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Micro-injection of inositol 1,3,4,5-tetrakisphosphate activates sea urchin eggs by a mechanism dependent on external Ca^{2+}

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Micro-injection of submicromolar concentrations of inositol 1,3,4,5-tetrakisphosphate caused a raising of the fertilization envelope in eggs of the sea urchin *Lytechinus variegatus*. This effect was dependent both on the presence of extracellular Ca^{2+} and on co-injection with a Ca^{2+}-mobilizing compound, inositol 2,4,5-trisphosphate. Inositol 1,3,4,5-tetrakisphosphate was the most potent compound tested in this assay; removal of the 3- or 5-phosphates or randomization of the phosphates in the inositol ring decreased its potency. These results show that inositol 1,3,4,5-tetrakisphosphate is an intracellular second messenger, and suggest that its function is to control cellular Ca^{2+} homoeostasis at the plasma membrane.

INTRODUCTION

Recent advances in our understanding of the role of inositides in cell signalling has led to the generally accepted hypothesis that stimulated phosphodiesteratic hydrolysis of phosphatidylinositol 4,5-bisphosphate occurs after receptor activation, giving rise to two intracellular messengers, diacylglycerol (Nishizuka, 1984) and Ins(1,4,5)P_3 (Berridge & Irvine, 1984). The function of the latter compound is to mobilize Ca^{2+} from intracellular stores, probably in the endoplasmic reticulum. However, Ca^{2+} stores inside the cell are limited, so Ca^{2+} mobilization is short-lived. When cells are stimulated by agonists that are coupled to inositide turnover, after the initial pulse of Ca^{2+} mobilization a prolonged raised level of Ca^{2+} can be seen which is entirely dependent on extracellular Ca^{2+} (e.g. Putney, 1978; Joseph *et al.*, 1985). This shows that a change in plasma membrane Ca^{2+} transport must also occur coincident with (and perhaps controlled by) inositide metabolism, as was first suggested by Michell (1975).

The mediator of this stimulated Ca^{2+} entry (which was directly demonstrated by, for example, Mauger *et al.*, 1984) is not known, although Putney (1986) has hypothesized that it might be Ins(1,4,5)P_3 acting on an intracellular Ca^{2+} pool with direct access to the extracellular space. Alternative candidates for controlling Ca^{2+} homoeostasis at the plasma membrane have emerged with the discovery of two novel inositol phosphates which are formed on cell stimulation, Ins(1,3,4)P_3 (Irvine *et al.*, 1984*a*, 1985; Burgess *et al.*, 1985) and its precursor Ins(1,3,4,5)P_4 (Batty *et al.*, 1985). The latter compound in particular, with its rapid rise after cell stimulation (Batty *et al.*, 1985; Heslop *et al.*, 1985; Hawkins *et al.*, 1986), and equally rapid decrease after receptor-blocking (Hawkins *et al.*, 1986), is a prime candidate for a role in controlling Ca^{2+} homoeostasis (Batty *et al.*, 1985; Irvine, 1986). Ins(1,3,4,5)P_4 has been shown to be completely inactive in mobilizing Ca^{2+} from intracellular stores in Swiss mouse 3T3 cells (Irvine *et al.*, 1986*a*), RINm5F insulinoma cells (Wollheim & Biden, 1986), *Limulus* photoreceptors (J. E. Brown & R. F. Irvine, unpublished work) and *Xenopus* oocytes (W. Busa, R. Nucitelli, M. J. Berridge & R. F. Irvine, unpublished work).

A possible way of testing directly the hypothesis that Ins(1,3,4,5)P_4 can control Ca^{2+} entry through the plasma membrane is by micro-injection into eggs. Ins(1,4,5)P_3 can activate sea urchin eggs (Whittaker & Irvine, 1984; Turner *et al.*, 1986; Slack *et al.*, 1986), and this Ins(1,4,5)P_3-induced activation has recently been shown to depend on extracellular Ca^{2+} (Slack *et al.*, 1986). Slack *et al.* (1986) demonstrated two distinct responses in membrane potential generated by micro-injection of Ins(1,4,5)P_3: an external Ca^{2+}-independent depolarization, and an external Ca^{2+}-dependent prolonged hyperpolarization which is presumably associated with the raising of the fertilization envelope. Ins(1,3,4,5)P_4 is now known to be formed from Ins(1,4,5)P_3 by a specific Ins(1,4,5)P_3 3-kinase (Irvine *et al.*, 1986*b*; Hawkins *et al.*, 1986; Stewart *et al.*, 1986; Hansen *et al.*, 1986; Biden & Wollheim, 1986) and this discovery opens up the possibility that Ins(1,4,5)P_3 may not activate sea urchin eggs directly by Ca^{2+} mobilization, but at least in part indirectly by being phosphorylated to Ins(1,3,4,5)P_4, which in turn stimulates Ca^{2+} entry. We report here experimental evidence consistent with this suggestion.

MATERIALS AND METHODS

Inositol phosphates

Ins(2,4,5)P_3 was prepared as in Irvine *et al.* (1984*b*). Ins(1,4,5)P_3, Ins(1,3,4)P_3 and Ins(1,3,4,5)P_4 were prepared as in Irvine *et al.* (1986*a*) and the latter two compounds were further purified by h.p.l.c. so that their Ins(1,4,5)P_3 content was less than 0.1%.

A *myo*-inositol tetrakisphosphate preparation was also made by partial acid hydrolysis of phytic acid and isolation of the InsP_4 fraction by ionophoresis (Seiffert & Agranoff, 1965; Batty *et al.*, 1985). The isomer content of this InsP_4 is unknown. From a purely random hydrolysis one would expect about 6% to be Ins(1,3,4,5)P_4; however, in view of the predominance of

Abbreviations used: InsP_3 and InsP_4, inositol trisphosphate and tetrakisphosphate respectively; locants are given in parentheses, e.g. Ins(1,2cyc4,5)P_3 is inositol 1,2-cyclic 4,5-trisphosphate.

inositol phosphates with a 2-phosphate resulting from acid hydrolysis of phytic acid (e.g. Pizer & Ballou, 1959), the actual Ins(1,3,4,5)P_4 content is probably less than that. This InsP_4 is designated Ins(w,x,y,z)P_4.

Injection of eggs and assessment of egg activation

Eggs of *Lytechinus variegatus* in artificial sea water [(430 mM-NaCl, 10 mM-KCl, 25 mM-$MgCl_2$, 25 mM-$MgSO_4$, 2 mM-$NaHCO_3$, 10 mM-$CaCl_2$, 10 mM-Hepes/NaOH (pH 8.0)] were held in an apparatus very similar to that described by Turner *et al.* (1986), and were injected with inositol phosphate solutions made up in 0.5 M-KCl/0.1 mM-EGTA (Whittaker & Irvine, 1984). Quantification of the amount of solution injected was by measurement of the diameter of an oil droplet injected at the same time (see Turner *et al.*, 1986). Egg activation was defined as the raising of a distinct fertilization envelope (e.g. Whittaker & Irvine, 1984; Turner *et al.*, 1986). Ten to twenty eggs were injected with the solution under trial, and the number of eggs showing a full fertilization envelope was counted; partially-activated eggs were excluded. Each batch of eggs was routinely tested for its ability to respond to 10^{-16} mol of Ins(1,4,5)P_3 or 10^{-17} mol of Ins(1,3,4,5)P_4 [co-injected with Ins(2,4,5)P_3; see below]. If a batch of eggs did not show a 100% response it was disregarded. All data are representative of a number of trials with different batches of eggs, and wherever two compounds are described in the text as being compared, the comparison was done at the same time on a single batch of eggs. Over many experiments we found some variation in the sensitivity of eggs to inositol phosphates, depending on the animal from which they were obtained and the time which had elapsed since they were taken out of the animals. Although these differences were to some extent corrected for by standardization with Ins(1,4,5)P_3 or Ins(1,3,4,5)P_4 (see above), because of them the assays for biological potency can only be regarded as semi-quantitative; in most instances the differences in potencies of inositol phosphates were of at least an order of magnitude, and so any relative activities recorded below are quantifiable, and were reproducible between batches of eggs of differing sensitivity.

RESULTS AND DISCUSSION

Effect of Ins(1,4,5)P_3

Preliminary trials injecting Ins(1,4,5)P_3 into eggs of *Lytechinus variegatus* showed that they behaved in our hands just as in others' experiments with the same species (Turner *et al.*, 1986) or other species (Whittaker & Irvine, 1984; Slack *et al.*, 1986), i.e. microinjection of about 10^{-17} mol of Ins(1,4,5)P_3 (e.g. 10 pl of 1 μM) would fully activate 100% of the eggs. We also confirmed the results of Slack *et al.* (1986) in that if EGTA (2 mM) was substituted for Ca^{2+} in the artificial sea water, no (or only partial) activation occurred, even with 10^{-16} mol of Ins(1,4,5)P_3. This effect was readily reversible by restoring the Ca^{2+} to the eggs. The practicalities of egg micro-injection prevented us from investigating in detail the time course of this EGTA effect, but less than 5 min without Ca^{2+} would abolish activation by Ins(1,4,5)P_3 in 90% of eggs, and a similar time of re-incubation in Ca^{2+} (after 30 min in EGTA) would restore it. This effect of EGTA was not instantaneous, and that is probably because the EGTA has to decrease Ca^{2+} in the perivitelline space before Ca^{2+} entry through the plasma membrane is affected. Slack *et al.* (1986) observed that long after EGTA had abolished the raising of fertilization envelopes by Ins(1,4,5)P_3, the earliest responses in membrane potential to Ins(1,4,5)P_3 (presumably caused by intracellular Ca^{2+} mobilization) were entirely unaffected. Thus in their hands even prolonged incubation with EGTA was not depleting intracellular stores, but was only preventing Ca^{2+} entry through the plasma membrane; therefore, as Ca^{2+} mediates the whole fertilization membrane response (Steinhardt & Epel, 1974; Steinhardt *et al.*, 1977), there is a very strong implication that stimulated Ca^{2+} entry through the plasma membrane is caused directly or indirectly by Ins(1,4,5)P_3, and is a prerequisite of the activation mechanism (see also Putney, 1986). We assume, though we cannot prove in these experiments, that the same applies here.

Effect of Ins(2,4,5)P_3 and Ins(1,3,4)P_3

When we injected eggs of *Lytechinus variegatus* [which respond rapidly and fully to 5×10^{-17} mol of Ins(1,4,5)P_3] with various doses of Ins(2,4,5)P_3 or Ins(1,3,4)P_3, there was very little response. At extremely high doses (10^{-14} mol; 20 pl of 0.5 mM) 20% of eggs raised envelopes in response to Ins(2,4,5)P_3, and at a similar high dose of Ins(1,3,4)P_3 a morphologically different (and very small) envelope was raised around some eggs, but no full envelopes were observed. The overall potency of Ins(2,4,5)P_3 was at least three orders of magnitude less than Ins(1,4,5)P_3, and Ins(1,3,4)P_3 was therefore even less effective.

These results were very surprising, as Ins(2,4,5)P_3 is known to be an efficient Ca^{2+} mobilizer with a potency about 6-fold less than Ins(1,4,5)P_3 in many vertebrate systems, e.g. permeabilized Swiss mouse 3T3 cells (Irvine *et al.*, 1984*b*), guinea-pig hepatocytes (Burgess *et al.*, 1984) or mouse pancreatic acinar cells (H. Streb, R. F. Irvine, M. J. Berridge & I. Schulz, unpublished work), and also on washed rat liver microsomes (Dawson & Irvine, 1984). Also, Ins(2,4,5)P_3 has been shown to compete with Ins(1,4,5)P_3 binding in permeabilized mammalian cells (Spät *et al.*, 1986). Furthermore, in invertebrate photoreceptors it is virtually indistinguishable from Ins(1,4,5)P_3 in inducing depolarization and adaptation (Brown *et al.*, 1984; Fein *et al.*, 1984), both of which are known to be mediated by Ca^{2+} mobilization (Rubin & Brown, 1985; Payne *et al.*, 1986). Ins(1,3,4)P_3 will also mobilize Ca^{2+} in Swiss mouse 3T3 cells (Irvine *et al.*, 1986*a*), RIN-insulinoma cells (Wollheim & Biden, 1986) or *Xenopus* oocytes (W. Busa, R. Nucitelli, M. J. Berridge & R. F. Irvine, unpublished work) albeit at a 20-fold higher dose than Ins(1,4,5)P_3. Pressure injection of Ins(1,3,4)P_3 mobilizes Ca^{2+} in invertebrate photoreceptors with a potency similar to that of Ins(1,4,5)P_3 (J. E. Brown & R. F. Irvine, unpublished work). Sea urchin eggs have also been reported to be unresponsive to a third inositol trisphosphate, Ins(1,2cyc4,5)P_3 (M. Whittaker & K. A. Swann, personal communication) which has calcium-mobilizing properties very similar to those of Ins(1,4,5)P_3 in both vertebrate and invertebrate tissues (Wilson *et al.*, 1985; Irvine *et al.*, 1986*a*).

Given the wide range of both vertebrate and invertebrate tissues in which Ins(2,4,5)P_3, Ins(1,3,4)P_3 and Ins(1,2cyc4,5)P_3 will mobilize Ca^{2+}, it therefore

seems extraordinary that they are so ineffective in activating sea urchin eggs. The most likely explanation is that, although they will indeed mobilize Ca^{2+} [i.e. they do induce the Ca^{2+}-independent phase recorded by Slack *et al.* (1986)], they do not induce the subsequent Ca^{2+} entry. Ins(1,4,5)P_3 is clearly capable of doing both, and the possible explanation for this, which we explore here, is that only Ins(1,4,5)P_3 can be phosphorylated to Ins(1,3,4,5)P_4 which in turn mediates the Ca^{2+} entry.

In view of the remarkable 3-hydroxyl specificity of Ins(1,4,5)P_3 3-kinase (Irvine *et al.*, 1986*a*), Ins(1,3,4)P_3 will be a very poor substrate for that enzyme. Furthermore, although Ins(1,2cyc4,5)P_3 may serve as a substrate for Ins(1,4,5)P_3 3-kinase (Irvine *et al.*, 1986*b*), subsequent studies by the laboratories of Downes (Hawkins *et al.*, 1987) and Majerus (Connolly *et al.*, 1987) have shown that it is also a poor substrate, with a K_m at least 100 times higher than that for Ins(1,4,5)P_3. Ins(2,4,5)P_3 is therefore likely to be an even poorer substrate for rat brain InsP_3 kinase, and we have confirmed this by comparing it directly with Ins(1,4,5)P_3 (both substrates at 100 μM) using [γ-^{32}P]ATP, under conditions described by Irvine *et al.* (1986*b*), and analysing the products by h.p.l.c. Even though the concentration of substrates was 150 times the K_m of the rat brain kinase for Ins(1,4,5)P_3 (Irvine *et al.*, 1986*b*), Ins(2,4,5)P_3 was phosphorylated at less than 10% of the rate of Ins(1,4,5)P_3, this being the limit of detection under these assay conditions.

Thus we can suggest that the inability of Ins(2,4,5)P_3 and Ins(1,3,4)P_3 (the present data) or of Ins(1,2cyc4,5)P_3 (M. Whittaker & K. A. Swann, personal communication) to activate sea urchin eggs lies in the fact that, unlike Ins(1,4,5)P_3, they are not readily phosphorylated to their corresponding InsP_4.

Effect of Ins(1,3,4,5)P_4

Our initial observations with injecting Ins(1,3,4,5)P_4 were disappointing with regard to this latter suggestion because microinjection of up to 10^{-14} mol of this compound (10 pl of 1 mM, h.p.l.c. pure) failed to activate eggs. However, it is likely that the second stage of egg activation (Slack *et al.*, 1986) would depend on the first; i.e. that Ins(1,3,4,5)P_4 might require prior Ca^{2+} mobilization to exert an effect on Ca^{2+} entry. We therefore undertook to test again all the inositol phosphates that we had prepared in eggs in which the intracellular Ca^{2+} had already been mobilized. There are two alternative ways open to us for doing this. Firstly, we could co-inject the compounds with Ca^{2+}; this would be difficult both to do and to interpret because we know neither the local Ca^{2+} concentrations nor the kinetics of Ca^{2+} release induced by Ins(1,4,5)P_3. A second, and more physiological, approach was to co-inject Ins(2,4,5)P_3, which as discussed above is a well-established Ca^{2+} mobilizer that does not raise fertilization envelopes in these eggs.

Effect of inositol phosphates co-injected with a Ca^{2+} mobilizer

In an extensive series of experiments we therefore injected into eggs inositol phosphate solutions all of which contained 50 μM-Ins(2,4,5)P_3; from studies on other vertebrate and invertebrate tissues (see above) we can be sure that this level of Ins(2,4,5)P_3 will maximally mobilize Ca^{2+} even if only 1% of cell volume [10 pl, 5×10^{-16} mol of Ins(2,4,5)P_3] is injected. Ten times this dose of Ins(2,4,5)P_3 does not activate eggs on its own (see above). The results of some of these experiments are recorded in Fig. 1. The difference between Ins(1,3,4,5)P_4 injected on its own (see above) and co-injected with Ins(2,4,5)P_3 (Fig. 1) can only be described as spectacular. Whereas 10^{-14} mol of Ins(1,3,4,5)P_4 was ineffective on its own, now as little as 2.1×10^{-18} mol [21 pl of 0.1 μM-Ins(1,3,4,5)P_4] would fully activate all of a batch of eggs. Ins(1,3,4,5)P_4 was slightly more potent than its precursor, Ins(1,4,5)P_3, though with this semiquantitative assay technique a much greater number of direct comparison experiments would be necessary for us to quantify the difference between them (results not shown). The breakdown product of Ins(1,3,4,5)P_4, Ins(1,3,4)P_3, was inactive up to 6×10^{-15} mol (Fig. 1). It is important to demonstrate that the effect of Ins(1,3,4,5)P_4 is not due to an artefact of injection of a substance more highly charged than Ins(1,4,5)P_3, and this we did by comparing directly Ins(1,3,4,5)P_4 with Ins(w,x,y,z)P_4 (see the Materials and methods section). Whereas Ins(1,3,4,5)P_4 (2.3×10^{-17} mol; 23 pl of 1 μM) activated eggs fully, the same dose of Ins(w,x,y,z)P_4 was inactive and a higher dose (2.1×10^{-16} mol; 21 pl of 10 μM) only partly effective (Fig. 1), which indicates a precise requirement for a correct distribution of the phosphates on the inositol ring. The effect of Ins(1,3,4,5)P_4 is dependent on

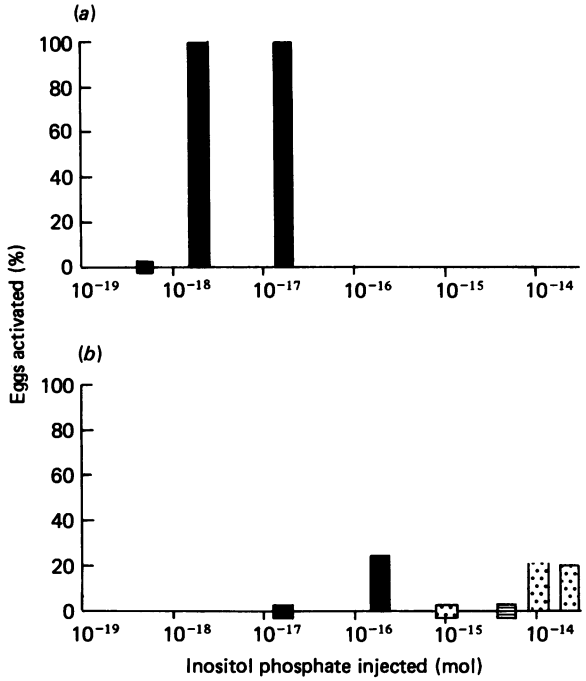

Fig. 1. Activation of sea urchin eggs by inositol phosphates co-injected with Ins(2,4,5)P_3

The data are the results of a number of independent experiments. All solutions contained 50 μM-Ins(2,4,5)P_3, and so in addition to the inositol phosphates shown, each egg received 5×10^{-16}–1.5×10^{-15} mol of Ins(2,4,5)P_3. (*a*) ■, Ins(1,3,4,5)P_4; (*b*) ■, Ins(w,x,y,z)P_4. Doses which produced no activation are shown by the histogram straddling the axis. On (*b*) are also shown Ins(1,3,4)P_3 ▤ and Ins(2,4,5)P_3 ⊡. For the latter two, only the highest concentrations tested that gave no activation are depicted, for simplicity.

extracellular Ca^{2+} by the same criteria as those used above, and, with the same reservations as above, we conclude that the activation by Ins(1,3,4,5)P_4 is likely to be a direct or indirect result of stimulated Ca^{2+} entry.

In conclusion, we should note that we have not yet directly demonstrated that Ins(2,4,5)P_3 is mobilizing Ca^{2+} in our experiments although, as discussed above, from known data it is the only probable explanation; exactly what Ins(2,4,5)P_3 is doing is not, strictly speaking, relevant to the data in Fig. 1, which clearly show the specificity and potency with which Ins(1,3,4,5)P_4 activates sea urchin eggs under these defined conditions. The remarkable requirement for Ins(2,4,5)P_3 to be present in order for Ins(1,3,4,5)P_4 to exert its effect raises the intriguing question of whether in other tissues also the promotion of Ca^{2+} entry is dependent on prior Ca^{2+} mobilization, or whether this is a unique feature of the acute response of these eggs. Experiments on parotid glands (Putney, 1977; see also Putney, 1986) do not support the idea that a raised Ca^{2+} level in the cytoplasm is a prerequisite for stimulated Ca^{2+} entry, although a priming mechanism stable for several minutes cannot be discounted. These parotid data could alternatively be interpreted to show that an empty intracellular Ca^{2+} store is the requirement for stimulated Ca^{2+} entry, and so the possibility that Ins(1,3,4,5)P_4 controls Ca^{2+} entry by modulating a mechanism of the sort proposed by Putney (1986) is just one of several possible modes of action of Ins(1,3,4,5)P_4 to be explored. It may be that in other tissues Ins(1,3,4,5)P_4 can act on its own, and if so then clearly Ins(1,4,5)P_3 kinase, by phosphorylating Ins(1,4,5)P_3 at resting or stimulated levels, will play a major role in cell physiology by its long-term control of cellular Ca^{2+}.

Whatever the possible subtleties and variations, our results show that Ins(1,3,4,5)P_4 joins Ins(1,4,5)P_3 and diacylglycerol as a second messenger generated from inositides. That this second messenger, Ins(1,3,4,5)P_4, is derived in turn from another second messenger, Ins(1,4,5)P_3, and that it may partly depend on the latter to exert its effect, is a fascinating new facet of the role of inositides in cell signalling.

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