The inositol 1,4,5-trisphosphate receptor binding sites of platelet membranes

pH-dependency, inhibition by polymeric sulphates, and the possible presence of arginine at the binding site

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The present study was initiated to characterize the inositol 1,4,5-trisphosphate (InsP₃)-binding site in human platelets that is involved in Ca²⁺ release. InsP₃ binding to platelet membranes was measured in two ways; (1) by displacement of labelled InsP₃ with unlabelled InsP₃, as in previous studies, and (2) directly, using only radioactive InsP₃ as ligand, over the concentration range 0.25–100 nM. At physiological pH (7.1) the binding data were best fitted by a model for a single saturable binding site, with $K_D = 11.8$ nM and $B_{max} = 1.4$ pmol/mg of protein. At alkaline pH values (8.3 and 9.4) binding was best fitted by a two-site model, the second site being of higher affinity ($K_D = 0.75$ – 1.2 nM) but lower concentration ($B_{max} = 0.195$ – 0.6 pmol/mg of protein). All binding of InsP₃ was blocked by polymeric sulphates (heparin, dextran sulphate, polyvinyl sulphate) regardless of pH. The specific arginine-modifying reagent *p*-hydroxyphenylglyoxal irreversibly blocked InsP₃ binding, suggesting the presence of arginine at the recognition site for InsP₃ binding. *NN'*-dicyclohexylcarbodi-imide (DCCD) and 1-ethyl-3-(3-dimethylaminopropyl)carbodi-imide (ECCD), which are carboxy-group-specific reagents, blocked Ca²⁺ release, but not InsP₃ binding, indicating the existence of another site that regulates Ca²⁺ release apart from the active centre for InsP₃.

INTRODUCTION

InsP₃ serves as a second messenger for Ca²⁺ mobilization in many cells (Berridge & Irvine, 1984). In order to release Ca²⁺, InsP₃ presumably must bind to a receptor site that exerts operational control over a Ca²⁺-permeable channel. InsP₃-gated Ca²⁺ channels have been demonstrated by patch-clamp techniques and in planar bilayer membranes with incorporated membrane vesicles (Kuno & Gardner, 1988; Ehrlich & Watras, 1988). An InsP₃ receptor has been characterized in cerebellum, which shows a single binding site for InsP₃ with $K_D = 20$ – 40 nM and $B_{max} = 10$ – 20 pmol/mg (Worley *et al.*, 1987; Willcocks *et al.*, 1987). InsP₃ binding in cerebellum was increased at alkaline pH, blocked by heparin (50% inhibition at 0.25– 0.5 μ M), and potentially inhibited by Ca²⁺ (50% inhibition at 0.3 μ M) through the mediation of a Ca²⁺-binding protein termed calmedin (Danoff *et al.*, 1988).

By using its affinity for heparin, an InsP₃-binding protein was purified from rat cerebellum by affinity chromatography (Supattapone *et al.*, 1988b). This InsP₃-binding protein (M_r 260 000) displayed most of the characteristics observed in cerebellar membranes, supporting the view that it is the physiological receptor. By using polyclonal antibodies raised against the purified brain protein, the InsP₃ receptor in cerebellar Purkinje neurons was localized in both smooth and rough endoplasmic reticulum, a portion of the sub-plasmalemmal cisternae and the nuclear membrane (Ross *et al.*, 1989).

The InsP₃ receptor has not been purified from peripheral tissues, but in most cases the K_D and B_{max} values are reported to be substantially less than reported for cerebellum. Binding of InsP₃ to a presumptive receptor has been measured in permeabilized hepatocytes, neutrophils and particulate fractions from other peripheral tissues, e.g. adrenal cortex, liver and anterior pituitary (Baukal *et al.*, 1985; Spat *et al.*, 1986; Worley

et al., 1987; Guillemette *et al.*, 1987; Willcocks *et al.*, 1987; Supattapone *et al.*, 1988a; Guillemette & Segui, 1988; Palmer *et al.*, 1989; Mauger *et al.*, 1989). Virtually all studies report a single class of binding sites, with K_D values of 1–5 nM and B_{max} in the range 0.1–0.2 pmol/mg. Binding of InsP₃ was increased at alkaline pH (Guillemette & Segui, 1988). In some studies (Spat *et al.*, 1986; Mauger *et al.*, 1989), the Scatchard plots of the binding data exhibit upward curvature, suggesting the existence of negative co-operativity, or a second class of lower-affinity binding sites. Peripheral tissues also differ from the cerebellum in that InsP₃ binding is virtually insensitive to Ca²⁺, corresponding to their much lower content of calmedin (Danoff *et al.*, 1988). The cerebellar InsP₃-binding protein was phosphorylated by the catalytic subunit of protein kinase A. This reaction was reported to cause an inhibition of the InsP₃ dose/response for release of Ca²⁺, although InsP₃ binding was unaffected (Supattapone *et al.*, 1988a). Protein kinase A did not affect Ca²⁺ release by a maximally effective concentration of InsP₃ in platelet membrane vesicles (O'Rourke *et al.*, 1989).

Little is known about the active site of the receptor. Binding of InsP₃ is inhibited competitively by heparin (e.g. Worley *et al.*, 1987; Supattapone *et al.*, 1988a; Mauger *et al.*, 1989), and some thiol reagents or disulphide-bond reagents also impair both InsP₃ binding and Ca²⁺ release in bovine adrenal-cortex microsomes and platelet membrane vesicles (Guillemette & Segui, 1988; Adunyah & Dean, 1986). InsP₃ binding was inhibited by high concentrations of *N*-ethylmaleimide, iodoacetamide and dithiothreitol, suggesting a role for thiol groups and disulphide bridges in InsP₃-recognition sites. InsP₃ binding to brain receptors was abolished by the mercurial *p*-chloromercuribenzenesulphate, but was not affected by dithiothreitol and mercaptoethanol (Supattapone *et al.*, 1988a).

InsP₃ causes the release of Ca²⁺ in permeabilized platelets (Brass & Joseph, 1985) and from Ca²⁺-loaded vesicles believed to

Abbreviations used: InsP₃, inositol 1,4,5-trisphosphate; DCCD, *NN'*-dicyclohexylcarbodi-imide; ECCD, 1-ethyl-3-(3-dimethylaminopropyl)-carbodi-imide.

be derived from the internal dense tubule membrane system (O'Rourke *et al.*, 1985; Brass & Joseph, 1985; Authi & Crawford, 1985; Adunyah & Dean, 1986). The amount of InsP_3 produced in intact platelets in response to thrombin appears sufficient to account for the mobilization of internal Ca^{2+} (Rittenhouse & Sasson, 1985). No studies on the binding of InsP_3 to platelet membranes have yet been reported. The present study describes the binding of InsP_3 , the effects of other inositol phosphates, and additional properties of the presumptive InsP_3 receptor in platelet membranes.

MATERIALS AND METHODS

Materials

Heparin (H-5640) (M_r 4000–6000), dextran sulphate (D-0768), polyvinyl sulphate (P-6000) (M_r 70000), dextran (D-9260) (M_r 9400), 1-ethyl-3-(3-dimethylaminopropyl)carbodi-imide (ECCD) (E-6383), polyvinyl alcohol of high M_r (P-1763) and low M_r (P-8136), polyvinyl chloride of high M_r (P-9562) and low M_r (P-9401) and chondroitin sulphate (C-8529) were all purchased from Sigma Chemical Co. NN' -Dicyclohexylcarbodi-imide (DCCD) and ECCD were from Aldrich Chemical Co., and *p*-hydroxyphenylglyoxal (20100) was purchased from Pierce Chemical Co. $[^3\text{H}]\text{InsP}_3$ (Net-911) (20 Ci/mmol) was obtained from New England Nuclear and $[^3\text{H}]\text{InsP}_3$ (TRK.999) (34.5 Ci/mmol) was from Amersham. Synthetic InsP_3 was given by Research Biochemicals, Natick, MA, U.S.A. Platelet-rich plasma was obtained from the Connecticut Red Cross Blood Center, Farmington, CT, U.S.A. The EBDA and LIGAND programs for analysis of binding data were purchased from Elsevier-Biosoft, Cambridge, U.K.

Methods

Binding studies. The platelet membrane vesicle fraction most sensitive to Ca^{2+} release by InsP_3 was prepared as described by O'Rourke *et al.* (1989) and washed twice in resuspension buffers. Three different buffers were used for studies at various pH values: (a) 50 mM-Hepes/5 mM-EDTA, pH 7.1, adjusted with KOH; (b) 50 mM-Tris/5 mM-EDTA, pH 8.3, adjusted with HCl; (c) 0.015 M- Na_2CO_3 /0.035 M- NaHCO_3 /1 mM-EDTA, pH 9.4. Binding of InsP_3 was measured by two methods. To obtain initial estimates of binding parameters, a low concentration of $[^3\text{H}]\text{InsP}_3$ was allowed to attain equilibrium binding to membranes and was then displaced by increasing concentrations of unlabelled InsP_3 . Millipore membrane filtration and centrifugation assays were compared. Some vesicles passed through Millipore GS (0.45 μm -pore-size) membranes, and filtration took an unacceptably long 20 s when 0.22 μm -pore-size membranes were used. Non-specific binding to Millipore membranes was also excessive, i.e. 35–50 % of the amount bound to platelet membranes. Furthermore, because the K_D was estimated to be ≥ 10 nM, we concluded that separation of membranes from the liquid phase would be best accomplished by centrifugation at 430000 *g* in a Beckman tabletop ultracentrifuge (Bennett & Yamamura, 1985). In the second method 8–14 concentrations of $[^3\text{H}]\text{InsP}_3$ were used to measure binding directly. Membrane vesicles resuspended at 1–2 mg of protein/ml were incubated with $[^3\text{H}]\text{InsP}_3$ at 4 °C for sufficient time to reach equilibrium (10 min). Duplicate samples (125–170 μl) were transferred into 7 $\mu\text{m} \times 20$ mm polycarbonate tubes. The samples were centrifuged for 8 min at 430000 *g* at 4 °C in a Beckman TL-100 ultracentrifuge. The resulting supernatants were aspirated, and 50 μl of 50 % (w/v) trichloroacetic acid was added to extract radiolabelled $[^3\text{H}]\text{InsP}_3$ from the pellets. Careful washing of the tubes and surface of the tightly packed pellets decreased the subsequently eluted radio-

activity by only 3 %, so that washing after complete aspiration of the supernatant was not routinely carried out. After extraction for 30 min with trichloroacetic acid, the $[^3\text{H}]\text{InsP}_3$ was measured in a liquid-scintillation counter. Non-specific binding was determined by adding unlabelled InsP_3 equal to 100 times the estimated high K_D to displace receptor-bound radioactive InsP_3 totally. Heparin was also used to displace receptor-bound $[^3\text{H}]\text{InsP}_3$, and yielded essentially the same value for non-specific binding. Non-specific binding was subtracted from control samples to determine net radioactivity specifically bound to receptors.

H.p.l.c. of $[^3\text{H}]\text{InsP}_3$. Batches of $[^3\text{H}]\text{InsP}_3$ used for binding studies were analysed by a modification of the h.p.l.c. method of Shayman & BeMent (1988) using a C_{18} reversed-phase column (Altex Ultrasphere ODS) and a mobile phase of 20 % (v/v) acetonitrile and 80 % (v/v) 0.5 M-phosphate/0.05 M-tetrabutylammonium dihydrogen phosphate, pH 3.5. Radioactivity of the column effluent was measured with a Radiomatic monitor equipped with a liquid-scintillation flow cell. ATP was added to samples to serve as an internal reference standard that was measured by u.v. absorbance with a Micromeritics monitor.

Treatment with protein-modification reagents. Platelet vesicles (1.0 mg of protein/ml) were washed twice with ice-cold incubation buffer (c) and treated with 1 mg of *p*-hydroxyphenylglyoxal reagent/ml for 60 min at 23 °C in the dark; untreated vesicles incubated in the same manner served as controls. The vesicles were cooled to 4 °C and incubated with 10 nM- $[^3\text{H}]\text{InsP}_3$ for 10 min and assayed for InsP_3 binding by the method previously described. Carbodi-imides were tested for their ability to affect Ca^{2+} release by InsP_3 from platelet vesicles. Vesicles loaded with $^{45}\text{Ca}^{2+}$ as described by O'Rourke *et al.* (1989) were incubated with DCCD or ECCD for 5 min. Ca^{2+} release was initiated by addition of 5 μM - InsP_3 or by raising pH to 7.7 with KOH. Vesicles were assayed for $^{45}\text{Ca}^{2+}$ content as described by O'Rourke *et al.* (1989).

RESULTS AND DISCUSSION

Binding of InsP_3 to platelet membranes and the effect of pH

In designing these experiments, we took into account several factors that can render the analysis of receptor–ligand binding assays inaccurate or misleading (Bennett & Yamamura, 1985; McPherson, 1985; Kermode, 1989): (1) when $K_D \geq 10$ nM, the usual filtration/washing method for separation of membranes from the aqueous phase can result in excessive loss of receptor-bound ligand, and separation by centrifugation is preferred (see also Willcocks *et al.*, 1987); (2) K_D can be greatly overestimated, and Hill plots show upward curvature even for a single class of binding sites, when the concentration of R_T (total receptor) is much larger than K_D ; (3) non-specific binding can be overestimated, and Scatchard plots show upward curvature, if the concentration of competing unlabelled ligand is excessive; and (4) the purity of the radioactive ligand is essential (Kermode, 1989).

To avoid these problems, initial estimates of K_D and B_{max} were made by plotting the data for displacement of a single low concentration of radioactive InsP_3 by unlabelled InsP_3 at pH 8.3 (Fig. 1). Binding of $[^3\text{H}]\text{InsP}_3$ was very rapid, and equilibrium was attained within a few minutes (results not shown). K_D was estimated to be about 20 nM, and the total number of binding sites estimated by Scatchard plot was 1.375 pmol/mg of protein (results not shown). The specificity of the InsP_3 -binding sites was also evaluated by displacement of bound $[^3\text{H}]\text{InsP}_3$ with unlabelled InsP_3 and other non-radioactive inositol phosphates.

Fig. 1. Displacement of $[^3\text{H}]\text{InsP}_3$ from platelet membrane binding sites by inositol phosphates

Platelet membranes were equilibrated with 15 nM- $[^3\text{H}]\text{InsP}_3$ at pH 8.3 and 1.0 mg of protein/ml. The graph shows the percentage displacement of $[^3\text{H}]\text{InsP}_3$ by various non-radioactive inositol phosphates: $\blacklozenge$, $\text{Ins}(1,4,5)\text{P}_3$; $\bullet$, $\text{Ins}(2,4,5)\text{P}_3$; ∇ , $\text{Ins}(1,3,4,5)\text{P}_4$; $\blacktriangledown$, $\text{Ins}(1,4,5,6)\text{P}_4$; $\circ$, $\text{Ins}(1,4)\text{P}_2$ (100% bound = 2028 c.p.m.; 0% bound = 273 c.p.m.).

The membranes were equilibrated with 15 nM- $[^3\text{H}]\text{InsP}_3$, and unlabelled inositol phosphates were added at increasing concentrations up to 5 μM .

In agreement with virtually all other studies, the optimal binding of inositol phosphates to platelet receptors requires three phosphate groups, at the 1, 4 and 5 positions. The 2,4,5 isomer produced a concentration-dependent displacement of $\text{Ins}(1,4,5)\text{P}_3$ that is consistent with its appreciable affinity for the site(s), as shown in other cells (Irvine *et al.*, 1984; Willcocks *et al.*, 1987). This isomer also has the ability to release Ca^{2+} (Spat *et al.*, 1987). $\text{Ins}(1,3,4,5)\text{P}_4$ produced only a partial displacement of InsP_3 at 5 μM , but did not release Ca^{2+} at that concentration (results not shown). The various inositol phosphates tested [including $\text{Ins}(4,5)\text{P}_2$, $\text{Ins}(1,4)\text{P}_2$ and $\text{Ins}(1,4,5,6)\text{P}_4$] had affinities for the InsP_3 -binding site that were in agreement with previous findings in other cells (Irvine *et al.*, 1984; Willcocks *et al.*, 1987; Worley *et al.*, 1987).

The equilibrium-binding experiments were 'hot' saturation studies in which the receptor population was determined by incubating membranes with increasing amounts of radioligand. The batches of $[^3\text{H}]\text{InsP}_3$ that we used had a purity of 97.3% ($\pm 0.4\%$ S.E.M.). It was important to verify purity of the radiolabelled compound, because occasional aberrant batches showed sufficient decomposition to render them unfit for accurate analysis of receptor binding. Binding of $[^3\text{H}]\text{InsP}_3$ was measured by equilibrating membranes with 8–14 concentrations of ligand at pH 7.1, 8.3 and 9.4 for 10 min, sufficient time to reach full equilibrium. The estimated total concentration of membrane receptors was maintained at ≤ 0.1 times the estimated K_D . Non-specific binding was determined at each concentration of radioligand by addition of unlabelled InsP_3 equal to 100 times the estimated high K_D value (Bennett & Yamamura, 1985). Displacement of $[^3\text{H}]\text{InsP}_3$ binding by an unrelated chemical species of competing ligand (i.e. heparin) gave virtually identical estimates of non-specific InsP_3 binding (i.e. 10–15%) as compared with displacement by unlabelled InsP_3 .

The binding data were fitted by using the computer program LIGAND, which employs a weighted non-linear least-squares curve-fitting technique (Munson & Rodbard, 1980). Initial estimates of the binding parameters to be used in LIGAND were

obtained from the original data by the iterative curve-fitting program EBDA (equilibrium-binding data analysis) as described by McPherson (1983, 1985). Goodness-of-fit was determined by the 'runs' test, which evaluates the randomness of points falling above and below the fitted curves, and by an appropriate F-test for reduction of the weighted sums of squares for one-site versus two-site models (Munson & Rodbard, 1980).

The binding of $[^3\text{H}]\text{InsP}_3$ was measured in a platelet membrane fraction that displays ATP-dependent phosphate- and oxalate-stimulated uptake of Ca^{2+} , and has the greatest specific activity for InsP_3 -induced Ca^{2+} release (O'Rourke *et al.*, 1985). Binding was measured at three pH values. At pH 7.1, which coincides with the intracellular pH of unstimulated platelets (Zavoico *et al.*, 1986), a single class of binding sites was observed, with a K_D of 11.8 nM and a B_{max} of 1.4 pmol/mg of protein (Fig. 2a). A Hill plot of the data (Fig. 2b) was linear, with a slope of 0.997 and correlation coefficient 0.93. Binding of $[^3\text{H}]\text{InsP}_3$ was inhibited by Ca^{2+} (50% inhibition at 40 μM). The affinity of the platelet sites is close to that in cerebellum, but is greater than that in most other peripheral tissues, except for the recent report of a low-affinity site in liver membranes with $K_D = 88$ nM (Mauger *et al.*, 1989). The receptor concentration of the platelet membrane

Fig. 2. Binding of $[^3\text{H}]\text{InsP}_3$ to platelet membranes at pH 7.1

(a) Scatchard plot of a single representative experiment with duplicate determinations of each point. The data were best-fitted (line) to a one-site model by the LIGAND program. In three experiments $K_D = 11.8$ nM (± 1.01 S.D.) and $B_{\text{max}} = 1.4$ (± 0.6 S.D.) pmol/mg of protein. (b) Hill plot of the binding data (concs. in M). The slope was 0.997, with correlation coefficient 0.93. Membranes were incubated with 0.25–100 nM- $[^3\text{H}]\text{InsP}_3$ for 10 min at 4 $^{\circ}\text{C}$, and binding was measured as described in the Materials and methods section.

fraction was about 7–10 times that reported in membranes of adrenal cortex, anterior pituitary and hepatocytes (Spat *et al.*, 1987; Guillemette & Segui, 1988; Mauger *et al.*, 1989), but significantly less than in cerebellum. The K_D of the InsP_3 -binding site at pH 7.1 is about one-tenth the EC_{50} for release of Ca^{2+} from membrane vesicles by InsP_3 . However, much of this discrepancy may be explained by two factors that decrease the effectiveness of InsP_3 in the Ca^{2+} -release experiments, i.e. metabolism of InsP_3 by membrane phosphatase, and the presence of millimolar concentrations of ATP, which inhibits InsP_3 binding (50% inhibition at 0.5 mM) (Willcocks *et al.*, 1987).

At elevated pH (i.e. 8.3 and 9.4) the Scatchard plots displayed a pronounced upward curvature, and the binding data were best fitted by a two-site model, the second site being of higher affinity ($K_D = 0.75\text{--}1.2\text{ nM}$) but low concentration; $B_{\text{max}} = 0.195\text{--}0.6\text{ pmol/mg}$ of protein (Fig. 3). These data suggest the presence of more than one set of sites, or of negative co-operativity. However, the binding data alone cannot distinguish between these two possibilities. At pH 7.1 and 8.3 the total binding capacity (high- and low-affinity sites) for InsP_3 was essentially constant at about 1.4 pmol/mg of protein, but total binding was about 5-fold greater at pH 9.4. The properties of the low-affinity binding site may also vary with pH, since the K_D increased from 11.8 nM at pH 7.1, to 23.3 nM at pH 8.3, and 61.3 nM at pH 9.4.

Fig. 3. Scatchard plots of $[^3\text{H}]\text{InsP}_3$ binding to platelet membranes, showing single representative experiments at pH 8.3 (a) and pH 9.4 (b)

Experiments were done with $1.0\text{--}1.4\text{ mg}$ of protein/ml. The curves were fitted to the data by the LIGAND program. The data were best fitted by a two-site model, shown by the straight lines. At pH 8.3 (six experiments): $K_{D1} = 1.23 (\pm 1.0)$, $K_{D2} = 23.4 (\pm 7.8)$, $B_{\text{max},1} = 0.195 (\pm 0.08)$, $B_{\text{max},2} = 1.21 (\pm 0.32)$. At pH 9.4 (means of two experiments): $K_{D1} = 0.75$, $K_{D2} = 61.3$, $B_{\text{max},1} = 0.58$; $B_{\text{max},2} = 6.07$. K_D values are in nM and B_{max} values pmol/mg of protein ($\pm$ s.d.).

We consider the single site measured at pH 7.1 to be the 'low-affinity' site, since its K_D was 10 times that of the high-affinity site measured at pH 8.3 and 9.4. At pH 8.3 and 9.4 the low-affinity site accounted for more than 85% of the total InsP_3 binding. We cannot conclude that the variations of pH are solely responsible for all of the observed effects, because the buffers used to control each pH properly were chemically different, i.e. Hepes, Tris and $\text{HCO}_3^-/\text{CO}_3^{2-}$ respectively. Further work is necessary to evaluate the effects of specific buffers.

Effects of heparin and other anionic polymers on InsP_3 binding

As noted previously in others cells (Worley *et al.*, 1987), heparin prevented binding of InsP_3 to platelet membranes, regardless of pH. At pH 7.1, because the binding site behaves as a single homogeneous class of sites it was possible to determine that heparin was a competitive inhibitor of InsP_3 binding, with $K_i = 0.56\text{ }\mu\text{M}$ (results not shown). Nevertheless, even at alkaline pH heparin was able to abolish totally binding of InsP_3 , although the complexity of the binding curves precluded determining whether the inhibition was strictly competitive. Assuming that the polyanionic nature of heparin is vital for this effect, we tested several other anionic polymers such as dextran sulphate, polyvinyl sulphate and chondroitin sulphate. Polyvinyl sulphate (Fig. 4) and dextran sulphate proved to be more potent than heparin, whereas chondroitin sulphate was ineffective (results not shown). The order of effectiveness for inhibition of InsP_3 binding was well correlated with the density of anionic charges in

Fig. 4. Displacement of $[^3\text{H}]\text{InsP}_3$ from platelet membranes by heparin ($\circ$, $\bullet$) and polyvinyl sulphate ($\square$, $\blacksquare$)

Conditions were similar to those for Fig. 1 (protein 1.5 mg/ml): (a) at pH 7.1, (b) at pH 8.3.

these molecules. Furthermore, the uncharged polymers polyvinyl chloride, polyvinyl alcohol and dextran were totally without effect on InsP_3 binding.

Surprisingly, heparin, dextran sulphate and polyvinyl sulphate cause extensive release of Ca^{2+} from platelet membrane vesicles. This effect has not been reported in other cells, and was not observed in reticular vesicles from vascular smooth muscle (F. O'Rourke, M. B. Feinstein & K. Soons, unpublished work). Whether the effect in platelets is on the InsP_3 -receptor/channel or on another Ca^{2+} -releasing system requires investigation.

Since InsP_3 is displaced from both sites by heparin, dextran sulphate and polyvinyl sulphate, it seems possible that only one actual binding site exists, but that with increasing pH there is some transition in ionization at, or near, the site which alters the affinity of a fraction of the sites. Also, the degree of ionization of InsP_3 phosphate groups may affect the binding affinities to a positively charged site (Irvine, 1989). The kinetic data alone cannot decide these issues. We also caution that the variation in buffer composition does not allow firm conclusions until the effects of different buffer components are evaluated.

Effects of protein-modification reagents on InsP_3 binding and Ca^{2+} release

The presence of a strong positively charged moiety at the InsP_3 -binding site is indicated by the ability of polymeric anions, such as heparin, to displace InsP_3 effectively. The known binding sites for heparin in various heparin-binding proteins are highly basic polypeptide regions that in almost all cases contain arginine residues, as well as lysine (Sun & Chang, 1989). The high pK_a (12.5) of arginine guanidino groups means they are positively charged sites even at pH 9.4 used in our studies. X-ray crystallography showed that guanidyl groups of guanidine form hydrogen bonds to phosphate oxygen atoms (Cotton *et al.*, 1973). Similarly, arginine residues probably serve as hydrogen-bonding binding sites with nucleotide phosphate groups (Riordan, 1979; Raess *et al.*, 1985). Therefore it is possible that InsP_3 phosphate groups interact with guanidyl side chains of certain arginine residues at the InsP_3 receptor. Phenylglyoxal and *p*-hydroxyphenylglyoxal have been widely used as reagents to modify, specifically and covalently, the guanidyl groups of arginine in proteins (Takahashi, 1968; Yamasaki *et al.*, 1980). To test this hypothesis, platelet membranes were incubated with *p*-hydroxyphenylglyoxal for various times up to 1 h, after which binding of $[^3\text{H}]\text{InsP}_3$ was measured in the usual way. *p*-Hydroxyphenylglyoxal produced a time-dependent inactivation of InsP_3 binding, which was not reversed by washing the membranes to remove the reagent (Fig. 5).

Ca^{2+} release from the isolated sarcoplasmic reticulum of muscle, induced by ryanodine and elevated pH, is blocked by millimolar concentrations of carboxy-group-modifying carbodi-imides. This effect occurred at a site distinct from the Ca^{2+} -transport ATPase (Argaman & Shoshan-Barmatz, 1988). To test the effects of carbodi-imides on the platelet Ca^{2+} -releasing system, we treated platelet membrane vesicles pre-loaded with $^{45}\text{Ca}^{2+}$ with 0.2–4.0 mM-DCCD or -ECCD and measured their effects on InsP_3 binding and Ca^{2+} release by InsP_3 . The carbodi-imides did not affect InsP_3 binding (results not shown), but they abolished release of Ca^{2+} by InsP_3 (Fig. 6). Elevation of pH to 7.7 also released Ca^{2+} (e.g. 27% in 5 min), which was blocked by 90% by 2 mM-DCCD. The inhibition of Ca^{2+} release by carbodi-imides indicates that the platelet InsP_3 -gated channel may have a region regulating Ca^{2+} permeation that is similar to the Ca^{2+} -efflux channel in sarcoplasmic reticulum. This region appears to be distinct from the InsP_3 -binding site.

After submission of this paper, Furuichi *et al.* (1989) reported the cloning and expression of the InsP_3 receptor of cerebellar

Fig. 5. Inhibition of $[^3\text{H}]\text{InsP}_3$ binding to platelet membranes by *p*-hydroxyphenylglyoxal

Vesicles were incubated with 1 mg of *p*-hydroxyphenylglyoxal/ml for 1 h at 23 °C in the dark. Membranes were cooled to 4 °C, and binding of 15 nM- $[^3\text{H}]\text{InsP}_3$ was determined. Values are means ($\pm$ s.d.) for duplicate determinations in seven experiments.

Fig. 6. Inhibition of Ca^{2+} release from platelet membrane vesicles by carbodi-imides (●, DCCD; ○, ECCD)

Membrane vesicles were preloaded with $^{45}\text{Ca}^{2+}$ as described by O'Rourke *et al.* (1989). Control uptake was 95 nmol of $^{45}\text{Ca}^{2+}$ /mg protein; 5 μM - InsP_3 released 19 nmol/mg of protein. Ca^{2+} -loaded membranes were incubated with 0.1–4.0 mM carbodi-imides for 5 min at 23 °C, and then $^{45}\text{Ca}^{2+}$ release by InsP_3 was initiated. Binding of 15 nM- $[^3\text{H}]\text{InsP}_3$ was unaffected by the carbodi-imides.

Purkinje fibres. It was pointed out that the InsP_3 receptor has some remarkable similarities to the ryanodine receptor of muscle, which may be related to Ca^{2+} -translocating properties.

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