Ins(1,3,4,5)P₄ is effective in mobilizing Ca²⁺ in mouse exocrine pancreatic acinar cells if phospholipase A₂ is inhibited

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In enzymically isolated mouse pancreatic acinar cells, under conditions of whole-cell patch-clamp current recording, the effect of phospholipase C-coupled agonists can be mimicked by internal perfusion of the intracellular second messenger Ins(1,4,5)P₃ (10 μM) or its analogue Ins(2,4,5)P₃ (10 μM). The inositol trisphosphates mimic receptor activation by releasing Ca²⁺ from intracellular stores and by promoting Ca²⁺ influx across the surface membrane. This Ca²⁺-mobilizing role of inositol polyphosphates seems to be confined to the inositol trisphosphates because internal perfusion of Ins(1,3,4,5)P₄ (10 μM) is not associated with any Ca²⁺-dependent current activation. In this study we investigate the effects of 4-bromophenacyl bromide (4BPB), a putative inhibitor of phospholipase A₂ and arachidonic acid production, on inositol polyphosphate-induced Ca²⁺ signalling. At 10 μM, 4BPB has no effect on unstimulated Ca²⁺-dependent membrane currents. However, if 4BPB is applied to

cells internally perfused with 10 μM Ins(1,4,5)P₃ or Ins(2,4,5)P₃ then the current responses are rapidly potentiated. In cells internally perfused with 10 μM Ins(1,3,4,5)P₄, which has itself no effect on membrane currents, application of 4BPB resulted in the activation of Ca²⁺-dependent currents, seen either as repetitive spikes of current or as sustained current activations. The application of arachidonic acid blocks the current responses evoked by the inositol trisphosphates and by Ins(1,3,4,5)P₄/4BPB. These results suggest that in enzymically isolated pancreatic acinar cells phospholipase A₂ activity is exerting an inhibitory effect on inositol polyphosphate-mediated Ca²⁺ mobilization. 4BPB removes this inhibition and potentiates the responses to internally perfused inositol trisphosphates and, importantly, makes 10 μM Ins(1,3,4,5)P₄ as effective as 10 μM Ins(1,4,5)P₃ in mobilizing intracellular Ca²⁺ and in promoting Ca²⁺ influx.

INTRODUCTION

The role of Ins(1,4,5)P₃ as a second messenger in the mobilization of Ca²⁺ is now unequivocally established [1] and the exocrine acinar cells of the rodent pancreas have played a key role in these investigations. It was in permeabilized pancreatic acinar cells that the ability of Ins(1,4,5)P₃ to release Ca²⁺ from intracellular stores was first demonstrated [2]. Pancreatic acinar cells from the mouse were also utilized in whole-cell patch-clamp recording protocols to demonstrate that internal perfusion of Ins(1,4,5)P₃ could, in a dose-dependent manner, mimic the effects of receptor-mediated stimulation of phospholipase C in the activation of Ca²⁺-dependent membrane currents [3,4]. At 10 μM, Ins(1,4,5)P₃ evokes brief, repetitive current spikes or oscillations. These current transients have now been shown to correspond to localized elevations in cytosolic Ca²⁺ owing to Ins(1,4,5)P₃-mediated Ca²⁺ release from stores at the secretory pole of the acinar cells [5]. The use of the non-metabolizable phosphorothiate analogue of Ins(1,4,5)P₃, [D,L-inositol 1,4,5-trisphosphorothiate (IPSt)], demonstrated that the Ins(1,4,5)P₃-mediated effects were not dependent on Ins(1,4,5)P₃ metabolism and that the repetitive current spikes could be evoked in the presence of a constant level of Ins(1,4,5)P₃ (IPSt) [3]. This latter point is particularly significant as it indicates that changes in the Ins(1,4,5)P₃ concentration (due either to changes in its rate of production and/or metabolism) do not provide the basis for oscillating Ca²⁺ signals. In the mouse pancreatic acinar cells the effects of receptor-mediated activation of phospholipase C can therefore readily be explained by the generation of Ins(1,4,5)P₃ and its subsequent Ca²⁺-mobilizing properties. There is, in this tissue at least, no

need to speculate on the involvement of any other second messenger in the basic mechanism of Ca²⁺ release from internal stores, Ca²⁺ oscillations and Ca²⁺ influx.

The experiments with IPSt reveal that the metabolism of Ins(1,4,5)P₃ is not necessary to evoke the full range of responses described above [3]. Endogenous Ins(1,4,5)P₃ is, however, the natural substrate for both phosphatase and kinase activity [6]. In particular a Ca²⁺-dependent Ins(1,4,5)P₃-3-kinase specifically converts Ins(1,4,5)P₃ to Ins(1,3,4,5)P₄ [7]. Putative Ins(1,3,4,5)P₄ receptors have been identified in a variety of tissues [8–11] and recently a specific Ins(1,3,4,5)P₄-binding protein has been cloned and characterized [12]. There has been much speculation as to the role of Ins(1,3,4,5)P₄ but although there are reports of effects of Ins(1,3,4,5)P₄ on Ca²⁺ mobilization in a variety of cell types there is as yet no clearly defined physiological role for Ins(1,3,4,5)P₄ (see [13,14]). In mouse pancreatic acinar cells it is found that internal perfusion of Ins(1,3,4,5)P₄ (10 μM), in patch-clamp protocols identical with those described above, is not associated with any activation of Ca²⁺-dependent membrane currents. This would seem to provide strong evidence against any role for this inositol polyphosphate in Ca²⁺ signalling. The only requirement for cellular signalling is, it seems, the production of the inositol trisphosphate, Ins(1,4,5)P₃. In the present study, however, we investigate the effects of 4-bromophenacyl bromide (4BPB), an inhibitor of phospholipase A₂ and arachidonic acid (AA) production [15], on inositol polyphosphate-mediated Ca²⁺ mobilization in mouse pancreatic acinar cells. When 4BPB is applied to the acinar cells, not only are the responses to internally perfused Ins(1,4,5)P₃ potentiated but Ins(1,3,4,5)P₄ (10 μM) is now effective in evoking current responses. In the

Abbreviations used: AA, arachidonic acid; 4BPB, 4-bromophenacyl bromide; IPSt, D,L-inositol 1,4,5-trisphosphorothiate; OEPC, oleoyloxyethyl phosphocholine.

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presence of 4BPB, $\text{Ins}(1,3,4,5)\text{P}_4$ can evoke a full range of current responses including repetitive current spikes and sustained responses. The results from our study indicate that under conditions of decreased phospholipase A_2 activity $\text{Ins}(1,3,4,5)\text{P}_4$ can function to mobilize Ca^{2+} from intracellular stores and to promote Ca^{2+} influx. The similarity of the responses to $\text{Ins}(1,4,5)\text{P}_3$ and $\text{Ins}(1,3,4,5)\text{P}_4$ suggest that the two inositol polyphosphates are activating identical mechanisms to elicit the Ca^{2+} -dependent currents. A preliminary report of this work has been published as an abstract [16].

MATERIALS AND METHODS

Cell preparation

Single mouse pancreatic acinar cells were prepared by dispersion with pure collagenase (Worthington; 200 units/ml at 37 °C) as described previously [3,5].

Patch-clamp electrophysiology

The standard patch-clamp technique was employed to record whole-cell currents from single pancreatic acinar cells [3–5]. Experiments were performed at room temperature (20–25 °C). Recording pipettes were pulled and fire-polished to a resistance of 2–4 M Ω . A List EPC7 amplifier was employed. Seals in excess of 10 G Ω were formed and suction was applied to establish the whole-cell configuration, indicated by the associated increase in capacitance and current noise. The cells were voltage-clamped at –30 mV, from which voltage steps to 0 mV were applied for 150 ms at 2 Hz. In the Figures presented the resolution is such that the current recordings at these two potentials appear continuous. The standard extracellular solution contained (in mM): NaCl 140, KCl 4.7, MgCl_2 1.13, Hepes 10, glucose 10 and CaCl_2 1.2 at pH 7.2. Ca^{2+} -free solutions contained no Ca^{2+} , and 0.1 mM EGTA was added. The standard pipette-filling solution contained (in mM): KCl 140, MgCl_2 1.13, Hepes 10, glucose 10, ATP 1 and EGTA 0.1 with no Ca^{2+} added. With the solutions employed the inward currents recorded at –30 mV comprised two components, due to simultaneous activation of Ca^{2+} -dependent Cl^- channels and Ca^{2+} -dependent cation channels with equivalent permeabilities for Na^+ and K^+ . The reversal potential for currents in both channels was approx. 0 mV.

The inositol polyphosphates were added to the internal solution at the concentrations indicated. $\text{Ins}(1,4,5)\text{P}_3$ and $\text{Ins}(1,3,4,5,6)\text{P}_5$ were supplied by Sigma, and $\text{Ins}(1,3,4,5)\text{P}_4$ was supplied by Sigma and by Alexis Corporation.

4BPB (Sigma) and thapsigargin (Sigma) were dissolved in DMSO, which was present in the final solution at a concentration of 0.1%. AA (Sigma) was dissolved in ethanol with a final concentration of 0.025% for the ethanol. The effects of both carrier vehicles were independently tested and they evoked no responses.

RESULTS

The internal perfusion of the inositol trisphosphates $\text{Ins}(1,4,5)\text{P}_3$ and $\text{Ins}(2,4,5)\text{P}_3$ is known [3,4] to be associated with the activation of Ca^{2+} -dependent membrane conductances in whole-cell patch-clamped mouse pancreatic acinar cells [$n = 66$ for $\text{Ins}(1,4,5)\text{P}_3$ and $n = 28$ for $\text{Ins}(2,4,5)\text{P}_3$ in the present study]. Figure 1 shows the typical repetitive current spikes evoked by these two agents when included in the pipette-filling solution at 10 μM . The figure also shows the lack of any effect of $\text{Ins}(1,3,4,5)\text{P}_4$ at the same concentration ($n = 42$). This lack of effect of $\text{Ins}(1,3,4,5)\text{P}_4$ in the mouse pancreas has been reported by others [17]. 4BPB is a

Figure 1 Whole-cell current recordings of mouse pancreatic acinar cells showing the effect of internal perfusion of inositol polyphosphates on Ca^{2+} -dependent currents

The cells were voltage-clamped at –30 mV and depolarizing jumps to a membrane potential of 0 mV were applied for 150 ms at 2 Hz. At the resolution shown the recordings at these two voltages appear continuous. The downward deflections seen in the traces at –30 mV represent inward currents that were due to the simultaneous activation of Ca^{2+} -dependent Cl^- and non-discriminating (Na^+ and K^+) channels. The traces show the effects of internal perfusion of 10 μM $\text{Ins}(1,4,5)\text{P}_3$ (top panel), 10 μM $\text{Ins}(2,4,5)\text{P}_3$ (middle panel) or 10 μM $\text{Ins}(1,3,4,5)\text{P}_4$ (bottom panel).

putative inhibitor of phospholipase A_2 . In control acinar cells in which no inositol polyphosphates are internally perfused, 4BPB has no effect on the unstimulated whole-cell currents ($n = 14$) (Figure 2). When the cells are internally perfused with either of the two inositol trisphosphates $\text{Ins}(1,4,5)\text{P}_3$ or $\text{Ins}(2,4,5)\text{P}_3$, the effect of 4BPB is to potentiate the current responses, transforming them from repetitive current spikes to sustained current activations ($n = 28$ and $n = 8$ respectively) (Figure 2). In cells that are internally perfused with 10 μM $\text{Ins}(1,3,4,5)\text{P}_4$ no current responses are seen until 4BPB is perfused externally when, after a latency of approx. 1 or 2 min, current activations are seen ($n = 30$). These generally take the form of current spikes that gradually fuse to give sustained current responses (Figure 3). If isolated mouse pancreatic acinar cells are preincubated with 4BPB for between 8 and 30 min before the establishment of whole-cell current recording, the inclusion of $\text{Ins}(1,3,4,5)\text{P}_4$ in the solution filling the patch-pipette is associated with current activations on initiation of whole-cell recording and internal dialysis of the cells ($n = 5$) (Figure 3).

The sustained current activations evoked by the inositol trisphosphates have been shown to be dependent on the presence of extracellular Ca^{2+} [4] ($n = 11$ in the present study) (Figure 4). Even the repetitive current spikes, which can persist for many minutes in the absence of extracellular Ca^{2+} , are ultimately abolished if Ca^{2+} is not restored. The Ca^{2+} -dependent current activations induced by the inositol trisphosphates are thus composed of Ca^{2+} released from intracellular stores and Ca^{2+}

Figure 2 Whole-cell current recordings showing the effect of 4BPB on Ins(1,4,5) P_3 - and Ins(2,4,5) P_3 -induced currents

These traces show the effect of external application of 4BPB on the Ca^{2+} -activated currents in control cells with no inositol polyphosphate in the recording pipette (top panel), with Ins(1,4,5) P_3 in the internal solution (middle panel) or with Ins(2,4,5) P_3 in the internal solution (bottom panel).

Figure 3 Whole-cell current recordings showing the effect of 4BPB in cells perfused with Ins(1,3,4,5) P_4

In both instances 10 μ M Ins(1,3,4,5) P_4 was in the internal solution. Upper panel: 4BPB was applied externally after several minutes of whole-cell recording and its application was associated with the development of Ca^{2+} -activated currents. Lower panel: the cell had been preincubated with 4BPB 15 min before establishing the whole-cell recording configuration, Ca^{2+} -activated currents developed within seconds.

Figure 4 Whole-cell current recordings showing the role of extracellular Ca^{2+} in sustained current responses

These traces show the effects of removal of extracellular Ca^{2+} on the Ca^{2+} -dependent current activations potentiated by 4BPB in the presence of internally perfused Ins(2,4,5) P_3 (upper panel) or Ins(1,3,4,5) P_4 (lower panel).

influx across the cell surface membrane. Figure 4 shows that the current activations evoked by Ins(1,3,4,5) P_4 in the presence of 4BPB are similarly dependent on the presence of extracellular Ca^{2+} ($n = 6$). The current responses persist for approx. 4–5 min but are ultimately abolished on the removal of extracellular Ca^{2+} . As with the inositol trisphosphates, the current responses to Ins(1,3,4,5) P_4 are mediated both by the release of Ca^{2+} from intracellular stores and by Ca^{2+} influx across the surface membrane. The effects of 4BPB were not manifested for all inositol polyphosphates and its application to cells internally perfused with Ins(1,3,4,5,6) P_5 (20 μ M) was not associated with any current activations ($n = 6$) (results not shown). This was not due to some general inhibitory effect of Ins(1,3,4,5,6) P_5 because 4BPB still induced current spikes when 10 μ M Ins(1,3,4,5) P_4 was combined with 20 μ M Ins(1,3,4,5,6) P_5 ($n = 4$).

The effect of another phospholipase A_2 inhibitor, oleoyloxyethyl phosphocholine (OEPIC) [18] was investigated. OEPIC (20 μ M) could mimic the effect of 4BPB in potentiating the responses to inositol trisphosphates and in making Ins(1,3,4,5) P_4 an effective activator of Ca^{2+} -dependent currents (Figure 5). The effects of this agent were much less reproducible than those of 4BPB. The effects described for 4BPB were seen invariably whereas those of OEPIC were seen in only six of eleven experiments with Ins(1,4,5) P_3 and in three of six experiments with Ins(1,3,4,5) P_4 . The inhibitor of AA oxidation, nordihydroguaiaretic acid, was tested and found to have no effect on cells internally perfused with either Ins(1,4,5) P_3 ($n = 4$) or Ins(1,3,4,5) P_4 ($n = 4$) (results not shown).

Acute external application of 25 μ M AA blocked the current responses evoked in the presence of 4BPB by both Ins(1,4,5) P_3 ($n = 11$) and Ins(1,3,4,5) P_4 ($n = 11$). This effect of AA was not due to a direct blockade of the ion channels because these were shown to be still capable of activation by elevated intracellular Ca^{2+} on application of the Ca^{2+} ionophore ionomycin ($n = 5$) (Figure 6). AA also blocked the effects of internally perfused

Figure 5 Whole-cell current recordings showing how OEPC can mimic the effects of 4BPB

These traces show the effect of the phospholipase A_2 inhibitor OEPC on the Ca^{2+} -activated currents in cells internally perfused with $Ins(2,4,5)P_3$ (upper panel) or $Ins(1,3,4,5)P_4$ (lower panel).

$Ins(1,4,5)P_3$ in the absence of 4BPB ($n = 6$), and the subsequent addition of the phospholipase A_2 inhibitor was without effect ($n = 3$) (Figure 6).

Thapsigargin is a tumour-promoting agent that has been demonstrated to inhibit the microsomal Ca^{2+} -ATPase and consequently to elevate Ca^{2+} by release from intracellular stores [19]. When thapsigargin ($2 \mu M$) was applied to mouse acinar cells, in the absence of extracellular Ca^{2+} , it resulted in a slow activation of Ca^{2+} -dependent currents that was small and transient. Figure 7 shows the largest current response evoked by thapsigargin in the 10 experiments performed. If cells were pretreated with 4BPB, the application of thapsigargin, again in the absence of Ca^{2+} , resulted in a more rapid and profound activation of the Ca^{2+} -dependent currents ($n = 6$) (Figure 7). Preincubation with 4BPB markedly potentiated the effects of thapsigargin in mobilizing Ca^{2+} from intracellular stores.

DISCUSSION

In mouse pancreatic acinar cells, patch-clamp investigations of the role of internally perfused inositol polyphosphates have revealed that that $Ins(1,4,5)P_3$ can mimic the effects of phospholipase C-coupled agonists in the activation of Ca^{2+} -dependent currents [3,4]. This inositol trisphosphate can induce a full range of responses from repetitive current spikes (which are now known to be due to discrete Ca^{2+} spikes localized to the secretory pole of these cells) to fully sustained current responses associated with a global elevation in Ca^{2+} across the cell that is dependent on the influx of Ca^{2+} from the extracellular to intracellular fluid [4,5]. $Ins(1,4,5)P_3$ is thus capable of mobilizing intracellular Ca^{2+} , giving rise to Ca^{2+} spikes or oscillations and of promoting Ca^{2+} influx. An inward Ca^{2+} current has been directly demonstrated in response to the depletion of intracellular Ca^{2+} stores mediated by internal perfusion of $Ins(1,4,5)P_3$ but not in response to $Ins(1,3,4,5)P_4$ [17]. In the present study, internally perfused $Ins(1,3,4,5)P_4$ is ineffective in the activation of the Ca^{2+} -dependent membrane currents. The mouse pancreatic acinar cell is then often used as an example illustrating that there is no essential or

Figure 6 Whole-cell current recordings showing how AA reverses or blocks the effects of 4BPB

Top panel: first, the potentiating effect of 4BPB on the current responses in a cell internally perfused with $Ins(1,4,5)P_3$ is seen. Then the inhibitory effect of the external application of $25 \mu M$ AA is seen. Subsequent application of the Ca^{2+} ionophore ionomycin restored the current response, showing that AA had not directly blocked the ion channels. Middle panel: the same protocol but with $Ins(1,3,4,5)P_4$ as the internally perfused inositol polyphosphate. Bottom panel: $Ins(1,4,5)P_3$ was perfused internally and AA is shown to block these currents in the absence of 4BPB. When 4BPB was subsequently applied in the presence of AA, it had no potentiating effect. Ionomycin was still capable of activating the currents. The arrows and the word 'saturation' indicate saturation of the patch-clamp amplifier, which led to the flattening of the current traces.

obligatory role for $Ins(1,3,4,5)P_4$ in Ca^{2+} signalling. This is in contrast with the situation even in other exocrine cells such as the mouse lacrimal gland, where a role for $Ins(1,3,4,5)P_4$ in synergism with $Ins(1,4,5)P_3$ has been reported [20–22].

The present investigation was prompted by two studies by Maruyama [23,24] on the effects of agonists and internally perfused inositol polyphosphates in isolated rat pancreatic acinar cells. The rat acinar preparation employed by Maruyama is notable in that the cells can give only a brief, transient response (in terms of Ca^{2+} -dependent current activation) to stimulation by either the externally applied phospholipase C-coupled agonist acetylcholine or to internally perfused $Ins(1,4,5)P_3$. These cells have clearly developed some refractoriness in the inositol polyphosphate/ Ca^{2+} signalling pathway. Those studies reported, however, that this refractoriness could be reversed by the application of a putative phospholipase A_2 inhibitor, 4BPB, after

Figure 7 Whole-cell current recordings showing how 4BPB potentiates currents induced by thapsigargin

These traces show the effect of the external application of thapsigargin in the absence of extracellular Ca^{2+} (0.1 mM EGTA). Upper panel: the largest current response obtained in any of the control experiments. Lower panel: a typical trace of the much more rapid and potentiated response seen if the cells were preincubated in the presence of 4BPB.

which sustained responses could be evoked both by receptor activation and Ins(1,4,5) P_3 stimulation. It was also reported that in the presence of 4BPB the internal perfusion of Ins(1,3,4,5) P_4 was now as successful in the activation of Ca^{2+} -dependent membrane currents as Ins(1,4,5) P_3 [24]. The refractoriness reported for the rat pancreatic acinar cells is unusual and is not present in the mouse pancreatic acinar cells. Our interest was in investigating whether 4BPB could be in any way effective in the mouse pancreatic acinar cells, where phosphoinositide metabolism and Ca^{2+} signalling are apparently fully intact and, importantly, in a tissue where oscillations in Ca^{2+} are a prominent feature of the responses.

4BPB potentiates the current responses evoked by internally perfused inositol trisphosphates, Ins(1,4,5) P_3 and Ins(2,4,5) P_3 . One possible explanation for this is that the effect of the phospholipase A_2 inhibitor is to increase the sensitivity of the Ins(1,4,5) P_3 receptor to the inositol trisphosphates; this would be similar to the effect already described in this tissue for the thiol reagent thimerosal [25]. This is also suggested to be the mechanism of action of 4BPB in rat pancreatic acinar cells [23,24]. Further evidence that this is so comes from the experiments with thapsigargin in the present study. Thapsigargin blocks the microsomal Ca^{2+} -ATPase resulting in the release of Ca^{2+} from the stores via a 'leak' pathway. In mouse pancreatic acinar cells [26] and in mouse submandibular salivary acinar cells [27] this 'leak' pathway has been shown to be due to the action of endogenous Ins(1,4,5) P_3 at the Ins(1,4,5) P_3 receptor, providing a background activity in the Ins(1,4,5) P_3 receptor Ca^{2+} channel. An increase in the sensitivity of the Ins(1,4,5) P_3 receptor would then be expected to increase the rate of Ca^{2+} efflux from the stores when the Ca^{2+} -ATPase has been blocked by thapsigargin. This is what was found. The effect of thapsigargin applied to whole-cell patch-clamped acinar cells, in the absence of extra-

cellular Ca^{2+} , was that modest and transient current activations were seen only after a latency of minutes. If the same cells were pretreated with 4BPB, the effects of applying thapsigargin were rapid and the degree of Ca^{2+} -dependent current activation was greatly potentiated. This would be consistent with an effect of 4BPB in increasing the sensitivity of the Ins(1,4,5) P_3 receptor. Unlike thimerosal, which can evoke current responses in control cells in the absence of internally perfused inositol polyphosphates [25], 4BPB had no effect on resting whole-cell currents.

Ins(1,3,4,5) P_4 was without effect unless cells were preincubated with 4BPB or unless it was applied acutely during whole-cell recording. The range of responses evoked by Ins(1,3,4,5) P_4 were identical with those in response to the inositol trisphosphates. The evidence is that, in the presence of 4BPB, Ins(1,3,4,5) P_4 is capable of giving rise to Ca^{2+} release from intracellular stores, Ca^{2+} oscillations and ultimately Ca^{2+} influx. The question that arises is whether the effect of internal perfusion of Ins(1,3,4,5) P_4 is a direct one (see [28]). Several indirect effects must be considered. Could the Ins(1,3,4,5) P_4 be acting as a substrate for the 5-phosphatase and thereby protecting endogenous Ins(1,4,5) P_3 from breakdown? This could lead to an elevation in Ins(1,4,5) P_3 that could then act at the Ins(1,4,5) P_3 receptor. We think this is not the case because the Ins(1,3,4,5) P_4 is ineffective in the absence of 4BPB, which would imply that the protective effect is manifested only in the presence of the phospholipase A_2 inhibitor. This could be the situation, however, if the effect of the 4BPB was to increase the sensitivity of the Ins(1,4,5) P_3 receptor to Ins(1,4,5) P_3 (as discussed above). This sensitization could then reveal the protective action of Ins(1,3,4,5) P_4 in the pipette. However, Ins(1,3,4,5,6) P_5 , even at 20 μ M, was without effect in either the absence or the presence of 4BPB. We consider that it should also act as a substrate for the 5-phosphatase [29] and should therefore mimic the effect of Ins(1,3,4,5) P_4 , which it did not. Another alternative is that the Ins(1,3,4,5) P_4 is being converted to Ins(1,4,5) P_3 . Because Ins(1,3,4,5) P_4 is effective only in the presence of 4BPB we have again to consider either that the 4BPB specifically enhances the 3-phosphatase-mediated conversion of Ins(1,3,4,5) P_4 to Ins(1,4,5) P_3 (unlikely) or that a sensitization of the Ins(1,4,5) P_3 receptor occurs that makes otherwise ineffective amounts of Ins(1,3,4,5) P_4 -derived Ins(1,4,5) P_3 markedly potent. There is no non-metabolizable Ins(1,3,4,5) P_4 available that would not itself be affected by 4BPB, so we cannot eliminate this possibility although we consider such an active, specific, conversion of Ins(1,3,4,5) P_4 to Ins(1,4,5) P_3 to be unlikely. Ins(1,3,4,5,6) P_5 should be a substrate for the 3-phosphatase [29,30], yet even when it was simultaneously present at double the concentration of Ins(1,3,4,5) P_4 it did not inhibit the effect of 4BPB. In addition, in the cells pretreated with 4BPB the effects of establishing the whole-cell configuration with Ins(1,3,4,5) P_4 in the recording pipette were rapid, similar to those of 10 μ M Ins(1,4,5) P_3 itself. The conversion of Ins(1,3,4,5) P_4 to Ins(1,4,5) P_3 would have to be extremely rapid at a time when the contents of the solution filling the recording pipette are still equilibrating with the cell interior. Another possibility for an indirect effect could arise if the Ins(1,3,4,5) P_4 that we employed were contaminated with Ins(1,4,5) P_3 . We employed Ins(1,3,4,5) P_4 from two sources and found no difference in effects. The purest of these was guaranteed to have less than 0.1% Ins(1,4,5) P_3 , the limit of detection. Bearing in mind that in the mouse pancreatic acinar cell 10 μ M Ins(1,4,5) P_3 is a threshold concentration for an effect, the sensitization of the Ins(1,4,5) P_3 receptor by 4BPB would have to be such as to make less than 10 nM (the maximum possible level of contamination) markedly effective in mobilizing Ca^{2+} , i.e. a 1000-fold shift in sensitivity. If this were the effect of 4BPB, we consider that it should give rise to responses in its own

right owing to sensitization of the receptors to endogenous $\text{Ins}(1,4,5)P_3$, as has been reported for the other sensitizing agent, thimerosal [25]. 4BPB was never observed to have any effect in quiescent cells in the absence of internally perfused inositol polyphosphates. In rat pancreatic acinar cells, where the inhibition of Ca^{2+} signalling is profound ($10 \mu\text{M}$ $\text{Ins}(1,4,5)P_3$ is virtually ineffective), 4BPB is suggested to cause only a 10–20-fold increase in the sensitivity of the $\text{Ins}(1,4,5)P_3$ receptor to the inositol trisphosphate. Overall we consider that the effect of 4BPB on cells internally perfused with $\text{Ins}(1,3,4,5)P_4$ is to alter the sensitivity of the $\text{Ins}(1,4,5)P_3$ receptor such that it is more sensitive to the inositol trisphosphates, but importantly $\text{Ins}(1,3,4,5)P_4$ is now rendered effective at the $\text{Ins}(1,4,5)P_3$ receptor. In the neuroblastoma cell line SH-SY5Y, an effect of $\text{Ins}(1,3,4,5)P_4$ in the mobilization of Ca^{2+} has been reported to be due to its action at the $\text{Ins}(1,4,5)P_3$ receptor [31,32]. We have preliminary results in permeabilized mouse pancreatic acinar cells that show, even in the absence of 4BPB, that $\text{Ins}(1,3,4,5)P_4$ is only about one order of magnitude less effective than $\text{Ins}(1,4,5)P_3$ in mobilizing Ca^{2+} from internal stores.

The mechanism of action of 4BPB is not known. Its effects cannot be considered to be specific for blockade of phospholipase A_2 , and as a potent alkylating agent it can be expected to alkylate thiol and amine groups of many proteins [33]. The effects of 4BPB were, however, mimicked by another inhibitor of phospholipase A_2 , OEPC [18]. This agent was less reproducible in its effect but it did potentiate responses to the inositol trisphosphates and rendered $\text{Ins}(1,3,4,5)P_4$ effective. Acute application of AA resulted in a blockade of the currents induced by both $\text{Ins}(1,4,5)P_3$ and $\text{Ins}(1,3,4,5)P_4$. This was not due to direct blockade of the Ca^{2+} -activated ion channels because they were shown to be activated by an elevation in Ca^{2+} concentration induced by application of the Ca^{2+} ionophore ionomycin. The exact effects of 4BPB are not established but there seems to be a link between its effects and their antagonism by AA. We consider that the evidence at hand indicates that the most likely explanation of the effects of 4BPB is that an inhibition of the production of AA results in a potentiation of the sensitivity of the $\text{Ins}(1,4,5)P_3$ receptor for both $\text{Ins}(1,4,5)P_3$ and $\text{Ins}(1,3,4,5)P_4$. Thus $\text{Ins}(1,3,4,5)P_4$ would be acting at the $\text{Ins}(1,4,5)P_3$ receptor, which could explain the identical nature of the current responses evoked by the two polyphosphates. $\text{Ins}(1,4,5)P_3$ and $\text{Ins}(1,3,4,5)P_4$ are, however, generally considered to act on specific and different receptors. It is possible that 4BPB acts to give rise to some functional association between the two receptors such that they now both regulate for Ca^{2+} release from the same stores. Regardless of its mechanism(s) of action the effects of 4BPB on inositol polyphosphate-mediated Ca^{2+} signalling are potent and indicate that even in an isolated tissue such as mouse pancreatic acinar cells, where phospholipase C-mediated events seem to be intact, the procedure of isolation might have resulted in the development of an inhibition of the inositol polyphosphate/ Ca^{2+} signalling pathway that can significantly influence results.

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REFERENCES

- Berridge, M. J. (1993) *Nature* (London) **361**, 315–325
- Streb, I., Irvine, R. F., Berridge, M. J. and Schultz, I. (1983) *Nature* (London) **306**, 67–69
- Wakui, M., Potter, B. V. L. and Petersen, O. H. (1989) *Nature* (London) **339**, 317–320
- Petersen, C. C. H., Toescu, E. C., Potter, B. V. L. and Petersen, O. H. (1991) *FEBS Lett.* **293**, 179–182
- Thorn, P., Lawrie, A., Smith, P. M., Gallacher, D. V. and Petersen, O. H. (1993) *Cell* **74**, 661–668
- Cullen, P. J., Irvine, R. F. and Dawson, A. P. (1990) *Biochem. J.* **271**, 549–553
- Irvine, R. F., Letcher, A. J., Heslop, J. P. and Berridge, M. J. (1986) *Nature* (London) **320**, 631–634
- Challis, R. A. J., Wilcocks, A. L., Mulloy, B., Potter, B. V. L. and Nahorski, S. R. (1991) *Biochem. J.* **274**, 861–867
- Cullen, P. J. and Irvine, R. F. (1992) *Biochem. J.* **288**, 149–154
- Thiebert, A. B., Estevez, V. V., Mourey, R. J., Maracek, J. F., Barrow, R. K., Prestwich, G. D. and Snyder, S. H. (1992) *J. Biol. Chem.* **109**, 173–186
- Irvine, R. F. and Cullen, P. J. (1993) *Curr. Biol.* **3**, 540–543
- 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
- Irvine, R. F. (1992) *Adv. Second Messenger Phosphoprot. Res.* **26**, 161–185
- Putney, Jr., J. W. and Bird, G. (1993) *Endocr. Rev.* **14**, 610–631
- Drenth, J., Enzing, C. M., Halk, K. H. and Vessles, J. C. A. (1976) *Nature* (London) **264**, 373–377
- Rowles, S.-J. and Gallacher, D. V. (1994) *J. Physiol.* **477**, 8P
- Bahnsen, T. D., Pandol, S. J. and Dionne, V. E. (1993) *J. Biol. Chem.* **268**, 10808–10812
- Magolda, R. L. (1985) in *Prostaglandins, Leukotrienes and Lipoxins* (Bailey, J. M., ed.), pp. 669–695, Plenum Press, New York
- Thastrup, O., Cullen, P. J., Drobak, B. K., Hanley, M. R. and Dawson, A. P. (1990) *Proc. Natl. Acad. Sci. U.S.A.* **87**, 2466–2470
- Morris, A. P., Gallacher, D. V., Irvine, R. F. and Petersen, O. H. (1987) *Nature* (London) **330**, 653–655
- Changya, L., Gallacher, D. V., Irvine, R. F. and Petersen, O. H. (1989) *FEBS Lett.* **251**, 42–48
- Smith, P. M. (1992) *Biochem. J.* **238**, 27–30
- Maruyama, Y. (1990) *J. Physiol. (London)* **430**, 471–482
- Maruyama, Y. (1993) *J. Physiol. (London)* **463**, 729–746
- Thorn, P., Brady, P., Llopis J., Gallacher, D. V. and Petersen O. H. (1992) *Pflügers Arch. Eur. J. Physiol.* **422**, 173–178
- Toescu, E. C. and Petersen, O. H. (1994) *Pflügers Arch. Eur. J. Physiol.* **427**, 325–331
- Smith, P. M. and Gallacher, D. V. (1994) *Biochem. J.* **299**, 37–40
- Loomis-Husselbee, J. W., Cullen, P. J., Dreikhausen, U. E., Irvine, R. F. and Dawson, A. P. (1996) *Biochem. J.* **314**, 811–816
- Hoer, A. and Oberdisse, E. (1991) *Biochem. J.* **278**, 219–224
- Hughes, P. J. and Shears, S. B. (1990) *J. Biol. Chem.* **265**, 9869–9875
- Wilcox, R. A., Challiss, R. A. J., Baudin, G., Vasella, A., Potter, B. V. L. and Nahorski, S. R. (1993) *Biochem. J.* **294**, 191–194
- Wilcox, R. A., Challiss, R. A. J., Liu, C. S., Potter, B. V. L. and Nahorski, S. R. (1993) *Mol. Pharmacol.* **44**, 810–817
- Chang, J., Meisser, J. H. and McGregor, H. (1987) *Biochem. Pharm.* **36**, 2429–2436