THE JOURNAL OF BIOLOGICAL CHEMISTRY

Vol. 268, No. 2, Issue of January 15, pp. 1032-1038, 1993
Printed in U.S.A.

Characterization of Metal Ion-induced [^{3}H]Inositol Hexakisphosphate Binding to Rat Cerebellar Membranes*

(Received for publication, May 15, 1992)

David R. Poyner‡, Frank Cooke, Michael R. Hanley§, D. John M. Reynolds¶, and Phillip T. Hawkins‖**

From the Medical Research Council Centre, Hills Road, Cambridge CB2 2QH, the ‖Institute of Animal Physiology and Genetics Research, Cambridge Research Station, Babraham, Cambridge CB2 4AT, and the ¶University Department of Clinical Pharmacology, John Radcliffe Infirmary, Oxford OX2 6HE, United Kingdom

The binding of [^{3}H]inositol hexakisphosphate ([^{3}H]$InsP_6$) to rat cerebellar membranes has been characterized with the objective of establishing the role, if any, of a membrane protein receptor. In the presence of EDTA, we have previously identified an $InsP_6$-binding site with a capacity of ~20 pmol/mg protein (Hawkins, P. T., Reynolds, D. J. M., Poyner, D. R., and Hanley, M. R. (1990) *Biochem. Biophys. Res. Commun.* 167, 819-827). However, in the presence of 1 mM Mg^{2+}, the capacity of [^{3}H]$InsP_6$ binding to membranes was increased ~9-fold. This enhancing effect of Mg^{2+} was reversed by addition of 10 μM of several cation chelators, suggesting that the increased binding required trace quantities of other metal cations. This is supported by experiments where it was possible to saturate binding by addition of excess membranes, despite not significantly depleting radioligand, pointing to removal of some other factor. Removal of endogenous cations from the binding assay by pretreatment with chelex resin also prevents the Mg^{2+}-induced potentiation. Consideration of the specificity of the chelators able to abolish this potentiation suggested involvement of Fe^{3+} or Al^{3+}. Both these ions (but not several others) were able to increase [^{3}H]$InsP_6$ binding to chelex-pretreated membranes at concentrations of 1 μM. It is possible to demonstrate synergy between Fe^{3+} and Mg^{2+} under these conditions. We propose that [^{3}H]$InsP_6$ may interact with membranes through non-protein recognition, possibly via phospholipids, in a manner dependent upon trace metals. The implications of this for $InsP_6$ biology are considered.

Inositol hexakisphosphate is usually found at concentrations of between 10 μM and 1 mM in most, if not all, plant and animal cells (*e.g.* Refs. 1-7). However, its functions remain largely mysterious. Studies of its metabolism suggest that the synthesis of $InsP_6$[1] is not directly linked to inositol phosphates or lipids involved in signal transduction (7). There are several reports which suggest that it has extracellular actions to excite nerve cells (8-10, 23). Additionally, a number of intracellular roles have been proposed for it, such as acting as a phosphate store or antioxidant (11, 12). In an effort to learn more about its biology, we and others (13, 14) have carried out studies with [^{3}H]$InsP_6$ to see if it can bind to membranes. A membrane-binding site might be expected to mediate any physiological extracellular actions of $InsP_6$ and might also be involved in a more general intracellular "housekeeping" role. In our previous study, working in a buffer containing 5 mM EDTA, we described a site of high capacity associated with most neuronal structures in the brain (13). Nicoletti *et al.* (14) also described a membrane-binding site for $InsP_6$, in rat cerebral cortical membranes, which was similar to the site found in the cerebellum. In an effort to understand the biological importance of these $InsP_6$-binding sites, we investigated the effects of various physiologically important cations (*e.g.* Mg^{2+} and Ca^{2+}) on [^{3}H]$InsP_6$ binding. This has led us to discover that the binding is extremely sensitive to trace quantities of certain metal ions and that this has important implications for assessing the significance of this binding.

MATERIALS AND METHODS

Membrane Preparation—Cerebella were removed from rats and homogenized in 10 volumes of 5 mM EDTA, 20 mM Tris, pH 7.7, and crude membrane fractions prepared by centrifugation (35,000 × *g*, 30 min). Membranes were resuspended, washed (1 volumes of 20 mM Tris, pH 7.7), and resuspended at ~0.2 mg protein/ml (in 20 mM Tris, pH 7.7) together with other ions or chelators as required (see the figure legends). All operations were carried out at 4 °C.

In certain experiments the membranes and radioligand were treated with chelex resin to remove endogenous cations. Membranes (40 ml, see above) were incubated with chelex resin slurry (10-ml packed volume) for 15 min at 4 °C, followed by removal of resin by a brief centrifugation (2000 × *g*, 5′, 4 °C). [^{3}H]$InsP_6$ (1 ml of 50 nM [^{3}H]$InsP_6$) was incubated with chelex resin (0.2-ml packed volume) for 45 min at room temperature and the supernatant recovered by brief centrifugation through a small plastic column (Kontes, 10-ml plastic column with 0.45-μM filter attached).

Binding Assays—Binding was carried out at 4 °C for 90 min in 20 mM Tris, pH 7.7, with additions as described under "Results" section and terminated by centrifugation, as described earlier (13). Routine assays were performed in a final volume of 1.0 ml with 0.5 nM [^{3}H]$InsP_6$ (~90,000 dpm/assay) and ~0.2 mg/ml membrane protein. All metal ion solutions were initially dissolved to a final concentration

* This work was supported in part by grants from Perstorp Pharma (to P. T. H. and M. R. H.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ Recipient of a grant from Celltech. To whom correspondence should be addressed: Dept. of Pharmaceutical Sciences, Aston University, Birmingham B4 7ET, U. K. Tel.: 021-359-3611; Fax: 021-359-0733.

§ Recipient of an International Life Sciences Institute Research Award. Present address: Dept. of Biological Chemistry, University of California School of Medicine, Davis, CA 95616-8635.

** Lister Fellow.

[1] The abbreviations used are: $InsP_3$, $InsP_4$, $InsP_5$, $InsP_6$, inositol tris-, tetrakis-, pentakis- and hexakisphosphate (isomers are numbered according to IUPAC recommendations (38)); HPLC, high pressure liquid chromotography.

of 1 M in H_20 and then used at the appropriate dilutions within 30 min.

Preparation of [^{3}H]InsP$_6$ and [^{3}H]InsP$_5$ Isomers—[^{3}H]InsP$_6$ and [^{3}H]InsP$_5$ isomers were prepared by phosphorylation of [^{3}H]inositol (Amersham) as described previously (13, 15). The resulting radioligands had specific activities of approximately 80 Ci/mmol. The unlabeled InsP$_5$ isomers were prepared as described previously (15).

HPLC Analysis of [^{3}H]InsP$_6$ Metabolism—This was done as described previously (13).

Analysis of Binding Data—Best values ± standard errors of the parameters were obtained from non-linear regression analysis using the Harwell Library routine VBØ1A (27) and the following equation.

$$\text{Relative amount bound } 1 - \left[\frac{x^{nH}}{x^{nH} + IC_{50}}\right] \qquad \text{(Eq. 1)}$$

IC_{50} = concentration of unlabeled ligand displacing 50% of the specifically bound radioligand, $\propto$ = concentration of unlabeled ligand, n_H = Hill coefficient.

RESULTS

In our initial study (13), the binding of [^{3}H]InsP$_6$ to cerebellar membranes was carried out under conditions previously used for Ins(1, 4, 5)P$_3$ binding (Ref. 16: 100 mM KCl, 20 mM NaCl, 5 mM EDTA, 20 mM Tris, pH 7.7, at 4 °C). While removal of the monovalent cations (K^+, Na^+) made little difference to [^{3}H]InsP$_6$ binding, replacement of EDTA by divalent cations caused an increase in the amount of [^{3}H]InsP$_6$ associated with the membranes (potency $Ba^{2+} > Ca^{2+} > Mg^{2+}$), see Table I).

Since high concentrations of Mg^{2+} (>1 mM) are found both inside and outside cells, we investigated the Mg^{2+}-induced binding further. The kinetics of [^{3}H]InsP$_6$ binding in the presence of 1 mM Mg^{2+} are shown in Fig. 1. This rate plot resembles that seen in 5 mM EDTA, 100 mM KCl (13) in that both association and dissociation rates appear to be biphasic, with the more rapid components occurring too quickly to be measured accurately by a microfuge binding assay. The specificity of binding in the presence of 1 mM Mg^{2+} is shown in Table II. The most potent compound in competing with [^{3}H]InsP$_6$ is InsP$_6$ itself with an IC_{50} of 0.1 μM, followed by the various InsP$_5$ isomers with IC_{50} values varying between 0.3 and 2 μM. Four InsP$_5$ isomers are chromatographically resolvable by non-chiral techniques (15, 24); the potency order for inhibition of InsP$_6$ binding is DL-Ins(1,2,3,5,6)P$_5$ ≥ Ins(1,2,3,4,6)P$_5$ > DL-Ins(2,3,4,5,6)P$_5$ ≥ Ins(1,3,4,5,6)P$_5$. It was not possible to assess the potency of Ins(1,3,4,5)P$_4$ and Ins(1,4,5)P$_3$ due to their rapid metabolism in the presence of 1 mM Mg^{2+} (data not shown). HPLC analysis of supernatants taken at the end of the binding experiments indicated that [^{3}H]InsP$_6$ was not significantly metabolized under these conditions (data not shown). It should be noted that the binding curves for the InsP$_5$ isomers all have Hill slopes significantly less than unity which suggests the presence of multiple or interacting binding sites.

As can be seen from Table I, in the presence of 1 mM Mg^{2+} there is a 9.4-fold increase in the amount of InsP$_6$ which is membrane-associated. Since [^{3}H]InsP$_6$ is present in these experiments at concentrations well below its apparent K_d (estimated at 60 nM in 5 mM EDTA, Ref. 13), the increased binding could, in principle, be due to either an increase in the affinity or the capacity of the sites, or some combination of these effects. However, although a contribution from a modest shift in affinity cannot be ruled out, the apparent IC_{50} of this site(s) for InsP$_6$ in the presence of 1 mM Mg^{2+} was found to be ~100 nM (Table II), and thus it is likely that Mg^{2+} increases the total capacity of the InsP$_6$-binding site(s).

TABLE I

Effects of metal cations on [^{3}H]InsP$_6$ binding

EDTA-washed membranes were prepared as described under "Experimental Procedures" and resuspended in buffer containing either 5 mM EDTA (control) or in the indicated concentration of metal ion (present as the chloride salt). Binding assays were carried out with 0.5 nM [^{3}H]InsP$_6$ for 90 min at 4 °C, as described under "Experimental Procedures." Values represent mean ± S.E. Control values were typically 2500 cpm/assay. Number of determinations is shown in brackets.

Cation	% [^{3}H]InsP$_6$ bound	Cation	% [^{3}H]InsP$_6$ bound
Control	100	100 μM Ca^{2+}	400 ± 47 (2)
10 μM Mg^{2+}	166 ± 24 (3)	1 mM Ca^{2+}	1608 ± 228 (2)
100 μM Mg^{2+}	444 ± 71 (3)	100 μM Ba^{2+}	530 (1)
1 mM Mg^{2+}	942 ± 144 (15)	1 mM Ba^{2+}	1717 (1)
10 μM Ca^{2+}	134 ± 28 (2)	100 mM Na^+	97 (1)

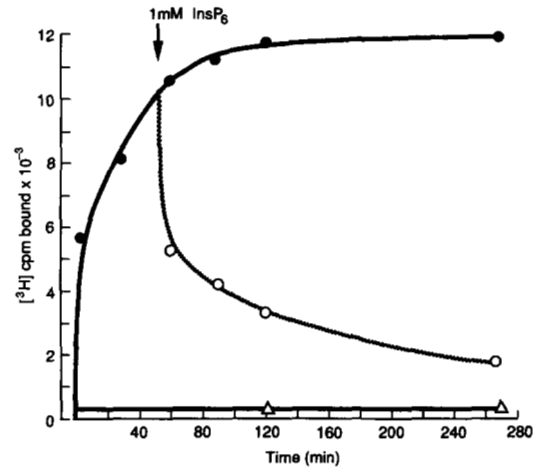

FIG. 1. **Association and dissociation of [^{3}H]InsP$_6$.** EDTA-washed cerebellar membranes were prepared and resuspended in buffer containing 1 mM $MgCl_2$ as described under "Materials and Methods." The membranes were then incubated at 4 °C with 0.5 nM [^{3}H]InsP$_6$ for the various times indicated to determine the association rate (*closed circles*). After 55 min, 1 mM unlabeled InsP$_6$ was added to a portion of the incubations and the dissociation rate followed (*open circles*). Nonspecific binding was defined in the presence of 1 mM unlabeled InsP$_6$ (*triangles*). Each point represents the mean of triplicate determinations from a single experiment, representative of two.

TABLE II

Binding of inositol phosphates in the presence of 1 mM Mg^{2+}

EDTA-washed membranes were prepared and resuspended in buffer containing 1 mM $MgCl_2$ as described under "Experimental Procedures." The binding of 0.5 nM [^{3}H]InsP$_6$ was measured in the presence of increasing concentrations of unlabeled inositol phosphates for 90 min at 4 °C. The data was analyzed as described under "Experimental Procedures" to obtain the IC_{50} and Hill coefficient (n_H). Values are mean ± S.E. of experiments carried out three to five times. Control values in the absence of any added inositol phosphate were typically 2500 cpm/assay.

Compound	IC_{50}	n_H
InsP$_6$	0.10 ± 0.02	0.87 ± 0.12
Ins(1,2,3,4,6)P$_5$	0.31 ± 0.06	0.55 ± 0.06
DL-Ins(1,2,3,5,6)P$_5$	0.25 ± 0.01	0.62 ± 0.02
DL-Ins(2,3,4,5,6)P$_5$	1.44 ± 0.12	0.54 ± 0.05
Ins(1,3,4,5,6)P$_5$	1.90 ± 0.19	0.63 ± 0.04

A variety of pharmacologically active substances were screened to see if any of them could alter [^{3}H]InsP$_6$ binding in the presence of 1 mM Mg^{2+}. Only isoprenaline inhibited binding (legend, Table III). This was not acting via β-adrenoceptors as deduced from three pieces of evidence: (i) the effect was not stereospecific (difference between inhibition produced by (+)isoprenaline and (±)isoprenaline = 9.3 ± 5.4%); (ii) the effect was not blocked by propanolol; and (iii) the structure activity relationship for the effect was not that predicted for activation of β-receptors (Table III). Indeed, the biologically active part of the molecule was the catechol moiety. The dose-response curves for isoprenaline and catechol are almost superimposable (Fig. 2, isoprenaline, IC_{50} = 0.84 ± 0.26 μM; catechol IC_{50} = 2.4 ± 0.84 μM).

Compounds such as catechol, with two vicinal hydroxyl groups are good chelators of divalent and trivalent metal ions (17). Clearly chelation of magnesium is unlikely to explain the effectiveness of these compounds, since they are active at concentrations which remove only 1% of this metal. However, in the presence of 1 mM Mg^{2+}, a second ion which is present in trace quantities could be essential for the observed increase in binding. Catechol would therefore produce its effect by removing this hypothetical second cation. To test this possibility, a variety of structurally distinct metal ion chelators were added to incubations at concentrations of 1–100 μM, to see if they could inhibit the Mg^{2+} potentiated binding. As can be seen in Table IV, all of the compounds tested were active at 10 μM to inhibit this binding. So far as we are aware, the only property they have in common is their ability to chelate metal ions. While EGTA, tetrakis-2-pyridylmethylethylenediamine and maltol are relatively non-selective, desferrioxamine shows a marked preference for trivalent cations such as Fe^{3+} and Al^{3+} (25).

Other evidence supports the notion that the key factor in the Mg^{2+} potentiated binding is a second metal cation found in the assay buffer and ligand preparation. In Fig. 3*a* it can be seen that the [^{3}H]InsP$_6$ binding saturated at concentrations of membranes greater than 1 mg/ml, despite the fact that the bulk (>80%) of the radioligand remained unhydrolyzed and available for binding (assessed by HPLC chromatography of the supernatant at the end of the binding assay, Fig. 3*b*). This is consistent with the second ion being limiting, such that it was depleted by adding more tissue before the free concentration of [^{3}H]InsP$_6$ was itself significantly reduced. If this "depleted" supernatant was removed from the membranes after the binding assay and added to fresh membranes (pretreated with 10 μM isoprenaline, 1 mM $MgCl_2$, to remove any endogenous source of the second ion), binding was greatly reduced compared to that seen when fresh supernatant and [^{3}H]InsP$_6$ were added, (Table V*A*). In contrast, if fresh [^{3}H]InsP$_6$ and assay buffer were added to membranes apparently saturated with bound radioligand (see Fig. 3*a*), then it was possible to get a further increase in binding, consistent with the addition of more of the limiting second ion (Table V*A*). If the membranes and the solution containing the radioligand were treated with chelex resin (to remove endogenous cations) prior to use in a binding study, then the observed Mg^{2+} enhancement was only 16 ± 2% of that seen normally (Table V*B*). It is interesting to note that if only the radioligand solution was chelex treated prior to addition to the binding assay, then the Mg^{2+} potentiation was still reduced by 50% (Table V*B*). This suggests that the radioligand solution itself may contribute the second ion in these experiments. Because [^{3}H]InsP$_6$ is prepared by lyophilization from 2 M ammonium formate (15), it is possible that the hypothetical ion becomes concentrated at this stage. (For example, using data provided for BDH Analar Grade ammonium for-

TABLE III

Effects of drugs on [^{3}H]InsP$_6$ binding

EDTA-washed membranes were resuspended in buffer containing 1 mM $MgCl_2$ and incubated for 90 min at 4 °C in the presence of 0.5 nM [^{3}H]InsP$_6$ with the various agents as indicated in the table. Values are the means ± S.E. of three to five determinations and are expressed as percentages of the binding seen in the absence of any drug addition (typically 2500 cpm/assay). The following did not inhibit binding: quisqualate, nitrendipine, cromoglycate, trifluoroperazine, dantrolene, histamine, carbachol (all at 10 μM), glycine, and glutamate (100 μM).

Drug	% [^{3}H]InsP$_6$ bound	Drug	% [^{3}H]InsP$_6$ bound
100 μM isoprenaline	12 ± 1	10 μM dopamine	22 ± 3
1 μM propranolol	101 ± 3	10 μM dichloroisoprenaline	113 ± 7
10 μM adrenaline	21 ± 6	10 μM tyrosine	110 ± 6
10 μM noradrenaline	18 ± 5	10 μM homovallinic acid	92 ± 6
10 μM normetradenaline	44 ± 2	100 μM phenylephrine	103 ± 11

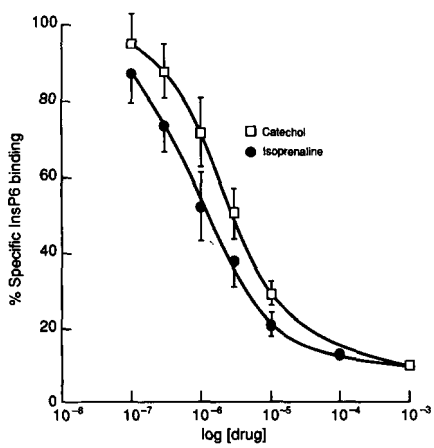

FIG. 2. **Inhibition of [^{3}H]InsP$_6$ binding by isoprenaline and catechol.** EDTA-washed cerebellar membranes were prepared and resuspended in buffer containing 1 mM $MgCl_2$ as described under "Materials and Methods." The membranes were then incubated with 0.5 nM [^{3}H]InsP$_6$ and increasing concentrations of catechol (*open squares*) or isoprenaline (*closed squares*) and the binding determined after 90 min at 4 °C as described under "Materials and Methods." Each point represents the mean ± S.E. of three to five determinations. Fitted values are as follows: catechol, IC_{50} = 2.4 ± 0.8 μM, Hill coefficient = 0.9 ± 0.2; isoprenaline, IC_{50} = 0.84 ± 0.26 μM, Hill coefficient = 0.85 ± 0.2.

TABLE IV

Effects of chelators on [^{3}H]InsP$_6$ binding

EDTA-washed membranes were resuspended in buffer containing 1 mM $MgCl_2$ and the binding of 0.5 nM [^{3}H]InsP$_6$ measured after incubation for 90 min at 4 °C in the presence of the various chelators indicated below. Values are means ± S.E. of three determinations, expressed as a percentage relative to the control binding in the absence of any chelator (typically 2500 cpm/assay). TPEN, tetrakis-2-pyridylmethylethylenediamine.

Chelator	% [^{3}H]InsP$_6$ bound
10 μM maltol	44 ± 7
10 μM catechol	31 ± 6
10 μM EDTA	35 ± 1
10 μM TPEN	53 ± 6
10 μM desferrioxamine	21 ± 12

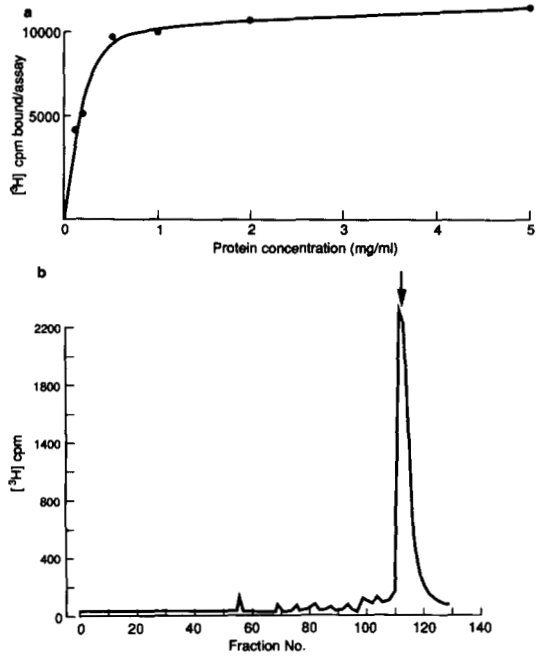

FIG. 3. *Panel a*, effect of membrane concentration on [^{3}H]InsP$_6$ binding. EDTA-washed cerebellar membranes were prepared and resuspended in buffer containing 1 mM $MgCl_2$ at up to 4 mg/ml, and incubated with 0.5 nM [^{3}H]InsP$_6$ for 90 minutes at 4 °C as described under "Materials and Methods." Each point is the mean of triplicate determinates from a single experiment, representative of two. Approximately 55,000 cpm of [^{3}H]InsP$_6$ were added to each incubation and at the end of the assay, 44,900 cpm were recovered in the supernatant from the 4 mg/ml incubation (see *b*) below). *Panel b*, HPLC analysis of [^{3}H]InsP$_6$ following a binding assay. The supernatant was taken from the 4 mg/ml incubation in *panel a* after the protein had been pelleted by microcentrifugation. This was analyzed by HPLC using a Partisil-10 SAX column as described previously (13). The *arrow* marks the elution point of a [^{3}H]InsP$_6$ standard run in a parallel analysis.

mate, the molar ratio of Fe^{3+} to InsP$_6$ in the radioligand solution could be as high as 1.5:1.).

To investigate the nature of the second ion involved in Mg^{2+}-potentiated [^{3}H]InsP$_6$ binding, various ions were added to membranes at concentrations between 10 μM and 1 mM, in the absence of Mg^{2+}, to see if they could promote binding. Zn^{2+}, Al^{3+}, Pb^{2+}, and Fe^{3+} were particularly effective in promoting up to 8-fold increases in binding at concentrations ≤10 μM (Table VI and data not shown), whereas Cu^{2+}, Ni^{2+}, Co^{2+}, Ba^{2+}, and Ca^{2+} were effective only at much higher concentrations (data not shown). The mechanism of action of the cations under these conditions is difficult to interpret; they could be mimicking the effect of the unknown cation, or synergizing with it in a more complicated interaction. In an attempt to provide a better reconstituted system, the membranes and radioligand were each pretreated with chelex resin to remove endogenous cations, and the effects of readdition of various cations was examined. When added back at 1 μM, only Al^{3+} and Fe^{3+} were able to cause a substantial increase in [^{3}H]InsP$_6$ binding (Table VI). Furthermore, it was possible to demonstrate a synergy between Mg^{2+} and Fe^{3+}. As can be seen in Fig. 4, the interactions between the two cations are complex. At high concentrations of Fe^{3+} (10 μM), addition of Mg^{2+} makes little difference to the increase in binding. However, at lower concentrations of Fe^{3+} (<1 μM), Mg^{2+} increases the binding markedly. In the absence of added Fe^{3+}, simply increasing the Mg^{2+} concentration will increase binding by ~2-fold. However, it is unclear whether this is a direct effect

TABLE V

Effects of metal ion removal on [^{3}H]InsP$_6$ binding

A, EDTA-washed membranes were resuspended at 4 mg protein/ml, in the presence of buffer containing 1 mM EGTA or 1 mM Mg^{2+} and distributed as 1-ml aliquots in Eppendorf microfuge tubes. A small volume of [^{3}H]InsP$_6$ (10 μl, ~50,000 cpm, giving a final concentration of [^{3}H]InsP$_6$ in the assay of 0.5 nM) was added to some of the samples at this stage. All samples were incubated for 90 min at 4 °C, after which time they were pelleted in a microfuge and the supernatants separated from the membrane pellets (see "Experimental Procedures"). For the assays which contained [^{3}H]InsP$_6$, the membrane pellets were counted to determine the amounts of [^{3}H]InsP$_6$ bound and the supernatants were used as "depleted" supernatant in the next stage of the experiment (the "depleted" supernatants contained 44,900 ± 100 cpm/ml of [^{3}H]InsP$_6$ at this stage). The membrane pellets from samples which did not contain [^{3}H]InsP$_6$ and which were originally resuspended in buffer containing 1 mM EGTA were used as the source of "resuspended membranes" in the next stage of the experiment. The resuspended membranes were then incubated in a final volume of ~1 ml with either "depleted supernatants" or "fresh supernatants" (fresh supernatants were prepared using incubation buffer containing 0.5 nM [^{3}H]InsP$_6$ and either 1 mM EGTA or 1 mM Mg^{2+}) for 90 min at 4 °C, and the amount of [^{3}H]InsP$_6$ bound then redetermined (see "Experimental Procedures"). Data represents means ± S.E. of triplicate assays from a single experiment. B, EDTA-washed membranes were resuspended at 1 mg/ml protein. Control membranes were not further treated. A portion of [^{3}H]InsP$_6$ was chelex treated as described in "Experimental Procedures," as was a portion of the membranes (chelexed membranes). Binding assays were set up with non-chelex-treated 0.5 nM [^{3}H]InsP$_6$ and controol membranes, chelex-treated 0.5 nM [^{3}H]InsP$_6$ and chelexed membranes, and chelex-treated 0.5 nM [^{3}H]InsP$_6$ and control membranes, all either in the presence of 1 mM EGTA or 1 mM Mg^{2+}, for 90 min at 4 °C. Data represents mean ± S.E. of triplicate assays from a single experiment.

A

Original Conditions		Resuspended Membranes	
Incubation medium	[^{3}H]InsP$_6$ bound, cpm/mg	Incubation medium	[^{3}H]InsP$_6$ bound, cpm/mg
1 mM EGTA	476 ± 33	Depleted supernatant, 1 mM EGTA	412 ± 18
		Depleted supernatant, 1 mM Mg^{2+}	495 ± 34
1 mM Mg^{2+}	2257 ± 72	Fresh supernatant, 1 mM EGTA	1212 ± 47
		Fresh supernatant, 1 mM Mg^{2+}	2020 ± 8

B

Membranes	Radioligand	Incubation medium	[^{3}H]InsP$_6$ bound, cpm/mg protein
Control	Control	1 mM EGTA	640 ± 77
		1 mM Mg^{2+}	4798 ± 368
Control	Chelexed	1 mM EGTA	285 ± 10
		1 mM Mg^{2+}	1173 ± 7
Chelexed	Chelexed	1 mM EGTA	253 ± 50
		1 mM Mg^{2+}	557 ± 38

of Mg^{2+} or whether it is "sensitizing" the membranes to traces of Fe^{3+} not removed by the chelex treatment (note: the selective iron-chelator desferrioxamine is able to reduce the basal binding obtained in the absence of any added ions by 55 ± 2%). (It is difficult to tell whether it is possible to saturate the binding by increasing the Fe^{3+} concentration, because at concentrations greater than 10 μM precipitation of the radioligand takes place (data not shown).)

Using chelex-pretreated membranes and radioligand solution, it is possible to demonstrate a synergy between added Fe^{3+} and Mg^{2+} in potentiating [^{3}H]InsP$_6$ binding (see above). It is thus proposed that Fe^{3+} is the "second-ion" that is

TABLE VI

Effects of cations on [^{3}H]InsP$_6$ binding to normal and chelex-treated membranes

EDTA-washed membranes were prepared. Normal membranes were not treated further and were incubated for 90 min at 4 °C with 0.5 nM [^{3}H]InsP$_6$ and various metal ions (chloride salts) as indicated. Chelex-treated membranes (prepared as described under "Experimental Procedures") were preincubated with chelex resin, and then incubated with chelex-treated 0.5 nM [^{3}H]InsP$_6$ (prepared as described under "Experimental Procedures") and metal cations, as for the normal membranes. Data represents the percentage of [^{3}H]InsP$_6$ bound relative to the binding seen with non-chelex-treated membranes suspended in 5 mM EDTA (1500 cpm/assay), and are means ± S.E. from three determinations.

Ion	Normal membranes % [^{3}H]InsP$_6$ bound	Chelex-treated membranes Ion	% [^{3}H]InsP$_6$ bound
5 mM EDTA	100	5 mM EDTA	67
10 μM Zn^{2+}	378 ± 67	1 μM Zn^{2+}	90 ± 10
10 μM Pb^{2+}	848 ± 221	1 μM Pb^{2+}	71 ± 11
10 μM Al^{3+}	807 ± 68	1 μM Al^{3+}	246 ± 15
10 μM Fe^{3+}	244 ± 34	1 μM Fe^{3+}	198 ± 16

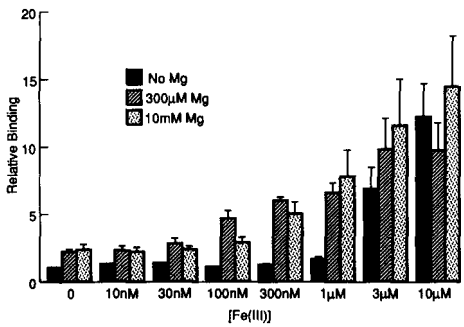

FIG. 4. **Effects of Fe^{3+} and Mg^{2+} on [^{3}H]InsP$_6$ binding.** Both [^{3}H]InsP$_6$ and EDTA-washed membranes were chelex treated as described under "Materials and Methods." The membranes were incubated with 0.5 nM [^{3}H]InsP$_6$ together with $Fe_2(SO_4)_3$ as indicated, in the presence of buffer containing 5 mM EDTA (*solid bars*), 300 μM $MgCl_2$ (*cross- hatched bars*), or 10 mM $MgCl_2$ (*stippled bars*) for 90 min at 4 °C. The level of binding was expressed relative to that seen in 5 mM EDTA in the absence of added Fe^{3+}. Each *bar* represent the means ± S.E. of three determinations.

responsible for Mg^{2+}-potentiated [^{3}H]InsP$_6$ binding in the non-chelex-pretreated binding assay. However, this explanation does not explain all the observations. Remarkably, addition of the iron chelator desferrioxamine (10 μM) does not inhibit the Fe^{3+}- or Al^{3+}-induced increase in binding in a chelex-pretreated binding assay but actually increases the binding (data not shown). This phenomenon implies a complex interaction between different metal ions, InsP$_6$, and the membrane-binding site.

We have investigated [^{3}H]InsP$_6$ binding to membranes prepared from a number of different rat tissues. In every tissue examined (heart, liver, kidney, spleen, lung, and brain (cerebellum, forebrain, hindbrain, and cortex)), we found significant InsP$_6$-displaceable [^{3}H]InsP$_6$ binding (data not shown). Evidence that similar binding sites were being measured in these tissues is provided by the similar amounts of radioligand displaced by 100 nM InsP$_6$ (between 32 and 48%). The degree of metal ion-potentiated binding appeared to vary quite widely between different tissues (*e.g.* isoprenaline inhibited binding by 88% in cerebellum but by only 44% in kidney). Autoradiographic examination of [^{3}H]InsP$_6$-binding sites in rat brain, in the presence of 1 mM Mg^{2+}, showed a distribution similar to that described previously in 5 mM EDTA (13, data not shown), suggesting that Mg^{2+} did not create new sites in regions not previously expressing [^{3}H]InsP$_6$ binding. However, without knowing the endogenous concentrations of the relevant metal ions or their buffering capacity in the various tissues it is obviously impossible to arrive at a more quantitative estimate of the degree of metal ion-potentiated binding in each tissue.

DISCUSSION

Specific [^{3}H]InsP$_6$ binding to cerebellar membranes can be dramatically potentiated by Mg^{2+} (1 mM), and this effect is dependent on limiting quantities of at least one further trace metal ion. The "metal ion potentiated" binding resembles the binding previously described in the presence of 5 mM EDTA (13), in that it has a similar affinity for InsP$_6$ and has similar kinetics. The mechanism of this potentiation is uncertain, but it is likely to result from an increase in the capacity of the [^{3}H]InsP$_6$-binding sites. Furthermore, with the addition of increasing amounts of cations it cannot be shown to be saturable. These results are in broad agreement with an observation made by Nicoletti *et al.* (14), who reported that various divalent cations (25 μM) potentiated [^{3}H]InsP$_6$ binding to membranes prepared from primary cultures of rat cerebellar granule cells and may explain why they were unable to observe saturable binding at high membrane protein concentrations (their membranes were not prepared in the presence of cation chelators).

The relationship between specific [^{3}H]InsP$_6$ binding in the presence of 5 mM EDTA and the metal ion potentiated binding is unclear. Given that InsP$_6$ is an excellent chelator of cations (*e.g.* 1, 18, 19), InsP$_6$ may be complexed to endogenous metal ions even in the presence of excess EDTA, and accordingly it might be that all InsP$_6$-membrane interactions require some form of metal ion participation (this would be analogous to kinase recognition of Mg ATP). However, chelators such as desferrioxamine, although they can reduce InsP$_6$ binding, do not abolish it. Therefore, the small contribution of a metal ion-independent binding site will be masked by the much greater metal ion-potentiated binding. A strong argument in favor of a separate membrane protein being responsible for [^{3}H]InsP$_6$ binding in the presence of EDTA is the recent purification of an inositol polyphosphate-binding protein from solubilized rat cerebellar membranes (26). This purified protein exhibits similar recognition characteristics in the presence of EDTA to [^{3}H]InsP$_6$ binding to intact membranes. We do not know whether InsP$_6$ binding to this purified protein is potentiated by transition metal ions, although we think it is unlikely that metal ion-potentiated InsP$_6$ binding is mediated by a specific protein (see below).

The data suggest multivalent cations influence the interactions of InsP$_6$ with biological membranes. The mechanism of the metal ion InsP$_6$ membrane association is unexpectedly complex. When chelex resin is used to remove endogenous cations from binding solutions and membrane preparations, then relatively high concentrations of Fe^{3+} and Al^{3+} (1 μM) can enhance InsP$_6$ binding. It may be that some divalent cations on their own are also able to promote binding when present at concentrations in excess of 1 mM. However, we have obtained clear evidence that in the case of 1 mM Mg^{2+}, the enhanced binding in non-chelex-treated binding assays is possibly due to small quantities of a second ion, perhaps Fe^{3+} or Al^{3+}. Although readdition of Fe^{3+} to chelex-treated membranes can mimic some aspects of the situation, we find in natural membranes that there are paradoxical potentiation effects of the iron chelator desferrioxamine, which indicate that the reconstituted ionic conditions may not match physiological conditions.

Although a number of models may explain how the different metal ions can act together to promote InsP$_6$ binding, a

significant constraint on these models is our ignorance of the nature of the [^{3}H]InsP$_6$-binding site. The metal ion-potentiated site is of very high capacity; we have been unable to obtain convincing data that it can be saturated, and it seems to be ubiquitous in membranes from mammalian tissues. Taken together, these data argue against a specific membrane protein being the site of metal ion-potentiated InsP$_6$ binding, and argues in favor of a more abundant membrane component, possibly negatively charged lipids or derivatives of them. This suggests that one explanation for the role of metal ions in InsP$_6$ binding is that they act as "bridges" between the InsP$_6$ and the negatively charged phospholipid phosphate groups of membranes. The more heavily charged trivalent metal ions might be expected to be particularly effective in taking part in the phosphate-metal ion phosphate complex required by this scheme (16–18). Mg^{2+} could interact directly with the InsP$_6$, increasing its affinity for the second ion, or perhaps allowing it to take part more readily in phosphate bridge complexes with the membranes. InsP$_6$ is an excellent chelator of metal ions, and in some cases the binding of one ion may increase its affinity for a second (20). However, an alternative mechanism is for large quantities of Mg^{2+} to saturate metal ion-binding sites of the membranes, in the process increasing the effective concentration of the second ion in solution. In this scenario, in the absence of Mg^{2+}, the second ion is all bound to the membrane at sites which are unable to allow the formation of the InsP$_6$-ion sandwich. InsP$_6$ by itself is unable to strip the second ion from these non-productive binding sites because it does not have a sufficiently high affinity for this metal ion. The "second ion" promoted potentiation of InsP$_6$ binding is consistent with most of our data. However, we cannot yet provide an explanation of why chelators can promote InsP$_6$ binding under conditions when certain ions are added back. A full explanation of the effects of metal ions on InsP$_6$ binding will require a much more extensive study of both the occurrence and concentrations of various metal ions in the binding assays and the chemistry of metal ion-InsP$_6$ interactions.

The biological significance of the metal ion-potentiated binding of [^{3}H]InsP$_6$ cannot be addressed without further study. It is not clear whether, under physiological conditions, traces of appropriate metal cations would allow significant amounts of InsP$_6$ to become membrane-bound. It seems likely, however, that this metal ion-potentiated binding may affect certain *in vitro* experiments. It may, for example, confound attempts to assess the true intracellular distribution of InsP$_6$ or it may lead to the unwitting introduction of InsP$_6$ (and/or its associated cations) into assays with membranes (*e.g.* as an inhibitor of inositol phosphate phosphatases, Ref. 21).

This work emphasizes the ability of InsP$_6$ to act as a quite remarkable ion chelator. It seems that certain metal ions can significantly modify the physiochemical properties of InsP$_6$ and the role of InsP$_6$ as a putative physiological chelating agent should be borne in mind when considering the biology of metals such as Al^{3+} or Fe^{3+}. In this regard, there are gaps in our knowledge of how cells handle iron which require a low molecular weight iron-binding molecule to shuttle iron between transport and storage proteins (*e.g.* transferrin and ferritin) and their ultimate destinations in the cell, the proteins which require iron to function (see for example, Refs. 28–30). A number of molecules have been postulated to exert such a role, *e.g.* nucleotides, citrate, glycine, and glucose, but the evidence seems somewhat unconvincing in view of their relatively low affinity and specificity for iron and their proven roles in other major areas of metabolism. InsP$_6$ would seem to be an attractive candidate for such a role since it both possesses a high affinity for iron (our preliminary experiments based on the solubilization of Fe(OH)$_3$ precipitates and the decolorization of various Fe^{3+} ligand complexes suggest that the affinity constant of InsP$_6$ for Fe^{3+} is in the range 10^{25}-10^{30} and the stoichiometry of binding is 4–5 Fe^{3+}/InsP$_6$, data not shown) and prevents the bound iron from participating in potentially damaging free radical reactions (12, 31). A further attractive feature of such speculation is that the rapid, "futile cycling" of specific phosphate groups on InsP$_6$ which is seen in cells (7) would open the door to rapid, directional transport of bound metal ions mediated by controlled and localized phosphorylation and dephosphorylation reactions.

InsP$_6$ has been reported to have a number of extracellular actions (8–10, 23, 32–34). Furthermore, two possible sites of intracellular action have recently been identified: 1) a highly selective interaction with the G-protein receptor regulatory protein, arrestin (35, 36) and 2) a novel inositol polyphosphate receptor which appears to be a gated potassium channel (37). The ubiquitous metal ion-dependent binding site described here is unlikely to mediate any physiological response to this compound, but will certainly mask any lower capacity binding site which might be involved in producing these responses. Consequently, radioligand binding to crude membranes may be limited in its applicability to the analysis of the membrane actions of InsP$_6$. A more productive approach may be the purification of inositol polyphosphate-binding proteins from detergent-solubilized membranes (26). However, the interaction of InsP$_6$ with cations does raise questions about studies on InsP$_6$-induced cellular ^{45}Ca accumulation (10, 34). Recently it has been claimed that metabolically dead cells accumulate ^{45}Ca when treated with InsP$_6$ (22). This may be another instance of the formation of a metal ion-InsP$_6$-membrane complex involving Ca^{2+}. In this regard, Nicoletti *et al.* (14) have noted a good correlation between the ability of divalent cations to both potentiate InsP$_6$-stimulated ^{45}Ca accumulation and InsP$_6$ binding. More generally, it is now clear that experiments designed to investigate membrane actions of InsP$_6$ should carefully control for the possibility that InsP$_6$ can bind to cell membranes via metal ions and thus alter their biological properties, in an apparently very specific, yet probably unphysiological manner.

Acknowledgments—We thank Dr. Ian Martin for bringing to our attention the high affinity of isoprenaline for di- and trivalent metal ions. We thank Kit Erlebach for technical assistance. We also thank Ed Constable, Len Stephens, Trevor Jackson, and Robin Irvine for many helpful discussions throughout the course of this work.

REFERENCES

1. Cosgrove, D. J. (1980) *Inositol Phosphates, Their Chemistry, Biochemistry and Physiology*, Elsevier, Amsterdam
2. Heslop, J. P., Irvine, R. F., Tashjian, A. H. & Berridge, M. J. (1985) *J. Exp. Biol.* **119,** 395–401
3. Jackson, T. R., Hallam, T. H., Downes, C. P. & Hanley, M. R. (1987) *EMBO J.* **6,** 49–54
4. French, P. J., Bunce, C. M., Brown, G., Creba, J. A. & Michell, R. H. (1988) *Biochem. Soc. Trans.* **16,** 985–986
5. Szwergold, B. S., Graham, R. A. & Brown, T. R. (1987) *Biochem. Biophys. Res. Commun.* **149,** 874–881
6. Pittet, D., Schlegel, W., Lew, D. P., Monod, A. & Mayr, G. W. (1989) *J. Biol. Chem.* **264,** 18489–18493
7. Stephens, L. R. & Irvine, R. F. (1990) *Nature* **346,** 580–583
8. Vallejo, M., Jackson, T. R., Lightman, S. & Hanley, M. R. (1987) *Nature* **330,** 656–658
9. Barraco, R. A., Phyllis, J. W. & Simpson, L. L. (1989) *Eur. J. Pharmacol.* **173,** 75–84
10. Nicoletti, F., Bruno, V., Fiore, L., Cavalloro, S. & Canonico, P. L. (1989) *J. Neurochem.* **53,** 1026–1030
11. Williams, S. G. (1970) *Plant Physiol.* **45,** 376–381
12. Graf, E., Empson, K. L. & Eaton, J. W. (1987) *J. Biol. Chem.* **262,** 11647–11650
13. Hawkins, P. T., Reynolds, D. J. M., Poyner, D. R. & Hanley, M. R. (1990) *Biochem. Biophys. Res. Commun.* **167,** 819–827
14. Nicoletti, F., Bruno, V., Cavalloro, S., Copani, A., Sortino, M. A. & Canonico, P. L. (1990) *Mol. Pharmacol.* **37,** 689–693
15. Stephens, L. R., Hawkins, P. T., Stanley, A. F., Moore, T., Poyner, D. R.,

Morris, P. J., Hanley, M. R., Kay, R. R. & Irvine, R. F. (1991) *Biochem. J.* **275,** 485–499
16. Worley, P. F., Baraban, J. M. & Snyder, S. H. (1989) *J. Neurosci.* **9,** 339–346
17. Martell, A. E. & Smith, R. M. (1982) *Critical Stability Constants,* Vol. 5, p. 340, Plenum Press, New York
18. Vohra, P. & Kraztner, J. E. (1965) *J. Poultry Sci.* **43,** 1164–1170
19. Maddaiah, V. Y., Kurnick, A. A., Reid, B. L. (1964) *Proc. Soc. Exp. Biol. Med.* **115,** 391–393
20. Wise, A. & Gilburt, D. J. (1982) *Toxicol Lett.* **11,** 49–54
21. Hughes, P. & Shears, S. (1990) *J. Biol. Chem.* **265,** 9869–9875
22. Mitchell, R., MacEwan, D., Dougan, L., Johnson, M. & Thomson, F. (1991) *Biochem. Soc. Trans.* **19,** 116S
23. Hanley, M. R., Jackson, J. R., Vallejo, M., Patterson, S. I., Thastrup, O., Lightman, S., Rogers, J., Henderson, G. & Pini, A. (1981) *Phil. Trans. R. Soc. Lond. Biol. Sci.* **320,** 381–398
24. Cosgrove, D. J. (1969) *Ann. N. Y. Acad. Sci.* **165,** 677–686
25. Tufano, T. A. & Raymond, K. N. (1981) *J. Am. Chem. Soc.* **103,** 6617–6624
26. Theibert, A. B., Estevez, V. A., Ferris, C. D., Danoff, S. K., Barrow, R. K., Prestwich, G. D. & Snyder, S. H. (1991) *Proc. Natl. Acad. Sci. U. S. A.* **88,** 3165–3169
27. Aceves, J., Mariscal, S., Morrison, K. E. & Young, J. M. (1985) *Br. J. Pharmacol.* **84,** 417–424
28. Jacobs, A. (1977) *CIBA Found. Symp.* **51,** 91–106
29. Grohlich, D., Morley, G. D. & Bezkorovainy, A. (1979) *Int. J. Biochem* **10,** 803–806
30. Crichton, R. R. (1979) *CIBA Found. Symp.* **65,** 57–75
31. Graf, E., Mahoney, J. R., Bryant, R. G. & Eaton, J. W. (1984) *J. Biol. Chem.* **259,** 3620–3524
32. Smith, S. E. & Dürmüller, N. (1990) *Eur. J. Pharmacol.* **191,** 337–343
33. Eggleton, P., Penhallow, J. & Crawford, N. (1991) *Biochim. Biophys. Acta* **1094,** 309–316
34. Regunathan, S., Reis, D. J. & Wahlestedt, C. (1991) *Biochem. Parmacol.* **43,** 1331–1336
35. Palczewski, K., Pulvermüller, A., Buczylko, J., Gutmann, C. & Hofmann, K. P. (1991) *FEBS Lett.* **295,** 195–199
36. Palczewski, K., Rispoli, G. & Detwiler, P. B. (1992) *Neuron* **8,** 117–126
37. Chadwick, C. C., Timerman, A. P., Saito, A., Mayrleitner, M., Schindler, H. & Fleischer, S. (1992) *J. Biol. Chem.* **267,** 3473–3481
38. I. U. B. Nomenclature Committee (1989) *Biochem. J.* **258,** 1–2