.G. and Atkins, T.W. (1986) Modification of insulin release from a cloned B-cell line (HIT-T15) by forskolin, imidazole and sulphonylureas. J.Endocr. 107(Suppl): 122. **P**

3/Lambert, D.G. and Atkins, T.W. (1986) The effects of calcium antagonists and IP3 on insulin release from a clonal B-cell line (HIT-T15). J.Endocr. 108(Suppl): 296. **P**

4/Lambert, D.G, Hughes, K. and Atkins, T.W. (1986) Biphasic insulin release from a cloned B-cell line (HIT-T15). Diabetic. Med. 3(4): 373A. **P**

5/Coleman, A., Lambert, D.G. and Atkins, T.W. (1986) The effect of colchicine on insulin release. Diabetic. Med. 3(4): 372A. **P**

6/Lambert, D.G., Hughes, K. and Atkins, T.W. (1986) Paracrine modulation of insulin release from cloned B-cell lines HIT-T15 and RINm5F. J.Endocr. 111(Suppl): 96. **O**

7/Lambert, D.G., Douglas, J.P. and Atkins, T.W. (1986) Cholinergic stimulationof insulin release from cloned B-cell lines, HIT-T15 and RINm5F. Diabetic. Med. 3(6): 569A. **P**

8/Lambert, D.G., and Atkins, T.W. (1987) Insulin release from a cloned B-cell line RINm5F: The effects of glyceraldehyde, forskolin, imidazole and calcium antagonists. J.Endocr. 112(Suppl): 81. **P**

9/Lambert, D.G., Hughes, K. and Atkins, T.W. (1987) Gastric inhibitory polypeptide stimulates insulin secretion from HIT-T15 cells in the absence of glucose. Diabetic. Med. 4(4): 338A. **P**

10/Lambert, D.G., Atkins, T.W., Hale, P., Nattrass, M., Baddeley, M. and Bailey, C.J. (1987) Human islet cell adenoma maintained in monolayer culture. Diabetic. Med. In Press. **P**

11/Lambert, D.G., Bistacchi, D. and Atkins, T.W. (1987) Tolbutamide stimulationof insulin release from HIT-T15 and UMR 407/3 cells does not involve glucose oxidation. Diabetic. Med. In Press. **P**

REFERENCES.

9. REFERENCES.

1. Harrison, R.G. (1907) Observations on the live developing nerve fiber. Proc. Soc. Exp. Biol. (NY) 4: 140-143.

2. Banting, F.G. and Best, C.H. (1922) The internal secretion of the pancreas. J. Lab. Clin. Med. 7: 251-266.

3. Kapel, O. (1926) Sur la culture in vitro du tissu hepatique et pancreatique. C.R. Soc. Biol. 95: 1108-1109.

4. Murray, M.R. and Bradley, C.F. (1935) Two island cell adenomas of the pancreas cultivated in vitro. Amer. J. Cancer. 25: 98-107.

5. Hofteizer, V. and Carpenter, A.M. (1973) Comparison of streptozotocin and alloxan induced diabetes in the rat, including volumetric quantitation of the pancreatic islets. Diabetologia. 9: 178-184.

6. Orci, L. (1976) Morphofunctional aspects of the islets of Langerhans. The microanatomy of the islets of Langerhans. Metabolism. 25: 1303-1313.

7. Leach, F.N., Achworth, M.S., Barson, A.J. and Milner, R.D.G. (1973) Insulin release from human foetal pancreas in tissue culture. J. Endocrinology. 59: 65-79.

8. Chen, J.M. (1954) Cultivation in fluid medium of organised liver, pancreas and other tissues of foetal rats. Exp. Cell. Res. 7: 518-529.

9. Corre, H.G. and Randle, P.J. (1964) Regulation of insulin secretion studied with pieces of rabbit pancreas incubated in vitro. Biochem. J. 93: 66-78.

10. Maitland, J.E., Parry, D.G. and Turtle, J.R. (1980) Perifusion and culture of human foetal pancreas. Diabetes. 29(Suppl 1): 57-63.

11. Agren, A., Andersson, A., Bjorken, C., Groth, C.G., Gunnarsson, R., Hellerstrom, C., Lindmark, G., Lundqvist, G., Petersson, B. and Swenne, I. (1980) Human foetal pancreas culture and function in vitro. Diabetes. 29(Suppl 1): 64-69.

12. Andersson, A., Christensen, N., Groth, C.G., Hellerstrom, C., Petersson, B. and Sandler, S. (1984) Survival of human foetal pancreatic explants in organ culture as reflected in insulin secretion and oxygen consumption. Transplantation. 37: 499-503.

13. Mandel, T.E., Carter, W.M. and Koulmanda, M. (1984) Organ culture of human foetal pancreas for transplantation. Transplant. Proc. 16: 1040-1042.

14. Hellerstrom, C. (1964) A method for the microdissection of intact pancreatic islets of mammals. Acta. Endocrinol. 45: 122-132.

15. Lacy, P.E. and Kostianovsky, M. (1967) Method for the isolation of intact islets of Langerhans from the rat pancreas. Diabetes. 16: 35-39.

16. Mosalewski, S. (1965) Isolation and culture of the islets of Langerhans of the guinea pig. Gen. Comp. Endocrinol. 5: 342-353.

17. Andersson, A. and Hellerstrom, C. (1972) Metabolic characteristics of isolated pancreatic islets in tissue culture. Diabetes. 21: 546-554.

18. Andersson, A., Gunnarsson, R. and Hellerstrom, C. (1976) Long term effects of low extracellular glucose concentration on glucose metabolism and insulin biosynthesis and release of mouse pancreatic islets maintained in tissue culture. Acta. Endocrinol. 82: 318-329.

19. Andersson, A., Westman, J. and Hellerstrom, C. (1974) Effects of glucose on the ultrastructure and insulin biosynthesis of isolated mouse pancreatic islets maintained in tissue culture. Diabetologia. 10: 743-753.

20. Andersson, A. (1974) Synthesis of DNA in isolated pancreatic islets maintained in tissue culture. Endocrinology. 96: 1051-1054.

21. Turcot-Lemay, L. and Lacy, P.E. (1975) Effects of glucose and anti insulin serum on insulin released from adult rat isolated islets of Langerhans in long term organ culture (Fourteen days). Diabetes. 24: 658-663.

22. Jonsson, L.E., Ponten, J. and Thorell, J. (1966) Long term production of insulin by adult rat pancreas in vitro. Diabetologia. 2: 157-161.

23. Mandel, T.E. and Georgiou, H.M. (1983) Insulin secretion by foetal human pancreatic islets of Langerhans in prolonged organ culture. Diabetes. 32: 915-920.

24. Paul, J. (1975) in 'Cell and tissue culture'. 5th edition. Churchill Livingstone. London. pp 219-231.

25. Halban, P., Wollheim, C., Blondel, B., Meda, P., Neisor, E. and Mintz, D. (1982) The possible importance of contact between islet cells for the control of insulin release. Endocrinology. 111: 86-94.

26. Pipeleers, D., Veld, P., maes, E. and Winkel, M. (1982) Glucose induced insulin release depends on functional cooperation between islet cells. Proc. Natl. Acad. Sci. (USA). 79: 7322-7325.

27. Bone, A. and Swenne, I. (1982) Microcarriers: A new approach to pancreatic islet cell culture. In Vitro. 18: 141-148.

28. Bailey, C.J., Flatt, P.R. and Atkins, T.W. (1982) Influence of genetic background and age on the expression of the obese hyperglycaemic syndrome in Aston ob/ob mice. Int. J. Obesity. 6: 11-21.

29. Braaken, J.T., Lee, M.J., Schenk, A. and Mintz, D.H. (1974) Removal of fibroblastoid cells from primary monolayer cultures of rat neonatal endocrine pancreas by sodium ethylmercurithiosalicylate. Biochem. Biophys. Res. Commun. 61: 476-482.

30. Lambert, A.E., Blondel, B., Kanazawa, Y., Orci, L. and Renold, A.E. (1972) Monolayer cell culture of neonatal pancreas. Light microscopy evidence for immunoreactive insulin synthesis and release. Endocrinology. 90: 239-248.

31. Chick, W.L., Like, A.A. and Lauris, V. (1975) Pancreatic beta cell culture: Preparation of purified monolayers. Endocrinology. 96: 637-643.

32. Chick, W.L. and Boston, M.D. (1973) Beta cell replication in rat pancreatic monolayer cultures. Effects of glucose, tolbutamide, glucocorticoid, growth hormone and glucagon. Diabetes. 22: 687-693.

33. Chick, W.L., Lauris, V., Flewelling, J.H., Andrews, K.A. and Woodruff, J.M. (1973) Effects of glucose on beta cells in pancreatic monolayer culture. Endocrinology. 92: 212-218.

34. Creutzfeldt, W., Arnold, R., Creutzfeldt, C., Deuticke, H., Frerichs, H. and Track, N,S. (1973) Biochemical and morphological investigations of 30 human insulinomas. Correlation between the tumour content of insulin and proinsulin-like components and the histological and ultrastructural appearance. Diabetologia. 9: 217-231.

35. Chick, W.L., Lauris, V.L., Soeldner, J.S., Tan, M.H. and Grinbergs, M. (1973) Monolayer culture of a human pancreatic beta cell adenoma. Metabolism. 22: 1217-1224.

36. Yip, C.C. and Schimmer, B.P. (1973) Human pancreatic islet tumour cells maintained in vitro. Diabetologia. 9: 251-254.

37. Adcock, K., Austin, W., Duckworth, W.C., Solomon, S.S. and Murrell, L.R. (1975) Human islet cell adenoma: Metabolic analysis of the patient and the tumour cells in monolayer culture. Diabetologia. 11: 527-534.

38. Saxe, A.W., Yoon, J-W., Gorden, P. and Brennan, M.F. (1982) Cell culture and in vitro studies of fresh and cryopreserved human insulinoma. In Vitro. 18: 884-890.

39. Chiba, T., Yamatani, T., Kadowaki, S., Yamaguchi, A., Inui, T., Saito, Y. and Fujita, T. (1987) Glucose stimulates insulin release without altering cyclic AMP production or inositolphospholipid turnover in freshly obtained human insulinoma cells. Biochem. Biophys. Res. Commun. 145: 263-268.

40. Bone, A.J., Gumpert, R.W., Howell, S.L., Sheldon, J., Yudilevich-Tellez, M., Tyhurst, M., Whittaker, P.G. and Zaheer, F (1977) Regulation of insulin and glucagon secretion from a human islet cell adenoma. J. Endocrinology. 74: 273-280.

41. Akagi, T. and Fujii, Y. (1981) Histology, ultrastructure, and tissue culture of human insulinomas. Cancer. 47: 417-424.

42. Rakieten, N., Gorden, B.S., Beaty, A., Cooney, D.A., Davis, R.D. and Schein, P.S. (1971) Pancreatic islet cell tumours produced by the combined action of streptozotocin and nicotinamide. Proc. Soc. Exp. Biol. Med. 137: 280-283.

43. Kazumi, T., Yoshino, G., Yoshida, Y., Doi, K., Yoshida, M., Kaneko, S. and Baba, S. (1978) Biochemical studies on rats with insulin secreting islet cell tumours induced by streptozotocin: With special reference to physiological response to oral glucose load in the course of and after tumour induction. Endocrinology. 103: 1541-1545.

44. Masiello, P., Wollheim, C.B., Blondel, B. and Renold, A.E. (1983) Studies in vivo and in vitro on chemically induced primary islet cell tumours and non tumour endocrine pancreatic tissue. Diabetologia. 24: 30-38.

45. Frigo, I., Younoszai, R., Dixit, P.K., Matta, S.G. and Bauer, G.E. (1981) Insulin release in vitro from tissues and adenomas of rats treated with nicotinamide and streptozotocin. The effects of glucose. Diabetes. 30: 302-309.

46. Warren, S., Carlstein, R.G., Steinke, J. and Chute, R.N. (1964) Island cell tumours in irradiated rats in parabiosis. Proc. Soc. Exp. Biol. Med. 115: 910-912.

47. Chick, W.L., Warren, S., Chute, R.N., Like, A.A., Lauris, V. and Kitchen W.L. (1977) A transplantable insulinoma of the rat. Proc. Natl. Acad. Sci. (USA). 74: 628-632.

48. Masiello, P., Wollheim, C.B., Janjic, D., Gjinovici, A., Blondel, B., Praz, G.A. and Renold, A.E. (1982) Stimulation of insulin release by glucose in a transplantable rat islet cell tumour. Endocrinology. 111: 2091-2095.

49. Kirkman, H.A. (1962) A preliminary report concerning tumours observed in Syrian hamsters. Stanford. Med. Bull. 20: 163-166.

50. Schubart, U.K., Udem, L., Baum, S.G. and Rosen, O.M. (1974) Characteristics of cyclic 3',5'-nucleotide phosphodiesterase activity in an islet cell tumour of the Syrian hamster. Mol. Cell. Endocrinology. 1: 227-247.

51. Schubart, U.K., Shapiro, S., Fleisher, N. and Rosen, O.M. (1977) Cyclic adenosine 3':5'-monophosphate mediated insulin secretion and ribosomal protein phosphorylation in a hamster islet cell tumour. J. Biol. Chem. 252: 92-101.

52. Schubart, U.K., Fleisher, N. and Erlichmann, J. (1980) Ca^{2+}-dependent protein phosphorylation and insulin release in intact hamster insulinoma cells. Inhibition by trifluoperazine. J. Biol. Chem. 255: 11063-11066.

53. Rae, P.A., Yip, C.C. and Schimmer, B.P. (1979) Isolation of cloned Syrian hamster insulinoma cell lines with limited capacity for insulin production. Can. J. Physiol. Pharmacol. 57: 819-824.

54. Uchida, S., Watanabe, S., Aizawa, T., Furuno, A. and Muto, T. (1979) Polyoncogenicity and insulinoma inducing ability of BK virus, a human papovavirus in Syrian gloden hamsters. J. Natl. Cancer. Inst. 63: 119-126.

55. Kikkawa, R., Haneda, M., Hatanaka, I., Yasuda, H., Shigeta, Y., Okuno, Y. and Wada, M. (1983) Insulin release from BK virus induced and in vitro maintained insulinoma cells of Syrian gloden hamsters. Horm. Metab. Res. 15: 221-224.

56. Gazdar, A.F., Chick, W.L., Oie, H.K., Sims, H.L., King, D.L., Weir, G.C. and Lauris, V. (1980) Continuous, clonal, insulin and somatostatin secreting cell lines established from a transplantable rat islet cell tumour. Proc. Natl. Acad. Sci. (USA). 77: 3519-3523.

57. Mc Evoy, R.C., Franklin, B.H. and Ginsberg-Fellner, F. (1986) Biological differences in a rat insulinoma cell line obtained from different laboratories do not affect binding of human anti-islet immunoglobulins. Diabetes. Res. 3: 339-344.

58. Praz, G.A., Halban, P.A., Wollheim, C.B., Blondel, B., Strauss, A.J. and Renold, A.E. (1983) Regulation of immunoreactive insulin release from a rat cell line (RINm5F). Biochem. J. 210: 345-352.

59. Giroix, M-H., Sener, A., Dufrane, S.P., Malaisse-Lagae, F. and Malaisse, W.J. (1985) Glucose metabolism in insulin producing tumoral cells. Arch. Biochem. Biophys. 241: 561-570.

60. Carrington, C.A., Rubery, E.D., Pearson, E.C. and Hales, C.N. (1986) Five new insulin producing cell lines with differing secretory properties. J. Endocrinology. 109: 193-200.

61. Neisor, E.T., Wollheim, C.B., Mintz, D.H., Blondel, B., Renold, A.E. and Weil, R. (1979) Establishment of rat pancreatic cell lines by infection with Simian virus 40. Biochem. J. 178: 559-568.

62. Santerre, R.F., Cook, R.A., Crisel, R.M.D., Sharp, J.D., Schmidt, R.J., Williams, D.C. and Wilson, D.C. (1981) Insulin synthesis in a clonal cell line of Simian virus 40 transformed hamster pancreatic beta cells. Proc. Natl. Acad. Sci. (USA). 78: 4339-4343.

63. Ng, K.W., Gummer, P.R., Grills, B.L., Michelangeli, V.P. and Dunlop, M.E. (1983) Insulin release from a cloned precursor beta cell line. J. Endocrinology. 113: 3-10.

64. Smith, J.S. and Karow, A.M. (1980) The effect of dimethyl sulphoxide on insulin release and phosphate efflux from isolated perifused islets of Langerhans. Res. Commun. Chem. Pathol. and Pharmacol. 30: 459-468.

65. Davis, B.D. (1980) Bacterial nutrition and growth. In 'Microbiology' 3rd Edition. Harper and Row. International. Editors, Davis, B.D., Dulbecco, R., Eisen, H.N. and Ginsberg, H.S. Chapter 5, pp 60-70.

66. Holley, R.W. (1975) Control of growth in mammalian cells. Nature. 258: 487-490.

67. Scherer, W.F. (1965) Practical applications and problems of low temperature preservation techniques for cultured animal cells. Fed. Proc. 24(Suppl 15): 5304-5305.

68. Rey, L-R. (1957) Studies on the action of liquid nitrogen on cultures in vitro of fibroblasts. Proc. R. Soc. (B). 147: 460-466.

69. Lovelock, J.E. (1953) The haemolysis of human red blood cells by freezing and thawing. Biochem. Biophys. Acta. 10: 414-426.

70. Lovelock, J.E. (1953) The mechanism of the protective action of glycerol against haemolysis by freezing and thawing. Biochem. Biophys. Acta. 11: 28-36.

71. Lovelock, J.E. (1954) The protective action of neutral solutes against haemolysis by freezing and thawing. Biochem. J. 56: 265-270.

72. Taylor, M.J. and Benton, M.J. (1987) Interaction of cooling rate, warming rate, and extent of permeation of cryoprotectant in determining survival of isolated rat islets of Langerhans during cryopreservation. Diabetes. 36: 59-66.

73. Hellerstrom, C., Andersson, A. and Sandler, S. (1984) Viability of cryopreserved cultured mouse pancreatic islets using a rapid freezing rate. Cryobiology. 21: 707A.

74. Sandler, S., Kojima, Y. and Andersson, A. (1987) Cryopreservation of mouse pancreatic islets. The effects of fast cooling on islet B-cell function and on the outcome of islet transplantation. Transplantation. In press. (quoted in ref 72)

75. Toledo-Pereyra, L.H., Gorden, D.A. and MacKenzie, G.H. (1983) Application of cryopreservation techniques to islet cell allotransplantation. Cryobiology. 20: 205-210.

76. Wise, M.H., Gorden, C. and Johnson, R.W.G. (1985) Intraportal autotransplantation of cryopreserved porcine islets of Langerhans. Cryobiology. 22: 359-366.

77. Lutz, G., Diller, K.R. and Kulbe, K.D. (1984) Cryopreservation of pig islets of Langerhans: effects of cooling rates and different cryoprotectants. Cryobiology. 21: 708A.

78. Samols, E. and Williams, H.S. (1961) Trace labelling of insulin with iodine. Nature. 109: 1211-1212.

79. Redshaw, M.R. and Lynch, S.S. (1974) An improved method for the preparation of iodinated antigens for radioimmunoassay. J. Endocrinology. 60: 527-528.

80. Pennisi, F. and Rosa, U. (1969) Preparation of radioiodinated insulin by constant current electrolysis. J. Nucl. Biol. Med. 13: 64-70.

81. Marchalonis, J.J. (1969) An enzymatic method for the trace iodination of immunoglobulins and other proteins. Biochem. J. 113: 299-305.

82. Hunter, W.M. and Greenwood, F.C. (1962) Preparation of iodine-131 labelled human growth hormone of high specific activity. Nature. 194: 495-496.

83. Freedlender, A. and Cathou, R.E. (1971) Iodination. In, 'Radioimmunoassay Methods' Churchill Livingstone, Edinburgh. Editors, Kirkham, K.E. and. Hunter, W.M. pp 94.

84. De Meyts, P. (1976) Insulin and growth hormone receptors in human cultured lymphocytes and peripheral blood monocytes. In 'Methods in receptor research' Marcel Dekker, New York. Editor, Belcher, M. Part 1 pp 301-383.

85. Ganguli, P.C. and Hunter, W.M. (1971) Iodination of gastrin. In, 'Radioimmunoassay methods' Churchill Livingstone, Edinburgh. Editors, Kirkman, K.E. and Hunter, W.M. pp 54.

86. Freychet, P., Roth, J. and Neville, D.M. Jnr. (1971) Monoiodoinsulin :demonstration of its biological activity and binding to fat cells and liver membranes. Biochem. Biophys. Res. Commun. 43: 400-408.

87. Rodbard, D. (1973) Mathematics of hormone receptor interaction. 1 Basic principles. In, 'Receptors for reproductive hormones' Plenum press New York, London. Editors, O'Malley, B.W. and Means, A.R. pp 289-326.

88. Yalow, R.S. and Berson, S.A. (1960) Immunoassay of endogenous plasma insulin in man. J. Clin. Invest. 39: 1157-1175.

89. Hales, C.N. and Randle, P.J. (1963) Immunoassay of insulin with insulin antibody precipitate. Biochem. J. 88: 137-146.

90. Heding, L.G. (1975) Radioimmunological determination of human C-peptide in serum. Diabetologia. 11: 541-548.

91. Fyles, J.M., Cawthorne, M.A. and Howell, S.L. (1986) The characteristics of ß-adrenergic binding sites on pancreatic islets of Langerhans. J. Endocrinology. 111: 263-270.

92. Hussain, M. and Atkins, T.W. (1985) The effect of digitoxose on insulin release, glucose oxidation and oxygen consumption by islets of lean and genetically obese diabetic (ob/ob) mice. Biochem. Biophys. Res. Commun. 129: 358-367.

93. Lindstrom, P. and Sehlin, J. (1986) Effect of intracellular alkalinization on pancreatic islet calcium uptake and insulin secretion. Biochem. J. 239: 199-204.

94. Hill, R.S. and Boyd, A.E III (1985) Perifusion of a clonal cell line of Simian virus 40-transformed beta cells. Insulin secretory dynamics in response to glucose, 3-isobutyl-1-methylxanthine, and potassium. Diabetes. 34: 115-120.

95. Ashcroft, S.J.H., Weerasinghe, L.C.C., Bassett, J.M. and Randle, P.J. (1972) The pentose cycle and insulin release in mouse pancreatic islets. Biochem. J. 126: 525-532.

96. Lessler, M.A. and Brierly, G.P. (1969) Oxygen electrode measurements in biochemical analysis. In, 'Methods of biochemical analysis' Willey, New York. Editor, Glick, D. Vol 17, pp 1-50.

97. Hedeskov, C.J. (1980) Mechanism of glucose induced insulin release. Physiol. Rev. 60: 442-509.

98. Ashcroft, S.J.H., Bassett, J.M. and Randle, P.J. (1972) Insulin secretion mechanisms. Diabetes. 21(Suppl 2): 538-545.

99. Malaisse, W.J., Sener, A., Herchuelz, A. and Hutton, J.C. (1979) Insulin release: the fuel hypothesis. Metabolism. 28: 373-386.

100. Niki, A. and Niki, H. (1980) Hexose anomers and insulin release in diabetes mellitus. Biomed. Res. 1: 189-206.

101. Ashcroft, S.J.H., Weerasinghe, L.C.C. and Randle, P.J. (1973) Interrelationships of islet metabolism, adenosine triphosphate content and insulin release. Biochem. J. 132: 223-231.

102. Ashcroft, S.J.H. and Lowry, M. (1979) B-cell recognition of sterioisomers of D-glucose. Diabetologia. 17: 165-168.

103. Ashcroft, S.J.H. (1980) Glucoreceptor mechanisms and the control of insulin biosynthesis and release. Diabetologia. 18: 5-15.

104. Malaisse, W.J., Sener, A., Koser, M. and Herchuelz, A. (1976) Identification of the L-steriospecific glucose sensor in the pancreatic B-cell. FEBS. Lett. 65: 131-134.

105. Hellman, B., Lernmark, A., Sehlin, J. and Taljedal, I-B. (1972) Effects of phloridzin on metabolism and function of pancreatic B-cells. Metabolism. 21: 60-66.

106. Ashcroft, S.J.H. and Nino, S. (1978) Effects of phloretin and dextran linked phloretin on pancreatic islet metabolism and insulin release. Biochem. Biophys. Acta. 538: 334-342.

107. Ashcroft, S.J.H. and Randle, P.J. (1970) Enzymes of glucose metabolism in normal mouse pancreatic islets. Biochem. J. 119: 5-15.

108. Sener, A. and Malaisse, W.J. (1984) Nutrient metabolism in islet cells. Experientia. 40: 1026-1035.

109. Giriox, M-H., Sener, A., Pipeleers, D.G. and Malaisse, W.J. (1984) Hexokinase metabolism in pancreatic islets: inhibition of hexokinase.. Biochem. J. 223: 447-453.

110. Meglasson, M.D. and Matschinsky, F.M. (1984) New perspectives on pancreatic islet glucokinase. Am. J. Physiol. 246: E1-E13.

111. Malaisse, W.J. and Sener, A. (1985) Glucokinase is not the pancreatic glucoreceptor. Diabetologia. 28: 520-527.

112. Ashcroft, S.J.H. (1976) The control of insulin release by sugars. In, 'Polypeptide hormones. Molecular and cellular aspects'. Ciba Foundation Symposium. 41: 117-139. North Holland, Elsiever. Excerpta Medica.

113. Niki, A., Niki, M. and Miwa, I. (1979) Effects of anomers of D-glucose on insulin release from perfused rat pancreas. Endocrinology. 105: 1050-1054.

114. Davis, B. and Lazarus, N. (1976) An in vitro system for studying insulin release caused by secretory granules-plasma membrane interaction of the system. J. Physiol (Lond). 256: 709-729.

115. Zawalich, W.S. and Beidler, L.M. (1973) Glucose and alloxan interaction in the pancreatic B-cells. Am. J. Physiol. 224: 963-966.

116. Tomita, T., Lacy, P.E. and Matschinsky, F.M. (1974) Effects of alloxan on insulin secretion in isolated rat islets perifused in vitro. Diabetes 23: 517-524.

117. Kawada, J., Okita, M., Nishida, M., Yoshimura, Y., Toyooka, K. and Kubota, S. (1987) Protective effect of 4,6-O-ethylidene glucose against the cytotoxicity of streptozotocin in pancreatic B-cells in vivo: indirect evidence for the presence of a glucose transporter in B-cells. J. Endocrinology. 112: 375-378.

118. Newton, D.F. (1979) A study of glucose stimulus insulin secretion coupling in pancreatic islets of lean and obese mice. Ph.D Thesis, University of Aston, Birmingham.

119. Price, S. (1973) Pancreatic islet cell membranes. Extraction of a possible glucoreceptor. Biochem. Biophys. Acta. 318: 459-463.

120. Kobayashi, K., Yoshida, K., Tsumura, Y. and Kagawa, S. (1980) Studies on glucoreceptor specific binding of D-glucose to partially purified membranes of rat islets of Langerhans. J. Pharm. Dyn. 3: 33-40.

121. McDaniel, M.L., Anderson, S. and Fink, J. (1975) Effect of alloxan on permeability and hexose transport in rat pancreatic islets. Endocrinology. 97: 68-75.

122. Floyd, C.J., Fajans, S.S., Conn, J.W., Knopf, R.F. and Rull, J. (1966) Insulin secretion in response to protein ingestion. J. Clin. Invest. 45: 1479-1486.

123. Floyd, J.C., Fajans, S.S., Conn, J.W., Knopf, R.F. and Rull, J. (1966) Stimulation of insulin secretion by amino acids. J. Clin. Invest. 45: 1487-1502.

124. Rapitis, S., Dollinger, H.C., Schroeder, K.E., Schleyer, M. and Rothenbuchner, G. (1973) Differences in insulin, growth hormone and pancreatic enzyme secretion after intravenous and intraduodenal administration of amino acids in man. New. Engl. J. Med. 288: 1199-1202.

125. Marks, V. and Samols, E. (1970) Intestinal factors in the regulation of insulin secretion. Advances. Metab. Disord. 4: 1-38.

126. Milner, R.D.G. (1970) The stimulation of insulin release by essential amino acids from rabbit pancreas in vitro. J. Endocrinology. 47: 347-356.

127. Lambert, A.E., Kanazawa, Y., Orci, L. and Christensen, H.N. (1970) Stimulation of insulin release by natural amino acids and their nonmetabolisable analogues. Diabetologia. 6: 635A

128. Lernmark, A. (1972) Effects of neutral and dibasic amino acids on the release of insulin. Hormones. 3: 22-30.

129. Sehlin, J. (1972) Uptake and oxidation of glutamic acid in mammalian pancreatic islets. Hormones. 3: 156-166.

130. Malaisse, W.J. and Malaisse-Lagae, F. (1968) Stimulation of insulin secretion by noncarbohydrate metabolites. J. Lab. Clin. Med. 72: 438-448.

131. Sehlin, J. (1972) Transport and oxidation of glycine in mammalian pancreatic islets with reference to the mechanism of amino acid induced insulin release. Hormones. 3: 144-153.

132. Lambert, A.E., Jeanrenaud, B., Junod, A. and Renold, A.E. (1969) Organ culture of foetal rat pancreas. II Insulin release induced by amino and organic acids, by hormonal prptides, by cationic alterations of the medium and by other agents. Biochem. Biophys. Acta. 174: 540-553.

133. Fajans, S.S., Floyd, J.C., Knopf, R.F. and Conn, J.W. (1967) Effects of amino acids and proteins on insulin secretion in man. Recent. Prog. Horm. Res. 23: 617-662.

134. Pfeiffer, E.F. and Telib, M. (1968) Insulin secretion in vitro: Studies in amphibians and mammals. Act. Diabet. Lat. 5(Suppl 1): 30-63.

135. Efendic, S., Cerasi, E. and Luft, R. (1971) Role of glucose in arginine induced insulin release in man. Metabolism. 20: 568-579.

136. Lernmark, A. (1972) Specificity of leucine stimulation of insulin release. Hormones: 14-21.

137. McChesney, T. and Schofield, J.G. (1969) Studies on the secretion of pancreatic glucagon. Diabetes. 18: 627-632.

138. Hellman, B., Sehlin, J. and Taljedal, I-B. (1971) Effects of glucose and other modifiers of insulin release on the oxidative metabolism of amino acids in microdissected pancreatic islets. Biochem. J. 123: 513-521.

139. Alberti, K.G.G.M. and Whalley, M.D. (1973) Short chain analogues of arginine: Potent stimulators of insulin secretion. In, 'Proceedings VIII Congress of the international diabetes federation' Amsterdam, Excerpta Medica. pp21.

140. Malaisse, W.J., Hutton, J.C., Carpinelli, A.R., Herchuelz, A. and Sener, A. (1980) The stimulus secretion coupling of amino acid induced insulin release: Metabolism and cationic effects of leucine. Diabetes. 29: 431-437.

141. Hellman, B., Sehlin, J. and Taljedal, I-B. (1971) Uptake of alanine, arginine and leucine by mammalian pancreatic B-cells. Endocrinology. 89: 1432-1439.

142. Panten, U., Kriegstein, E.V., Poser, W., Schonborn, J. and Hasselblatt, A. (1972) Effects of leucine and alpha-ketoisocaproic acid upon insulin secretion and metabolism of isolated pancreatic islets. FEBS. Lett. 20: 225-228.

143. Panten, U., Christians, J., Kriegstein, E.V., Poser, W. and Hasselblatt, A. (1974) Studies on the mechanism of L-leucine and alpha-ketoisocaproic acid induced insulin release from perifused isolated pancreatic islets. Diabetologia. 10: 149-154.

144. Sener, A., Malaisse-Lagae, F. and Malaisse, W.J. (1981) Stimulation of pancreatic islet metabolism and insulin release by a nonmetabolisable amino acid. Proc. Natl. Acad. Sci. (USA). 78: 5460-5464.

145. Malaisse-Lagae, F., Brison, G.R. and Malaisse, W.J. (1971) The stimulus secretion coupling of glucose induced insulin release VI. Analogy between the insulinotrophic mechanisms of sugars and amino acids. Horm. Metab. Res. 3: 374-378.

146. Henquin, J.C. and Lambert, A.E. (1975) Cobalt inhibition of insulin secretion and calcium uptake by isolated rat islets. Am. J. Physiol. 228: 1669-1677.

147. Leclercq-Meyer, V., Marchand, J. and Malaisse, W.J. (1978) The role of calcium in insulin release. Studies with verapamil. Diabetes. 27: 996-1004.

148. Howell, S.L. and Montague, W. (1973) Adenylate cyclase activity in isolated rat islets of Langerhans, effect of agents which alter rates of insulin secretion. Biochem. Biophys. Acta. 320: 44-52.

149. Feinglos, M.N. and Lebovitz, H.E. (1980) Sulphonylurea treatment of insulin independent diabetes mellitus. Metabolism. 29: 488-494.

150. Maden J. (1967) Extrapancreatic and intrapancreatic action of sulphonylureas. A review. Acta. Med. Scand. [Suppl] 476: 109-122.

151. Maloff, B.L. and Lockwood, D.H. (1981) In vitro effects of a sulphonylurea on insulin action in adipocytes. Potentiation of insulin stimulated hexose transport. J. Clin. Invest. 68: 85-91.

152. Pfeifer, M.A., Halter, J.B. and Porte, D. Jr. (1981) Insulin secretion in diabetes mellitus. Am. J. Med. 70: 579-588.

153. Hellman, B., Sehlin, J. and Taljedal, I-B. (1984) Glibenclamide is exceptional among hypoglycaemic sulphonylureas in accumulating progressively in B-cell rich pancreatic islets. Acta. Endocrinologica. 105: 385-390.

154. Hellman, B., Lernmark, A., Sehlin, J. and Taljedal, I-B. (1973) The pancreatic B-cell recognition of insulin secretagogues. Inhibitory effects of a membrane probe on the islet uptake and insulin releasing action of glibenclamide. FEBS. Lett. 34: 347-349.

155. Borg, H. and Andersson, A. (1981) Long term effects of glibenclamide on insulin production, oxidative metabolism and quantitative ultrastructure of mouse pancreatic islets maintained in tissue culture at different glucose concentrations. Acta. Diabet. Lat. 18: 65-83.

156. Hellman, B. (1982) The mechanism of sulphonylurea stimulation of insulin release. Acta. Biol. Med. Germany. 41: 1211-1219.

157. Matthews, E.K. and Dean, P.M. (1970) The biophysical effects of insulin releasing agents on islet cells. Postgrad. Med. J. 46: 21-23.

158. Meissner, H.P. and Attwater, I.J. (1976) The kinetics of electrical activity of beta cells in response to a 'square wave' stimulation with glucose or glibenclamide. Horm. Metab. Res. 8: 11-16.

159. Henquin, J.C. and Meissner, H.P. (1982) Opposite effects of tolbutamide and diazoxide on $^{86}Rb^{+}$ fluxes and membrane potential in pancreatic B-cells. Biochem. Pharmacol. 31: 1407-1415.

160. Lebrun, P., Malaisse, W.J. and Herchuelz, A. (1985) Do hypoglycaemic sulphonylureas inhibit Na^{+},K^{+}-ATP ase activity in pancreatic islets. Am. J. Physiol. 248: E411-E419.

161. Grill, V. (1980) Role of cyclic AMP in insulin release evoked by glucose and other secretagogues. Horm. Metab. Res. 10: 43-49.

162. Curry, D.L., Bennett, L.L. and Grodsky, G.M. (1968) Requirements for calcium ion in insulin secretion by the perfused rat pancreas. Am. J. Physiol. 214: 174-178.

163. Malaisse, W.J., Malaisse-Lagae, F. and Brisson, G. (1971) Combined effects of glucose and sulphonylureas on insulin secretion by the rat pancreas in vitro. In, 'Recent hypoglycaemic sulphonylureas mechanism of action and clinical implications' Bern Huber. Editors, Dubach, U.C. and Buckert, A. pp114-126.

164. Henquin, J.C. (1980) Tolbutamide stimulation and inhibition of insulin release: studies on the underlying ionic mechanisms in isolated rat islets. Diabetologia. 18: 151-160.

165. Basabe, J.C., Farina, J.M.S. and Chieri, R.A. (1976) Studies on the dynamics and mechanism of glibenclamide induced insulin secretion. Horm. Metab. Res. 8: 413-419.

166. Lacy, P.E., Walker, M.M. and Fink, C.J. (1972) Perifusion of isolated rat islets in vitro. Participation of the microtubular system in the phasic mechanism of insulin release. Diabetes. 21: 987-998.

167. Ashcroft, S.J.H., Hammonds, P. and Harrison, D.E. (1986) Insulin secretory responses of a clonal cell line of simian virus 40 transformed B-cells. Diabetologia. 29: 727-733.

168. Pipellers, D., Veld, P., Maes, E. and Winkel, M. (1982) Glucose induced insulin release depends upon functional cooperation between islet cells. Proc. Natl. Acad. Sci. (USA). 79: 7322-7325.

169. Meglasson, M.D., Manning, C.D., Najafi, H. and Matschinsky, F.M. (1986) Glucose transport by radiation induced insulinoma and clonal pancreatic B-cells. Diabetes. 35: 1340-1344.

170. Halban, P.A., Praz, G.A. and Wollheim, C.B. (1983) Abnormal glucose metabolism accompanies failure of glucose to stimulate insulin release from a rat pancreatic cell line (RINm5F). Biochem. J. 212: 439-443.

171. Sener, A., Leclercq-Meyer, V., Giriox, M-H., Malaisse, W.J. and Hellerstrom, C. (1987) Opposite effects of D-glucose and a nonmetabolized analogue of L-leucine on respiration and secretion in insulin producing tumoral cells (RINm5F). Diabetes. 36: 187-192.

172. Vischer, U., Blondel, B., Wollheim, C.B., Hoppner, W., Seitz, H.J. and Iynedjian, P.B. (1987) Hexokinase isoenzymes of RINm5F insulinoma cells. Expression of glucokinase gene in insulin producing cells. Biochem. J. 241: 249-255.

173. Malaisse, W.J., Herchuelz, A., Levy, J., Sener, A., Pipeleers, D.G., Devis, G. and Van Obberghen, E. (1976) The stimulus secretion coupling of glucose induced insulin release XLX. The insulinotrophic effect of glyceraldehyde. Mol. Cell. Endocrinol. 4: 1-12.

174. Knudson, P., Kofod, H., Lernmark, A. and Hedeskov, C.J. (1983) L-leucine methyl ester stimulates insulin secretion and islet glutamate dehydrogenase. Am. J. Physiol. 245: E338-E346.

175. Khalid, Q. and Rahman, M.A. (1975) Studies on the synergistic effect of glucose on insulin release from isolated rat islets. Biochem. Soc. Trans. 3: 1043-1044.

176. Ashcroft, S.J.H., Hedeskov, C.J. and Randle, P.J. (1970) Glucose metabolism in mouse pancreatic islets. Biochem. J. 118: 143-154.

177. Kawazu, S., Sener, A., Couturier, E. and Malaisse, W.J. (1980) Metabolic, cationic and secretory effects of hypoglycaemic sulphonylureas in pancreatic islets. Naunyn. Schmiedebergs. Arch. Pharmak. 312: 277-283.

178. Grill, V. and Cerasi, E. (1978) Immediate and time dependent effects of glucose on insulin release from rat pancreatic tissue. J. Clin. Invest. 61: 1034-1043.

179. Wollheim, C.B. and Sharp, G.W.G. (1981) Regulation of insulin release by calcium. Physiol. Rev. 61: 914-973.

180. Simar, L.J., Sodoyez, J.C., Grillo, A.I., Nagano, T., Lefebvre, P.J. and Foa, P.P. (1968) Biological properties of a transplantable islet cell tumour of the golden hamster. Electron microscopic studies. Diabetes. 17: 582-587.

181. Dustin, P. (1984) In, 'Microtubules' 2nd Edition. Springer-verlag, Berlin. pp 482.

182. Williams, J.A. and Wolff, J. (1970) Possible role of microtubules in thyroid secretion. Proc. Natl. Acad. Sci. (USA). 67: 1901-1908.

183. Orci, L., Malaisse-Lagae, F., Rouiller, D., Renold, A.E., Perrelet, A. and Unger, R.H. (1975) A morphological basis for intercellular communication between alpha and beta cells in the endocrine pancreas. J. Clin. Invest. 56: 1066-1070.

184. Pipeleers, D. (1984) Islet cell interactions with pancreatic B-cells. Experientia. 40: 1114-1126.

185. Samols, E. and Harrison, J. (1976) Intraislet negative insulin glucagon feedback. Metabolism. 25: 1443-1447.

186. Weir, G.C., Knowlton, S.D., Atkins, R.F., McKennan, K.X. and Martin, D.B. (1976) Glucagon secretion from the perfused pancreas of streptozotocin treated rats. Diabetes. 25: 275-282.

187. Asplin, C.M., Paquette, T.L. and Palmer, J.D. (1981) In vivo inhibition of glucagon secretion by paracrine beta cell activity in man. J. Clin. Invest. 68: 314-318.

188. Service, F.J., Nelson, R.L., Rubenstein, A.H. and Go, V.L.W. (1978) Direct effect of insulin on the secretion of insulin, glucagon, gastric inhibitory polypeptide and gastrin during maintenance of normoglycaemia. J. Clin. Endocrinol. Metab. 47: 488-493.

189. Weir, G.C., Samols, E., Loo, S., Patel, Y.C. and Gabbay, K.H. (1979) Somatostatin and pancreatic polypeptide secretion. Effects of glucagon, insulin and arginine. Diabetes. 28: 35-40.

190. Gerber, P.P.G., Trimble, E.R., Wollheim, C.B. and Renold, A.E. (1981) Effect of insulin on glucose and arginine stimulated somatostatin secretion from the isolated perfused rat pancreas. Endocrinology. 109: 279-283.

191. Patel, Y.C., Amherdt, M. and Orci, L. (1982) Quantitative electron microscopic autoradiography of insulin, glucagon and somatostatin binding sites on islets. Science. 217: 1155-1156.

192. Toyota, T., Abe, K., Kudo, M., Kimura, K. and Goto, Y. (1975) Inhibitory effects of synthetic rat C-peptide-1 on insulin secretion in the isolated perfused rat pancreas. Tohku. J. Exp. Med. 117: 79-83.

193. Samols, E., Marri, G. and Marks, V. (1965) Promotion of insulin secretion by glucagon. Lancet 2: 415-416.

194. Trimble, E.R. and Renold, A.E. (1981) Ventral and dorsal areas of rat pancreas. islet hormone content and secretion. Am. J. Physiol. 240: E422-E427.

195. Beatens, D., Malaisse-Lagae, F., Perrelet, A. and Orci, L. (1979) Endocrine pancreas: three dimensional reconstruction shows two types of islets of Langerhans. Science. 206: 1323-1325.

196. Patton, G.S., Ipp, E., Dobbs, R.E., Orci, L., Vale, W. and Unger, R.H. (1977) Pancreatic immunoreactive somatostatin release. Proc. Natl. Acad. Sci. (USA). 74: 2140-2143.

197. Weir, G.C., Samols, E., Day, J.A. and Patel, Y.C. (1978) Glucose and glucagon stimulate the secretion of somatostatin from the perfused canine pancreas. Metabolism[Suppl 1] 27: 1223-1226.

198. Ipp, E., Dobbs, R.E., Arimura, A., Vale, W., Harris, V. and Unger, R.H. (1977) Release of immunoreactive somatostatin from the pancreas in response to glucose, amino acids, pancreozymin -cholecystokinin and tolbutamide. J. Clin. Invest. 60: 760-765.

199. Samols, E., Weir, G.C. and Bonner-Weir, S. (1983) Intraislet insulin-glucagon-somatostatin relationships. In, 'Handbook of experimental pharmacology Vol 66/II, Glucagon' Springer-Verlag, Editor, Lefebvre, P.J. pp 133-173.

200. Alberti, K.G.G.M., Christensen, N.J., Christensen, S.E., Hansen, A.A.P., Iversen, J., Lundbaek, K., Seyer-Hansen, K. and Orskov, H. (1973) Inhibition of insulin secretion by somatostatin. Lancet 2: 1299-1301.

201. Johnson, D., Ensinck, J., Koerker, D., Palmer, J. and Goodner, C.J. (1975) Inhibition of glucagon and insulin secretion by somatostatin in the rat pancreas perfused in situ. Endocrinology. 96: 370-374.

202. Hermansen, K. and Schwartz, T.W. (1979) Differential sensitivity to somatostatin of pancreatic polypeptide, glucagon and insulin secretion from the isolated perfused canine pancreas. Metabolism. 28: 1229-1233.

203. Patel, Y. and Reichlin, S. (1978) Somatostatin in hypothalamus, extrahypothalamic brain and peripheral tissues of the rat. Endocrinology. 102: 523-530.

204. Benoit, R., Bohlen, P., Brazeau, P., Ling, N. and Guillemin, R. (1980) Isolation and characterisation of rat pancreatic somatostatin. Endocrinology. 107: 2127-2129.

205. Mandarino, L., Stenner, D., Blanchard, W., Nissen, S., Gerich, J., Ling, N., Brazeau, P., Bohlem, P., Esch, F. and Guillemin, R. (1981) Selective effects of somatostatin-14, -25 and -28 on in vitro insulin and glucagon secretion. Nature. 291: 76-77.

206. Murphy, W.A., Fries, J.L., Meyers, C.A. and Coy, D.H. (1981) Human pancreatic polypeptide inhibits insulin release in the rat. Biochem. Biophys. Res. Commun. 101: 189-193.

207. Arimura, A., Meyers, C.A., Case, W.L., Murphy, W.A. and Schally, A.V. (1979) Suppression of somatostatin levels in the hepatic portal and systemic plasma of the rat by synthetic human pancreatic polypeptide. Biochem. Biophys. Res. Commun. 89: 913-918.

208. Elahi, D., Andersen, D.K., Brown, J.C., Debas, H.T., Hershcopf, R.J., Raizes, G.S., Tobin, J.D. and Andres, R. (1979) Pancreatic alpha- and beta-cell responses to GIP infusion in normal man. Am. J. Physiol. E185-E191.

209. Szecowka, J., Lins, P.E. and Efendic, S. (1982) Effects of cholecystokinin, gastric inhibitory polypeptide, and secretin on insulin and glucagon secretion in rats. Endocrinology. 110: 1268-1272.

210. Elahi, D., Andersen, D.K., Tobin, J.D. and Andres, R. (1981) Discrepant performance of oral and intravenous glucose tolerance tests: The role of gastric inhibitory polypeptide. J. Clin. Endocrinol. Metab. 52: 1199-1203.

211. LaBarre, J. (1932) Sur les possibilities d'un traitement du diabete par l'incretine. Bull. Acad. R. Med. Belg. 12: 620-634.

212. Catland, S., Crockett, S.E., Brown, J.C. and Mazzaferri, E.L. (1974) Gastric inhibitory polypeptide (GIP) stimulation by oral glucose in man. J. Clin. Endocrinol. Metab. 39: 223-228.

213. Creutzfeldt, W. (1979) The incretin concept today. Diabetologia. 16: 75-85.

214. Creutzfeldt, W. and Ebert, R. (1985) New developments in the incretin concept. Diabetologia. 28: 565-573.

215. Caspary, W.F., Elsenhans, B., Sufke, J., Ptok, M., Blume, R., Lembcke, B. and Creutzfeldt, W. (1980) In, 'Frontiers of hormone research'. Basel, S. Karger. Editor, Creutzfeldt, W. pp 202-217.

216. Smith, P.H., Mechant, F.W., Johnson, D.G., Fujimoto, W.L. and Williams, R.H. (1977) Immunocytochemical localisation of a gastric inhibitory polypeptide like material within the A-cells of the endocrine pancreas. Am. J. Anat. 149: 585-590.

217. Brown, J.C., Dryburgh, J.R., Ross, S.A. and Dupre, J. (1975) Identification and actions of gastric inhibitory polypeptide. Rec. Prog. Horm. Res. 31: 487-531.

218. Montague, W. (1983) The function of the islets of Langerhans. In, 'Diabetes and the endocrine pancreas. A biochemical approach.' Croom Helm, London. pp116-131.

219. Korman, L.Y., Bhathena, S.J., Voyles, N.R., Oie, H.K. and Recant, L. (1985) Characteristics of the interaction of the glucagon receptor, cAMP and insulin secretion in parent cells and clone 5F of a cultured rat insulinoma. Diabetes. 34: 717-722.

220. Fujimoto, W,Y., Kawazu, S., Ikeuchi, M. and Kanazawa, Y. (1983) In vitro paracrine regulation of islet B-cell function by A and D cells. Life. Sciences. 32: 1873-1878.

221. Turcot-Lemay, L., Lemay, A. and Lacy, P.E. (1975) Somatostatin inhibition of insulin release from freshly isolated and organ cultured rat islets of Langerhans in vitro. Biochem. Biophys. Res. Commun. 63: 1130-1138.

222. Swope, S.L. and Schonbrunn, A. (1984) Bombesin stimulates insulin secretion by a pancreatic islet cell line. Proc. Natl. Acad. Sci. (USA). 81: 1822-1826.

223. Sarson, D.L., Wood, S.M., Kansal, P.C. and Bloom, S.R. (1984) Glucose dependent insulinotrophic polypeptide augmentation of insulin release. Physiology or pharmacology. Diabetes. 33: 389-393.

224. Raizes, G.S., Elahi, D., Andersen, D.K., Hershcopf, R.J., Tobin, J.D. and Andres, R. (1977) Insulin release in man by gastric inhibitory polypeptide and by arginine: Evidence for a common mechanism. Clin. Res. 25: 379-402.

225. Samols, E., Marri, G. and Marks, V. (1965) Promotion of insulin secretion by glucagon. Lancet. 2: 415-416.

226. Brisson, G.R., Malaisse-Lagae, F. and Malaisse, W.J. (1972) The stimulus secretion coupling of glucose induced insulin release. VII A proposed site of action for adenosine-3',5'-cyclic monophosphate. J. Clin. Invest. 51: 232-241.

227. Cerasi, E. and Luft, R. (1969) The effect of adenosine 3',5' monophosphate diesterase inhibitor (aminophylline) on the insulin respone to glucose infusion in prediabetic and diabetic subjects. Horm. Metab. Res. 1: 162-168.

228. Maldonato, A., Renold, A.E., Sharp, G.W.G. and Cerasi, E. (1977) Glucose induced proinsulin biosynthesis. Role of islet cyclic AMP. Diabetes. 26: 538-545.

229. Somers, G., Devis, G., Van Obberghen, E. and Malaisse, W.J. (1976) Calcium antagonists and islet function II Interaction of theophylline and verapamil. Endocrinology 99: 114-124.

230. Howell, S.L. and Whitfield, M. (1972) Cytochemical localisation of adenyl cyclase activity in rat islets of Langerhans. J. Histochem. Cytochem. 20: 873-879.

231. Malaisse, W.J., Garcia-Morales, P., Dufrane, S.P., Sener, A. and Valverde, I. (1984) Forskolin induced activation of adenylate cyclase, cyclic AMP production and insulin release in rat pancreatic islets. Endocrinology. 115: 2015-2020.

232. Smigel, M., Katada, T., Northup, J.K., Bokoch, G.M., Ui, M. and Gilman, A.G. (1983) Mechanisms of guanine nucleotide mediated regulation of adenylate cyclase activity. Adv. Cyclic. Nucl. Prot. Phos. Res. 17: 1-19.

233. Kuo, W.N., Hodgins, D.S. and Kuo, J.F. (1973) Adenylate cyclase in islets of Langerhans. Isolation of islets and regulation of adenylate cyclase activity by various hormones and agents. J. Biol. Chem. 248: 2705-2711.

234. Goldfine, I.D., Perlman, R. and Roth, J (1971) Inhibition of cyclic 3',5'-AMP phosphodiesterase in intact islet cells and other tissues by tolbutamide. Nature. 234: 295-297.

235. Sams, D.J. and Montague, W. (1972) The role of adenosine-3':5'-cyclic monophosphate in the regulation of insulin release. Properties of islet cell adenosine-3':5'-cyclic monophosphate phosphodiesterase. Biochem. J. 129: 945-952.

236. Dods, R.F. and Burdowski, A. (1973) Adenosine 3',5'-cyclic monophosphate dependent protein kinase and phosphoprotein phosphatase in rat islets of Langerhans. Biochem. Biophys. Res. Commun. 51: 421-427.

237. Sugden, M.C., Ashcroft, S.J.H. and Sugden. P.H. (1979) Protein kinase activities in rat pancreatic islets of Langerhans. Biochem. J. 180: 219-229.

238. Malaisse, W.J. and Malaisse-Lagae, F. (1984) The role of cyclic AMP in insulin release. Experientia. 40: 1068-1075.

239. Howell, S.L (1984) The mechanism of insulin secretion. Diabetologia. 26: 319-327.

240. Henquin, J.C. and Meissner, H.P. (1983) Dibutyryl cyclic AMP triggers Ca^{2+} influx and Ca^{2+}-dependent electrical activity in pancreatic B-cell. Biochem. Biophys. Res. Commun. 112: 614-620.

241. Brisson, G.R. and Malaisse, W.J. (1973) The stimulus secretion coupling of glucose induced insulin release. XI Effects of theophylline and epinephrine on ^{45}Ca efflux from perifused islets. Metabolism. 22: 455-465.

242. Curry, D.L., Bennett, L.L. and Grodsky, G.M. (1986) Requirement for calcium ion in insulin secretion by the perfused rat pancreas. Am. J. Physiol. 214: 174-178.

243. Hellman, B. (1975) The significance of calcium for glucose stimulation of insulin release. Endocrinology. 97: 392-398.

244. Grodsky, G.M. and Bennett, L.L. (1960) Cation requirements for insulin secretion in the isolated perfused pancreas. Diabetes. 15: 910-913.

245. Blaustein, M.P. and Nelson, M.T. (1982) Sodium calcium exchange: its role in thre regulation of cell calcium. In, 'Membrane transport of calcium.' Academic press, London. Editor, Carafoli, E. pp 217-236.

246. Schatzmann, H.J. (1982) The plasma membrane calcium pump of erythrocytes and other animal cells. In. 'Membrane transport of calcium.' Academic Press, London. Editor, Carafoli, E. pp 41-108.

247. Kotagal, N., Colca, J.R. and McDaniel, M.L. (1983) Activation of an islet cell plasma membrane (Ca^{2+} + Mg^{2+})-ATPase by calmodulin and Ca-EGTA. J. Biol. Chem. 258: 4808-4813.

248. Siegel, E.G., Wollheim, C.B., Renold, A.E. and Sharp, G.W.G. (1980) Evidence for the involvement of Na/Ca exchange in glucose induced insulin release from rat pancreatic islets. J. Clin. Invest. 66: 996-1003.

249. Borle, A.B. (1981) Control, modulation and regulation of cell calcium. Rev. Physiol. Biochem. Pharmacol. 90: 14-153.

250. Meisheri, K.D., Hwang, O. and Van Breeman, C. (1981) Evidence for two separate Ca^{2+} pathways in smooth muscle plasmalemma. J. Memb. Biol. 59: 19-25.

251. Atwater, L., Dawson, C.M., Eddlestone, G.T. and Rojas, E. (1981) Voltage noise measurements across the pancreatic B-cell membrane: calcium channel characteristics. J. Physiol. (Lond). 314: 195-212.

252. Prentki, M. and Wollheim, C.B. (1984) Cytosolic free Ca^{2+} in insulin secreting cells and its regulation by isolated organelles. Experientia. 40: 1052-1060.

253. Cheung, W.Y. (1970) Cyclic 3',5'-nucleotide phosphodiesterase. Evidence for and properties of a protein activator. J. Biol. Chem. 246: 2859-2869.

254. Sugden, M.C., Christie, M.R. and Ashcroft, S.J.H. (1979) Presence and possible role of calcium dependent regulator (calmodulin) in rat islets of Langerhans. FEBS. Lett. 105: 95-100.

255. Valverde, I., Vandermeers, A., Anjaneyulu, R. and Malaisse, W.J. (1979) Calmodulin activation of adenylate cyclase in pancreatic islets. Science 206: 225-227.

256. Klee, C.B., Crouch, T.H. and Richman, P.G. (1980) Calmodulin. Ann. Rev. Biochem. 49: 489-515.

257. Cheung, W.Y. (1982) Calmodulin. Sci. Am. 246: 62-82.

258. Sugden, M.C. and Ashcroft, S.J.H. (1981) Cyclic nucleotide phosphodiesterase of rat pancreatic islets. Effects of calcium, calmodulin and trifluoperazine. Biochem. J. 197: 459-464.

259. Owen, A., Sener, A. and Malaisse, W.J. (1983) Stimulus secretion coupling of glucose induced insulin release. LI Divalent cations and ATP-ase activity in pancreatic islets. Enzyme. 29: 2-14.

260. Harrison, D.E. and Ashcroft, S.J.H. (1982) Effects of calcium, calmodulin and cyclic AMP on the phosphorylation of endogenous proteins by homogenates of rat islets of Langerhans. Biochem. Biophys. Acta. 714: 313-319.

261. Colca, J.R., Brooks, C.L., Landt, M. and McDaniel, M.L. (1983) Correlation of Ca^{2+} and calmodulin dependent protein kinase activity with secretion of insulin from rat islets of Langerhans. Biochem. J. 212: 819-827.

262. MacDonald, M.J. and Kowluru, A. (1982) Calcium calmodulin dependent myosin phosphorylation by pancreatic islets. Diabetes. 31: 566-570.

263. Watkins, D.T. and Cooperstein, S.J. (1983) Role of calcium and calmodulin in the interaction between islet cell secretion granules and plasma membranes. Endocrinology. 112: 766-768.

264. Hokin, M.R. and Hokin, L.E. (1954) Effects of acetyl choline on phospholipids of the pancreas. J. Biol. Chem. 209: 549-558.

265. Best, L. and Malaisse, W.J. (1983) Phospholipids and islet function. Diabetologia. 25: 299-305.

266. Fex, G. and Lernmark, A. (1972) Effects of D-glucose on the incorporation of ^{32}P into phospholipids of mouse pancreatic islets. FEBS. Lett. 25: 287-291.

267. Freinkel, N., El Younsi, C. and Dawson, R.M.C. (1975) Interrelations between the phospholipids of rat pancreatic islets during glucose stimulation and their response to medium inositol and tetracaine. Eur. J. Biochem. 59: 245-252.

268. Best, L. and Malaisse, W.J. (1983) Stimulation of phosphoinositide breakdown in rat pancreatic islets by glucose and carbamylcholine. Biochem. Biophys. Res. Commun. 116: 9-16.

269. Nishizuka, Y. (1984) The role of protein kinase C in cell surface signal transduction and tumour promotion. Nature. 308: 693-698.

270. Kishimoto, A., Takai, Y., Mori, T., Kikkawa, U. and Nishizuka, Y. (1980) Activation of calcium and phospholipid dependent protein kinase by diacylglycerol, its possible relation to phosphatidylinositol turnover. J. Biol. Chem. 255: 2273-2276.

271. Berridge, M.J. (1984) Inositol trisphosphate and diacylglycerol as second messengers. Biochem. J. 220. 345-360.

272. Biden, T.J., Prentki, M., Irvine, R.F., Berridge, M.J. and Wollheim, C.B. (1984) Inositol 1,4,5-trisphosphate mobilises intracellular calcium from permeabilised insulin secreting cell. Biochem. J. 223: 467-473.

273. Laychock, S. (1982) Phospholipase A_2 activity in pancreatic islets is calcium dependent and stimulated by glucose. Cell. Calcium. 3: 43-54.

274. Robertson, R.P. (1979) Prostaglandins as modulators of islet function. Diabetes. 28: 943-948.

275. Robertson, R.P., Garaveski, D.J., Porte, D, Jr. and Bierman, E.L. (1974) Inhibition of in vivo insulin secretion by prostaglandin E1. J. Clin. Invest. 54: 310-315.

276. Lacy, P.E., Howell, S.L., Young, D.A. and Fink, C.J. (1968) New hypothesis of insulin secretion. Nature. 219: 1177-1179.

277. Van Obberghen, E., Somers, G., Devis, G., Ravazzola, M., Malaisse-Lagae, F., Orci, L. and Malaisse, W.J. (1974) Dynamics of insulin release and microtubular microfilamentous system: VI Effects of D_2O. Endocrinology. 95: 1518-1528.

278. Howell, S.L., Hii, C.S.T., Shaikh, S. and Tyhurst, M. (1982) Effects of taxol and nocodazole on insulin secretion from isolated rat islets of Langerhans. Biosci. Rep. 2: 795-801.

279. Dean, P.M. (1973) Ultrastructural morphometry of the pancreatic B-cell. Diabetologia. 9: 115-119.

280. Mc Daniel, M., Roth, C. and Fink, J. (1975) Effects of cytochalasins B and D on alloxan inhibition of insulin release. Biochem. Biophys. Res. Commun. 66: 1089-1096.

281. Montague, W., Howell, S.L. and Green, I.C. (1975) Insulin release and the microtubular system of the islets of Langerhans. Identification and characterisation of tubulin like protein. Biochem. J. 148: 237-243.

282. Swanston-Flatt, S.K., Carlsson, L. and Gylfe, E. (1980) Actin filament formation in pancreatic B-cells during glucose stimulation of insulin secretion. FEBS. Lett. 117: 299-302.

283. Ostlund, R.E., Leung, J.T. and Kipnis. D.M. (1978) Myosins of secretory tissues. J. Cell. Biol. 77: 827-836.

284. Dedman, J.R., Brinkley, B.R. and Means, A.R. (1979) Regulation of microfilaments and microtubules by calcium and cyclic AMP. Adv. Cyclic. Nucl. Res. 11: 131-174.

285. Sandoval, I.V. and Cuatrecasas, P. (1976) Protein kinase associated with tubulin. Affinity chromatography and properties. Biochemistry. 15: 3424-3432.

286. Mimura, N. and Asano, A. (1979) Ca^{2+} sensitive gelation of actin filaments by a new protein factor. Nature. (Lond) 282: 44-47.

287. Yin, H.L. and Stossl, T.P. (1979) Control of cytoplasmic actin gel-sol transformation by gelsolin, a calcium dependent regulatory protein. Nature. 281: 583-586.

288. Hasegawa, T., Takahashi, S., Hayashi, H. and Hatano, S. (1980) Fragmin: a calcium ion sensitive regulatory factor on the formation of actin filaments. Biochemistry. 19: 2677-2683.

289. Bretscher, A. and Weber, K. (1980) Villin is a major protein of the microvillous cytoskeleton which binds both G and F actin in a calcium dependent manner. Cell. 20: 839-847.

290. Howell, S.L. and Tyhurst, M. (1980) Regulation of actin polymerisation in rat islets of Langerhans. Biochem. J. 192: 381-383.

291. Howell, S.L. (1984) The mechanism of insulin secretion. Diabetologia: 319-327.

292. Howell, S.L. and Tyhurst, M. (1979) Interaction between insulin storage granules and F-actin in vitro. Biochem. J. 178: 367-371.

293. Seamon, K.B. and Daly, J.W. (1981) Forskolin: A unique diterpene activator of cyclic AMP generating systems. J. Cyclic. Nucl. Res. 7: 201-224.

294. Butcher, R.W. and Sutherland, E.W. (1962) Adenosine 3',5'-phosphate in biological material. 1. Purification and properties of cyclic 3',5' nucleotide phosphodiesterase and use of this enzyme to characterise adenosine 3',5' phosphate in human urine. J. Biol. Chem. 237: 1244-1250.

295. Litosch, I., Hudson, T.H., Mills, I., Li, S-Y. and Fain, J.N. (1982) Forskolin as an activator of cyclic AMP accumulation and lipolysis in rat adipocytes. Mol. Pharmacol. 22: 109-115.

296. Yamamoto, S., Nakadate, T., Uzumaki, H. and Kato, R. (1985) Lipoxygenase inhibitors and cyclic AMP mediated insulin secretion caused by forskolin, theophylline and dibutyryl cyclic AMP. J. Pharmacol. Exp. Ther. 233: 176-180.

297. Wollheim, C.B., Ullrich, S. and Pozzan, T. (1984) Glyceraldehyde but not cyclic AMP stimulated insulin release is preceeded by a rise in cytosolic free Ca^{2+}. FEBS. Lett. 177: 17-21.

298. Kleisbauer, J.P., Ytiner, L. and Nahas, G. (1968) Effect of imidazole on in vitro lipolysis of rat epididymal adipose tissue. C.R. Soc. Biol. 162: 497-501.

299. Heerche, J.N.M. (1969) Effect of thyrocalcitonin and parathyroid hormone on bone metabolism in tissue culture. Metabolism of bone in tissue culture: Differences between the mouse and rat in sensitivity to parathyroid hormone and thyrocalcitonin and the effects of imidazole. Proc. Kon. Ned. Akad. Wetensch. 72: 578-593.

300. Takagi, K., Takayanogi, I. and Tsuchida, Y. (1972) The effects of caffeine and imidazole on the actions of beta and alpha adrenergic stimulants, papaverine and cyclic 3',5'-AMP. Jap. J. Pharmacol. 22: 403-409.

301. Amer, M.S. and Kreighbaum, D. (1975) Cyclic nucleotide phosphoidiesterases. Properties activators, inhibitors, structure activity relationships and possible role in drug development. J. Pharmaceutical Sci. 64: 1-37.

302. Goldberg, N.D., Lust, W.D., O'Pea, R.F., Wei, S. and O'Toole, A.G. (1970) A role for cyclic nucleotides in brain metabolism. Adv. Biochem. Psychopharmacol. 3: 67-87.

303. Klotz, U. and Stock, K. (1972) Influence of cyclic guanosine 3',5' monophosphate on the enzymatic hydrolysis of adenosine 3',5' monophosphate. Naunyn. Schmiedebergs. Arch. Pharmacol. 274: 54-62.

304. Rasmussen, H. and Goodman, D.B.P. (1977) Relationships between calcium and cyclic nucleotides in cell activation. Physiol. Rev. 57: 421-509.

305. Devis, G., Somers, G., Van Obberghen, E. and Malaisse, W.J. (1975) Calcium antagonists and islet function: I Inhibition of insulin release by verapamil. Diabetes. 24: 547-551.

306. Boyd, A.E. III., Hill, R.S., Oberwetter, J.M. and Berg, M. (1986) Calcium dependency and free Ca^{2+} concentration during insulin secretion in a hamster beta cell line. J. Clin. Invest. 77: 774-781.

307. Triggle, D.J. (1981) Calcium antagonists: some basic chemical and pharmacological aspects. In, 'New perspectives on calcium antagonists' American Physiological Society, Bethseda. Editor, Weiss, G.B. pp 1-18.

308. Perris, A.D., Cade, C. and Atkinson, M.J. (1985) Hormones, ions and mitotic control in thymic lymphocytes. In, 'Peptide hormones as mediators in immunology and oncology, vol 19' Raven Press. Editor, Hesch, R.D. pp 75-113.

309. Henquin, J.C. (1981) Effects of trifluoperazine and pimozide on stimulus secretion coupling in pancreatic B-cells. Biochem. J. 196: 771-780.

310. Krausz, Y., Wollheim, C.B., Siegal, E. and Sharp, G.W.G. (1980) Possible role for calmodulin in insulin release: Studies with trifluoperazine in rat pancreatic islets. J. Clin. Invest. 66: 603-607.

311. Fleischer, N., Schubart, U.K., Fleckman, A. and Erlichman, J. (1981) Calcium antagonists and the secretion of insulin and thyrotrophin. In, 'New perspectives on calcium antagonists.' American Physiological Society, Bethseda. Editor, Weiss, G.B. pp 177-190.

312. Chiou, C.Y. and Malagodi, C.M. (1975) Studies on the mechanism of action of a new Ca^{2+} antagonist, 8-(N,N-diethylamino) octyl 3,4,5-trimethoxybenzoate hydrochloride in smooth muscle and skeletal muscles. Br. J. Pharmacol. 53: 279-285.

313. Weidenkeller, D.G. and Sharp, G.W.G. (1984) Unexpected potentiation of insulin release by the calcium blocker TMB-8. Endocrinology. 114: 116-119.

314. Castagna, M., Takai, Y., Kaibuchi, K., Sano, K., Kikkawa, U. and Nishizuka, Y. (1982) Direct activation of calcium activated phospholipid dependent protein kinase by tumour promoting phorbol esters. J. Biol. Chem. 257: 7847-7851.

315. Virji, M.A.G., Steffs, M. and Estensen, R.D. (1978) Phorbol myristate acetate: Effects of a tumour promotor on insulin release from isolated rat islets of Langerhans. Endocrinology. 102: 706-711.

316. Malaisse, W.J., Sener, A., Herchuelz, A., Carpinelli, A.R., Poloczek, P., Winand, J. and Castagna, M. (1980) Insulinotrophic effect of the tumour promotor 12-O-Tetradecanoyl phorbol-13-acetate in rat pancreatic islets. Cancer. Res. 40: 3827-3831.

317. Hutton, J.C., Peshavaria, M. and Brocklehurst, K.W. (1984) Phorbol ester stimulation of insulin release and secretory granule protein phosphorylation in a transplantable rat insulinoma. Biochem. J. 224: 483-490.

318. Arcoleo, J.P. and Weinstein, I. B. (1985) Activation of protein kinase C by tumour promoting phorbol esters, teleocidin and aplysiatoxin in the absence of added calcium. Carcinogenesis. 6: 213-217.

319. Hill, R.S., Oberwetter, J.M. and Boyd, A.E. III. (1987) Increase in cAMP levels in a B-cell line potentiates insulin secretion without altering cytosolic free calcium concentrations. Diabetes. 36: 440-446.

320. Karl, R.C., Zawalich, W.S., Ferrendelli, J.A. and Matschinsky, F.M. (1975) The role of Ca^{2+} and cyclic adenine 3 : 5 monophosphate in insulin release induced in vitro by the divalent cation ionophore A23187. J. Biol. Chem. 250: 4575-4579.

321. Sharp, G.W.G., Weidenkeller, D.E., Kaelin, D., Siegel, E.G. and Wollheim, C.B. (1980) Stimulation of adenylate cyclase by Ca^{2+} and calmodulin in rat islets of Langerhans. Explanation for the glucose induced increase in cyclic AMP levels. Diabetes. 29: 74-77.

322. Valverde, T., Vandermeers, A., Anjaneyalow, R. and Malaisse, W.J. (1979) Calmodulin activation of adenylate cyclase in pancreatic islets. Science. 206: 225-227.

323. Clark, G.A. (1925) The influence of the vagus on the islets of Langerhans. Part 1. Vagus hypoglycaemia. J. Physiol. 59: 466-471.

324. LaBarre, J. (1927) Sur l'augmentation de la teneur en insuline de sang veineux pancreatique excitation de nerf vague. C. R. Soc. Biol. (Paris) 96: 193-196.

325. Coupland, R. (1958) The innervation of the pancreas of the rat, cat, and rabbit as revealed by the cholinesterase technique. J. Anat. 92: 143-149.

326. Miller, R.E. (1981) Pancreatic neuroendocrinology: Peripheral neural mechanisms in the regulation of the islets of Langerhans. Endocrinol. Rev. 4: 471-494.

327. Ahren, B. and Taborsky, J.G. Jr. (1986) The mechanism of vagal nerve stimulation of glucagon and insulin secretion in the dog. Endocrinology. 118: 1551-1557.

328. Holst, J.J., Gronholt, R., Schaffalitzky de Muckadell, O.B. and Fahrenkrug, J. (1981) Nervous control of pancreatic endocrine secretion in pigs. 2. The effect of pharmacological blocking agents on the response to vagal nerve stimulation. Acta. Physiol. Scand. 111: 9-14.

329. Bloom, S.R. and Edwards, A.V. (1981) Pancreatic endocrine responses to stimulation of the vagus nerves in conscious calves. J. Physiol. 315: 31-41.

330. Hermansen, K. (1980) Secretion of somatostatin from the normal and diabetic pancreas. Studies in vitro. Diabetologia. 19: 492-504.

331. Holst, J.J., Jensen, S.L., Knuhtsen, S. and Nielsen, O.V. (1983) Autonomic nervous control of pancreatic somatostatin secretion. Am. J. Physiol. 245: E542-E548.

332. Schwartz, T.W., Holst, J.J., Fahrenkrug, J., Jensen, S.L., Nielsen, O.V., Rehfeld, J.F., Schaffalitzky de Muckadel, O.B. and Stadil, F. (1978) Vagal cholinergic regulation of pancreatic polypeptide secretion. J. Clin. Invest. 61: 781-789.

333. Malaisse, W,J., Malaisse-Lagae, F., Wright, P.H. and Ashmore, J. (1967) Effects of adrenergic and cholinergic agents upon insulin secretion in vitro. Endocrinology. 80: 975-978.

334. Kajinuma, H., Kaneto, A., Kuzuya, T. and Nakao, K. (1968) Effects of methacholine on insulin secretion in man. J. Clin. Endocrinology. 28: 1384-1388.

335. Ahren, B. (1981) On the peptidergic and aminergic regulation of insulin secretion. PhD Thesis, University of Lund, Sweden.

336. Kaneto, A., Sata, H., Kaneko, T., Yanaihara, C., Yanaihara, N. and Kosaka, K. (1981) Stimulation of somatostatin, pancreatic polypeptide, glucagon and insulin secretion by acetyl choline infused intrapancreatically in dogs. Biomed. Res. 2: 57-66.

337. Welsh, J.H. and Taub, R. (1960) The action of choline and related compounds on the heart of Venus merceniaria. Biol. Bull. 95: 346-349.

338. Waser, P.G. (1960) The cholinergic receptor. J. Pharmacol. 12: 577-589.

339. Grill, V. and Ostenson, C.G. (1983) Muscarinic receptors in pancreatic islets of the rat: demonstration and dependence on long term glucose environment. Biochem. Biophys. Acta. 756: 159-162.

340. Malaisse, W.J., Mahy, M. and Mathias, P.C.F. (1985) Binding of [^{3}H]-methylscopolamine to rat pancreatic islets. IRCS. Med. Sci. 13: 503-504.

341. Ostenson, C.G. and Grill, V. (1985) Glucose exerts opposite effects on muscarinic receptor binding to A and B cells of the endocrine pancreas. Endocrinology. 116: 1741-1744.

342. Triggle, D.J. (1976) Antagonists at muscarinic receptor sites. In, 'Chemical pharmacology of the synapse' Academic Press, New York. Editors, Triggle, D.J. and Triggle, C.R. pp 335-361.

343. Stagner, J.I. and Samols, E. (1986) Modulation of insulin secretion by pancreatic ganglionic nicotinic receptors. Diabetes. 35: 849-854.

344. Marliss, E.B., Girardier, L., Seydoux, J., Wollheim, C.B., Kanazawa, Y., Orci, L., Renold, A.E. and Porte, D. Jr. (1973) Glucagon release induced by pancreatic nerve stimulation in the dog. J. Clin. Invest. 52: 1246-1259.

345. Bloom, S.R. and Edwards, A.V. (1978) Certain pharmacological characteristics of the release of pancreatic glucagon in response to stimulation of the splanchnic nerves. J. Physiol. 280: 25-35.

346. Ahren, B., Veith, R.C., Paquette, T.L. and Taborsky, G.J. Jr. (1987) Sympathetic nerve stimulation versus pancreatic norepinephrine infusion in the dog. 2. Effects on basal release of somatostatin and pancreatic polypeptide. Endocrinology. 121: 332-339.

347. Bloom, S.R. and Edwards, A.V. (1984) Characteristics of the neuroendocrine responses to stimulation of the splanchnic nerves in bursts in the conscious calf. J. Physiol. 346: 533-545.

348. Burr, I.M. and Sharp, R. (1974) Effects of prostaglandin E1 and of epinephrine on the dynamics of insulin release in vitro. Endocrinology. 94: 835-839.

349. Burr, I.M., Balant, L., Stauffacher, W. and Renold, A.E. (1971) Adrenergic modification of glucose induced insulin release from perifused rat pancreas. Europ. J. Clin. Invest. 1: 216-224.

350. Itoh, M. and Gerich, J.E. (1982) Adrenergic modulation of pancreatic somatostatin, insulin and glucagon secretion: Evidence for differential sensitivity of islet A, B and D cells. Metabolism. 31: 715-720.

351. Samols, E. and Weir, G. (1976) Adrenergic modulation of pancreatic A, B and D cells: alpha adrenergic suppression and beta adrenergic stimulation of somatostatin secretion, alpha adrenergic stimulation of glucagon secretion in the perfused dog pancreas. J. Clin. Invest. 63: 230-238.

352. Ahlquist, R.P. (1948) A study of the adrenotrophic receptors. Am. J. Phssiol. 153: 586-600.

353. Lands, A.M., Arnold, A., McAuliff, J.P., Luduena, F.P. and Brown, T.G. (1967) Differentiation of receptor systems activated by sympathomimetic amines. Nature. 214: 5970598.

354. Drew, G.M. and Whiting, S.B. (1979) Evidence for two distinct types of postsynaptic alpha-adrenoreceptor in vascular smooth muscle. Br. J. Pharmacol. 67: 207-215.

355. Hoffman, B.B. and Lefkowitz, R.J. (1980) Alpha adrenergic receptor subtypes. N. Engl. J. Med. 302: 1390-1396.

356. Leclercq-Meyer, V., Herchuelz, A., Valverde, I., Couturier, E., Marchand, J. and Malaisse, W.J. (1980) Mode of action of clonidine upon islet function. Dissociated effects upon the time course and magnitude of insulin release. Diabetes. 29: 193-200.

357. Nakaki, T., Nakadate, T. and Kato, R. (1980) Alpha$_2$ adrenoreceptors modulating insulin release from isolated pancreatic islets. Naunyn. Schmiedebergs. Arch. Pharmacol. 313: 151-153.

358. Malaisse, W.J. and Moratinos, J. (1986) Are pancreatic B-cells equipped with alpha$_1$ adrenoreceptors?. IRCS. Med. Sci. 14: 1194-1195.

359. Lundquist, I. and Ericson, L.E. (1978) Beta adrenergic insulin release and adrenergic innervation of mouse pancreatic islets. Cell. Tissue. Res. 193: 73-85.

360. Bernard, C. (1949) Chiens redus diabetiques. C.R. Soc. Biol. (Paris). 1: 60-78.

361. Shimazu, T., Fukuda, A. and Ban, T. (1966) Reciprocal influences of the ventromedial and lateral hypothalamic nuclei on blood glucose levels and liver glycogen content. Nature. 210: 1178-1179.

362. Niijima, A. (1975) Studies on the nervous regulatory mechanism of blood sugar levels. Pharmacol. Biochem. Behaviour. 3(Suppl 1): 139-143.

363. Pansepp, J. (1974) Hypothalamic regulation of energy balance and feeding behaviour. Fed. Proc. 33: 1150-1165.

364. Szabo, O. and Szabo, A. (1975) Neuropharmacologic characterisation of insulin sensitive CNS glucoreceptor. Am. J. Physiol. 229: 663-668.

365. Anand, B., Chhina, G., Sharma, K., Dua, S. and Singh, B. (1964) Activity of single neurons in the hypothalamic feeding centres: Effect of glucose. Am. J. Physiol. 207: 1146-1154.

366. Frohman, L. and Bernardis, L. (1971) Effect of hypothalamic stimulation on plasma glucose, insulin and glucagon levels. Am. J. Physiol. 221: 1596-1607.

367. Rohner-Jeanrenaud, F., Bobbioni, E., Ionescu, E., Sauter, J-F. and Jeanrenaud, B. (1983) In, 'CNS regulation of carbohydrate metabolism'. Advances in metabolic disorders 10. Academic Press. Editor, Szabo, A.J. pp 193-220.

368. Ahren, B., Taborsky, G.J. and Porte, D. Jr. (1986) Neuropeptidergic versus cholinergic and adrenergic regulation of islet hormone secretion. Diabetologia. 29: 827-836.

369. Bishop, A.E., Polak, J.M., Green, I.C., Bryant, M.G. and Bloom, S.R. (1980) The localisation of VIP in the pancreas of man and rat. Diabetologia. 18: 73-78.

370. Rehfeld, J.F., Larson, L.I., Goltermann, N.R., Schwartz, T.W., Holst, J.J., Jensen, S.L. and Morley, J.S. (1980) Neural regulation of pancreatic hormone secretion by the C-terminal tetra peptide of CCK. Nature. 284: 33-38.

371. Moghimzadeh, E., Ekman, R., Hakensen, R., Yanaihara, N. and Sundler, F. (1983) Neuronal gastrin releasing peptide in the mammalian gut and pancreas. Neuroscience. 10: 553-563.

372. Dunning, B.E., Ahren, B., Veith, R.C., Bottcher, G., Sundler, F. and Taborsky, G.J. Jr. (1986) Galanin: A novel pancreatic neuropeptide. Am. J. Physiol. 251: E127-E133.

373. Pettersson, M., Ahren, B., Lundquist, I., Botcher, G. and Sundler, F. (1986) Neuropeptide-Y: Intrapancreatic neuronal localisation and inhibitory effects on glucose stimulated insulin secretion in the mouse. Cell. Tissue. Res. 248: 43-48.

374. Pettersson, M., Ahren, B., Bottcher, G. and Sundler, F. (1986) Calcitonin gene related peptide: Occurrence in pancreatic islets in the mouse and the rat and inhibition of insulin secretion in the mouse. Endocrinology. 119: 865-869.

375. Larsson, L.I. (1979) Innervation of the pancreas by substance P, enkephalin, vasoactive intestinal polypeptide and gastrin-CCK immunoreactive nerves. J. Histochem. Cytochem. 27: 1283-1284.

376. Holst, J.J., Fahrenkrug, J., Knuhtsen, S., Jensen, S.L., Poulsen, S.S. and Nielsen, O.V. (1984) Vasoactive intestinal polypeptide (VIP) in the pig pancreas: Role of VIPergic fibers in control of fluid and bicarbonate secretion. Regul. Pept. 8: 245-249.

377. Knuhtsen, S., Holst, J.J., Jensen, S.L. and Nielsen, O.V. (1985) Gastrin releasing peptide: Effect on exocrine secretion and release from isolated perfused pig pancreas. Am. J. Physiol. 248: G281-G287.

378. Szecowka, J., Lins, P.E., Tatemoto, K. and Efendic, S. (1983) Effects of porcine intestinal heptacosapeptide and vasoactive intestinal polypeptide on insulin and glucagon secretion in rats. Endocrinology. 112: 1469-1473.

379. Bloom, S.R. and Edwards, A.V. (1983) Endocrine responses to exogenous bombesin and gastrin releasing peptide in conscious calves. J. Physiol. 344: 37-48.

380. Hermansen, K. (1980) Effects of substance P and other peptides on the release of somatostatin, insulin and glucagon in vitro. Endocrinology. 107: 256-261.

381. Green, I.C., Ray, K. and Pervin, D. (1983) Opioid peptide effects on insulin release and cAMP in islets of Langerhans. Horm. Metab. Res. 15: 1240128.

382. Nenquin, M., Awouter, P., Mathot, F. and Henquin, J.C. (1984) Distinct effects of acetyl choline and glucose on ^{45}calcium and ^{86}rubidium efflux from mouse pancreatic islets. FEBS. Lett. 176: 457-461.

383. Kimura, H., Katagiri, K., Ohno, T., Harada, N., Imanishi, H., Iwasaki, M., Ito, M. and Takeuchi, T. (1982) Effect of acetyl choline and a new cholinergic derivative on amylase output, insulin, glucagon and somatostatin secretion from perfused isolated rat pancreas. Horm. Metab. Res. 14: 356-360.

384. Wollheim, C.B. and Biden, T.J. (1986) Second messenger function of inositol 1,4,5 trisphosphate. Early changes in inositol phosphates, cytosolic Ca^{2+} and insulin release in carbamylcholine stimulated RINm5F cells. J. Biol. Chem. 261: 8314-8319.

385. Gagerman, E., Idahl, L-A., Meissner, H.P. and Taljedal, I-B. (1978) Insulin release, cGMP, cAMP and membrane potential in acetyl choline stimulated islets. Am. J. Physiol. 235: E493-E500.

386. Malaisse, W.J. (1986) Stimulus secretion coupling in the pancreatic B-cell: The cholinergic pathway for insulin release. Diabete. Metab. Rev. 2: 243-259.

387. Dunlop, M.E. and Malaisse, W.J. (1980) Phosphoinositide phosphorylation and hydrolysis in pancreatic islet cell membrane. Arch. Biochem. Biophys. 244: 421-429.

388. Mathias, P.C.F., Carpinelli, A.R., Billuadel, B., Garcia-Morales, P., Valverde, I. and Malaisse, W.J. (1985) Cholinergic stimulation of ion fluxes in pancreatic islets. Biochem. Pharmacol. 34: 3451-3457.

389. Malaisse, W.J., Dunlop, M.E., Mathias, P.C.F., Malaisse-Lagae, F. and Sener, A. (1985) Stimulation of protein kinase-C and insulin release by 1-oleyl-2-acetyl glycerol. Eur. J. Biochem. 149: 23-27.

390. Ullrich, S. and Wollheim, C.B. (1985) Expression of both alpha1 and alpha2 adrenoreceptors in an insulin secreting cell line. Parallel studies of cytosolic free Ca^{2+} and insulin release. Mol. Pharmacol. 28: 100-106.

391. Bylund, D.B. and Pritchard, D.C. (1983) Characterisation of alpha1 and alpha2 adrenergic receptors. Int. Rev. Neurobiol. 24: 343-431.

392. Garcia-Morales, P., Dufrane, S.P., Sener, A., Valverde. and Malaisse, W.J. (1984) Inhibitory effect of clonidine upon adenylate cyclase activity, cyclic AMP production, and insulin release in rat pancreatic islets. Bioscience. Reports. 4: 511-521.

393. Wollheim, C.B., Kikuchi, M., Renold, A.E. and Sharp, G.W.G. (1978) The roles of intracellular and extracellular Ca^{2+} in glucose stimulated biphasic insulin release by rat islets. J. Clin. Invest. 62: 451-458.

394. Hellman, B., Idahl, L.A., Lernmark, A. and Taljedal, I-b. (1974) The pancreatic B-cell recognition of insulin secretagogues. Does cAMP mediate the effect of glucose. Proc. Natl. Acad. Sci. (USA). 71: 3405-3409.

395. Gardner, S.D., Field, A.M., Coleman, B. and Hulme, A. (1971) New human papovavirus (BK) isolated from urine after renal transplantation. Lancet. 1: 1253-1257.

396. Uchida, S., Watanabe, S. and Aizawa, T. (1976) Induction of papillary ependyomas and insulinomas in the Syrian golden hamsters by BK virus, a human papovavirus. Gan. 67: 857-865.

397. Corallini, A., Altavilla, G., Cecchetti, M.G., Fabris, G., Grossi, M.P., Balboni, P.G., Lanza, G. and Barbanti-Brodano. (1978) Ependymomas, malignant tumours of pancreatic islets and osteosarcomas induced in hamsters by BK virus, a human papovavirus. J. Natl. Cancer. Inst. 61: 875-883.

398. Senn, J.S., McCulloch, E.A. and Till, J.E. (1967) Comparison of the colony forming ability of normal and leukaemic human marrow in cell culture. Lancel. 2: 597-598.

399. Johnson, G.R. (1984) Haemopoeitic multipotential stem cells in culture. Clinics in Haematology. 13: 309-315.

400. Panten, U., Zunkler, B.J., Scheit, S., Kirchoff, K. and Lenzen, S. (1986) Regulation of energy metabolism in pancreatic islets by glucose and tolbutamide. Diabetologia. 29: 648-654.

401. Ashcroft, S.J.H. and Stubbs, M. (1987) The glucose sensor in HIT-cells is the glucose transporter. FEBS. Lett. 219: 311-316.

402. Boyd, A.E. III., Riser, M.E., Bolton, W.E. and Siciliano, M.J. (1982) Human insulinoma hybrids produce proinsulin-like material. Diabetes. 31: 1035-1040.