Biochem. J. (1992) 283, 27–30 (Printed in Great Britain)

RESEARCH COMMUNICATION

Ins(1,3,4,5)P_4 promotes sustained activation of the Ca^{2+}-dependent Cl^- current in isolated mouse lacrimal cells

Peter M. SMITH
Department of Physiology, University of Liverpool, Liverpool L69 3BX, U.K.

Infusion of 50 μM-Ins(1,3,4,5)P_4 in addition to 500 μM-Ins(1,4,5)P_3 into mouse lacrimal cells via a patch-clamp pipette promoted sustained activation of the Ca^{2+}-dependent Cl^- current, which could not be achieved with 500 μM-Ins(1,4,5)P_3 alone. It has been proposed that Ins(1,3,4,5)P_4 facilitates Ca^{2+} influx in the presence of Ins(1,4,5)P_3 [Morris, Gallacher, Irvine & Petersen (1987) Nature (London) **330**, 653–655], but a subsequent study in mouse lacrimal cells [Bird, Rossier, Hughes, Shears, Armstrong & Putney (1991) Nature (London) **352**, 162–165] showed that a high concentration of Ins(1,4,5)P_3 could mobilize both intra- and extra-cellular Ca^{2+} in the absence of Ins(1,3,4,5)P_4. My data confirm these findings, but also show that Ins(1,3,4,5)P_4 can stimulate additional Ca^{2+} influx even when the Ins(1,4,5)P_3-dependent intracellular Ca^{2+} pools have been depleted.

INTRODUCTION

Receptor-mediated activation of phospholipase C and cleavage of phosphatidylinositol 4,5-bisphosphate to give Ins(1,4,5)P_3 and diacylglycerol is the first step of the inositol phosphate cascade, which has been widely implicated in stimulus–secretion coupling. Of the 63 or so possible inositol phosphate derivatives [1], only Ins(1,4,5)P_3 has a clearly defined function, as a soluble second messenger communicating receptor activation to release of Ca^{2+} from intracellular Ca^{2+} pools by means of an Ins(1,4,5)P_3-dependent Ca^{2+} channel [2,3]. The role of Ins(1,3,4,5)P_4 in Ca^{2+} mobilization is less clear. Receptors for Ins(1,3,4,5)P_4 [4–7] have been identified, and there have been reports that Ins(1,3,4,5)P_4 is able to cause mobilization of intracellular Ca^{2+}, either by acting in synergism with Ins(1,4,5)P_3, to release Ca^{2+} from Ca^{2+} stores which are inaccessible to Ins(1,4,5)P_3 alone [8,9], or by releasing Ca^{2+} directly by a mechanism independent of Ins(1,4,5)P_3 [10–12]. However, there are some cell types which mobilize Ca^{2+} without any Ins(1,3,4,5)P_4 involvement [13]. Previous work with mouse lacrimal cells revealed that they were unusually insensitive to Ins(1,4,5)P_3 and that 10–100 μM-Ins(1,4,5)P_3 applied by internal perfusion via a patch-clamp recording pipette produced only small transient Ca^{2+} release [8,14]. In the same cells, Ins(1,3,4,5)P_4 together with Ins(1,4,5)P_3 allowed entry of extracellular Ca^{2+} under conditions where Ins(1,4,5)P_3 alone did not, and it was concluded that Ins(1,3,4,5)P_4 was necessary to gate the influx of extracellular Ca^{2+} in these cells. More recently Bird *et al.* [15] have suggested that complete Ca^{2+} mobilization may also be achieved in lacrimal cells without the assistance of Ins(1,3,4,5)P_4, by increasing the intracellular concentration of Ins(1,4,5)P_3 to 500 μM. It is surprising that such a high concentration of Ins(1,4,5)P_3 was required for complete Ca^{2+} mobilization, as Ins(1,4,5)P_3 concentrations as low as 5 μM have been found to be maximally effective in other exocrine cells [16], and in hepatocytes maximal binding of Ins(1,4,5)P_3 to the Ins(1,4,5)P_3 receptor under near physiological conditions is slightly less than 1 μM [17]. It is clear, however, that the previous studies [8,14] did not apply Ins(1,4,5)P_3 at the sufficiently high concentration necessary to mobilize intra- and extra-cellular Ca^{2+} fully in this preparation. In the present study the patch-clamp whole-cell technique has been employed both to introduce inositol polyphosphates into the cell and to measure Ca^{2+}-activated membrane currents, in order to assess Ca^{2+} mobilization caused by high concentrations of Ins(1,4,5)P_3 and to determine whether or not Ins(1,3,4,5)P_4 is effective in Ca^{2+} mobilization when the Ins(1,4,5)P_3 concentration has been raised to a level at which it alone can stimulate entry of extracellular Ca^{2+}.

MATERIALS AND METHODS

Adult male outbred Swiss mice were killed by cervical dislocation and lacrimal cells were isolated by collagenase (Worthington Diagnostic, Freehold, NJ, U.S.A.) digestion [14]. Cells were allowed to attach to a plastic Petri dish and were viewed at $\times 400$ magnification. The whole-cell configuration [14] was achieved with single cells by using 2–5 MΩ patch-clamp pipettes pulled from Assistant haematocrit tubing. Access resistance through the patch pipette was approx. 3 times that of the pipette itself. Cells were voltage-clamped to -30 mV by using the List EPC7 (List Electronics, Darmstadt, Germany) patch-clamp amplifier. K^+ and Cl^- currents were measured by pulsing to 0 mV and -80 mV respectively for 100 ms twice a second. Currents were digitized by using the CED 1401 interface (Cambridge Electronics Design, Cambridge, U.K.) and stored and analysed with an IBM AT compatible computer [18]. The mean steady-state current elicited in response to each voltage step was calculated and these values were then plotted against time. Fig. 1 shows data averaged over several experiments by using a spreadsheet program. The digitized currents from 5–15 experiments were averaged to give the mean and S.E.M. Values in the text show means $\pm$ S.E.M. (n = number of experiments). Probabilities were calculated by Student's t test.

Lacrimal cells have both Cl^- channels and non-selective channels which, in the standard intra- and extra-cellular bathing solutions used in these experiments, could contribute to the inward current measured at -80 mV. A series of experiments were performed in which the outward current was blocked by 10 mM-tetraethylammonium and the Cl^- and non-selective currents were separated by lowering the extracellular Na^+ concentration (Na^+ was replaced by *N*-methyl-D-glucamine). With this protocol, the inward current induced by acetylcholine and Ins(1,4,5)P_3 or Ins(1,4,5)P_3/Ins(1,3,4,5)P_4 was observed to be a Cl^- current which could be clearly distinguished from current through the non-selective channel activated by ATP [19] (results not shown).

Ins(1,3,4,5)P_4 was used at 50 μM throughout this study, as preliminary experiments showed this concentration to be slightly

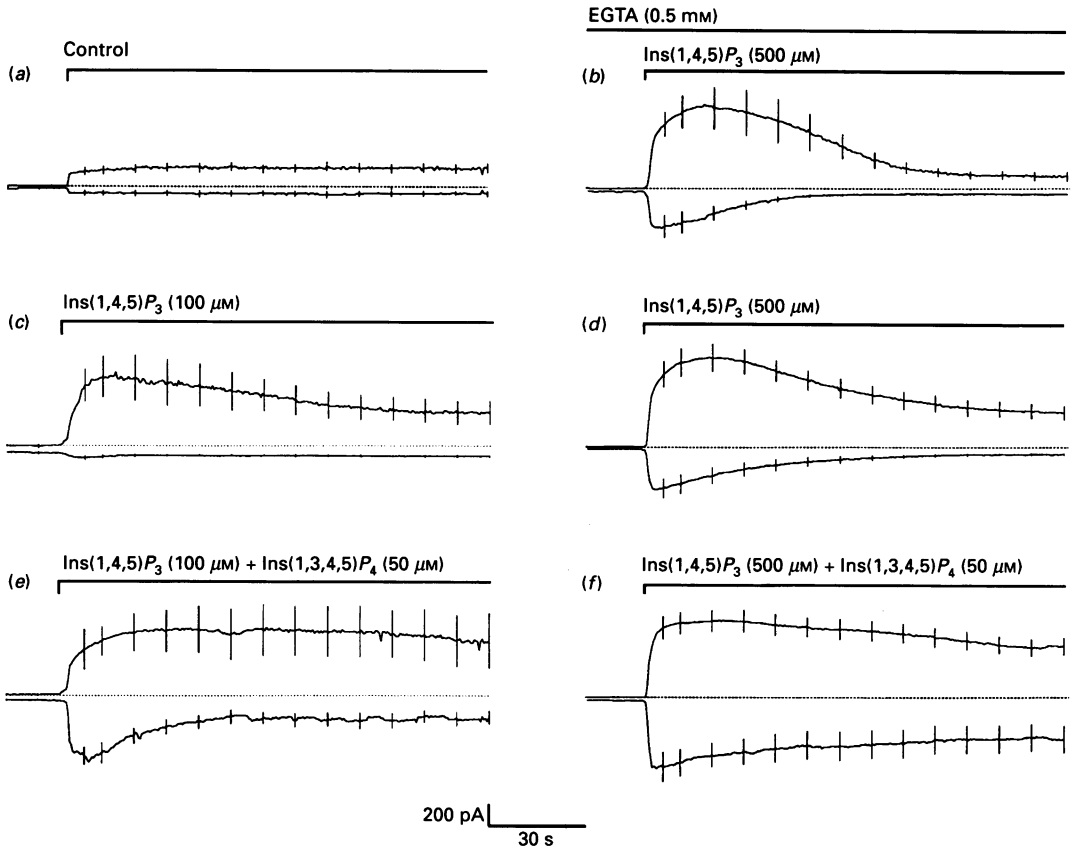

Fig. 1. Averaged outward K^+ currents measured at 0 mV and inward Cl^- currents measured at −80 mV in acutely isolated mouse lacrimal cells over 2.5 min immediately after establishing the whole-cell configuration (shown by the bar)

The broken line indicates the zero current level. The S.E.M. is shown at 10 s intervals. Panel (*a*) shows the average of 15 controls in which the patch pipette contained no inositol polyphosphates. In (*b*) the cells were preincubated for 3–5 min in a Ca^{2+}-free solution containing 0.5 mM-EGTA. EGTA was present for the duration of the experiment and the patch-clamp pipette contained 500 μM-Ins(1,4,5)P_3 ($n = 5$). Panel (*c*) shows the response to 100 μM-Ins(1,4,5)P_3 alone in the patch pipette ($n = 5$) and (*d*) the response to 500 μM-Ins(1,4,5)P_3 ($n = 14$). In (*e*) and (*f*) the patch pipette contained 50 μM-Ins(1,3,4,5)P_4 in addition to 100 μM- ($n = 5$) and 500 μM-Ins(1,4,5)P_3 ($n = 11$) respectively.

more effective than 10 μM and no less effective than 100 μM. However, no systematic attempt was made to determine the maximally effective Ins(1,3,4,5)P_4 concentration (results not shown).

The patch-clamp pipette contained (in mM) 140 KCl, 1.13 $MgCl_2$, 10 glucose, 0.5 EGTA and 1 ATP, buffered to pH 7.2 with 10 mM-Hepes. The external bathing solution contained (in mM) 140 NaCl, 4.7 KCl, 1.13 $MgCl_2$, 1.2 $CaCl_2$ and 10 glucose buffered to pH 7.2 with 10 mM-Hepes. Cells were superfused continuously at 0.5 ml/min from one of several parallel superfusion pipettes. The solution bathing the cell could be changed in 1–2 s. All experiments were carried out at 24±2 °C.

RESULTS AND DISCUSSION

In lacrimal cells both the K^+ and the Cl^- channels are Ca^{2+}-dependent, but the K^+ channel is sensitive to Ca^{2+} in a lower range than the Cl^- current [20,21]. Therefore it is possible to see small changes in $[Ca^{2+}]_i$ reflected in the K^+ current, and elevation of $[Ca^{2+}]_i$ past the point where the K^+ current is maximally activated is manifest as changes in the Cl^- current. Fig. 1(*a*) shows that in the absence of any inositol polyphosphate both the K^+ and Cl^- currents stabilized within 2–3 s of establishing the whole-cell configuration, and stable currents were maintained over the experimental period. Inclusion of 50 μM-Ins(1,3,4,5)P_4 in the patch-clamp pipette did not alter either the K^+ or Cl^- currents from those seen under control conditions (results not shown). The data in Fig. 1(*b*) were obtained in the absence of extracellular Ca^{2+} and confirm that Ins(1,4,5)P_3 mobilizes intracellular Ca^{2+}. Following a lag of 0.5–1 s after establishment of the whole cell, which is probably the time required for the Ins(1,4,5)P_3 to diffuse into the cell, the K^+ current rose rapidly and stabilized in 20–30 s. The Cl^- current also increased rapidly and reached a peak in 2–3 s. Activation of both the K^+ and the Cl^- currents was transient, and both currents declined to control values within 2 min. In the presence of 1.2 mM extracellular Ca^{2+} both 100 μM- and 500 μM-Ins(1,4,5)P_3 induced a small sustained K^+-current component in addition to the transient activation of both the K^+ and Cl^- currents. The mean K^+ current measured after 2.5 min was 156.1±24 pA ($n = 15$) under control conditions, 98.8±41 pA ($n = 5$) after stimulation by 500 μM-Ins(1,4,5)P_3 in the absence of extracellular Ca^{2+} and 335.1±63 pA ($n = 14$) after stimulation by 500 μM-Ins(1,4,5)P_3 in the presence of extracellular Ca^{2+}. The K^+ current was significantly ($P < 0.01$) elevated above control values after 2.5 min and remained elevated on average for 4–5 min and on some occasions for up to 10–15 min. Ins(1,4,5)P_3 did not activate any significant Cl^- current when applied at 100 μM (Fig. 1*c*), and 500 μM-Ins(1,4,5)P_3 produced only a transient activation of the Cl^- current (Fig. 1*d*); preincubation in the absence of extracellular Ca^{2+} had no effect on this transient Cl^- current (Fig. 1*b*). Ins(1,3,4,5)P_4 (50 μM) potentiated the action of both 100 μM- and 500 μM-Ins(1,4,5)P_3. When applied with 100 μM-Ins(1,4,5)P_3, the predominant effect of 50 μM-Ins(1,3,4,5)P_4 was to stimulate a transient Cl^- current;

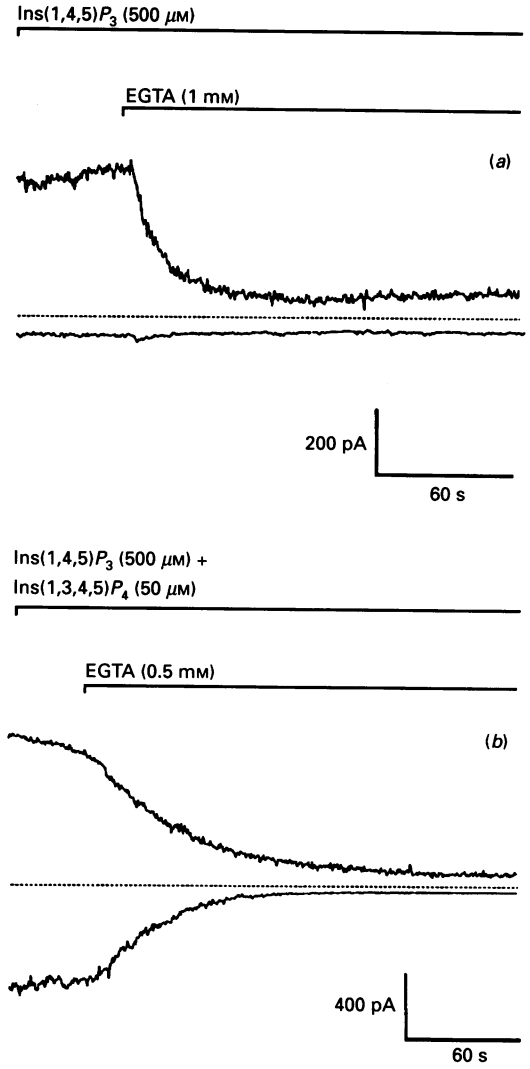

Fig. 2. K^+ and Cl^- currents stimulated by 500 μM-Ins(1,4,5)P_3 (*a*) and 500 μM-Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4 together (*b*), showing that the sustained component of both the K^+- and the Cl^--current responses could be abolished by removing extracellular Ca^{2+}

Results shown in (*a*) and (*b*) are each typical of three experiments.

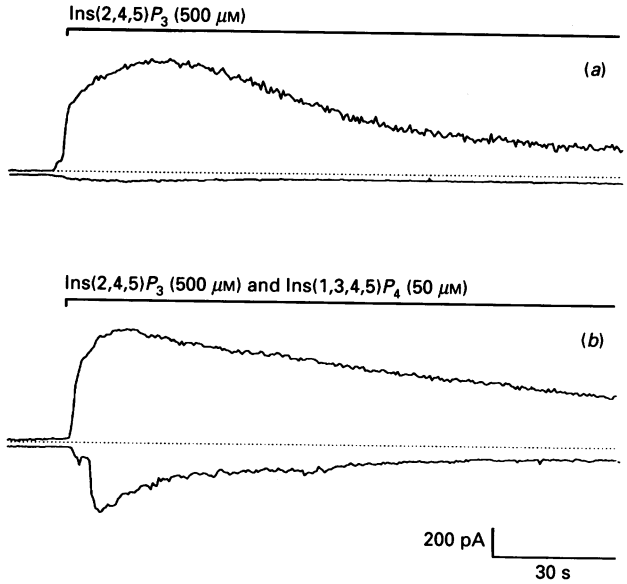

Fig. 3. (*a*) K^+ current and no Cl^- current stimulated by 500 μM-Ins(2,4,5)P_3 in the patch pipette and (*b*) activation of the Cl^- current in addition to the K^+ current by inclusion of 50 μM-Ins(1,3,4,5)P_4 in addition to 500 μM-Ins(2,4,5)P_3 in the patch pipette

Results shown in (*a*) and (*b*) are each typical of three experiments.

given that the transient component of the Cl^- current was independent of extracellular Ca^{2+}, these data suggest that Ins(1,3,4,5)P_4 can assist in mobilization of intracellular Ca^{2+}. The potentiating effect of 50 μM-Ins(1,3,4,5)P_4 on the action of 500 μM-Ins(1,4,5)P_3 is shown in Fig. 1(*f*). The duration of both the K^+- and the Cl^--current responses was extended in the presence of Ins(1,3,4,5)P_4; this enhancement was most pronounced in the Cl^- current, where Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4 together stimulated a significant sustained component which was not seen with Ins(1,4,5)P_3 alone. The Cl^- current measured after 2.5 min was -51.8 ± 11 pA ($n = 15$) under control conditions, -77.8 ± 10 pA ($n = 14$) after stimulation by 500 μM-Ins(1,4,5)P_3 alone and 376 ± 128 pA ($n = 11$) ($P < 0.01$ compared with control) after stimulation by 500 μM-Ins(1,4,5)P_3 and 50 μM-Ins(1,3,4,5)P_4 together. The Cl^- current measured after 10 min was -290 ± 133 pA ($n = 5$), still significantly ($P < 0.01$) elevated above control.

Fig. 2 shows directly that the sustained component of the K^+-current response, induced by Ins(1,4,5)P_3 alone, and the sustained K^+ and Cl^- currents induced by Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4 together were dependent on extracellular Ca^{2+}. All the sustained currents were abolished by replacement of Ca^{2+} in the external bathing solution by 0.5 mM-EGTA. The effects of a short (1–5 min) exposure to EGTA were always reversible, but on some occasions the effects of a prolonged (5–10 min) exposure were not, and the currents did not recover when Ca^{2+} was re-admitted to the bathing solution.

It has been suggested that Ins(1,3,4,5)P_4 does not have any specific role in Ca^{2+} mobilization, but rather that it enhances the action of Ins(1,4,5)P_3 by protecting it from degradation. This was tested by using Ins(2,4,5)P_3, which is poorly metabolized [22] and therefore would not benefit from any protection by Ins(1,3,4,5)P_4. Fig. 3(*a*) shows that 500 μM-Ins(2,4,5)P_3 evoked transient and sustained K^+ currents, but caused no stimulation of the Cl^- current. These data differ from the response to 500 μM-Ins(1,4,5)P_3 because of the lower activity of Ins(2,4,5)P_3. Ins(2,4,5)P_3 is thought to be approx. 4–6-fold less effective in mobilizing Ca^{2+} than is Ins(1,4,5)P_3 [12,22]; this is consistent with the data in Fig. 1(*c*), which show that a 5-fold lower Ins(1,4,5)P_3 concentration (100 μM) also produced K^+-current activation but no significant stimulation of the Cl^- current. The data in Fig. 3(*b*) show that the additional presence of 50 μM-Ins(1,3,4,5)P_4 with 500 μM-Ins(2,4,5)P_3 stimulated a significant transient Cl^--current activation, very similar to the potentiation of the Cl^- current caused by 50 μM-Ins(1,3,4,5)P_4 with 100 μM-Ins(1,4,5)P_3 (Fig. 1*e*). Potentiation of Ins(2,4,5)P_3-dependent currents by Ins(1,3,4,5)P_4 is unlikely to be due to any non-specific protection of Ins(2,4,5)P_3 against degradation by Ins(1,3,4,5)P_4, therefore this action of Ins(1,3,4,5)P_4 is likely to be the result of a real synergism between the two inositol polyphosphates.

A common factor in most models to account for Ca^{2+} influx into non-electrically excitable cells is depletion of the Ca^{2+} contained in the intracellular Ca^{2+} pools. In the 'capacitance model', emptying of intracellular Ca^{2+} pools, by whatever mechanism, is deemed a full and sufficient signal for influx of intracellular Ca^{2+} [23,24]. Experiments using thapsigargin have shown that this Ca^{2+}-ATPase inhibitor [25] can mobilize intracellular Ca^{2+} from pools insensitive to Ins(1,4,5)P_3 as well as

from Ins(1,4,5)P_3-sensitive pools, and that increased mobilization of intracellular Ca^{2+} causes increased Ca^{2+} influx [23]. This is taken into account in the most recent version of the 'capacitance model' [23], where it was suggested that the Ca^{2+} pools which regulate Ca^{2+} influx could include a population which lack Ins(1,4,5)P_3 receptors. My data show that Ins(1,3,4,5)P_4 is able to enhance both the release of intracellular Ca^{2+} caused by 100 μM-Ins(1,4,5)P_3 [9,8] and the Ca^{2+} influx evoked by 500 μM-Ins(1,4,5)P_3. These data can be accommodated by the 'capacitance model' if the apparently Ins(1,4,5)P_3-insensitive population of Ca^{2+} pools includes those which are stimulated to release Ca^{2+} by Ins(1,4,5)P_3 when Ins(1,3,4,5)P_4 is also present. Thus, like thapsigargin, Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4 together (Fig. 1*f*) can cause greater Ca^{2+} influx than Ins(1,4,5)P_3 alone (Fig. 1*d*) by causing greater release of intracellular Ca^{2+}.

Other models allow for control of Ca^{2+} influx by factors in addition to depletion of the Ca^{2+} pools; Irvine [26] has suggested that the Ins(1,4,5)P_3 receptor connects intracellular Ca^{2+} pools to the plasma membrane by interacting with a plasma-membrane protein, possibly the Ins(1,3,4,5)P_4 receptor. Ca^{2+} influx is stimulated by dissociation of the Ins(1,4,5)P_3 receptor from the plasma membrane. This mechanism predicts that low [Ca^{2+}] within the Ca^{2+} pool and raised concentrations of Ins(1,4,5)P_3 or Ins(1,3,4,5)P_4 will all contribute to the degree of dissociation of the two receptors, and therefore Ca^{2+} influx. My data are equally well explained by this model, which contains a fundamental, but not obligate, role for Ins(1,3,4,5)P_4. A very high concentration of Ins(1,4,5)P_3 could deplete the Ca^{2+} pools to the point at which the two receptors dissociate and allow Ca^{2+} influx in the absence of Ins(1,3,4,5)P_4 (Fig. 1). In both models Ins(1,3,4,5)P_4 cannot cause Ca^{2+} influx in the absence of Ins(1,4,5)P_3, because Ins(1,3,4,5)P_4 alone does not cause the Ca^{2+} pool to empty.

The key difference between these two hypotheses, i.e. whether potentiation of Ca^{2+} influx by Ins(1,3,4,5)P_4 is a direct result of Ca^{2+} gating at the plasma membrane by Ins(1,3,4,5)P_4, or is secondary to increased mobilization of intracellular Ca^{2+} by Ins(1,3,4,5)P_4 acting synergistically with Ins(1,4,5)P_3, cannot be resolved by my data.

These studies and those of Bird *et al.* [15] have employed inositol phosphate concentrations far in excess of those ever likely to be produced by receptor activation, in order to mimic the release of intracellular Ca^{2+} and influx of Ca^{2+} stimulated by agonist. Although it is possible that there are variations in Ins(1,4,5)P_3 concentration throughout the cell, and the Ins(1,4,5)P_3 concentration immediately adjacent to the Ins(1,4,5)P_3-sensitive store could rise to 500 μM after agonist stimulation, it seems more likely that 500 μM-Ins(1,4,5)P_3 represents a supramaximal stimulus which has no physiological counterpart. Therefore, although these data may provide useful insights into the pharmacology of Ins(1,4,5)P_3- and Ins(1,3,4,5)P_4-receptor activation and may even help resolve what can stimulate Ca^{2+} influx, the observation that very high concentrations of Ins(1,4,5)P_3 can stimulate Ca^{2+} influx in the absence of Ins(1,3,4,5)P_4 probably has little physiological relevance. These data, in conjunction with previous studies using lower Ins(1,4,5)P_3 concentrations, do show that Ins(1,3,4,5)P_4 potentiates both release of intracellular Ca^{2+} and influx of extracellular Ca^{2+} stimulated by a wide range of Ins(1,4,5)P_3 concentrations and that, particularly in lacrimal cells, which have a low sensitivity to Ins(1,4,5)P_3, the synergism between Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4 is likely to be a vital part of the physiological response to agonist stimulation.

This work was supported by a grant from the Wellcome Trust to D. V. Gallacher. I thank D. V. Gallacher, O. H. Petersen and P. Thorn for help and advice throughout this study.

REFERENCES

1. Downes, C. P. (1989) Biochem. Soc. Trans. **17**, 259–268
2. Spat, A., Bradford, P. G., McKinney, J. S., Rubin, R. P. & Putney, J. W., Jr. (1986) Nature (London) **319**, 514–516
3. Ferris, C. D., Huganir, R. L., Supattapone, S. & Snyder, S. H. (1989) Nature (London) **342**, 87–89
4. Challiss, R. A. J., Willcocks, A. L., Mulloy, B., Potter, B. V. L. & Nahorski, S. R. (1989) Biochem. J. **274**, 861–867
5. Donie, F., Hulser, E. & Reiser, G. (1990) FEBS Lett. **268**, 194–198
6. Theibert, A. B., Supattapone, S., Ferris, C. D., Danoff, S. K., Evans, R. K. & Snyder, S. H. (1990) Biochem. J. **267**, 441–445
7. Theibert, A. B., Estevez, V. A., Ferris, C. D., Danoff, S. K., Barrow, R. K., Prestwich, G. D. & Snyder, S. H. (1991) Proc. Natl. Acad. Sci. U.S.A. **88**, 3165–3167
8. Changya, L., Gallacher, D. V., Irvine, R. F., Potter, B. V. L. & Petersen, O. H. (1989) J. Membr. Biol. **109**, 85–93
9. Cullen, P. J., Irvine, R. F. & Dawson, A. P. (1990) Biochem. J. **271**, 549–553
10. Ivorra, I., Gigg, R., Irvine, R. F. & Parker, I. (1991) Biochem. J. **273**, 317–321
11. Ely, J. A., Hunyady, L., Baukal, A. J. & Catt, K. J. (1990) Biochem. J. **268**, 333–338
12. Ferguson, J. E., Han, J. K., Kao, J. P. Y. & Nuccitelli, R. (1991) Exp. Cell Res. **192**, 352–356
13. Matthews, G., Neher, E. & Penner, R. (1989) J. Physiol (London) **418**, 105–130
14. Morris, A. P., Gallacher, R. F., Irvine, R. F. & Petersen, O. H. (1987) Nature (London) **330**, 653–655
15. Bird, G. St. J., Rossier, M. F., Hughes, A. R., Shears, S. B., Armstrong, D. L. & Putney, J. W., Jr. (1991) Nature (London) **352**, 162–165
16. Muallem, S., Pandol, S. J. & Beeker, T. G. (1989) J. Biol. Chem. **264**, 205–212
17. Nunn, D. L. & Taylor, C. W. (1990) Biochem. J. **270**, 227–232
18. Smith, P. M. (1992) J. Physiol. (London), in the press
19. Sasaki, T. & Gallacher, D. V. (1990) FEBS Lett. **264**, 130–134
20. Findlay, I. & Petersen, O. H. (1985) Pflugers Arch. **403**, 328–330
21. Marty, A., Tan, Y. P. & Trautmann, A. (1984) J. Physiol. (London) **357**, 293–325
22. Irvine, R. F. & Moor, R. M. (1986) Biochem. J. **240**, 917–920
23. Putney, J. W., Jr. (1986) Cell Calcium **7**, 1–12
24. Putney, J. W., Jr. (1990) Cell Calcium **11**, 611–624
25. Thastrup, O., Cullen, P. J., Brobak, B. K., Hanley, M. R. & Dawson, A. P. (1990) Proc. Natl. Acad. Sci. U.S.A. **87**, 2466–2470
26. Irvine, R. F. (1990) FEBS Lett. **263**, 5–9

Received 8 January 1992/22 January 1992; accepted 23 January 1992