Modulation of $\text{Ins}(2,4,5)\text{P}_3$ -stimulated Ca^{2+} mobilization by $\text{Ins}(1,3,4,5)\text{P}_4$: enhancement by activated G-proteins, and evidence for the involvement of a GAP1 protein, a putative $\text{Ins}(1,3,4,5)\text{P}_4$ receptor

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We have previously shown that addition of $\text{Ins}(1,3,4,5)\text{P}_4$ to permeabilized L1210 cells increases the amount of Ca^{2+} mobilized by a submaximal concentration of $\text{Ins}(2,4,5)\text{P}_3$, and we suggested that, in doing this, $\text{Ins}(1,3,4,5)\text{P}_4$ is not working via an InsP_3 receptor but indirectly via an InsP_4 receptor [Loomis-Husselbee, Cullen, Dreikhausen, Irvine and Dawson (1996) *Biochem. J.* **314**, 811–816]. Here we have investigated whether this effect might be mediated by $\text{GAP1}^{\text{IP4BP}}$, recently identified as a putative receptor for $\text{Ins}(1,3,4,5)\text{P}_4$. $\text{GAP1}^{\text{IP4BP}}$ is a protein that interacts with one or more monomeric G-proteins, so we sought evidence for involvement of monomeric G-proteins in the effects of $\text{Ins}(1,3,4,5)\text{P}_4$ in permeabilized L1210 cells. Guanosine 5'-[γ -thio]triphosphate (GTP[S]) enhanced the effect of $\text{Ins}(1,3,4,5)\text{P}_4$ on $\text{Ins}(2,4,5)\text{P}_3$ -stimulated Ca^{2+} mobilization, but had no effect

on the action of $\text{Ins}(2,4,5)\text{P}_3$ alone. A specific enhancement of only the action of $\text{Ins}(1,3,4,5)\text{P}_4$ was also seen with GTP[S]-loaded R-Ras or Rap1a (two G-proteins known to interact with $\text{GAP1}^{\text{IP4BP}}$), whereas H-Ras was inactive at similar concentrations. Guanosine 5'-[β -thio]diphosphate (GDP[S]) did not alter the action of either $\text{Ins}(2,4,5)\text{P}_3$ or $\text{Ins}(1,3,4,5)\text{P}_4$. Finally, the addition of exogenous $\text{GAP1}^{\text{IP4BP}}$, purified from platelets, markedly enhanced the effect of $\text{Ins}(1,3,4,5)\text{P}_4$, and again, the amount of Ca^{2+} mobilized by $\text{Ins}(2,4,5)\text{P}_3$ alone was unaltered. We conclude that the increase in $\text{Ins}(2,4,5)\text{P}_3$ -stimulated Ca^{2+} mobilization by $\text{Ins}(1,3,4,5)\text{P}_4$ may be mediated by $\text{GAP1}^{\text{IP4BP}}$ or a closely related protein (such as GAP1^{m}), and if so, the action of the GAP1 is not solely to regulate GTP loading of a G-protein, but rather it acts with a G-protein to cause its effect.

INTRODUCTION

Despite many studies on the possible role of inositol 1,3,4,5-tetrakisphosphate [$\text{Ins}(1,3,4,5)\text{P}_4$] in cellular physiology, its function remains unclear and, indeed, there is still debate over whether it has a function at all (see e.g. [1] and [2] for reviews). As the only known route of synthesis of $\text{Ins}(1,3,4,5)\text{P}_4$ *de novo* is from $\text{Ins}(1,4,5)\text{P}_3$ [3], it would seem probable that were $\text{Ins}(1,3,4,5)\text{P}_4$ to have a physiological function, it may be closely related to that of $\text{Ins}(1,4,5)\text{P}_3$, i.e. it would be involved either with the regulation or the effects of intracellular Ca^{2+} . Recently, we have made two advances that significantly advance our knowledge of the proposed second-messenger function of $\text{Ins}(1,3,4,5)\text{P}_4$.

First, we have studied the effect of $\text{Ins}(1,3,4,5)\text{P}_4$ on intracellular Ca^{2+} mobilization in permeabilized L1210 cells, where $\text{Ins}(1,3,4,5)\text{P}_4$ alone does not mobilize Ca^{2+} , but increases the amount of Ca^{2+} mobilized by a suboptimal concentration of $\text{Ins}(2,4,5)\text{P}_3$ [4,5]. We have shown that the effect of $\text{Ins}(1,3,4,5)\text{P}_4$ specifically increases the magnitude and decreases the rate constant of the slow phase of $\text{Ins}(2,4,5)\text{P}_3$ -stimulated Ca^{2+} release, while leaving the magnitude of the fast phase unaltered [5]. Moreover, the effect of $\text{Ins}(1,3,4,5)\text{P}_4$ may not be attributed to increasing $\text{Ins}(1,4,5)\text{P}_3$ by conversion of added $\text{Ins}(1,3,4,5)\text{P}_4$ into $\text{Ins}(1,4,5)\text{P}_3$ by the 3-phosphatase, or to added $\text{Ins}(1,3,4,5)\text{P}_4$ and $\text{Ins}(2,4,5)\text{P}_3$ protecting endogenous $\text{Ins}(1,4,5)\text{P}_3$ against hydrolysis, or to micromolar concentrations of $\text{Ins}(1,3,4,5)\text{P}_4$ acting as a weak (or partial) agonist on $\text{Ins}(1,4,5)\text{P}_3$ receptors [5]. These studies demonstrate that, in permeabilized L1210 cells,

$\text{Ins}(1,3,4,5)\text{P}_4$ is interacting primarily with a specific $\text{Ins}(1,3,4,5)\text{P}_4$ -binding protein, which is in turn exerting either a direct or an indirect effect on the action of $\text{Ins}(2,4,5)\text{P}_3$.

Secondly, we have purified from porcine platelets, and then cloned from a human circulating blood cDNA library, a highly specific $\text{Ins}(1,3,4,5)\text{P}_4$ -binding protein which, as indicated by its amino acid sequence to be a member of the GAP1 family, we have called $\text{GAP1}^{\text{IP4BP}}$ [6]. While this work was in progress, Maekawa et al. [7] cloned a closely related protein, GAP1^{m} , which may also be considered as a putative $\text{Ins}(1,3,4,5)\text{P}_4$ receptor [8].

A key question that emerges from these two independent advances is whether they are related. More precisely, are the effects of $\text{Ins}(1,3,4,5)\text{P}_4$ in L1210 cells mediated by $\text{GAP1}^{\text{IP4BP}}$ (or its close relative, GAP1^{m})? The experiments described here represent an attempt to answer this question, and also to gain some insight into the functional inter-relationship between both exogenous and endogenous G-proteins, and the action of $\text{Ins}(1,3,4,5)\text{P}_4$.

EXPERIMENTAL

Materials and methods

Materials and experimental procedures of cell growth, Ca^{2+} uptake and measurement of Ca^{2+} mobilization induced by inositol phosphates were essentially as described by Loomis-Husselbee et al. [5]. Digitonin was added at a concentration of 20 μg (in 2 μl of DMSO) per ml of suspended cells at a density of

Abbreviations used: GTP[S], guanosine 5'-[γ -thio]triphosphate; GDP[S], guanosine 5'-[β -thio]diphosphate; DTT, dithiothreitol; G38V and R12V, mutant proteins bearing either a Gly³⁸ → Val³⁸ or an Arg¹² → Val¹² replacement respectively; wt, wild-type; GST, glutathione S-transferase; Gpp[NH]p, guanosine 5'-[β , γ -imido]triphosphate.

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1.0×10^7 cell/ml in the assay medium. The assay medium consisted of 100 mM KCl/20 mM Hepes/KOH (pH 7.0)/5 mM succinate (K^+ salt)/50 μ g/ml creatine kinase/10 mM phosphocreatine/1 mM $MgCl_2$ /0.7 mM ATP/1 μ M Fluo3 (penta-ammonium salt) at 30 °C.

Preparation and purification of Ras subfamily proteins

Gly³⁸ $\rightarrow$ Val³⁸ (G38V) R-Ras

Constitutively active oncogenic mutant G38V R-Ras [25,26] expressed from a T7 expression system in *Escherichia coli* [27] was purified by a modified procedure described by Rey et al. [25]. Cells (from a 4 litre culture) were resuspended in Buffer A (50 mM Tris/HCl (pH 8.0)/10 mM NaCl/5 mM $MgCl_2$ /5 mM dithiothreitol (DTT)/1 mM PMSF) and lysed by a 3 $\times$ freeze-thaw process (solid CO_2 /ethanol bath, 37 °C water bath), then sonicated on ice 10 times for 10 s bursts over 10 min. Cell debris was pelleted by centrifugation (2745 g, 10 min) and nucleic acids were precipitated by slow addition of polyethylenimine in Buffer A [final concentration of 1% (v/v)]. The precipitated nucleic acids were removed by centrifugation (12000 g, 10 min) and the supernatant was applied to a Q-Sepharose column (50 ml, 4 °C, 1 ml/min, Pharmacia) and washed with 100 ml of Buffer A. The protein was eluted from the column with an NaCl gradient (10–460 mM NaCl) in Buffer A. [³H]GTP-binding assays were carried out on the eluted fractions (see below). The pooled R-Ras-containing fractions were concentrated by ultrafiltration using an Amicon Ultrafiltration cell (Model 8003, 5 ml) and R-Ras was precipitated by ammonium sulphate (30%–60% cut). The precipitate was resuspended in Buffer B [50 mM Tris/HCl (pH 7.6)/100 mM NaCl/5 mM $MgCl_2$ /5 mM DTT/1 mM PMSF] and dialysed against the same buffer (4 °C). The protein was then applied to a G75 Sephadex column (Sigma) and eluted with Buffer B. [³H]GTP-binding assays were carried out on the eluted fractions. The pool of R-Ras fractions was concentrated by ultrafiltration (as above) and dialysed against Buffer FD [50 mM Tris/HCl (pH 7.6)/100 mM NaCl/0.1 mM EDTA/1 mM DTT], where Buffer FD had been pretreated with Chelex 100 (Sigma) to reduce contaminating Ca^{2+} ($Ca^{2+}_{free} < 10$ nM). The purified protein concentration (95%, as estimated by SDS/PAGE) was adjusted to 0.4 mg/ml with buffer FD, and stored at –20 °C.

Arg¹² $\rightarrow$ Val¹² (R12V) H-Ras and wild-type (wt) Rap1a

The pGEX-2T expression vectors for R12V H-Ras and wt Rap1a proteins were generously provided by Professor A. Hall and Dr. J. Bos respectively. Constitutively active mutant R12V H-Ras and wt Rap1a were expressed as glutathione S-transferase (GST)-H-Ras and GST-Rap1a fusion proteins in *E. coli*, and purified using GST 4B (Sigma) using a method essentially as described by the manufacturer (Sigma Protocols). Fusion proteins were cleaved from glutathione-agarose beads at the thrombin-cleavage site between the GST-tag and the monomeric G-protein by bovine thrombin (Sigma; 1 unit/0.1 ml). Contaminating proteins, including thrombin, were separated from fusion protein by loading on a Q-sepharose column (Pharmacia, 10 ml) prewashed with 50 mM Tris/HCl (pH 8.0)/10 mM NaCl/5 mM $MgCl_2$ /1 mM DTT. The column was washed with 4 column vol. of the same buffer. Proteins were eluted by a 10–500 mM NaCl gradient. Identification of fractions containing fusion protein was carried out by correlating the amount of specific [³H]GTP binding with the qualitative amount of protein, of the correct molecular mass, as resolved by SDS/PAGE. Fractions containing fusion protein with the greatest level of specific [³H]GTP binding were pooled and concentrated by

ultrafiltration (as above), and dialysed against Buffer C [50 mM Tris/HCl (pH 7.6)/100 mM NaCl/1 mM DTT]. To reduce contaminating Ca^{2+} , Buffer C had been pretreated with Chelex 100 (Sigma). Protein purity was estimated to be > 95% by SDS/PAGE.

Guanine-nucleotide-binding assays

GTP-binding assays were as described by Porfiri et al. [29]. Aliquots (5 μ l or 2 μ l) from eluted fractions were labelled in 20 mM Tris/HCl (pH 7.5)/50 mM NaCl/5 mM $MgCl_2$ /1 mM DTT/10 mM EDTA/1% (w/v) BSA/0.05% (w/v) sodium cholate with 1 mM [³H]GTP (31.5 Ci/mmol; Amersham) in a 40 μ l assay vol. Following a 10 min incubation at room temperature, the loading was stopped by adding 10 mM $MgCl_2$. After a further 5 min, 1 ml of ice-cold 20 mM Tris/HCl (pH 7.5)/50 mM NaCl/5 mM $MgCl_2$ was added to each tube, and filtered through a nitrocellulose filter (pore size, 45 μ m). Filters were washed with 10 ml of ice-cold 20 mM Tris/HCl (pH 7.5)/50 mM NaCl/5 mM $MgCl_2$, dried and counted in a scintillation counter to determine the position of monomeric G-protein elution from the column.

GTP-analogue loading of monomeric G-proteins

Monomeric G-proteins (G38V R-Ras, R12V H-Ras and wt Rap1a) were loaded with one of two non-hydrolysable GTP analogues [guanosine 5'-[γ -thio]triphosphate (GTP[S]) or guanosine 5'-[β , γ -imido]triphosphate (Gpp[NH]pp)], as specified in the Figure legends. The required G-protein was loaded with GTP analogue by incubation at an equal concn. for 10 min at room temperature, and followed by an addition of 1 mM $MgCl_2$. Samples were loaded fresh each day, stored at 4 °C and used for experimentation within 2 h.

Preparation of platelet membranes and purification of GAP1^{IP4BP}

GAP1^{IP4BP} was purified from porcine platelet membranes, prepared exactly as described by Cullen et al. [14]. Purified GAP1^{IP4BP} was dialysed overnight against 100 vol. of final dialysis buffer [25 mM Tris/HCl (pH 7.2)/1 mM EGTA/1 mM EDTA/1 mM 2-mercaptoethanol/1 mM PMSF/0.25% (w/v) CHAPS], and diluted to give a concentration of 0.5 mg/ml in 50% (v/v) glycerol and final dialysis buffer. The final dialysis buffer was pretreated with Chelex 100 (Sigma) to reduce contaminating Ca^{2+} ($Ca^{2+}_{free} < 10$ nM).

RESULTS AND DISCUSSION

The experimental system used has been described previously [4,5,9]. A key feature of the protocol is that ATP-dependent Ca^{2+} uptake into the permeabilized cells takes place in a stock suspension, from which aliquots are removed for investigating the effects of inositol phosphates. This enables controls to be inserted between experimental assays so as to ensure a highly reproducible set of data within a given batch of cells. There is some variability between the size of the effect of $Ins(2,4,5)P_3$ in different batches of cells, and also variability in the effect of $Ins(1,3,4,5)P_4$, but within a given batch, even a small change in the Ca^{2+} release generated by $Ins(2,4,5)P_3$ in the presence or absence of $Ins(1,3,4,5)P_4$ is easily quantifiable.

The aim of our experiments was to identify any possible change in the magnitude of the action of $Ins(1,3,4,5)P_4$ caused by various additions (e.g. GAP1^{IP4BP}, GTP analogues, etc.). Thus for all the experiments reported here, we have altered the assay conditions from our previous study by reducing the number of

Figure 1 Enhancement of Ins(2,4,5) P_3 -stimulated Ca^{2+} release by Ins(1,3,4,5) P_4 in the presence and absence of thapsigargin

Assay conditions were as described in the Experimental section. Each panel shows three superimposed Ca^{2+} -efflux traces. In (A), trace (a) shows the absence of any Ca^{2+} release when 3 μ M Ins(1,3,4,5) P_4 was added at the arrow (1) with no subsequent addition of Ins(2,4,5) P_3 . Trace (b) shows Ca^{2+} release stimulated by 1 μ M Ins(2,4,5) P_3 alone, and trace (c) shows the enhancement of the extent of Ca^{2+} release by 1 μ M Ins(2,4,5) P_3 following a 30 s preincubation with 3 μ M Ins(1,3,4,5) P_4 . (B) shows that the Ins(1,3,4,5) P_4 response is retained in thapsigargin-treated permeabilized L1210 cells. Assay conditions were as described for (A), except thapsigargin (TG; 300 nM) was added 10 s before the addition of 1 μ M Ins(2,4,5) P_3 , as indicated by the arrow. Experiments were carried out in triplicate at 30 °C.

cells per assay so that the endogenous Ins(1,3,4,5) P_4 effect was minimized [5].

Kinetics of Ca^{2+} release

We have shown previously that the effect of Ins(1,3,4,5) P_4 in permeabilized L1210 cells has a very recognizable feature: of the two phases of Ca^{2+} release generated by Ins(2,4,5) P_3 (see e.g. [10,11]), the effect of Ins(1,3,4,5) P_4 is apparent only on the slow phase [5]. It is detectable at all Ins(2,4,5) P_3 concentrations, but is most easily studied at low levels of Ins(2,4,5) P_3 , where it results in a larger overall release of Ca^{2+} (see [5] for further discussion). Strictly speaking, we have not proved that the effect is on Ca^{2+} release by Ins(2,4,5) P_3 rather than on Ca^{2+} pumping by the endoplasmic-reticulum Ca^{2+} -ATPase. It is conceivable that a decrease in the apparent rate constant for Ca^{2+} release could be due to an indirect effect of Ins(1,3,4,5) P_4 on the Ins P_3 receptor, or an inhibition of Ca^{2+} re-uptake. To address this issue directly, we studied the effect of Ins(1,3,4,5) P_4 on Ca^{2+} release in the presence of a maximal concentration of thapsigargin to block any Ca^{2+} re-uptake. Figure 1 shows that Ins(1,3,4,5) P_4 in itself has no effect on the leak rate of Ca^{2+} from intracellular Ca^{2+} stores in the presence of thapsigargin. However, under these conditions, the effect of Ins(1,3,4,5) P_4 in enhancing the release of Ca^{2+} induced by Ins(2,4,5) P_3 is retained. Thus the modulation of Ins(2,4,5) P_3 -stimulated Ca^{2+} release is on the release process, and not on the Ca^{2+} pumps. Note that quantification of the Ins(1,3,4,5) P_4 response is more difficult with thapsigargin present (because of the large extent and fast rate of Ca^{2+} release), so all the experiments reported below were conducted in its absence, exactly as described by Loomis-Husselbee et al. [5].

Guanine nucleotides

As the primary sequence of GAP1^{IP4BP} shows that it is likely to interact with a monomeric G-protein [6,12], we first investigated

whether the addition of guanine nucleotides had any effect on Ca^{2+} mobilization in permeabilized L1210 cells. We added guanosine 5'-[β -thio]diphosphate (GDP[S]) at 100 μ M to several batches of cells and tried three separate batches of GDP[S], but were never able to detect any effect on Ca^{2+} mobilization by Ins(2,4,5) P_3 , with or without Ins(1,3,4,5) P_4 (results not shown). To investigate the effect of activating G-proteins, we added either GTP[S] (100 μ M) or Gpp[NH]p (100 μ M), and these two compounds gave indistinguishable results. The effect of the GTP analogues was specific (Figure 2); they had no detectable effect on the kinetics of Ca^{2+} release caused by Ins(2,4,5) P_3 alone [i.e. they did not mimic the effect of Ins(1,3,4,5) P_4], but markedly enhanced the effect of Ins(1,3,4,5) P_4 on Ins(2,4,5) P_3 -stimulated Ca^{2+} release. It should be noted that, as with the control effect of Ins(1,3,4,5) P_4 [5], in the presence of GTP analogues addition of Ins(1,3,4,5) P_4 after Ins(2,4,5) P_3 had no apparent effect on Ins(2,4,5) P_3 to release more Ca^{2+} . Thus the guanine-nucleotide-enhanced action of Ins(1,3,4,5) P_4 is probably an enhancement of the same Ins(1,3,4,5) P_4 -induced mechanism [5], rather than a separate effect that is only seen with GTP[S] (or Gpp[NH]p) plus Ins(1,3,4,5) P_4 present.

These data imply that a GTP-binding protein is interacting with the protein transducing the Ins(1,3,4,5) P_4 signal (see also below). The lack of effect of GDP[S] could be interpreted as suggesting that the G-protein is not essential for the action of Ins(1,3,4,5) P_4 . However, we cannot be sure that all GTP-loaded G-proteins will have exchanged with GDP[S] during the preincubation; an interaction between a GTP-loaded G-protein and its effector can stabilize the G-protein in its GTP-bound conformation (for example, see [30]). Therefore a GTP-loaded G-protein could still be an essential component in the mechanism of Ins(1,3,4,5) P_4 action, despite the lack of effect of GDP[S]. The observation that GTP[S] can increase the effect of Ins(1,3,4,5) P_4 implies that if a GAP1 protein is involved, its action cannot be solely to inactivate a G-protein by stimulating its GTPase activity, but rather it may combine with a GTP-loaded G-protein to exert

Figure 2 Effect of GTP[S] pretreatment on Ins(1,3,4,5) P_4 -induced potentiation of Ins(2,4,5) P_3 -dependent Ca^{2+} release

Conditions were essentially as described in Figure 1(A). Each panel shows two superimposed Ca^{2+} -efflux traces: (a) 1 μ M Ins(2,4,5) P_3 , and (b) 1 μ M Ins(2,4,5) P_3 following a 30 s incubation with 3 μ M Ins(1,3,4,5) P_4 . (A) The control responses to 1 μ M Ins(2,4,5) P_3 , both with and without Ins(1,3,4,5) P_4 pretreatment, for that particular batch of cells. (B) GTP[S]-induced enhancement of the action of Ins(1,3,4,5) P_4 . Assay conditions for (B) were as described for (A), except a 3 min incubation with 100 μ M GTP[S] before the addition of Ins(2,4,5) P_3 was included. Experiments were carried out in triplicate at 30 °C.

the effect of Ins(1,3,4,5) P_4 . Furthermore, the observation that GTP[S] only enhances, and does not mimic, the effect of Ins(1,3,4,5) P_4 suggests that the participation of the G-protein in its GTP-bound conformation is not in itself sufficient, but that the GAP1 protein with Ins(1,3,4,5) P_4 bound to it is also required for the effect on Ca^{2+} mobilization.

Monomeric G-proteins

We have shown that GAP1^{IP4BP} can both interact *in vitro* with and stimulate the GTPase activity of several members of the Ras subfamily [6]. These include R-Ras and, indeed, Yamamoto et al. [13] independently purified and cloned GAP1^{IP4BP} from bovine brain (and called it R-RasGAP, on the basis of its ability to interact with R-Ras; in their hands, it favoured R-Ras over H-Ras). One surprising observation in this context is that we found that GAP1^{IP4BP} was also able to interact with Rap1a, suggesting some difference in specificity between GAP1^{IP4BP} and e.g. p120-rasGAP [6]. None of these experiments *in vitro* gives us an unequivocal answer to the question of which monomeric G-protein(s) GAP1^{IP4BP} interact(s) with *in vivo*, but they do suggest that it might be informative to investigate the effect of some of these members of the Ras subfamily in their GTP-bound conformation (GTP[S]- or Gpp[NH]p-loaded) in permeabilized L1210 cells.

Because these experiments are taking place in final volumes of 1 ml, a full series of tests investigating any G-protein requires large amounts of recombinant protein, so we confined our investigations to only three monomeric G-proteins, and chose R-Ras, Rap1a and H-Ras for the reasons outlined in the preceding paragraph. Figures 3(A) and 3(B) show representative traces (for R-Ras) and Figure 3(C) pooled data for R-Ras and H-Ras. At 20 pmol/ml, R-Ras loaded with GTP analogues enhanced threefold the effect of Ins(1,3,4,5) P_4 . By itself, Ins(1,3,4,5) P_4 increased the amount of Ca^{2+} released by Ins(2,4,5) P_3 by $33 \pm 6\%$ ($n = 24$; $P < 0.001$), i.e. from 100% to 133% (Figure 3C). However, following preincubation with R-Ras loaded with GTP

analogue (either GTP[S] or Gpp[NH]p) for 3 min, Ins(1,3,4,5) P_4 increased the Ca^{2+} release by Ins(2,4,5) P_3 by $106 \pm 12\%$ ($n = 5$; $P < 0.001$), i.e. from 100% to 206%. It is important to note that in the absence of Ins(1,3,4,5) P_4 , R-Ras loaded with GTP analogues had no effect on the action of Ins(2,4,5) P_3 . At 20 pmol/ml, Rap1a loaded with GTP analogues also enhanced the action of Ins(1,3,4,5) P_4 twofold from 33% to 65% [i.e. the extent of Ca^{2+} released by Ins(2,4,5) P_3 following incubation with both Rap1a loaded with GTP analogues (3 min) and Ins(1,3,4,5) P_4 (30 s) was $165 \pm 11\%$ ($n = 3$; $P < 0.05$) of that released by Ins(2,4,5) P_3 alone]. Unloaded or GDP β S-loaded R-Ras and Rap1a had no effect on the action of either Ins(2,4,5) P_3 or Ins(1,3,4,5) P_4 (results not shown). Furthermore, H-Ras loaded with GTP analogues (20 pmol/ml) had no effect on the action of Ins(1,3,4,5) P_4 or Ins(2,4,5) P_3 (Figure 3C), consistent with the preference *in vitro* of GAP1^{IP4BP} for R-Ras reported by Yamamoto et al. [13].

Taken together, these data corroborate those found in Figure 2 and strengthen our interpretation of that Figure, i.e. that a monomeric G-protein of the Ras subfamily is indeed mediating the effect of the GTP analogues. Moreover, although the full gamut of the Ras family has not been tested (either here or on purified GAP1^{IP4BP}) and Figure 3(C) is, at best, a semi-quantitative pharmacological study, the relative efficacy of the three monomeric G-proteins employed is consistent with the known properties of GAP1^{IP4BP} [6,13].

Exogenous GAP1^{IP4BP}

The effects of guanine nucleotides and monomeric G-proteins provided some information about some of the possible interactions in the transduction pathways of Ins(1,3,4,5) P_4 in this system (see above). However, when considered in the context of evidence that GAP1^{IP4BP} (or a closely related protein) is involved, the data are indirect and circumstantial. Knocking the protein out (e.g. by antisense) is a strategy to be pursued in the future, and as we have, as yet, no inhibitory antibodies, ablating

Figure 3 Effect of R-Ras, Rap1a and H-Ras on Ins(1,3,4,5) P_4 -induced potentiation of Ins(2,4,5) P_3 -dependent Ca^{2+} release

Conditions were as described in Figure 2(A). Each panel shows two superimposed Ca^{2+} -efflux traces: (a) 1 μ M Ins(2,4,5) P_3 alone; (b) 1 μ M Ins(2,4,5) P_3 following a 30 s incubation with 3 μ M Ins(1,3,4,5) P_4 . (A) Control responses to Ins(2,4,5) P_3 , both with and without Ins(1,3,4,5) P_4 pretreatment, for that particular batch of cells. (B) R-Ras (20 pmol/ml) loaded with GTP[S] enhances the action of Ins(1,3,4,5) P_4 . Assay conditions were as described for (A), except a 3 min incubation with GTP[S]-loaded R-Ras before the addition of 1 μ M Ins(2,4,5) P_3 was included. Monomeric G-protein loaded with p[NH]ppG gave indistinguishable results. (C) is the accumulated data from at least 5 experiments, of which (A) and (B) are examples, plotted as axis: ordinate, % Ins(2,4,5) P_3 -stimulated Ca^{2+} release in the presence of 3 μ M Ins(1,3,4,5) P_4 , where submaximal Ins(2,4,5) P_3 -stimulated Ca^{2+} release by 1 μ M Ins(2,4,5) P_3 alone was designated as 100%; abscissa, pmol Ras/ml for R-Ras (●) or H-Ras (□), incubated for 3 min before the addition of 1 μ M Ins(2,4,5) P_3 . Values are plotted as the means $\pm$ S.E.M. The data are pooled from three different R-Ras and H-Ras preparations.

Figure 4 Effect of exogenous GAP1^{IP4BP} on Ins(1,3,4,5) P_4 -induced potentiation of Ins(2,4,5) P_3 -dependent Ca^{2+} release

Conditions were essentially as described in Figure 1(A). Each panel shows two superimposed Ca^{2+} -efflux traces: (a) 1 μ M Ins(2,4,5) P_3 alone; and (b) 1 μ M Ins(2,4,5) P_3 following a 30 s incubation with 3 μ M Ins(1,3,4,5) P_4 . (A) shows control responses to Ins(2,4,5) P_3 , both with and without Ins(1,3,4,5) P_4 pretreatment, for that particular batch of cells. (B) shows that GAP1^{IP4BP} enhances the action of Ins(1,3,4,5) P_4 . Assay conditions were as described for (A), but with a 3 min preincubation with 25 pmol/ml GAP1^{IP4BP}. Experiments were carried out in triplicate on at least two separate purifications of GAP1^{IP4BP}.

GAP1^{IP4BP} in this system is not an immediate option. However, we are able to purify GAP1^{IP4BP} in reasonable quantities from porcine platelets [14], and as we had set up conditions to decrease the magnitude of the endogenous effect of Ins(1,3,4,5) P_4 in L1210 cells (see above), we could investigate the effect of exogenous GAP1^{IP4BP} protein.

The addition of 25 pmol/ml of GAP1^{IP4BP} purified from porcine platelets (see [14] for data on the purification) caused a robust increase in the Ins(1,3,4,5) P_4 response (Figure 4), where the effect of Ins(1,3,4,5) P_4 is on the slow phase of Ins(2,4,5) P_3 -stimulated Ca^{2+} release, and the extent of the fast phase is apparently unaffected [5]. It is crucial to note that only the effect

of $\text{Ins}(1,3,4,5)P_4$ is increased, and the action of $\text{Ins}(2,4,5)P_3$ alone is unchanged. Thus the effect of $\text{Ins}(1,3,4,5)P_4$ in the presence of exogenous $\text{GAP1}^{\text{IP4BP}}$ here is indistinguishable from the endogenous $\text{Ins}(1,3,4,5)P_4$ effect documented in detail in Loomis-Husselbee et al. [5]. Similarly, GTP[S] (or Gpp[NH]p) and guanine-nucleotide-loaded small G-proteins also extend the enhancement of the effect of $\text{Ins}(1,3,4,5)P_4$'s effects on the slow phase of $\text{Ins}(2,4,5)P_3$ -stimulated Ca^{2+} release, without affecting the fast phase of Ca^{2+} release. Thus overall, these observations seem to suggest that these various exogenous agents enhance the endogenous effect of $\text{Ins}(1,3,4,5)P_4$ rather than cause an entirely different and independent effect.

The data presented above strongly suggests that the effects of $\text{Ins}(1,3,4,5)P_4$ on $\text{Ins}(2,4,5)P_3$ -mediated Ca^{2+} mobilization in permeabilized L1210 cells involve $\text{GAP1}^{\text{IP4BP}}$, which has been reported to be a putative $\text{Ins}(1,3,4,5)P_4$ receptor on the basis of its specificity for $\text{Ins}(1,3,4,5)P_4$ binding [14,15]. However, at present, we cannot be sure whether $\text{GAP1}^{\text{IP4BP}}$ or GAP1^{m} (or both) modulates the endogenous $\text{Ins}(1,3,4,5)P_4$ effect, i.e. although different proteins, they show a high level of homology in their primary sequence [6,12,13,16], so it may seem reasonable that, *in vitro*, one could substitute one for the other. Recent evidence [17] suggesting that GAP1^{m} may be in the endoplasmic reticulum, whereas $\text{GAP1}^{\text{IP4BP}}$, as previously inferred [12,18,19], is mostly in the plasma membrane, makes GAP1^{m} arguably the more likely of the two, but this requires further study. Also, whether GAP proteins interact directly or indirectly with $\text{Ins}(1,4,5)P_3$ receptors to modify their kinetic behaviour, or whether their function is to change the availability of Ca^{2+} to the receptor by altering the communication among intracellular Ca^{2+} pools [20,21] (which could also be argued to lead to changes in the kinetics of Ca^{2+} release) remains to be explored. There are other reports of $\text{Ins}(1,3,4,5)P_4$ affecting $\text{Ins}(1,4,5)P_3$ -stimulated Ca^{2+} mobilization [22,23,31]. Finally, while the physiological correlate of this effect of $\text{Ins}(1,3,4,5)P_4$ in L1210 cells remains unknown, it must surely lie in the complexities of Ca^{2+} signal generation [24], and we now have indications of a way forward to investigate this.

These findings present a meeting of two major experimental threads of evidence concerning the possible second-messenger function of $\text{Ins}(1,3,4,5)P_4$: a well-documented [5] modulation by $\text{Ins}(1,3,4,5)P_4$ of the second-messenger action of its precursor, $\text{Ins}(1,4,5)P_3$ [3], with the identification and cloning of a specific $\text{Ins}(1,3,4,5)P_4$ -binding protein [6]. These data give us the first insights into the inter-relationship between $\text{GAP1}^{\text{IP4BP}}$ and G-protein(s) implicated with the action of $\text{Ins}(1,3,4,5)P_4$, and we suggest that they also greatly increase the likelihood that $\text{GAP1}^{\text{IP4BP}}$ and/or GAP1^{m} is indeed a physiological receptor for $\text{Ins}(1,3,4,5)P_4$.

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REFERENCES

- Irvine, R. F. (1992) *Faseb J.* **6**, 3085-3091
- Putney, Jr., J. W. and Bird, G. S. (1993) *Endocrinol. Rev.* **14**, 610-631
- Irvine, R. F., Letcher, A. J., Heslop, J. P. and Berridge, M. J. (1986) *Nature (London)* **320**, 631-634
- Cullen, P. J., Irvine, R. F. and Dawson, A. P. (1990) *Biochem. J.* **271**, 549-553
- Loomis-Husselbee, J. W., Cullen, P. J., Dreikhausen, U. E., Irvine, R. F. and Dawson, A. P. (1996) *Biochem. J.* **314**, 811-816
- Cullen, P. J., Hsuan, J. J., Truong, O., Letcher, A. J., Jackson, T. R., Dawson, A. P. and Irvine, R. F. (1995) *Nature (London)* **376**, 527-530
- Maekawa, M., Li, S., Iwamatsu, A., Morishita, T., Yokata, K., Imai, Y. K. S. and Nakamura, S. (1994) *Mol. Cell Biol.* **14**, 6879-6885
- Fukuda, M. and Mikoshiba, K. (1996) *J. Biol. Chem.* **271**, 18838-18842
- Loomis-Husselbee, J. W., Cullen, P. J., Irvine, R. F. and Dawson, A. P. (1991) *Biochem. J.* **277**, 883-885
- Loomis-Husselbee, J. W. and Dawson, A. P. (1993) *Biochem. J.* **289**, 861-866
- Hirota, J., Michikawa, T., Miyawaki, A., Furuichi, T., Okura, I. and Mikoshiba, K. (1995) *J. Biol. Chem.* **270**, 9046-19051
- Cullen, P. J., Loomis-Husselbee, J. W., Dawson, A. P. and Irvine, R. F. (1997) *Biochem. Soc. Trans.* **25**, 991-996
- Yamamoto, T., Matsui, T., Nakafu, M. and Iwamatsu, A. (1995) *J. Biol. Chem.* **270**, 30557-30561
- Cullen, P. J., Dawson, A. P. and Irvine, R. F. (1995) *Biochem. J.* **305**, 139-143
- Cullen, P. J., Chung, S. K., Chang, Y. T., Dawson, A. P. and Irvine, R. F. (1995) *FEBS Lett.* **358**, 240-242
- Baba, H., Fuss, B., Urano, J., Watson, J. B., Tamanoi, F. and Macklin, W. B. (1995) *J. Neurosci. Res.* **41**, 846-858
- Lockyer, P. J., Bottomley, J. R., Reynolds, J. S., McNulty, T. J., Venkateswarlu, K., Potter, B. V. L., Dempsey, C. E. and Cullen, P. J. (1997) *Curr. Biol.* **7**, 1007-1010
- Cullen, P. J., Patel, Y., Kakkar, V. V., Irvine, R. F. and Authi, K. S. (1994) *Biochem. J.* **3**, 739-742
- O'Rourke, F., Matthews, E. and Feinstein, M. B. (1996) *Biochem. J.* **315**, 1027-1034
- Irvine, R. F. (1989) *Biochem. Soc. Trans.* **17**, 6-9
- Soriano, S. and Banting, G. (1997) *FEBS Lett.* **403**, 1-4
- Changya, L., Gallacher, D. V., Irvine, R. F., Potter, B. V. L. and Petersen, O. H. (1989) *J. Membr. Biol.* **109**, 85-93
- Van der Zee, L., Sipma, H., Nelemans, A. and Hertog, A. D. (1995) *Eur. J. Pharmacol.* **289**, 463-469
- Berridge, M. J. (1993) *Nature (London)* **361**, 315-325
- Rey, I., Taylor-Harris, P., van Erp, H. and Hall, A. (1994) *Oncogene* **9**, 685-692
- Saez, R., Chan, A. M.-L., Miki, T. and Aaronson, S. A. (1994) *Oncogene* **9**, 2977-2982
- Studier, F. W. and Moffatt, B. A. (1986) *J. Mol. Biol.* **189**, 113-130
- Self, A. J. and Hall, A. (1995) *Methods Enzymol.* **256**, 3-10
- Porfiri, E., Evans, T., Bollag, G., Clark, K. and Hancock, J. F. (1995) *Methods Enzymol.* **255**, 13-21
- Herrmann, C., Martin, G. A. and Wittinghofer, A. (1995) *J. Biol. Chem.* **270**, 2901-2905
- Bird, G. St. J. and Putney, J. W. (1996) *J. Biol. Chem.* **271**, 6766-6770