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DISTINCT BINDING SITES FOR Ins(1,4,5)P_3 AND Ins(1,3,4,5)P_4 IN BOVINE PARATHYROID GLANDS

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Summary: We utilized high specific activity, [^{32}P]-labelled ligands to measure the binding of Ins(1,3,4,5)P_4 and Ins(1,4,5)P_3 to membranes prepared from bovine parathyroid glands. [^{32}P]Ins(1,3,4,5)P_4 bound rapidly and reversibly to parathyroid membranes, and the binding data could be fitted by the interaction of the ligand with two sites, one with K_d=6.8x10^{-9} M and B_{max}=26 fmol/mg protein and a second, lower affinity site, with K_d=4.1x10^{-7} M and B_{max}=400 fmol/mg protein. InsP_5 was 10-20 fold less potent than InsP_4, and Ins(1,3,4)P_3 and Ins(1,4,5)P_3 were nearly 1000-fold less potent in displacing [^{32}P]Ins(1,3,4,5)P_4. [^{32}P]Ins(1,4,5)P_3, on the other hand, bound to a single class of sites with K_d=7.6x10^{-9} M and B_{max}=34 fmol/mg. While the binding of [^{32}P]Ins(1,4,5)P_3 increased markedly on raising pH from 5 to 8, the binding of [^{32}P]Ins(1,3,4,5)P_4 decreased by 75% over this range of pH. Thus, [^{32}P]-labelled Ins(1,3,4,5)P_4 and Ins(1,4,5)P_3 may be used to identify distinct binding sites which may represent physiologically relevant intracellular receptors for InsP_3 and InsP_4 in parathyroid cells.

Receptor-mediated activation of phosphoinositide hydrolysis by Ca^{++}-mobilizing hormones leads to the formation of inositol (1,4,5)trisphosphate (InsP_3)(cf.1) and inositol (1,3,4,5)tetrakisphosphate (InsP_4)(2). The former releases Ca^{++} from non-mitochondrial stores (3), leading to transient increases in cytosolic Ca^{++}, while the latter is probably also involved in the regulation of the cytosolic Ca^{++} concentration by promoting the refilling of intracellular Ca^{++}-stores, either from the extracellular space (4,5) or from the cytosol (6). The effects of InsP_3 and InsP_4 on intracellular Ca^{++} dynamics may be mediated by binding to putative intracellular receptors (7,8), which regulate transmembrane movement of calcium ions.

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The parathyroid cell is unusual in that high extracellular Ca^{++} concentrations per se promote transient, followed by sustained, increases in cytosolic Ca^{++} (9,10), as well as the intracellular accumulation of $InsP_3$ (11) and $InsP_4$ (12). In the present studies, we have used recently developed, [^{32}P]-labelled $InsP_3$ (13) and $InsP_4$ (14) to determine whether distinct binding sites for these biologically active inositol phosphates could be identified in membranes from bovine parathyroid glands.

MATERIALS AND METHODS

4,5-[^{32}P]Ins(1,4,5)trisphosphate (specific activity of 41-107 Ci/mmol by self displacement determination) was obtained from New England Nuclear Corp. (Boston, MA) (thanks to J. G. Breuning) and the unlabelled Ins(1,4,5)P_3 and $InsP_5$ from Calbiochem (San Diego, CA). Ins(1,3,4)P_3 was a generous gift from R. F. Irvine, Cambridge, U.K. Other reagents were from sources previously cited (14).

[^{32}P]Ins(1,3,4,5)tetrakisphosphate was prepared as previously described, by phosphorylation of [^{32}P]Ins(1,4,5)trisphosphate, using a partially purified Ins(1,4,5)-kinase prepared from rat brain (14). The radiochemical purity, as determined by HPLC, was always at least 95%, with less than 1% contamination by [^{32}P]Ins(1,4,5)P_3.

Membranes were prepared from either fresh or frozen parathyroid glands from calves or adult cows as follows: After the tissue was carefully trimmed of fat at 0°C, it was finely minced, and any fat was aspirated from the supernatant prior to homogenization. The minced tissue was then resuspended in 0.25 M sucrose, 1 mM dithiothreitol, 0.5 mM EGTA, 5 mM Hepes-KOH pH 7.0 and homogenized, first in a polytron and then with a Potter homogenizer. The homogenate was sedimented for 10 minutes at 1000xg and the supernatant saved. The supernatant was then sedimented at 35000xg for 20 minutes to yield a crude membrane pellet, which was resuspended in 0.25 M sucrose, 10 mM KCl, 1 mM dithiothreitol, 5 mM Hepes-KOH pH 7.0, washed once with the same buffer, recentrifuged and kept at -70°C until use.

The buffer for binding studies contained the following components in a final volume of 250 ul/tube: KCl (100 mM), NaCl (20 mM), EDTA (1 mM), BSA (0.1%), sodium phosphate, pH 7.2 (25 mM) as well as membranes (0.8-1.4 mg protein/tube), [^{32}P]Ins(1,3,4,5)P_4 (4.5-7.2 nCi/tube) or [^{32}P]Ins(1,4,5)P_3 (4.1-7.3 nCi/tube) and varying concentrations of the appropriate unlabelled inositol phosphates. Binding studies were conducted at 0°C for the indicated time intervals, and the reaction was stopped by diluting the samples with 3 ml of ice-cold phosphate-buffered saline (pH 7.0), containing phytic acid (1 mM) and filtering through Whatman GF/B filters, followed by rapid washing with 2 x 3 ml of the same solution.

K_d values and the number of binding sites were estimated using the "LIGAND" data analysis computer program (15).

RESULTS

Binding of [^{32}P]Ins(1,3,4,5)P_4 to bovine parathyroid membranes was rapid, with apparent equilibrium reached by 20 minutes at 0°C (Figure 1).

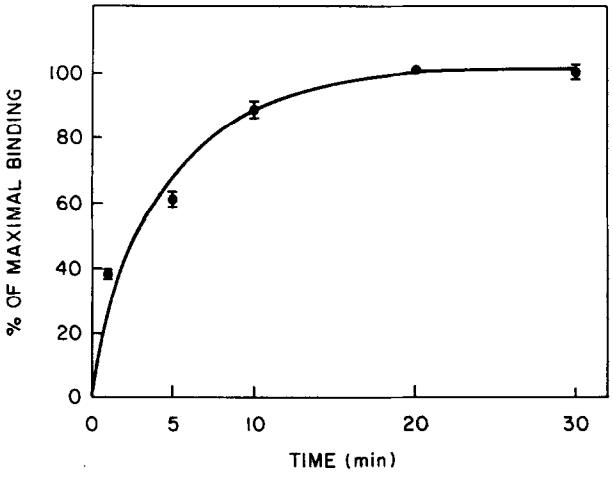

Figure 1: Association rate for the binding of [^{32}P]Ins(1,3,4,5)P_4 to bovine parathyroid membranes. [^{32}P]Ins(1,3,4,5)P_4 was incubated with bovine parathyroid membranes at 0°C for the times shown, and bound and free tracer were separated as described in Materials and Methods. Points represent the mean ± SEM for 3-4 determinations in two separate membrane preparations. If not shown, error bars were smaller than the point. Maximal specific binding averaged 282 cpm. Specific binding was calculated by subtracting the counts bound in the presence of an excess (2 x 10^{-6} M) of unlabelled Ins(1,3,4,5) from the total counts bound. Nonspecific binding averaged 33% of total bound counts under these conditions.

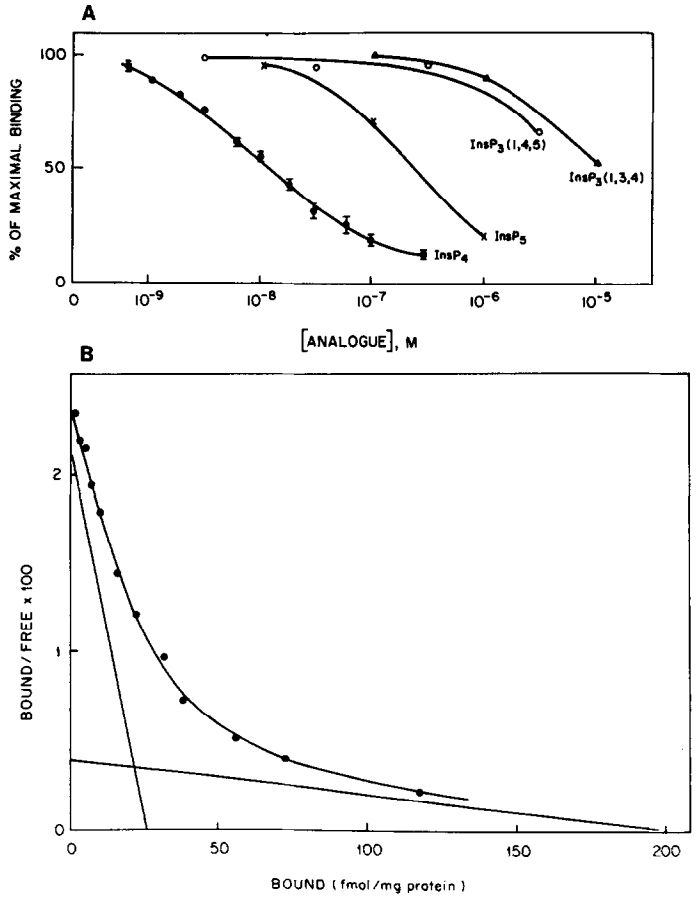

Figure 2: A. Displacement of [^{32}P]Ins(1,3,4,5)P_4 from bovine parathyroid membranes by Ins(1,3,4,5)P_4 and other inositol phosphates. Membranes were incubated with [^{32}P]Ins(1,3,4,5)P_4 as well as the indicated concentrations of the inositol phosphates shown for 20 minutes at 0°C. Results are expressed as the percent of the maximal binding of [^{32}P]Ins(1,3,4,5)P_4 in the absence of added unlabelled inositol phosphates and represent the mean of 5-6 determinations in 3 separate membrane preparations. Error bars (± SEM) shown for $InsP_4$ are representative of those observed with the other inositol phosphates, which were in all cases less than ± 10%.
B. The Scatchard plot of the Ins(1,3,4,5)P_4 binding data from one representative experiment.

Under these conditions, nonspecific binding was 33% of total binding. Subsequent addition of unlabelled Ins(1,3,4,5)P_4 ($2x10^{-6}$ M) resulted in rapid dissociation of bound [^{32}P]Ins(1,3,4,5)P_4, with 50% loss of bound counts within 1 minute and 90% loss within 10 minutes (not shown). Under equilibrium conditions, with a fixed concentration of [^{32}P]Ins(1,3,4,5)P_4 and increasing concentrations of unlabelled $InsP_4$, the IC_{50} for displacement of bound label was approximately 10^{-8} M (Figure 2A). Similar results were obtained using membranes from calf and adult cow parathyroid glands, and the

results, therefore, were combined. The displacement curve was flat, occurring over about a 1000-fold range of concentrations of unlabelled Ins(1,3,4,5)P_4, and Scatchard plot of these data was curvilinear upwards (Figure 2B). Analysis by the LIGAND computer program showed that the data could be fitted by assuming two classes of binding sites, with apparent K_d's and densities of $6.81 \pm .46 \times 10^{-9}$ M and 26.4 ± 3.7 fmol/mg protein and $4.12 \pm 0.91 \times 10^{-7}$ M and 400 ± 171 fmol/mg protein, respectively. $InsP_5$ was about 20 fold less potent in reducing [^{32}P]Ins(1,3,4,5)P_4 binding to bovine parathyroid membranes, while Ins(1,4,5)P_3 and Ins(1,3,4)P_3 were about 1000-fold less potent than unlabelled Ins(1,3,4,5)P_4.

The binding of [^{32}P]Ins(1,4,5)P_3 was examined under conditions identical to those used with [^{32}P]Ins(1,3,4,5)P_4 (Figure 3). Data for the competition of unlabelled Ins(1,4,5)P_3 with [^{32}P]$InsP_3$ for binding to bovine parathyroid membranes could be fitted with a single class of binding sites, with $K_d = 7.67 \pm 2.03 \times 10^{-9}$ M and receptor density of 34.3 fmol/mg protein. The affinity of Ins(1,4,5)P_3 for this binding site was markedly different from

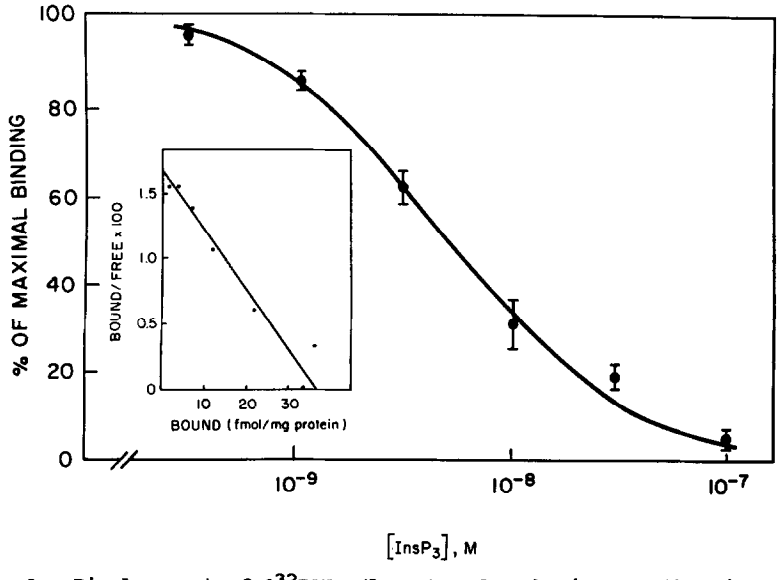

Figure 3: Displacement of [^{32}P]Ins(1,4,5)P_3 from bovine parathyroid microsomes by Ins(1,4,5)P_3. The experimental conditions were the same as in Figure 2, except that [^{32}P]Ins(1,4,5)P_3 was used as the tracer and nonspecific binding was determined with 10^{-6} M Ins(1,4,5)P_3. Points are the mean ± SEM for 5-6 determinations in three separate membrane preparations. The Scatchard plot of the binding data from one representative experiment is shown in the inset.

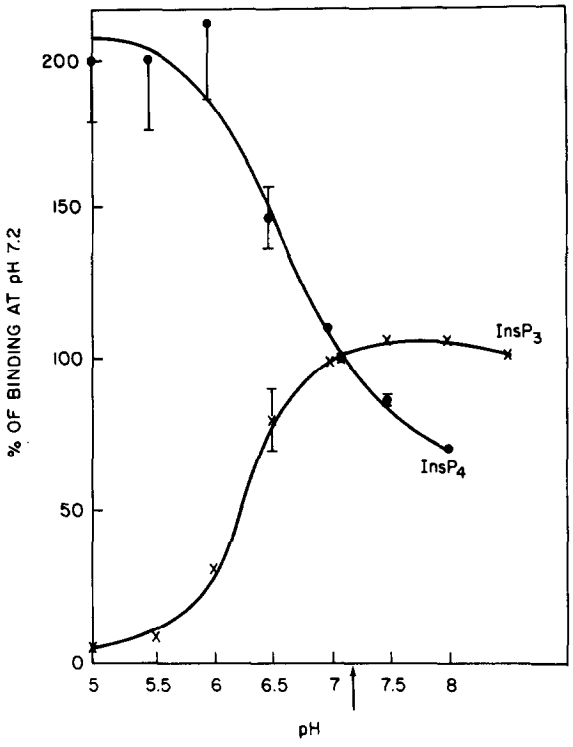

Figure 4: Binding of [^{32}P]Ins(1,3,4,5)P_4 and [^{32}P]Ins(1,4,5)P_3 to bovine parathyroid membranes as a function of pH. Binding was carried out for 20 minutes at 0°C as described in Materials and Methods. Specific binding is shown for each pH (total binding minus nonspecific binding with 2 x 10^{-6}M $InsP_3$ or $InsP_4$). Points represent the mean of 4-6 determinations in 2 separate membrane preparations for $InsP_3$ and 3 preparations for $InsP_4$. Data are expressed as percent of the binding at pH 7.2 [171 cpm for [^{32}P]Ins(1,3,4,5)P_4 and 194 cpm for [^{32}P]Ins(1,4,5)P_3].

that defined by using [^{32}P]Ins(1,3,4,5)P_4 as tracer, suggesting different high affinity binding sites for $InsP_3$ and $InsP_4$. This conclusion was also supported by determining the pH dependence of the binding of [^{32}P]Ins(1,3,4,5)P_4 and [^{32}P]Ins(1,4,5)P_3 to bovine parathyroid membranes (Fig. 4). With increasing pH from 5 to 8, the binding of Ins(1,4,5)P_3 increased markedly, while that for Ins(1,3,4,5) decreased by nearly 75%.

DISCUSSION

The present results demonstrate that membranes from calf and cow parathyroid glands contain binding sites for both Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4. We detected a single class of binding sites for Ins(1,4,5)P_3. These sites were of relatively high affinity (K_d=7.6 x 10^{-9} M)

for IP_3 and could be distinguished in several ways from the binding sites for Ins(1,3,4,5)P_4. In contrast to the sites which bound [^{32}P]Ins(1,4,5)P_3, the sites binding [^{32}P]Ins(1,3,4,5)P_4 appeared to fall into at least two classes--a high affinity, low capacity site and a lower affinity, high capacity site. Although our data are closely fitted by assuming two different binding sites, other models are also possible, such as a single class of sites showing negative cooperativity. With the available data, we cannot definitively discriminate between independent and interacting sites. Neither of the sites binding Ins(1,3,4,5)P_4, however, appears to correspond to the site binding Ins(1,4,5)P_3, since very high concentrations of $InsP_3$, well above those saturating the site binding $InsP_3$, were required to displace [^{32}P]Ins(1,3,4,5)P_4. The sites interacting with $InsP_4$ and $InsP_3$ could also be differentiated by the effects of pH on binding: $InsP_3$ binding is increased at higher pH, while the binding of $InsP_4$ decreased markedly on raising pH from 5 to 8. The presence of distinct binding sites for Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4 in bovine parathyroid membranes is consistent with data on $InsP_3$ and $InsP_4$ binding to bovine adrenal cortical microsomes (14), although the sites in adrenal and parathyroid differ in a 4-6 fold higher density of sites for $InsP_4$ in the latter.

Since whole tissue was employed to prepare membranes in the present studies, it is possible that the binding sites for $InsP_3$ and $InsP_4$ were present in a cell type other than parathyroid cells per se. Parathyroid glands from adult cows, for example, contain about 50% fat. We obtained similar data using hyperplastic parathyroid tissue from neonatal calves, however, in which there is little or no fat, suggesting that the binding sites for the inositol phosphates were probably not in fat cells. We have not, however, ruled out the possibility that $InsP_3$ and $InsP_4$ bind to other cell types present in smaller numbers, such as vascular cells or fibroblasts.

Our data do not establish whether the binding sites identified here correspond to the putative receptors for Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4 which are thought to mediate the effects of these inositol phosphates on

translocation of Ca^{++} in the intact cell (3-5). The sites labelled by the high specific activity [^{32}P]Ins(1,4,5)P_3 and [^{32}P]Ins(1,3,4,5)P_4 used here, however, show rapid kinetics for association and dissociation, saturability, specificity, high affinity and low capacity, characteristics exhibited by many bona fide receptors.

It is of interest that high extracellular Ca^{++} concentrations promote the accumulation of $InsP_3$ (11) and $InsP_4$ (12) in parathyroid cells Since Ins(1,4,5)P_3 has previously been shown to release Ca^{++} from nonmitochondrial stores in permeabilized parathyroid cells (16), the high extracellular Ca^{++}-induced spikes in cytosolic Ca^{++} in intact parathyroid cells might arise from binding of Ins(1,4,5)P_3 to a site such as that identified here, which mediates release of intracellular Ca^{++}. Similarly, the sustained rise in cytosolic Ca^{++} observed at high extracellular Ca^{++} concentrations, which is due to uptake of extracellular Ca^{++} (9,10,17,18), might result, in part, from interaction of $InsP_4$ with intracellular binding sites which promote uptake of extracellular Ca^{++}.

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