The Biological Relevance of the Binding of Calcium Ions by Inositol Phosphates*

Brian M. Luttrell
From the Department of Endocrinology, Royal North Shore Hospital, St. Leonards,
New South Wales 2065, Australia

The binding of Ca\(^{2+}\) (chelation) by myo-inositol polyphosphates at pH 7.0 was studied using a Ca\(^{2+}\)-sensitive electrode. Glucose 6-phosphate (used as a model for a monophosphate) bound Ca\(^{2+}\) with an affinity of 152 ± 91 liters/mol and a molar ratio of 0.94 ± 0.02. Inositol 1,4,5-trisphosphate, inositol 1,3,4,5-tetrakisphosphate, inositol 1,3,4,5,6-pentakisphosphate, and inositol hexakisphosphate showed affinities of 9.0 ± 2.1 \times 10^3, 6.3 ± 1.5 \times 10^3, 6.2 ± 10^4, and 1.92 ± 0.47 \times 10^5 liters/mol, respectively, and molar ratios of 0.92 ± 0.49, 0.95 ± 0.10, 0.75, and 2.5 ± 0.5. In general, the affinity increased with the number of phosphate substituents on the inositol ring, although the stereochemistry is also expected to be important. This suggests that for the physiologically relevant inositol phosphates (tris-, tetrakis-, pentakis-, and hexakis-) half-maximal Ca\(^{2+}\) binding will occur in the Ca\(^{2+}\)-concentration range of approximately 5 \times 10^{-4} to 2 \times 10^{-4} M. This range lies between the basal intracellular and the free extracellular Ca\(^{2+}\) levels (10^{-7} and 10^{-3} M), respectively, and may therefore be of physiological importance. Chelation provides a possible simple explanation for the inhibition by Ca\(^{2+}\) of inositol 1,4,5-trisphosphate binding to its receptor in rat cerebellum and other tissues. It may also have a role in limiting inositol phosphate-mediated increases in intracellular Ca\(^{2+}\).

A variety of low molecular weight compounds are considered to function as intracellular second messengers of hormone action, including cAMP (1), calcium ion (2), and polyphosphates of myo-inositol. Of these, inositol 1,4,5-trisphosphate (Ins(1,4,5)P\(_3\))\(^1\) and inositol 1,3,4,5-tetrakisphosphate (Ins(1,3,4,5)P\(_4\)) are considered to be involved in the influx of ionic calcium (Ca\(^{2+}\)) into the cell cytosol from intracellular stores and from the cell exterior (3, 4). Various roles in intracellular regulation have been proposed for a variety of other inositol polyphosphates including inositol 1,2,6-trisphosphate acting as an antagonist of neuropeptide Y (5) and as an inhibitor of glucose-stimulated insulin secretion in the mouse (6); inositol 1,3,4,6-tetrakisphosphate mobilizing Ca\(^{2+}\) in Xenopus oocytes (7); 1,2-(cyclic)4,5-trisphosphate inducing membrane currents in Xenopus oocytes (8); and inositol hexakisphosphate (InsP\(_6\)) priming stimulated respiratory bursts in human neutrophils (9) and modifying the affinity of hemoglobin for oxygen (10).

One property of the inositol polyphosphates that has not received due attention is their ability to bind Ca\(^{2+}\) directly to form chelates. This is a general property of organic phosphates and has been previously demonstrated for nucleotides (11), but the 6-membered myo-inositol ring bearing multiple phosphate substituents provides particularly favorable stereochemistry for the formation of polydentine Ca\(^{2+}\)-binding sites. The stability of these complexes has not been measured directly, but Hendrickson and Reinertsen (12) demonstrated in 1969 that the inositolate 1-(glycerylphosphoryl)-1-myoinositol 4,5-bisphosphate bound Ca\(^{2+}\) with an affinity of 1.6 \times 10^6 l/mol. They also studied a model compound trans-1,2-dihydroxycyclohexane bisphosphate and observed an affinity for Ca\(^{2+}\) of 5.5 \times 10^5 l/mol. These appeared to be bidentate chelates, and it might be expected that the participation of additional phosphate groups would increase the binding affinity.

Since Ca\(^{2+}\) is present within the cell, it is reasonable to postulate that the inositol polyphosphates will form chelates under certain physiological conditions. This study was designed to provide direct evidence for these binding properties and to attempt to show how chelation may be important in the mode of action of second messengers. Because of the limited availability of the milligram quantities of inositol phosphates required for this purpose, a series of representative compounds were chosen. These included myo-inositol hexaphosphate (phytic acid, InsP\(_6\)), Ins(1,3,4,5)P\(_4\), Ins(1,4,5)P\(_3\), and a trans-bisphosphate, myo-inositol 3,4-bisphosphate (Ins(3,4)P\(_2\)). A model compound glucose 6-phosphate was also studied as an example of a monophosphate since a suitable inositol monophosphate was unavailable. Phytic acid is already known as a strong chelator of Ca\(^{2+}\) (13) and of ferrous ion (Fe\(^{2+}\)), and this latter property has been exploited in its use as an antioxidant (14).

**MATERIALS AND METHODS**

Phytic acid as the dodecasodium salt and glucose 6-phosphate monosodium salt were obtained from Sigma, while Ins(3,4)P\(_2\) as the cyclohexylamine salt was a generous gift from Emeritus Professor S. J. Angyal (University of New South Wales, Sydney, Australia). Ins(1,3,4,5)P\(_4\) as the ammonium salt was purchased from Boehringer Mannheim (Sydney, Australia), and the lithium salt of Ins(1,4,5)P\(_3\) was obtained from Calbiochem-Novabiochem Pty. Ltd. (Sydney, Australia). Calcium binding studies were performed with a Radiometer model PHM62 pH meter equipped with a Ca\(^{2+}\) ion-selective electrode model 2002 and a calomel reference electrode model K401 (Radiometer, Copenhagen, Denmark). Standard solutions of calcium chloride were also obtained from Radiometer.

Unless otherwise indicated, all studies were conducted at pH 7.0 and at room temperature (22 ± 2°C). The total ionic concentrations were within the ranges of: 1–50 \times 10^{-3} M (glucose 6-phosphate studies); 4–10 \times 10^{-2} M for Ins(3,4)P\(_2\); 1–2 \times 10^{-3} M for Ins(1,3,4,5)P\(_4\); 3–10 \times 10^{-4} M for Ins(1,3,4,5)P\(_4\); and 1–50 \times 10^{-4} M (phytic acid studies); and these ranges were chosen to accommodate the varying affinities of these ligands. Solutions of the inositol phosphates and glucose 6-phosphate were mixed with progressively increasing amounts of calcium chloride solution, and the potential difference (in millivolts) between the calcium and calomel electrodes was measured. Free Ca\(^{2+}\) concentrations were determined by reference to a dose-response curve determined from parallel experiments in which the...
organic phosphates were replaced by solutions of equivalent molarity of sodium, lithium, or ammonium chlorides, depending upon the cation present with the phosphate ligand. Scatchard plots (15) were then used to determine the binding affinity of and capacity of the Ca\(^{2+}\) ligands. Molar binding ratios were determined by dividing the observed Ca\(^{2+}\) binding capacity of each ligand by its known concentration.

Riacalc software from LKB-Wallac (Turku, Finland) was used for curve fitting and data interpolation of the Ca\(^{2+}\) dose curves, while Fig-P software from Fig-P Corp. (Durham, NC) was used for curve fitting the Scatchard plots.

RESULTS

The binding of Ca\(^{2+}\) by all of the phosphate ligands was readily demonstrable. Binding was unaffected by the total ionic concentration within the limited ranges used (shown above) provided that concentration adjustments were made with inert ions (monovalent cations and non-oxygenated anions). Binding studies were performed routinely at room temperature (22 °C), but a test of Ins(1,4,5)P3 binding at 37 °C revealed no significant alteration in binding characteristics. This was as expected since the interaction is a chemical one involving no protein component. As seen in Fig. 1, essentially linear Scatchard plots were obtained, indicating the occurrence of predominantly one population of binding sites for each ligand, although there was some evidence for additional weaker binding in the case of InsP6 in the form of a departure from linearity at low bound/free values. The affinity constants for the principal interactions and the molar ratios observed are listed in Table I. These data were obtained as the mean of the results of multiple experiments (four for InsP6 and two each for glucose 6-phosphate, Ins(3,4)P2, and Ins(1,4,5)P3. An exception was Ins(1,3,4,5)P4, which was studied only once due to the limited amount of material available. In general, the binding affinity for Ca\(^{2+}\) increased progressively with the number of phosphate residues present on the ligand ring, with the highest value 1.92 ± 0.47 × 10^4 l/mol being observed for the hexakisphosphate phytic acid.

It is noted that the affinity constant for the hexakisphosphate is higher than the previous estimate of 8.7 × 10^4 l/mol obtained by Gosselin and Coghlan (13), who used a method of equilibration with an ion-exchange resin.

There was a minor inconsistency in that the estimate of the affinity constant for Ins(1,4,5)P3 of 6.5 ± 1.5 × 10^4 l/mol was slightly lower than that for Ins(3,4)P2 (9.2 ± 2.1 × 10^3 l/mol), and this is unexplained. Both values are reasonably consistent with the value of 5.5 × 10^5 l/mol for trans-1,2-dihydroxycyclohexane bisphosphate by Hendrickson and Reinersen (12). Each of these compounds includes vicinal bisphosphate sequences with the two substituents having a trans configuration. The stability of the chelates may also be affected by weak interactions between Ca\(^{2+}\) and hydroxyl groups around the ring and by the free energy required to optimize the conformation of the myo-inositol ring for binding. As a generalization it can be said that the binding affinity is dependent upon the number of phosphate groups associated with the ligand and on their stereochemistry.

The molar ratios of Ca\(^{2+}\) ions to ligand molecules also varied. The mono-, bis-, tris-, and tetrakis phosphates each had ratios approximating unity, indicating the presence of only one binding site per molecule. The hexakisphosphate had a molar ratio of 2.5 ± 0.5, indicating the capacity to bind two and possibly three Ca\(^{2+}\) ions. Since InsP6 has six available phosphate substituents distributed around the ring, the possibility of accommodating two Ca\(^{2+}\) ions, one above and one below the plane of the inositol ring, can be readily visualized. An alternative binding model which allowed for three cations to be accepted is less obvious but might involve a satellite donation from oxygen to the metal ion. A strong binding site can be created if several of these functional groups are positioned around the metal ion in an appropriate geometrical formation. Thus the widely used chelating agent EDTA binds Ca\(^{2+}\) with an affinity constant of approximately 10\(^{-15}\) l/mol with four strategically positioned carboxylate groups.

The present data demonstrate that the affinity of binding
Inositol Phosphate Chelation

increases with the number of phosphate substituents on the myo-inositol ring. Glucose 6-phosphate provided an indication of the activity of a monophosphate although it is recognized that the pyranose ring of glucose provides a slightly different stereochemical environment to that of myo-inositol. The ligands of biological interest (trisphosphate and above) may be expected to have binding affinities within the range estimated in this study, i.e. from $6.3 \times 10^{-5}$ to $1.92 \times 10^{-5}\text{M}$ for Ins(1,4,5)P$_3$.

It follows from the Law of Mass Action that half-maximal binding for these compounds will occur at $Ca^{2+}$ concentrations in the approximate range of $5 \times 10^{-8}$ to $2 \times 10^{-5}\text{M}$. It is notable that this range is positioned between the concentrations found for intracellular $Ca^{2+}$ in the basal state and the extracellular free $Ca^{2+}$ concentration ($10^{-7}$ to $10^{-3}\text{M}$, respectively, in approximate figures).

**The Physiological Importance of $Ca^{2+}$ Binding**—There are two main arguments for considering that chelation may be of physiological importance within the cell. The first is that $Ca^{2+}$ and inositol phosphates co-exist there and appear to interact, at least indirectly, in the facilitation of $Ca^{2+}$ release from intracellular stores by Ins(1,4,5)P$_3$ and the activation of calcium channels by Ins(1,3,4,5)P$_4$.

The second argument lies in the large number of biochemical steps involved in the synthesis, function, and metabolism of inositol polyphosphates that have been demonstrated to be sensitive to divalent cation concentrations, notably $Ca^{2+}$ and Mg$^{2+}$.

The inositol phosphates are initially produced by cleavage of phosphatidylinositol by phospholipase C. The specificity of this enzyme is influenced by $Ca^{2+}$ such that at low concentrations of the ion Ins(1,4)P$_3$ and Ins(1,4,5)P$_3$ are the predominant products while inositol 1-monophosphate is favored at higher concentrations (17). The phosphorylation of Ins(1,4,5)P$_3$ by its kinase enzyme is stimulated 5-fold by $Ca^{2+}$ (18, 19). Inositol polyphosphate 1-phosphomonoesterase is dependent on Mg$^{2+}$ for activity and is inhibited by $Ca^{2+}$ and Li$^+$ (20). The binding of Ins(1,4,5)P$_3$ to its receptor in rat cerebellum has been shown to be inhibited by $Ca^{2+}$ (21).

These considerations suggest a variety of mechanistic possibilities for the involvement of $Ca^{2+}$ chelation in intracellular second messenger regulation both inhibitory and stimulatory. In this communication, attention is focused on a possible mechanism for the regulation of intracellular $Ca^{2+}$ which is suggested by the present data but draws additionally on the previous observations of others.

**Calcium Autoregulation**—The observation that the chelation of Ins(1,4,5)P$_3$ and Ins(1,3,4,5)P$_4$ is likely to be optimal at $Ca^{2+}$ concentrations between basal intracellular and extracellular levels suggests a regulatory role for the chelates. Specific receptors for Ins(1,4,5)P$_3$ and Ins(1,3,4,5)P$_4$ have been described (reviewed by Taylor and Richardson (22)), and these are likely to be involved in the process of $Ca^{2+}$ mobilization stimulated by these two inositol phosphates. If it is assumed that their chelates are not bound by the respective receptors, then chelation would be antagonistic to the process of $Ca^{2+}$ release by reducing the concentration of active ligand.

Basal intracellular $Ca^{2+}$ concentrations would not promote chelation, but rising localized $Ca^{2+}$ concentrations could be expected at the point of release from intracellular stores, in the Ins(1,4,5)P$_3$-mediated process, and in the vicinity of calcium channels in the process initiated by Ins(1,3,4,5)P$_4$. Chelation could then occur resulting in the loss of the ligands’ ability to bind to their receptors and therefore their ability to sustain $Ca^{2+}$ mobilization, and the influx of the cation would cease. Presumably dilution within the cell cytosol of the localized high $Ca^{2+}$ concentration would follow, with dissociation of the chelates and a return to basal conditions. Thus a self-regulatory mechanism for the maintenance of intracellular $Ca^{2+}$ homeostasis is consistent with the binding properties of the inositol polyphosphates demonstrated in this study.

This is a self-regulatory mechanism, which may also explain the quantal nature of $Ca^{2+}$ release from Ins(1,4,5)P$_3$-sensitive stores as described by Mualllem et al. (23), whereby submaximal concentrations of the ligand were able to initiate a rapid release of the cation from intracellular stores but unable to deplete these stores, even under conditions of prolonged incubation. Thus the rapid initial release of $Ca^{2+}$ may lead to inactivation of the limited amount of ligand present and termination of the process. Under this mechanism the cell, after a brief refractory period, would become sensitive to a subsequent addition of Ins(1,4,5)P$_3$, as in fact was observed by Meyer and Stryer (24).

Experiments by Finch et al. (25), who superfused microsomal vesicles from rat brain synaptosomes loaded with $Ca^{2+}$, demonstrated a biphasic effect of $Ca^{2+}$ on $45Ca^{2+}$ release. At a constant concentration of Ins(1,4,5)P$_3$, they were able to show an increase in the release rate as $Ca^{2+}$ in the superfusion medium increased from $10^{-5}\text{M}$, peaking when it was between $10^{-6}$ and $10^{-5}\text{M}$ and decreasing sharply when $Ca^{2+}$ was $10^{-4}\text{M}$. The inhibitory effect of the higher $Ca^{2+}$ levels is quantitatively consistent with the conditions for $Ca^{2+}$ chelation demonstrated in the present study, suggesting that chelation becomes the limiting factor in vesicular $Ca^{2+}$ release.

There is experimental evidence in the work of others both for the occurrence of “hot spots” of high $Ca^{2+}$ concentration in the vicinity of calcium channels and for the inhibition of Ins(1,4,5)P$_3$ binding to its receptor by $Ca^{2+}$. Thus, Linas et al. (26), working with the presynaptic terminal of the giant squid, detected transient intracellular microdomains with $Ca^{2+}$ concentrations in the range of $2.0\rightarrow 3.0 \times 10^{-4}\text{M}$. These coincided with transmitter secretions and were located against the cytoplasmic surface of the plasmalemma and were presumed to be at the “mouth” of calcium channels. While these neurons are highly specialized cells, they provide an example of what localized $Ca^{2+}$ concentrations may be achievable in other cells.

Two alternative explanations have been advanced by others for the inhibition of Ins(1,4,5)P$_3$ receptor binding. Thus Danoff et al. (27) reported that in rat cerebellum, the inhibitory activity was dissociable from the purified receptor but was associated with a neutral membrane protein, which they suggested may bind $Ca^{2+}$ and which they named calmedin. Reassociation of calmodulin to the purified receptor restored $Ca^{2+}$ sensitivity. By contrast, Mignery et al. (28) suggested that the inhibitory substance was actually freshly synthesized inositol 1,4,5-trisphosphate produced by the action of a $Ca^{2+}$-sensitive phospholipase C. The chelation of $Ca^{2+}$ by inositol phosphates thus provides a third possible explanation for this phenomenon.

The suggestion that $Ca^{2+}$ inhibition of Ins(1,4,5)P$_3$ receptor interaction can “turn off” further $Ca^{2+}$ release after an initial action of Ins(1,4,5)P$_3$ was first made by Worley et al. (21). The present hypothesis supports this basic concept but proposes that the inhibition is a pre-receptor event, associated with the inactivation of the inositol polyphosphate ligand by chelation.

This study demonstrates that myo-inositol polyphosphates bind $Ca^{2+}$ with modest affinity, and the strength of the interaction is dependent upon the number of phosphate substituents on the inositol ring and (presumably) their stereochemistry. For those polyphosphates considered to be of physiological importance (tris-, tetrakis-, pentakis-, and hexakis-
phosphates) affinity constants appear to fall within the range of $6.3 \times 10^3$ to $1.92 \times 10^5$ l/mol. These values appear to be high enough to support a physiological role within the cell for chelation and may provide a simple mechanism for the previously described inhibition of Ins(1,4,5)P$_3$ binding to its receptor. Further investigation will be required to resolve this question.

Acknowledgments—I wish to thank Emeritus Professor S. J. Angyal, Department of Organic Chemistry, University of New South Wales (Kensington, Australia) for the donation of compounds for study and for much helpful advice. I also gratefully acknowledge the help of Dr. R. Gigg of the M. R. C. Institute, Ridgeway (Mill Hill, London) in supplying additional inositol phosphates.

REFERENCES
1. Ross, E. M., and Gillman, A. G. (1980) Annu. Rev. Biochem. 49, 533-564
2. Griffith, L. C., and Schulman, H. (1986) J. Biol. Chem. 261, 9542-9549
3. Beveridge, M. J., and Irvine, R. F. (1984) Nature 312, 315-321
4. Irvine, R. F., and Moor, R. M. (1986) Biochem. J. 240, 917-920
5. Heilig, M., Edvinsson, L., and Wahlestedt, C. (1991) Eur. J. Pharmacol. 209, 27-32
6. Ahren, B. (1992) Neuropeptides 21, 163-166
7. Ivorra, I., Gigg, R., Irvine, R. F., and Parker, I. (1991) Biochem. J. 273, 317-321
8. Stith, B. J., and Proctor, W. R. (1989) J. Cell. Biochem. 40, 321-330
9. Eggleton, P., Penhallow, J., and Crawford, N. (1991) Biochim. Biophys. Acta Mol. Cell. Res. 1094, 309-316
10. Benesch, R., and Benesch, R. E. (1969) Nature 221, 618-622
11. Luttrell, B. M., and Henniker, A. J. (1991) J. Biol. Chem. 266, 21626-21630
12. Hendrickson, H. S., and Reinertsen, J. L. (1969) Biochemistry 8, 4855-4858
13. Gosselin, R. E., and Coghlan, E. R. (1953) Arch. Biochem. Biophys. 45, 301-304
14. Graf, E., Empson, K. L., and Eaton, J. W. (1987) J. Biol. Chem. 262, 11647-11650
15. Scatchard, G. (1949) Ann. N. Y. Acad. Sci. 51, 660-672
16. Cohn, M., and Hughes, T. R. (1982) J. Biol. Chem. 254, 176-179
17. Wilson, D. B., Blois, T. E., Hoffmann, S. L., and Majerus, P. W. (1981) J. Biol. Chem. 256, 11718-11724
18. Biden, T. J., and Wolheim, L. B. (1986) J. Biol. Chem. 261, 11931-11934
19. Imboden, J. B., and Pattison, G. (1984) J. Clin. Invest. 73, 1538-1541
20. Ichinoh, R. C., and Majerus, P. W. (1984) J. Biol. Chem. 262, 15946-15952
21. Worley, P. F., Baraban, J. M., Supattapone, S., Wilson, V. S., and Snyder, S. H. (1987) J. Biol. Chem. 262, 12159-12166
22. Taylor, C. W., and Richardson, A. R. (1991) Pharmacol. Ther. 51, 97-137
23. Muallim, S., Pandol, S. J., and Beeker, T. G. (1988) J. Biol. Chem. 264, 206-212
24. Meyer, T., and Stryer, L. (1990) Proc. Natl. Acad. Sci. U. S. A. 87, 3841-3845
25. Finch, E. A., Turner, T. J., and Goldin, S. M. (1991) Science 252, 443-448
26. Llina, R., Sugimori, M., and Silver, R. B. (1992) Science 256, 677-679
27. Danoff, S. K., Supattapone, S., and Snyder, S. H. (1988) Biochem. J. 254, 701-705
28. Mignery, G. A., Johnston, P. A., and Sudhof, T. C. (1992) J. Biol. Chem. 267, 7450-7455