Inositol 1,3,4,6-tetrakisphosphate mobilizes calcium in *Xenopus* oocytes with high potency

Isabel IVORRA,*§ Roy GIGG,† Robin F. IRVINE‡ and Ian PARKER*‖

*Laboratory of Cellular and Molecular Neurobiology, Department of Psychobiology, University of California, Irvine, CA 92717, U.S.A., †National Institute for Medical Research, Mill Hill, London NW7 1AA, U.K., and ‡AFRC Institute of Animal Physiology and Genetics Research, Cambridge Research Station, Babraham Hall, Cambridge CB2 4AT, U.K.

Injection of Ins(1,3,4,6)P_4 into *Xenopus* oocytes evoked Ca^{2+}-dependent membrane currents with a potency 5–10 times less than Ins(1,4,5)P_3, whereas Ins(1,3,4)P_3 and Ins(1,3,4,5,6)P_5 were almost ineffective. Responses to Ins(1,3,4,6)P_4 arose through liberation of intracellular Ca^{2+} and through entry of extracellular Ca^{2+}. These results, together with the observation that Ins(1,3,4,6)P_4 facilitated responses to Ins(1,4,5)P_3, suggests that both of these compounds may act on the same intracellular receptors.

INTRODUCTION

Ins(1,4,5)P_3 is now well established as a ubiquitous intracellular messenger molecule, which functions by increasing cytoplasmic free Ca^{2+} (Berridge & Irvine, 1989; Rana & Hokin, 1990). However, it also serves as a substrate for complex metabolic pathways, leading to the formation of a bewildering array of other inositol phosphates, the levels of many of which are sensitive to agonist stimulation (Berridge & Irvine, 1989; Shears, 1989*a*,*b*; Stephens & Downes, 1990). Definitive physiological roles have not yet been established for any of these compounds.

We have used the *Xenopus* oocyte as a convenient assay system with which to look for messenger functions of various inositol polyphosphates. The larger size (> 1 mm diameter) of this cell facilitates intracellular microinjection. Furthermore, the presence of Ca^{2+}-activated chloride channels in the plasma membrane (Miledi & Parker, 1984) allows voltage-clamp recording of chloride membrane currents to be used as a monitor of intracellular Ca^{2+}. Previous studies using the oocyte have examined the actions of inositol phosphates including Ins(1)P, Ins(1,4)P_2, Ins(4,5)P_2, Ins(1,4,5)P_3, Ins(1,2-(cyclic)-4,5)P_3, Ins(2,4,5)P_3, Ins(1,3,4)P_3, Ins(1,3,4,5)P_4 and Ins(1,3,4,5,6)P_5 (Oron *et al.*, 1985; Parker & Miledi, 1986, 1987*a*,*b*; Snyder *et al.*, 1988; Stith & Proctor, 1989; Parker & Ivorra, 1991). A complication in interpreting some of these data is that commercial preparations may be appreciably contaminated by other active inositol phosphates (Parker & Ivorra, 1991). Accordingly, the experiments described here were performed with highly purified compounds. The present paper is concerned principally with Ins(1,3,4,6)P_4, since we find that this InsP_4 isomer induces responses with a potency greater than that of Ins(1,3,4,5)P_4, and only a few times less than Ins(1,4,5)P_3 itself.

MATERIALS AND METHODS

Preparation of oocytes and electrophysiological recording

Recordings were made from oocytes of *Xenopus laevis*, which were treated with collagenase to remove enveloping cells. Details of the preparation of oocytes and of voltage-clamp recording procedures were as described by Sumikawa *et al.* (1989). Briefly, oocytes were penetrated by two microelectrodes for voltage-clamping, and membrane currents were recorded at a clamp potential of −60 mV. During recording, oocytes were continually superfused with Ringer's solution at room temperature (22–24 °C). The normal solution had the composition (in mM): NaCl, 120; KCl, 2; $CaCl_2$, 1.8; Hepes, 5, at pH about 7. High-Ca^{2+} solution contained 12 mM-$CaCl_2$. Ca^{2+}-free solution was made by omitting $CaCl_2$ and adding 1 mM-EGTA and 5 mM-$MgCl_2$.

Intracellular injections were made by pneumatic pressure pulses through a third micropipette (Sumikawa *et al.*, 1989). The volumes of fluid ejected were estimated by measuring the sizes of fluid droplets expelled with the pipette tip in the air. Compounds for injection were dissolved in an aqueous solution including 5 mM-Hepes (at pH 7.0) and 50 μM-EDTA. Ins(1,3,4,6)P_4 and Ins(1,4,5)P_3 were injected at concentrations of 100 μM, and Ins(1,3,4)P_3 and Ins(1,3,4,5,6)P_5 at concentrations of 1 mM. Unless otherwise noted, injection pipettes were inserted into the oocytes at a position about mid-way between the animal pole and the equator. In some experiments Ins(1,4,5)P_3 was released in the cytoplasm by applying flashes of u.v. light to an oocyte loaded with caged InsP_3 [*myo*-inositol 1,4,5-trisphosphate $P^{4,5}$-1-(2-nitrophenyl)ethyl ester], by using procedures described previously (Parker & Miledi, 1989; Parker, 1991). The photolysis light was focused as a spot of about 100 μm diameter, positioned on the vegetal hemisphere close to the equator. Injections of Ins(1,3,4,6)P_4 were made through a micropipette positioned in the centre of the light spot.

Inositol phosphates

Ins(1,4,5)P_3 was obtained from Calbiochem. Ins(1,3,4,5)P_4 was prepared by phosphorylation of Ins(1,4,5)P_3 by using a rat brain supernatant exactly as described by Irvine *et al.* (1986). It was finally purified by h.p.l.c. (Irvine *et al.*, 1986) and, after removal of P_i on Bio-Rad AG 1-X 200–400 mesh (formate form) anion-exchange resin as in Irvine *et al.* (1986), was desalted by repeated freeze-drying to remove the ammonium formate. Analysis by ionophoresis (Seiffert & Agranoff, 1965) showed < 1% contamination by P_i as detected by a molybdate spray (Clarke & Dawson, 1981). Ins(1,3,4)P_3 was prepared from Ins(1,3,4,5)P_4

Abbreviations used: InsP_3, InsP_4, InsP_5, inositol tris-, tetrakis- and pentakis-phosphates respectively, with assignment of phosphate locants where appropriate; caged InsP_3, *myo*-inositol 1,4,5-trisphosphate $P^{4,5}$-1-(2-nitrophenyl)ethyl ester.

§Present address: Departmento de Fisiologia, Facultad de Medicine, Universidad de Alicante, Alicante, Spain.

‖To whom correspondence and reprint requests should be addressed.

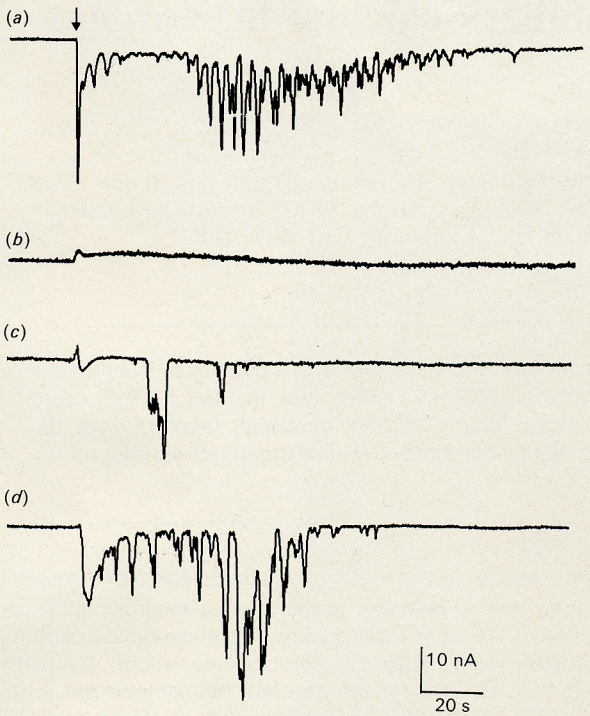

Fig. 1. Membrane currents evoked by intracellular injections of Ins(1,3,4,6)P_4 and other inositol polyphosphates

Traces show currents at a clamp potential of −60 mV; downward deflections correspond to inward currents. Injections were made by pneumatic pressure pulses at the times marked by the arrow. (*a*) Injection of 4.5 fmol of Ins(1,3,4,6)P_4. (*b*) Injection of 1000 fmol of Ins(1,3,4)P_3. (*c*) Injection of 600 fmol of Ins(1,3,4,5,6)P_5. (*d*) Injection of 4 fmol of Ins(1,4,5)P_3. Records in (*a*) and (*b*) are from a single oocyte, and those in (*c*) and (*d*) are from different oocytes. The transient outward currents immediately after the injections in (*b*) and (*c*) are probably artefacts arising from the relatively large volumes of fluid injected.

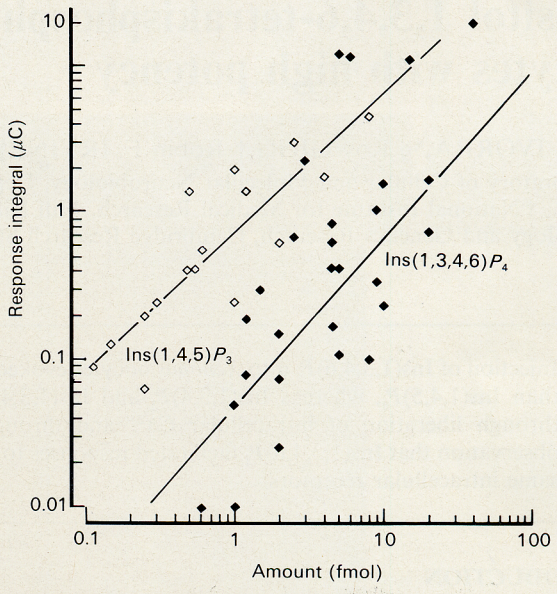

Fig. 2. Dose–response relationships for responses evoked by injections of Ins(1,4,5)P_3 (◇) and Ins(1,3,4,6)P_4 (◆)

Horizontal axis shows the amounts of each compound injected (in fmol). Vertical axis shows the integral under the current responses (i.e. charge displacement) in μC. Data are from seven oocytes, three of which were injected with both compounds. Regression lines are fitted to the data.

exactly as described by Irvine *et al.* (1986) and finally purified by h.p.l.c. as described above for Ins(1,3,4,5)P_4 with, as an internal marker, [^{32}P]Ins(1,4,5)P_3 prepared as in Letcher *et al.* (1990). It was desalted as for Ins(1,3,4,5)P_4, and quantitative analysis by ionophoresis revealed that it contained 17 % (as % phosphorus) P_i. Ins(1,3,4,5,6)P_5 was obtained from Calbiochem, and was further purified by h.p.l.c. as described by Stephens *et al.* (1991).

Ins(1,3,4,6)P_4 was synthesized chemically by deprotection of the crystalline (m.p. 109 °C) analytically pure octa-(2-cyanoethyl) ester of 2,5-di-*O*-benzyl-*myo*-inositol 1,3,4,6-tetrakisphosphate (Desai *et al.*, 1991) and treated by preparative ionophoresis in pyridine/acetic acid on Whatman no. 1 paper (Dawson & Clarke, 1972) to remove an impurity, which was probably a partially deprotected precursor [a mono-benzyl ether of Ins(1,3,4,6)P_4]. The Ins(1,3,4,6)P_4 was detected by a phosphorus spray (Clarke & Dawson, 1981), and eluted from the paper with water. Ionophoretic analysis indicated that there was < 0.5 % contamination by inositol phosphates other than InsP_4. Contamination by other isomers of InsP_4 was estimated to be < 0.1 %.

RESULTS

Oscillatory responses evoked by Ins(1,3,4,6)P_4

Injection of Ins(1,3,4,6)P_4 into *Xenopus* oocytes evoked a series of oscillatory inward membrane currents (Fig. 1*a*), closely resembling the currents evoked by Ins(1,4,5)P_3 (Fig. 1*d*; and see Oron *et al.*, 1985; Parker & Miledi, 1986, 1987*b*). The currents reversed direction at −31 mV (mean value from three oocytes), which corresponds to the chloride equilibrium potential in the oocyte (Kusano *et al.*, 1982). Furthermore, they were abolished after intracellular injection of the Ca^{2+}-chelating agent EGTA, but persisted when Ca^{2+} was removed from the bathing solution (e.g. Fig. 3). Thus the currents almost certainly arise through the liberation of Ca^{2+} from intracellular stores, which then activates Ca^{2+}-dependent chloride channels in the oocyte membrane (Miledi & Parker, 1984).

The responses to Ins(1,3,4,6)P_4 usually comprised two components: a transient inward current 'spike' beginning almost immediately after injection, followed by a more slowly rising and decaying series of oscillatory inward currents (Fig. 1*a*). The relative sizes of these components varied greatly between different oocytes, and with different doses of Ins(1,3,4,6)P_4 in the same oocyte. Increasing doses evoked larger and more prolonged oscillatory currents, whereas the size of the initial 'spike' usually increased little.

Relative potencies of Ins(1,3,4,6)P_4 and other inositol polyphosphates

To compare the potency of Ins(1,3,4,6)P_4 to evoke membrane currents in relation to Ins(1,4,5)P_3, oocytes were injected with various amounts of each, so as to be able to construct dose–response relationships. Injections were made at a consistent location roughly half-way between the animal pole and equator, to minimize errors due to the variation in sensitivity across the surface of the oocyte (Berridge, 1988). Because of the fluctuating nature of the responses to each compound, and because the responses became longer as well as larger with increasing doses, it seemed more appropriate to quantify the currents by their integrals (i.e. total charge displacement), rather than by measuring peak sizes. A double-logarithmic plot of dose–response relationships derived in this way is shown in Fig. 2. Regression lines are fitted to the pooled data for Ins(1,4,5)P_3 and

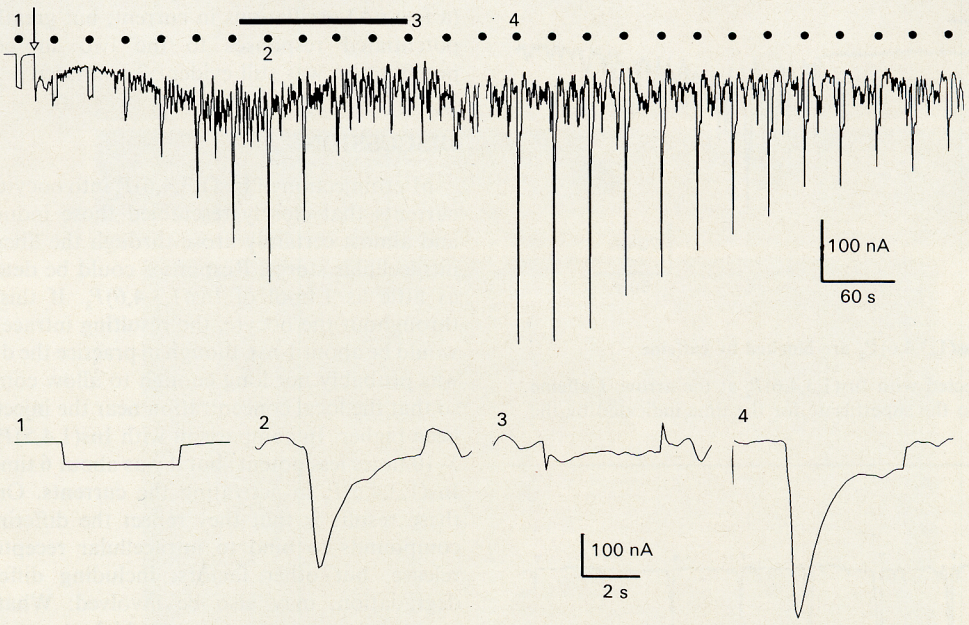

Fig. 3. Ins(1,3,4,6)P_4 induces entry of extracellular Ca^{2+}, in addition to mobilizing intracellular Ca^{2+}

Upper trace shows membrane current recorded at a holding potential of −60 mV, with periodic steps to −130 mV as marked by the dots. The oocyte was injected with 50 fmol of Ins(1,3,4,6)P_4 where marked by the arrow. The bathing solution contained 12 mM-Ca^{2+}, except when indicated by the bar, during which time the solution was exchanged for zero-Ca^{2+} Ringer. Lower traces show selected responses to hyperpolarization at a faster sweep speed; numbers correspond to the marked stimuli in the upper record.

Ins(1,3,4,6)P_4. The slopes of these lines differ slightly, so that it is not possible to give a single value for the relative potencies. However, the threshold amount of Ins(1,3,4,6)P_4 required to evoke a response was about 10 times greater than the corresponding amount of Ins(1,4,5)P_3, whereas with the largest responses obtained in this study the difference in potency was only by a factor of about 5.

The data in Fig. 2 were obtained by injecting Ins(1,4,5)P_3 and Ins(1,3,4,6)P_4 into oocytes obtained from the same three donor frogs, so that variations in sensitivity between donors would not affect the assessment of their relative potencies. In a previous, more extensive, study of responses evoked by Ins(1,4,5)P_3, we obtained a slightly lower estimate of potency (Parker & Ivorra, 1991); the mean dose required to evoke a response with integral of 1 μC was 6.1 fmol, as compared with 1.5 fmol in the present study. If that value is used for comparison, Ins(1,3,4,6)P_4 is less potent than Ins(1,4,5)P_3 by only a factor of about 2.

A comparison between Ins(1,3,4,6)P_4 and Ins(1,3,4,5)P_4 similarly was made by injecting various amounts of both compounds into five oocytes obtained from a single frog. The relative potency was estimated from the ratios of the (interpolated) amounts required to evoke a response in each oocyte with an integral of 1 μC. On average, the amount of Ins(1,3,4,5)P_4 required was 6.6 (±2.8; S.E.M.) times greater than for Ins(1,3,4,6)P_4.

We also examined the sensitivities of oocytes to Ins(1,3,4)P_3 and Ins(1,3,4,5,6)P_5, which may be, respectively, the precursor and product of Ins(1,3,4,6)P_4 metabolism in the cell (Shears, 1989*b*). Ins(1,3,4)P_3 was almost ineffective to evoke oscillatory current responses. Fig. 1(*b*) shows that injection of 1000 fmol of Ins(1,3,4)P_3 evoked only a slight (2 nA) outward current, even though injection of 4.5 fmol of Ins(1,3,4,6)P_4 into the same oocyte gave a prominent oscillatory response (Fig. 1*a*). Measurements on ten oocytes showed that some failed to respond to doses of a few thousand fmol, whereas others gave small oscillatory currents with several hundred fmol of Ins(1,3,4)P_3 (Parker & Ivorra, 1991). Ins(1,3,4,5,6)P_5 was also of very low potency. Some oocytes showed small oscillatory responses to several hundred fmol (Fig. 1*c*), whereas others showed no response, or a small transient inward current, to doses larger than 1000 fmol.

Ins(1,3,4,6)P_4 induces Ca^{2+} entry

Ins(1,4,5)P_3 induces an entry of extracellular Ca^{2+} into the oocyte, as well as mobilizing the release of sequestered Ca^{2+} (Parker & Miledi, 1987*a*). We were therefore interested to determine whether Ins(1,3,4,6)P_4 also induces Ca^{2+} entry. This was monitored by applying repetitive hyperpolarizing steps to increase the driving force for Ca^{2+} across the membrane. As shown in Fig. 3, a step from −60 to −130 mV evoked only passive 'leakage' currents in the resting oocyte. However, identical steps applied after injecting Ins(1,3,4,6)P_4 evoked in addition a transient inward (T_{in}) current that grew progressively over several minutes. This current depended on the presence of Ca^{2+} in the bathing solution, since it was abolished after removal of extracellular Ca^{2+} but subsequently recovered when Ca^{2+} was replaced.

T_{in} currents like that in Fig. 3 have previously been described during activation of the oocyte by agonists that stimulate phosphoinositide turnover (Parker *et al.*, 1985) and after injection of Ins(1,4,5)P_3 (Parker & Miledi, 1987*a*). They arise through the opening of a class of Ca^{2+} channels in the plasma membrane, so that the increased driving force on hyperpolarization leads to an influx of Ca^{2+} into the cytoplasm and the subsequent activation of a transient chloride current (Parker & Miledi, 1987*a*). Thus Ins(1,3,4,6)P_4 appears to act on the Ca^{2+}-influx channels in a manner similar to Ins(1,4,5)P_3.

Caffeine blocks responses to Ins(1,3,4,6)P_4

The oscillatory currents activated by Ins(1,4,5)P_3 in the oocyte are decreased or blocked by millimolar concentrations of caffeine, which acts by inhibiting the liberation of intracellular Ca^{2+} rather

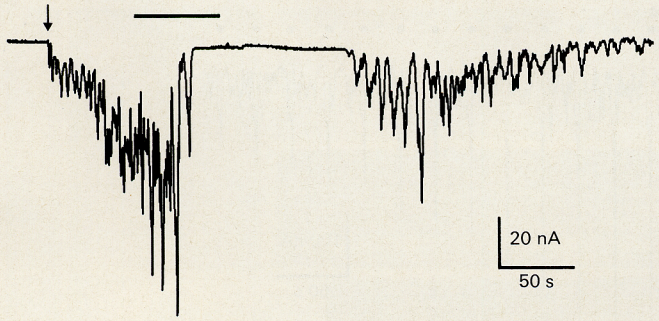

Fig. 4. Responses to Ins(1,3,4,6)P_4 are blocked by caffeine

The oocyte was injected with Ins(1,3,4,6)P_4 at the arrow. Caffeine (5 mM) was added to the superfusate for the time indicated by the bar.

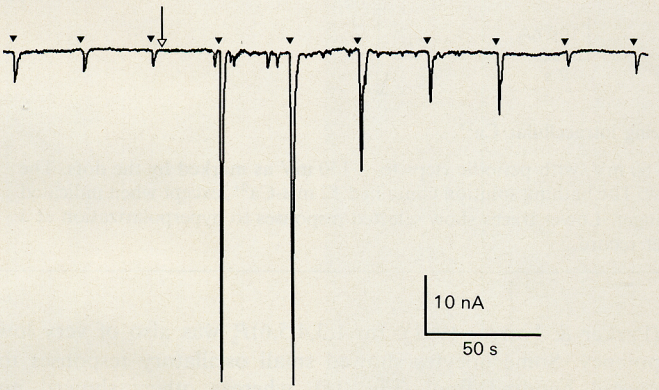

Fig. 5. Ins(1,3,4,6)P_4 facilitates responses evoked by photoreleased Ins(1,4,5)P_3

The record is from an oocyte that was loaded with caged InsP_3, and was stimulated by identical flashes of u.v. light at 30 s intervals (marked by arrowheads). An intracellular injection of about 0.3 fmol of Ins(1,3,4,6)P_4 was given at the arrow.

than by blocking the Ca^{2+}-activated chloride channels (Parker & Ivorra, 1990*a*). Fig. 4 shows that caffeine has a similarly powerful blocking action on responses evoked by injection of Ins(1,3,4,6)P_4.

Potentiation of responses to Ins(1,4,5)P_3

Light-flash photolysis of caged Ins(1,4,5)P_3 loaded into oocytes provides a means by which precisely controlled amounts of Ins(1,4,5)P_3 can be released into the cytoplasm. By use of this technique, we have demonstrated that a threshold amount of Ins(1,4,5)P_3 is required before any Ca^{2+} is liberated from intracellular stores (Parker & Miledi, 1989; Parker & Ivorra, 1990*b*). Associated with this threshold phenomenon, a marked facilitation of the response to a light flash is seen when it is preceded by injection of a low dose of Ins(1,4,5)P_3 (Parker & Miledi, 1989). The question thus arose of whether injection of Ins(1,3,4,6)P_4 would similarly facilitate a subsequent response to photo-release of Ins(1,4,5)P_3.

An experiment to test this point is shown in Fig. 5. A regular train of light flashes was applied to an oocyte previously loaded with caged InsP_3. The intensity and duration of the flashes were set so that they evoked small and roughly constant membrane current responses. After the third flash, about 0.3 fmol of Ins(1,3,4,6)P_4 was injected into the oocyte, through a pipette centred in the photolysis light spot. This dose evoked only small (a few nA) oscillations in current, but greatly (15-fold or more) potentiated responses to the two subsequent flashes, and potentiation was still evident 2 min later.

DISCUSSION

Microinjection of Ins(1,3,4,6)P_4 into oocytes evoked oscillatory currents that closely resembled those induced by Ins(1,4,5)P_3, and almost certainly arose through the liberation of Ca^{2+} from intracellular stores. Responses could be detected to injection of as little as 1 fmol of Ins(1,3,4,6)P_3. If this were to distribute throughout the oocyte, the resulting intracellular concentration would be about 1 nM, though in practice the duration of responses was probably not long enough to allow complete equilibration, so that the local concentration near the injection site would have been higher. In comparison with Ins(1,4,5)P_3, Ins(1,3,4,6)P_4 was 5–10 times less potent; but it was about 6 times more potent than Ins(1,3,4,5)P_4 in activating the currents. One interpretation of these results is that they reflect the differing abilities of these compounds to bind to intracellular receptors mediating Ca^{2+} release, but other factors, including differences in rates of degradation, may also be involved. Whatever the case, the integrated current response which we used as the basis for comparison should provide a good reflection of the physiological activities of the compounds.

As well as causing liberation of intracellular Ca^{2+}, Ins(1,3,4,6)P_4 showed other properties that closely mimicked those of Ins(1,4,5)P_3. Specifically: (i) it facilitated near-threshold responses to photo-released Ins(1,4,5)P_3, (ii) it activated an influx of extracellular Ca^{2+} across the plasma membrane, and (iii) the oscillatory responses to Ins(1,3,4,6)P_4 were reversibly blocked by caffeine. All of these results may be most simply explained if Ins(1,3,4,6)P_4 binds to the same intracellular receptors that mediate the actions of Ins(1,4,5)P_3, albeit with a slightly lower affinity. Such a suggestion is not unreasonable, given that the spatial distribution of the phosphate groups in Ins(1,3,4,6)P_4 is similar to that in Ins(1,4,5)P_3 and Ins(2,4,5)P_3 (Irvine *et al.*, 1988).

The physiological significance of these observations remains an open question. Ins(1,3,4,6)P_4 increases slowly after stimulation in some cells (for references see Stephens *et al.*, 1991), but, apart from chick erythrocytes, its mass levels are not known; in the latter case the resting concentration is about 12 μM and increases to about 28 μM after stimulation (Stephens *et al.*, 1991). Were similar resting levels to be found in the cytosol of *Xenopus* oocytes, one would expect them to be perpetually activated. However, the same applies to Ins(1,4,5)P_3. That is, measured resting levels are frequently in excess of 1–2 μM (e.g. Nogimori *et al.*, 1990; Challiss *et al.*, 1990), suggesting that inositol polyphosphates may be compartmentalized within the cell. Ins(1,3,4,6)P_4 is also an obligatory intermediate in the synthesis of InsP_6, at least in *Dictyostelium* (Stephens & Irvine, 1990), and the full significance of its surprising potency in mobilizing Ca^{2+} in *Xenopus* oocytes must await further information on its distribution and susceptibility to agonist activation in other tissues.

We thank A. Letcher and D. Lander for help in purifying the inositol phosphates, and L. Stephens for the InsP_5. This work was supported by grant GM 39831 from the U.S. Public Health Services to I.P.

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Received 26 September 1990/6 November 1990; accepted 13 November 1990