Synergistic effects of inositol 1,3,4,5-tetrakisphosphate on inositol 2,4,5-trisphosphate-stimulated Ca^{2+} release do not involve direct interaction of inositol 1,3,4,5-tetrakisphosphate with inositol trisphosphate-binding sites

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We have previously found that for permeabilized L1210 cells, low micromolar concentrations of Ins(1,3,4,5)P_4 added prior to Ins(2,4,5)P_3 enhance the effects of suboptimal concentrations of Ins(2,4,5)P_3 in causing Ca^{2+} release from InsP_3-sensitive Ca^{2+} stores [Cullen, Irvine and Dawson (1990) Biochem. J. **271**, 549–553]. If this was due either to some conversion of added Ins(1,3,4,5)P_4 into Ins(1,4,5)P_3 by the 3-phosphatase, or to Ins(1,3,4,5)P_4 acting as a weak (or partial) agonist on the InsP_3 receptor it would be expected that, in the presence of thimerosal to sensitize the InsP_3 receptor, the dose–response curve to Ins(1,3,4,5)P_4 would be left-shifted by the same extent as that of Ins(1,4,5)P_3. This was found not to be the case; the dose–response curve to Ins(1,3,4,5)P_4 was not shifted at all by thimerosal. Furthermore, L-Ins(1,3,4,5)P_4, which can displace radiolabelled D-Ins(1,3,4,5)P_4 but not D-Ins(1,4,5)P_3 from their respective high-affinity binding sites, mimicked the effects of D-Ins(1,3,4,5)P_4 in enhancing the slow phase of Ins(2,4,5)P_3-stimulated Ca^{2+} release. Ins(1,3,4,5)P_4 caused an increase in magnitude of the slow phase of InsP_3-stimulated Ca^{2+} release leaving the magnitude of the fast phase unaltered, in contrast to increasing Ins(2,4,5)P_3 concentrations which increased the size of both phases. In addition, Ins(1,3,4,5)P_4 decreased the rate constant for the slow phase of Ca^{2+} release These findings point strongly to the conclusion that InsP_4 is not working directly via the InsP_3 receptor but indirectly via an InsP_4 receptor.

INTRODUCTION

There has been controversy for several years about the physiological function of InsP_4 and whether or not it has a second messenger function in some aspect of the regulation of intracellular Ca^{2+} movements [1,2]. A very positive piece of evidence for a second messenger function has been the demonstration (reviewed in [3]) of specific high-affinity InsP_4-binding sites in a wide variety of neuronal and non-neuronal cells. We have recently purified to homogeneity a high-affinity InsP_4-binding protein from pig platelets [4] which we have now shown has GTPase-activating protein (GAP) activity and which we have suggested is named GAP1^{IP4BP} [5]. However, the function of such binding proteins, and the downstream effects of InsP_4 on cell signalling remain ill-defined.

Effects of InsP_4 on Ca^{2+} signals have been observed in sea urchin eggs [6], perfused lachrymal cells [7–9] and *ras*-transformed fibroblasts [10]. In the first two of these systems, synergy was observed between InsP_4 and InsP_3, suggesting that the presence of InsP_3 was required in order to observe any effects of InsP_4. A variety of other systems have also been shown to respond to InsP_4 (reviewed in [1]) and, notably, an InsP_4-sensitive Ca^{2+} channel has been demonstrated in endothelial cells [11]. In permeabilized cells, and microsomal preparations, two effects have been seen. In permeabilized L1210 cells, we have found that there is a synergistic effect of InsP_4 in enhancing release of Ca^{2+} by Ins(2,4,5)P_3 [12]. A similar effect has been shown in pituitary microsomes [13]. In contrast, for permeabilized SH-SY5Y neuroblastoma cells, Gawler et al. [15,16] found, besides some synergistic effect, that InsP_4 was able to release Ca^{2+} from intracellular stores by itself. The interpretation of InsP_4 effects is complicated by potential artefacts such as the conversion of InsP_4, via 3-phosphatase activity, into Ins(1,4,5)P_3 [17,18], as well as contamination of InsP_4 preparations by traces of InsP_3 [12,15]. However, under conditions where care was taken to avoid these problems Wilcox and co-workers [19,20], using SH-SY5Y cells, have shown that Ins(1,3,4,5)P_4 can act as a rather weak InsP_3-receptor agonist, causing Ca^{2+} release by direct interaction with the InsP_3 receptor. In contrast, in our previous work on L1210 cells [12], where we could detect no conversion of Ins(1,3,4,5)P_4 into Ins(1,4,5)P_3 and where we used HPLC-purified InsP_4 throughout, InsP_4 did not cause any Ca^{2+} mobilization in the absence of added InsP_3. The detection limit for conversion of InsP_4 into Ins(1,4,5)P_3 in our previous work was such that we would have only detected InsP_3 if it had been produced at greater than about 20 nM. However, given the extreme degree of co-operativity exhibited by the InsP_3 receptor [21], 20 nM InsP_3, present as a steady-state background level, might have significant effects on Ca^{2+} mobilization. Similarly, if InsP_4 acts as an agonist at InsP_3 receptors in L1210 cells, subthreshold levels of InsP_4 might synergize with InsP_3 to enhance Ca^{2+} release. It has thus become important to determine whether or not the effects of InsP_4 which we have observed in permeabilized L1210 cells can be explained by a direct effect of InsP_4 on InsP_3 receptors, or whether they are mediated by a different mechanism.

We have adopted several experimental approaches in order to address this problem. As an experimental system, we have used L1210 cells which have been permeabilized either with digitonin or by electroporation using silver electrode plates. Neither of these treatments leads to activation of detectable inositol polyphosphate 3-phosphatase activity. As previously, we have used Ins(2,4,5)P_3 as an InsP_3-receptor agonist since, unlike

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$Ins(1,4,5)P_3$, it cannot be converted into $Ins(1,3,4,5)P_4$ by the 3-kinase and is poorly metabolized by the 5-phosphatase [12]. We have attempted to make all measurements of the effects of $InsP_4$ as rapidly as possible (usually within 15 s) after the addition of $InsP_4$ to the experimental medium, to minimize the possibility of conversion of $InsP_4$ into $Ins(1,4,5)P_3$. Under these conditions we have compared the effect of thimerosal on the dose–response curves for $InsP_4$ and $Ins(2,4,5)P_3$. Thimerosal has been shown, in a variety of systems [22,23] to sensitize the $InsP_3$ receptor by shifting the dose–response curve by up to a factor of ten to the left. If $InsP_4$ were to work via the $InsP_3$ receptor, then a similar shift in the dose–response curve of $InsP_4$ would be expected. We have also examined the effect of L-$Ins(1,3,4,5)P_4$, which has been shown to displace D-$Ins(1,3,4,5)P_4$ from high-affinity binding sites [4,19] but to be without effect on the binding of $Ins(1,4,5)P_3$ to the $InsP_3$ receptor. Finally, we have looked at the kinetics of Ca^{2+} release brought about by $Ins(2,4,5)P_3$ by itself and in combination with $InsP_4$.

EXPERIMENTAL

Materials

Fluo-3 pentammonium salt, a membrane-impermeable Ca^{2+} indicator, was purchased from Molecular Probes Inc., Eugene, OR, U.S.A. Ca^{2+} ionophore ETH1001 was purchased from Fluka Chemicals Ltd., Glossop, Derbyshire, U.K. Phosphocreatine, creatine kinase and EGTA were purchased from Sigma Chemical Co., Poole, Dorset, U.K.; ATP and dithiothreitol were from B.C.L., Lewes, Sussex, U.K.; silver foil, used as electrode plates for cell permeabilization by electroporation, was obtained from Aldrich, Gillingham, Dorset, U.K.; digitonin from B.D.H., Poole, Dorset, U.K. and tissue-culture materials from GIBCO, Uxbridge, U.K. $Ins(2,4,5)P_3$ and D-$Ins(1,3,4,5)P_4$ were prepared as previously described [12] and L-$Ins(1,3,4,5)P_4$ [in D numbering, $Ins(1,3,5,6)P_4$] was a gift from Professor S. R. Nahorski, Department of Pharmacology and Therapeutics, University of Leicester, Leicester, U.K.

Cell growth

Mouse lymphoma L1210 cells were grown essentially as described previously [24] using a system of gas-permeable batch culture. Cells were grown over a 4 day period to a density of 6.0×10^5 cells/ml, as measured on a Coulter electronic cell counter. A 42 ml sample of cells (6.0×10^5 cells/ml) were centrifuged at 2000 ***g*** for 5 min in a bench centrifuge at 25 °C. The pellet was resuspended in 25 ml of wash buffer consisting of 0.9 % (w/v) NaCl, 20 mM Hepes/KOH, pH 7.0, at 30 °C and centrifuged as above. This wash was repeated before resuspending the final pellet of cells in 1 ml of assay buffer to give a cell density of 2.5×10^7 cells/ml.

Cell permeabilization

Permeabilization by electroporation was carried out with a Bio-Rad Gene Pulser using a custom-designed electroporation cuvette. Cells were subjected to five pulses of 0.8 kV (2 kV/cm) with a capacitance of 25 μF and a time constant of 0.3 ms. Unlike commercially available electroporation cuvettes, the custom-designed cuvettes had electrode plates made from silver foil. This avoided possible artefacts shown to be due to solubilization of cations from the electrode plates [18].

Permeabilization by digitonin was performed by adding 10 μg of digitonin (in 1 μl of DMSO) per ml of 2.5×10^7 cells suspended in the assay medium.

Ca^{2+} uptake

To increase reproducibility between successive experiments, permeabilized cells were loaded with Ca^{2+} on a large scale (10–15 ml of 2.5×10^7 cells/ml). The assay medium consisted of 100 mM KCl, 20 mM Hepes/KOH, pH 7.0, 1 mM dithiothreitol, 5 mM succinate (K^+ salt), 50 mg/ml creatine kinase, 10 mM phosphocreatine, 1 mM $MgCl_2$, 0.7 mM ATP and 1 μM Fluo-3 (pentammonium salt). Experiments were carried out at 30 °C. Ca^{2+} uptake was routinely measured with a Ca^{2+}-sensitive electrode. Ca^{2+}-sensitive membranes contained the neutral ionophore ETH1001 and were prepared by the method described by Clapper and Lee [25]. After the cells had loaded to steady state, 1 ml samples of the incubation mixture were transferred to a Shimadzu RF-5000 or Perkin-Elmer LS50B fluorimeter coupled to an IBM-compatible computer. Ca^{2+} release was measured by Fluo-3 fluorimetry. The Ca^{2+} release properties of the cells loaded in large scale were constant for up to 2 h, allowing experiments to be carried out in rapid succession on identical 1 ml samples of cells.

Under the experimental conditions described above, the $InsP_3$-sensitive pool was 76 ± 3 % ($n = 10$) of the total ionomycin-releasable Ca^{2+} pool.

Kinetic analysis

Digitized output from the fluorimeter was processed first by Microsoft Excel. The time course of Ca^{2+} efflux was found to fit to a double-exponential function. Enzfitter (Biosoft) was used to determine rate constants and extents for fast and slow phases of Ca^{2+} release.

Immunoblotting of $InsP_4$-binding protein

Immunological identification of the specific $InsP_4$-binding protein in L1210 cells was carried out using a polyclonal rabbit antibody raised against a C-terminal peptide of human $GAP1^{IP4BP}$ [5]. After SDS/PAGE and electroblotting on to poly(vinylidene difluoride) membranes, blots were probed with anti-$GAP1^{IP4BP}$ antibody at a dilution of 1:4000 (or rabbit pre-immune serum), followed by peroxidase-conjugated goat anti-(rabbit IgG) secondary antibody (Dako). The blot was developed using enhanced chemiluminescence detection reagents (Amersham) and exposure to X-ray film.

RESULTS AND DISCUSSION

Figure 1 shows the experimental protocol used to measure the dose–response characteristics of $Ins(2,4,5)P_3$ and $Ins(1,3,4,5)P_4$. The data shown in Figure 1 were from electroporated cells, but similar data were also obtained from cells permeabilized with digitonin. Under these conditions, low doses of $Ins(2,4,5)P_3$ cause a biphasic release of Ca^{2+}, similar to that described by Meyer and Stryer [26]. $Ins(1,3,4,5)P_4$ causes no Ca^{2+} release by itself (Figure 1) even after 8 min (results not shown) but, as previously found [12], increases the response to $Ins(2,4,5)P_3$ added 5 s after $InsP_4$. Typically, $Ins(1,3,4,5)P_4$ does not change the magnitude of the fast phase of Ca^{2+} release, but increases the size of the slow phase (Table 1 and see also the traces in Figures 5 and 6). As found previously [12], the effect of $Ins(1,3,4,5)P_4$ on the extent of Ca^{2+} release is only readily observable at $Ins(2,4,5)P_3$ concentrations which cause mobilization of 50 % or less of the total $InsP_3$-sensitive pool. To quantify the effect of $Ins(1,3,4,5)P_4$, the rate of Ca^{2+} release was measured 10 s after the addition of $Ins(2,4,5)P_3$, to avoid complications due to effects of mixing and the fast phase of release. The rates taken are shown as dotted

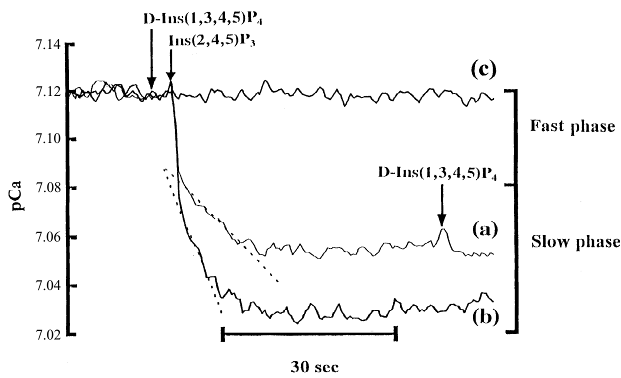

Figure 1 Synergistic effects of D-Ins(1,3,4,5)P_4 and Ins(2,4,5)P_3 at low concentrations of Ins(2,4,5)P_3

Assay conditions were as described in the Experimental section. Cells were permeabilized by electroporation using silver electrode plates. Two Ca^{2+} efflux traces are superimposed: to show enhancement of both the rate (– – –) and extent of the 'slow phase' of Ca^{2+} efflux induced by 1 μM Ins(2,4,5)P_3 after addition of 2 μM Ins(1,3,4,5)P_4 (trace b), and the lack of effect when Ins(1,3,4,5)P_4 was added after Ins(2,4,5)P_3 (trace a). Trace (c) shows the absence of any Ca^{2+} release when 2 μM Ins(1,3,4,5)P_4 was added at the arrow with no subsequent addition of Ins(2,4,5)P_3. Addition times are indicated by the arrows. Identical traces were obtained under assay conditions where thimerosal (100 μM) was added 1 min prior to the addition of Ins(2,4,5)P_3. Under conditions of thimerosal pretreatment, a lower concentration of Ins(2,4,5)P_3 (0.25 μM) was added to give the same change in cytosolic free Ca concentration as that shown in the absence of thimerosal. Experiments were carried out in triplicate at 30 °C.

lines in Figure 1. Also illustrated in Figure 1 is the 'order of addition' phenomenon observed previously [12], where addition of InsP_4 after InsP_3 is without effect.

Application of 100 μM thimerosal to the incubation mixture results in sensitization of the InsP_3 receptor [22,23] and, in these cells, the dose–response curve for Ins(2,4,5)P_3 is shifted about 4-fold to the left (Figure 2A). The resultant EC_{50} values are approx. 0.4 μM and 1.6 μM in the presence and absence of thimerosal respectively. Thimerosal causes a complementary leftward shift of the dose–response curve to Ins(2,4,5)P_3 when the latter is expressed in terms of the initial rate of Ca^{2+} release in the slow phase (Figure 2B). Incubation of cells for up to 4 min with thimerosal alone caused no Ca^{2+} release, suggesting that production of endogenous Ins(1,4,5)P_3 is very small (cf. [27]). Routinely, thimerosal was added 1 min before Ins(2,4,5)P_3. In order to compare the dose–response to InsP_4 under conditions where Ca^{2+} release was similar, it was necessary to adjust the concentration of Ins(2,4,5)P_3 to give similar control Ca^{2+} release. By decreasing the Ins(2,4,5)P_3 from 1 μM to 0.25 μM while maintaining the InsP_4 concentration at 2 μM, in the presence of thimerosal, a set of Ca^{2+} release traces superimposable on those shown in Figure 1 were obtained. The effect of InsP_4 concentration on the rate of Ca^{2+} release, measured as indicated above and in Figure 1, in the presence and absence of thimerosal, is shown in Figure 3. It is clear that the concentration dependence of the effect of InsP_4 is unaffected by the presence of thimerosal, the EC_{50} being about 1.2 μM under both conditions.

Wilcox et al. [19] have shown that L-Ins(1,3,4,5)P_4 [equals D-Ins(1,3,5,6)P_4] displaces [^{32}P]D-Ins(1,3,4,5)P_4 from specific binding sites on pig cerebellar membranes with a potency only 10-fold less than D-Ins(1,3,4,5)P_4, while it was > 1000-fold weaker than Ins(1,4,5)P_3 at displacing [^{3}H]Ins(1,4,5)P_3 from specific InsP_3-binding sites. We have found a similar displacement of D-Ins(1,3,4,5)P_4 by L-Ins(1,3,4,5)P_4 from a putative InsP_4 receptor ($GAP1^{IP4BP}$) purified from pig platelets [4]. Using permeabilized SH-SY5Y cells, Wilcox et al. [19], showed that while D-Ins(1,3,4,5)P_4 would release Ca^{2+} from intracellular stores, the L-isomer was essentially inactive at concentrations of less than 100 μM, thus supporting their interpretation that in their cells Ins(1,3,4,5)P_4 is indeed acting as a direct agonist on Ins(1,4,5)P_3 receptors. Measurement of binding of inositol phosphates to

Table 1 Kinetics of the Ca^{2+} release in the presence of InsP_4

Results from progress curves were fitted using Enzfitter to two exponentials. InsP_4 had no effect on the rate constant or extent of the initial fast phase of Ca^{2+} release. The parameters for the slow phase in the presence of 2 μM Ins(1,3,4,5)P_4 were expressed as a percentage of the paired value for the given concentration of Ins(2,4,5)P_3 in the absence of Ins(1,3,4,5)P_4 (±S.E.M.). n is the number of separate batches of cells used for each data set.

Ins(2,4,5)P_3 (μM)	Extent of slow phase + InsP_4 (% of Control)	Slow phase rate constant + InsP_4 (% of Control)	n
0.50	164±11	60±10	5
0.75	159±32	76±08	4
1.00	156±12	82±06	10
8.00	99±14	76±10	3

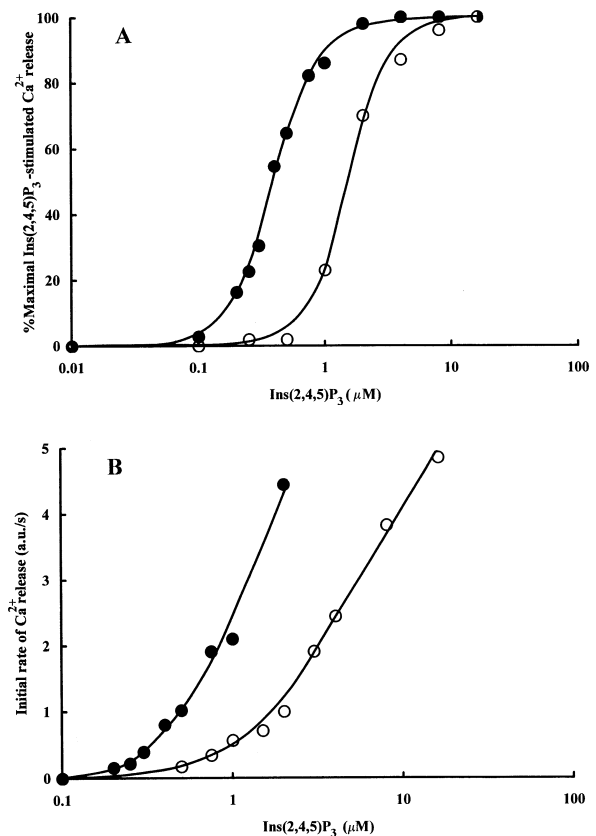

Figure 2 Effect of thimerosal pretreatment on Ins(2,4,5)P_3-dependent Ca^{2+} release from electroporated cells

Assay conditions were as described for Figure 1. In (**A**), Ins(2,4,5)P_3-induced Ca^{2+} release was expressed as a percentage of the Ca^{2+} release caused by a supramaximal Ins(2,4,5)P_3 concentration (16 μM). In (**B**), the initial rate of the slow phase of Ins(2,4,5)P_3-induced Ca^{2+} release was measured as described in Figure 1. The effect of thimerosal on the dose-dependence of Ins(2,4,5)P_3-stimulated Ca^{2+} release was measured at 0 μM (○) and 100 μM (●) thimerosal. Experiments were carried out in triplicate at 30 °C.

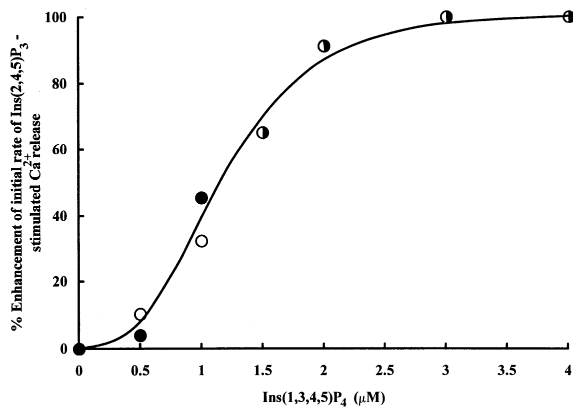

Figure 3 Effect of thimerosal pretreatment on D-Ins(1,3,4,5)P_4 potentiation of Ins(2,4,5)P_3-dependent Ca^{2+} release from electroporated cells

Experimental conditions were as described for Figure 1. The effect of thimerosal pretreatment on the dose-dependence of Ins(1,3,4,5)P_4 potentiation of Ins(2,4,5)P_3-dependent Ca^{2+} release was measured at 0 μM (○) and 100 μM (●) thimerosal. Ins(1,3,4,5)P_4 potentiation of the initial rate of the 'slow phase' of Ins(2,4,5)P_3-stimulated Ca^{2+} release was expressed as a percentagae of the maximum enhancement induced by 4 μM Ins(1,3,4,5)P_4 (maximal concentration) added prior to Ins(2,4,5)P_3. Experiments were carried out at least in triplicate at 30 °C.

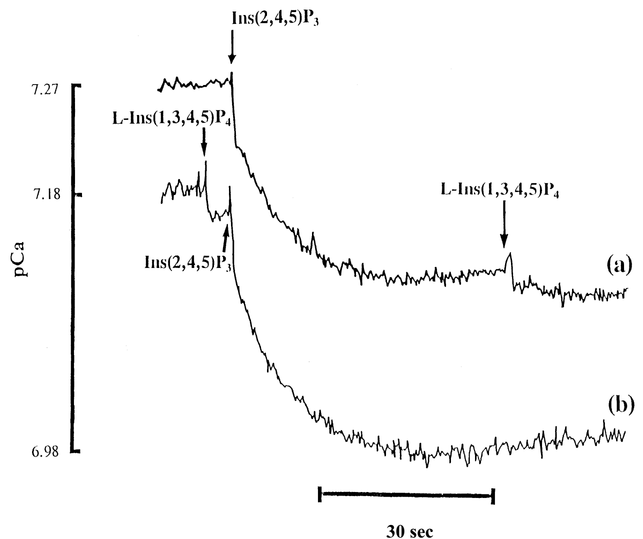

Figure 5 Synergism between L-Ins(1,3,4,5)P_4 and Ins(2,4,5)P_3 at low concentrations of Ins(2,4,5)P_3

Conditions were as described in Figure 1. Ins(2,4,5)P_3 (1 μM) and L-Ins(1,3,4,5)P_4 (40 μM) were added as indicated by the arrows. These traces are typical of five independent experiments.

sonicated membranes from L1210 cells has proved difficult, due to the presence of high levels of non-specific binding. However, immunoblotting (Figure 4) shows that the antibody against the C-terminal sequence of human $GAP1^{IP4BP}$ recognizes a very similar protein in L1210 cells. There is an intensely stained band migrating at approx. 100 kDa (cf. human platelets). The two weaker bands at lower molecular masses are due to low-affinity reactions with very abundant proteins. It is thus likely that L1210 cells contain a mouse equivalent of $GAP1^{IP4BP}$ and hence that L-Ins(1,3,4,5)P_4 will be able to displace D-Ins(1,3,4,5)P_4 from its specific binding sites. Figure 5 shows the effect of 40 μM L-Ins(1,3,4,5)P_4 on Ca^{2+} release from permeabilized L1210 cells. Under these conditions, 40 μM L-Ins(1,3,4,5)P_4 is capable of mimicking the effect of 2 μM D-Ins(1,3,4,5)P_4 in enhancing the action of Ins(2,4,5)P_3, consistent with the approx. 10-fold higher K_d found for the L-isomer compared with D-Ins(1,3,4,5)P_4 [4,19].

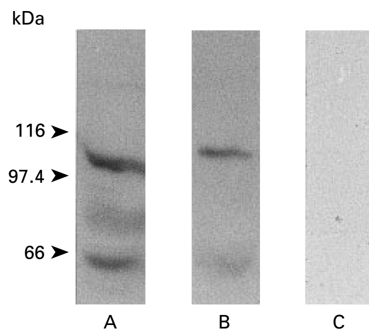

Figure 4 Identification of $GAP1^{IP4BP}$ in L1210 cells

Samples (20 μg) of whole-cell lysates of human platelets (lane B) and mouse L1210 cells (lanes A and C) were analysed on SDS/PAGE, transferrred to PVDF membranes and probed with either the polyclonal rabbit anti-$GAP1^{IP4BP}$ antibody (lanes A and B) or with rabbit pre-immune serum (lane C). Numbers on the left indicate the positions of the molecular-mass markers (kDa). Further details were as described in the Experimental section.

Like the D-isomer, it also shows an order of addition effect; while it enhances the action of Ins(2,4,5)P_3 if added beforehand, it has no effect (other than a small addition artefact) if added after InsP_3.

As indicated in Figures 1 and 5, for both D- and L-Ins(1,3,4,5)P_4, the enhancement of Ins(2,4,5)P_3-induced Ca^{2+} release is due to a substantial increase in the size of the slow phase of Ca^{2+} release. It should be noted that on this time scale and with this mixing protocol it is not possible to comment on the rate of the fast phase, only that its size is not changed by the presence of InsP_4. However, increasing concentrations of Ins(2,4,5)P_3 increase the magnitude of both fast and slow phases of Ca^{2+} release (J. Loomis-Husselbee, unpublished work). If InsP_4 was having its effect by acting as an agonist directly on the InsP_3 receptor or by conversion into Ins(1,4,5)P_3 it would be expected to cause a similar kinetic pattern to an increase in InsP_3 concentration. Figure 6 shows the results of an experiment to test this. The traces compare the time course of Ca^{2+} release caused by 0.5 μM Ins(2,4,5)P_3 alone, 0.5 μM Ins(2,4,5)P_3 in the presence of 2 μM InsP_4 and a concentration of Ins(2,4,5)P_3 (0.57 μM) which has been chosen to give the same overall magnitude of Ca^{2+} release as that caused by 0.5 μM Ins(2,4,5)P_4 in the presence of Ins(1,3,4,5)P_4. It is clear that the time courses are different. Increasing the concentration of Ins(2,4,5)P_3 increases the extent of both fast and slow phases, while as shown in the previous Figures, Ins(1,3,4,5)P_4 addition alters the extent of the slow phase alone. It also appears that the rate constant for the slow phase of Ca^{2+} release has been changed by the presence of Ins(1,3,4,5)P_4. The slow phase is well fitted by a single exponential, allowing determination of the first-order rate constant. In the particular example shown, the rate constant for the slow phase at 0.5 μM Ins(2,4,5)P_3 in the absence of Ins(1,3,4,5)P_4 is $0.14 \pm 0.003\ s^{-1}$ while in the presence of Ins(1,3,4,5)P_4 this rate constant is decreased to $0.074 \pm 0.0006\ s^{-1}$. At 0.57 μM Ins(2,4,5)P_3, the rate constant for the slow phase is $0.135 \pm 0.002\ s^{-1}$. The results of a more detailed analysis of the various kinetic parameters are shown in Table 1. It should be

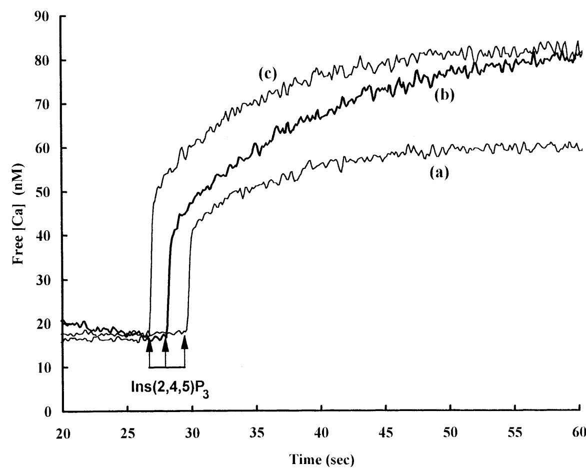

Figure 6 Different time courses of Ca^{2+} release caused by Ins(2,4,5)P_3 in the absence and presence of Ins(1,3,4,5)P_4

Conditions were similar to those described for Figure 1. For clarity traces have been separated by time (2.5 s). For trace (**a**) 0.5 μM Ins(2,4,5)P_3 was added at the arrow. Trace (**b**) was similar to (**a**) except that 2 μM Ins(1,3,4,5)P_4 was added 20 s before Ins(2,4,5)P_3. For trace (**c**) the concentration of Ins(2,4,5)P_3 was increased to 0.57 μM, to give the same overall Ca^{2+} release as that found for trace (**b**).

noted that because there is some variation in absolute values of rate constants and extents between different batches of cells, the values in the presence of Ins(1,3,4,5)P_4 are expressed as a percentage of the paired values in the absence of Ins(1,3,4,5)P_4 for a series of different batches of cells. However, in agreement with the recent results on purified InsP_3 receptors in vesicles [28], we found that the absolute value of the rate constant of the slow phase did not increase significantly with InsP_3 concentration [$0.11 \pm 0.04\ s^{-1}$ ($n = 6$) at 0.5 μM Ins(2,4,5)P_3, $0.085 \pm 0.015\ s^{-1}$ ($n = 10$) at 1.0 μM Ins(2,4,5)P_3] although the extent of the slow phase was changed very substantially (approx. 4-fold) over this concentration range. In agreement with the results shown in Figure 6, the combined results from a series of batches of cells show that Ins(1,3,4,5)P_4, as well as increasing the size, consistently decreased the rate constant for the slow phase of Ca^{2+} release (Table 1). Although the effect is most marked at low concentrations of Ins(2,4,5)P_3, this decrease in rate constant, unlike the effect on size, is observed even at supramaximal Ins(2,4,5)P_3 concentrations [e.g. the data for 8 μM Ins(2,4,5)P_3 in Table 1].

The kinetic properties of Ca^{2+} release by the InsP_3 receptor are very complex and transitions from fast to slow phases of release may involve positive and negative feedback of released Ca^{2+} [29] and/or slow channel inactivation phenomena [30]. The rate constant for the slow phase of Ca^{2+} release is likely to be made up of a series of individual rate constants reflecting (among other possible components) the intrinsic rate of Ca^{2+} release, the rate of channel inactivation and the rate of Ca^{2+} re-uptake into InsP_3-insensitive stores. The decrease in rate constant and increase in extent of release caused by InsP_4 could be due to an effect on any one of these processes, although it seems unlikely that it could be due to InsP_4acting directly as a weak agonist on the InsP_3 receptor since an increase in InsP_3 concentration does not cause a decrease in rate constant for Ca^{2+} release. Despite this, it remains possible that, if Ins(1,3,4,5)P_4 were to act as a weak (subthreshold) InsP_3 agonist, or were to generate a subthreshold steady-state Ins(1,4,5)P_3 concentration, Ins(1,3,4,5)P_4 pretreatment would lead to subtle kinetic changes of the sort shown in Figure 6 and Table 1. However, the other lines of evidence presented in this paper [the failure of thimerosal to shift the dose–response curve for D-Ins(1,3,4,5)P_4 and the ability of L-Ins(1,3,4,5)P_4 to mimic the effects of D-Ins(1,3,4,5)P_4] do not support the idea of Ins(1,3,4,5)P_4 acting directly on the InsP_3 receptor but instead indicate that Ins(1,3,4,5)P_4 is acting indirectly via its own binding sites. The simplest hypothesis is therefore that the effects on the kinetics of release are due to interactions at the same, specific Ins(1,3,4,5)P_4-binding sites. As previously described, the synergy between InsP_3 and InsP_4 is most readily observed at low concentrations of InsP_3. This may be simply because an increase in the extent of the slow phase of Ca^{2+} release, such as is found at low InsP_3 concentrations (see above), is the most easily detected difference. On the other hand it may be that only a subpopulation of InsP_3 receptors is sensitive to modulation by Ins(1,3,4,5)P_4 (discussed in [1]). Our recent finding that the specific Ins(1,3,4,5)P_4-binding protein of platelets ($GAP1^{IP4BP}$) has GAP activity towards Ras and Rap, coupled with the demonstration here of an immunologically similar protein in L1210 cells, raises intriguing questions, which we are currently starting to address, about the mechanisms by which Ins(1,3,4,5)P_4 might exert its effects on Ca^{2+} movements.

The reasons for the substantial differences between our findings and those of Wilcox et al. [19,20] who did not, in their recent experiments, find any synergy between InsP_3 and InsP_4, are not clear at present. Synergistic effects of InsP_4 on InsP_3-stimulated Ca^{2+} release where InsP_4 is binding at its own receptor, separate from the InsP_3 receptor, would be expected to depend upon a high level of structural organization. Such organization might be easily lost during cell harvesting and solubilization, and in this respect suspension cultured L1210 cells might be considerably more robust than cells, like SH-SY5Y, which have to be harvested from monolayers. However, there are other, possibly substantial, differences in experimental procedures and systems which may also make a contribution. Wilcox et al. [19,20] show a direct action of Ins(1,3,4,5)P_4 causing Ca^{2+} release via InsP_3 receptors. Such an effect of Ins(1,3,4,5)P_4 has also been observed in various microsomal preparations and in *Xenopus* oocytes [30a,31,32]. At concentrations of InsP_4 (1–10 μM) which should cause Ca^{2+} mobilization given the concentration-dependence found for SH-SY5Y cells [19], we see no Ca^{2+} release by InsP_4, even after the addition of Ins(2,4,5)P_3 (Figure 1) or after thimerosal treatment which should decrease the concentration of InsP_4 needed to mobilize Ca^{2+} by a factor of four. Perhaps the most likely explanation is in the type of InsP_3 receptors present in the different cell types. It seems likely that SH-SY5Y cells will express neuronal-type receptors, while L1210 lymphoma cells will not. Environment of the receptor, Ca^{2+} loading and association of the receptor with other proteins are all further possible sources of variation. Clearly direct effects of InsP_4 on the InsP_3 receptor would be a considerable complication in attempts to unravel possible interactions between InsP_4 receptors and InsP_3 receptors and in this respect L1210 cells, with their lack of Ca^{2+} mobilization by InsP_4 alone, seem to be a useful experimental system.

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