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

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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 (I$[^3$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 $\approx 20$ 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 $\approx 9$-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$_6$ 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 “house-keeping” 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 X 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 chelax resin to remove endogenous cations. Membranes (40 ml, see above) were incubated with chelax resin slurry (10-ml packed volume) for 15 min at 4 °C, followed by removal of resin by a brief centrifugation (2000 X g, 5', 4°C). $[^3$H]InsP$_6$ (1 ml of 50 nM $[^3$H]InsP$_6$) was incubated with chelax 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

$^{*}$The abbreviations used are: InsP$_3$, InsP$_4$, InsP$_6$, InsP$_8$, inositol tris-, tetrakis-, pentakis- and hexakisphosphate (isomers are numbered according to IUPAC recommendations (38)); HPLC, high-pressure liquid chromatography.
metal ion-potentiated \(^{3}H\)InsP\(_{6}\) binding

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of 1 mM in H\(_{2}\)O and then used at the appropriate dilutions within 30 min.

Preparation of \(^{3}H\)InsP\(_{6}\) and \(^{3}H\)InsP\(_{6}\) Isomers—\(^{3}H\)InsP\(_{6}\) and \(^{3}H\)InsP\(_{6}\) 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\(_{6}\) 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 VD01A (27) and the following equation.

Relative amount bound

\[
1 - \frac{x_{0}^{nH}}{x_{0}^{nH} + IC_{50}} 
\]

(Eq. 1)

IC\(_{50}\) = concentration of unlabeled ligand displacing 50% of the specifically bound radioligand, \(n_{H}\) = 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 protein or divalent cations caused an increase in the amount of \(^{3}H\)InsP\(_{6}\) associated with the membranes (potency Ba\(^{2+}\) > Ca\(^{2+}\) > Mg\(^{2+}\)).

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 ìM, followed by the various InsP\(_{6}\) isomers with IC\(_{50}\) values varying between 0.3 and 2 ìM. Four InsP\(_{6}\) 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\(_{5}\) > DL-Ins(1,2,3,4,5,6)P\(_{6}\) > Ins(1,2,3,4,5,6)P\(_{6}\) > Ins(1,2,3,4,6)P\(_{6}\) (triangles). Each point represents the mean of triplicate determinations from a single experiment, representative of two.

TABLE II

| 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\(_{6}\) | 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 |

Effects of metal cations on \(^{3}H\)InsP\(_{6}\) binding

EDTA-washed 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 up to 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.

TABLE I

| Cation | \(^{3}H\)InsP\(_{6}\) bound | \(^{3}H\)InsP\(_{6}\) bound |
|--------|------------------|------------------|
| Control | 100 | 100 µM Ca\(^{2+}\) | 400 ± 47 (2) |
| 10 ìM Mg\(^{2+}\) | 166 ± 24 (3) | 1 mM Ca\(^{2+}\) | 1608 ± 228 (2) |
| 100 ìM 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 ìM Ca\(^{2+}\) | 134 ± 28 (2) | 100 µM Na\(^{+}\) | 97 (1) |

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\(_{6}\) 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 IC\(_{50}\) (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 it is likely that Mg\(^{2+}\) increases the total capacity of the InsP\(_{6}\)-binding site(s).
A variety of pharmacologically active substances were screened to see if any of them could alter $[\text{H}]\text{InsP}_6$ binding in the presence of 1 mM Mg$^{2+}$. Only isoprenaline inhibited binding (legend, Table III). This was not acting via $\beta$-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 $\beta$-receptors (Table III). Indeed, the structure activity relationship for the effect was not that predicted for activation of $\delta$-receptors (Table I). The dose-response curves for isoprenaline and catechol are almost superimposable (Fig. 2, isoprenaline, IC$_{50}$ = 0.84 ± 0.26 $\mu$M; catechol IC$_{50}$ = 2.4 ± 0.84 $\mu$M).

Compounds such as catechol, with two vicinal hydroxyl groups are good chelators of divalent and trivalent metal ions.

| Table III |
| Effects of drugs on $[\text{H}]\text{InsP}_6$ 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 $[\text{H}]\text{InsP}_6$ with the various agents as indicated in the table. Values are the means ± S.E. of five 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, nitrrendipine, cromoglycate, trifluoroperazine, dantrolene, histamine, carbachol (all at 10 $\mu$M), glycine, and glutamate (100 $\mu$M).

| Drug          | % $[\text{H}]\text{InsP}_6$ bound | Drug          | % $[\text{H}]\text{InsP}_6$ bound |
|---------------|----------------------------------|---------------|----------------------------------|
| 100 $\mu$M isoprenaline | 12 ± 1                           | 10 $\mu$M dopamine | 22 ± 3                           |
| 1 $\mu$M propranolol | 101 ± 3                          | 10 $\mu$M dichloroisoprenaline | 113 ± 7                          |
| 10 $\mu$M adrenaline | 21 ± 6                           | 10 $\mu$M tyrosine | 110 ± 6                          |
| 10 $\mu$M noradrenaline | 18 ± 5                           | 10 $\mu$M homovanilllic acid | 92 ± 6                           |
| 10 $\mu$M normetradenidine | 44 ± 2                           | 100 $\mu$M phenylephrine | 103 ± 11                          |

Fig. 2. Inhibition of $[\text{H}]\text{InsP}_6$ binding by isoprenaline and catechol. EDTA-washed cerebellar membranes were prepared and resuspended in buffer containing 1 mM MgCl$_2$, and incubated for 90 min at 4 °C as described under "Materials and Methods." The membranes were then incubated with 0.5 nM $[\text{H}]\text{InsP}_6$ 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. The differences between the % of the binding seen in the absence of any chelator were significantly reduced by 50% (Table VB). It is interesting to note that if only the radioligand was not a chelator prior to addition to the binding assay, then the Mg$^{2+}$ potentiation was still reduced by 50% (Table VB). This suggests that the radioligand solution itself may contribute the second ion in these experiments. Because $[\text{H}]\text{InsP}_6$ is prepared by biophosphorization from 2 M ammonium formate (15), it is possible that the hypotetical ion becomes concentrated at this stage. (For example, using data provided for BDH Analar Grade ammonium for-

Table IV

Effects of chelators on $[\text{H}]\text{InsP}_6$ binding

EDTA-washed membranes were resuspended in buffer containing 1 mM MgCl$_2$ and the binding of 0.5 nM $[\text{H}]\text{InsP}_6$ 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-pyrindimethyltetraethylenediamine.

| Chelator          | % $[\text{H}]\text{InsP}_6$ bound |
|-------------------|----------------------------------|
| 10 $\mu$M maltol  | 44 ± 7                           |
| 10 $\mu$M catechol| 31 ± 6                           |
| 10 $\mu$M EDTA    | 35 ± 1                           |
| 10 $\mu$M TPEN    | 53 ± 6                           |
| 10 $\mu$M desferrioxamine | 21 ± 12                          |
ever, at lower concentrations of Fe$^{3+}$ (<1 mM), Mg$^{2+}$ increases to remove endogenous cations, and the effects of readdition to demonstrate a synergy between Mg$^{2+}$ and Fe$^{3+}$. Mg$^{2+}$ makes little difference to the increase in binding. How-

seen in Fig. 3., increasing the Mg$^{2+}$ concentration will increase binding by

membranes and radioligand were each pretreated with chelax resin to attempt to provide a better reconstituted system, the mem-

branes and radioligand were 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 [3H]InsP$_6$ standard run in a parallel analysis.

To investigate the nature of the second ion involved in Mg$^{2+}$-potentiated [H]InsP$_6$ binding, various ions were added to membranes at concentrations between 10 mM 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 of 10 mM (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 chelax resin to remove endogenous cations, and the effects of readdition of various cations was examined. When added back at 1 mM, only Al$^{3+}$ and Fe$^{3+}$ were able to cause a substantial increase in [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 mM), addition of Mg$^{2+}$ makes little difference to the increase in binding. How-

ever, at lower concentrations of Fe$^{3+}$ (<1 mM), 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 of Mg$^{2+}$ or whether it is "sensitizing" the membranes to traces of Fe$^{3+}$ not removed by the chelax 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 mM precipitation of the radioligand takes place (data not shown).)

Using chelax-pretreated membranes and radioligand solution, it is possible to demonstrate a synergy between added Fe$^{3+}$ and Mg$^{2+}$ in potentiating [H]InsP$_6$ binding (see above). It is thus proposed that Fe$^{3+}$ is the "second-ion" that is
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 [3H]InsP6 binding to cerebellar membranes can be dramatically potentiated by Mg2+ (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 InsPs 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 [3H]InsP6-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 [3H]InsP6 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 chelator chelators).

The relationship between specific [3H]InsP6 binding in the presence of 5 mM EDTA and the metal ion potentiated binding is unclear. Given that InsPs is an excellent chelator of cations (e.g., 1, 18, 19), InsPs may be complexed to endogenous metal ions even in the presence of excess EDTA, and accordingly it might be that all InsPs-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 InsPs 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 [3H]InsP6 binding in the presence of EDTA is the recent purification of an inositol phosphophosphate-binding protein from solubilized rat cerebellar membranes (26). This purified protein exhibits similar recognition characteristics in the presence of EDTA to [3H]InsP6 binding to intact membranes. We do not know whether InsPs binding to this purified protein is potentiated by transition metal ions, although we think it is unlikely that metal ion-potentiated InsPs binding is mediated by a specific protein (see below).

The data suggest multivalent cations influence the interactions of InsPs with biological membranes. The mechanism of the metal ion InsP6 membrane association is unexpectedly complex. When chelate is used to remove endogenous cations from binding solutions and membrane preparations, then relatively high concentrations of Fe3+ and Al3+ (1 µM) can enhance InsP6 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 Mg2+, the enhanced binding in non-chelate-treated binding assays is possibly due to small quantities of a second ion, perhaps Fe3+ or Al3+. Although readdition of Fe3+ to chelate-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 InsP6 binding, a

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### Table VI

**Effects of cations on [3H]InsP6 binding to normal and chelex-treated membranes**

| Ion        | Normal membranes | Chelex-treated membranes |
|------------|------------------|--------------------------|
| 5 mM EDTA  | 100 ± 77         | 5 mM EDTA                |
| 10 µM Zn2+ | 378 ± 67         | 1 µM Zn2+                |
| 10 µM Pb2+ | 840 ± 221        | 1 µM Pb2+                |
| 10 µM Al3+ | 807 ± 68         | 1 µM Al3+                |
| 10 µM Fe3+ | 244 ± 34         | 1 µM Fe3+                |

**FIG. 4. Effects of Fe3+ and Mg2+ on [3H]InsP6 binding.** Both [3H]InsP6 and EDTA-washed membranes were chelex treated as described under "Materials and Methods." The membranes were incubated with 0.5 nM [3H]InsP6 and EDTA-washed membranes were chelex treated as described under "Experimental Procedures" and metal cations, as for the normal membranes. Data represents the percentage of [3H]InsPs 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.

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We have investigated [3H]InsP6 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 InsPs-displaceable [3H]InsP6 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 InsPs (between 32 and 48%). The degree of metal ion-potentiated binding appeared to vary quite widely between different tissues (e.g., isoprostane inhibited binding by 88% in cerebellum but by only 44% in kidney). Autoradiographic examination of [3H]InsP6 binding sites in rat brain, in the presence of 1 mM Mg2+, showed a distribution similar to that described previously in 5 mM EDTA (13, data not shown), suggesting that Mg2+ did not create new sites in regions not previously expressing [3H]InsP6 binding. However, without knowing the endogenous concentrations of the
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 bound to the membranes 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. 2). 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 A$^{1+}$ 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 unconvinving 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.

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