Vol. 146, No. 1, 1987 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
July 15, 1987 Pages 284-290

INOSITOL(1,3,4,5)TETRAKISPHOSPHATE-INDUCED ACTIVATION OF SEA URCHIN EGGS REQUIRES THE PRESENCE OF INOSITOL TRISPHOSPHATE

R.F. Irvine* and R.M. Moor+

Dept. of Biochemistry* and Molecular Embryology+,
AFRC Institute of Animal Physiology and Genetics Research,
Babraham, Cambridge CB2 4AT, U.K.

Received June 3, 1987

We have earlier reported that Inositol(1,3,4,5)tetrakisphosphate microinjection will activate eggs of the sea urchin Lytechinus variegatus provided that it is co-injected with inositol(2,4,5)trisphosphate (Irvine and Moor, Biochem. J. 240, 917-920, 1986). Here we extend these observations to show that (1) inositol(1,3,4,5,6)pentakisphosphate is a partial agonist in this assay and (2) the requirement for the presence of inositol(2,4,5)trisphosphate cannot be bypassed by raised, but sub-threshold, Ca^{2+} concentrations. A mechanism for the proposed stimulation of Ca^{2+} entry into the cell requiring both inositol tris- and tetrakisphosphates is presented.

Our recent experiments (1) show that fertilization membranes are raised in the eggs of the sea urchin Lytechinus variegatus by the microinjection of Inositol tetrakisphosphate [Ins(1,3,4,5)P_4]. This effect is specific for Ins(1,3,4,5)P_4 which is active at submicromolar concentrations, and its effectiveness depends both upon the presence of extracellular Ca^{2+} and on the co-injection of the Ca^{2+}-mobilising inositol phosphate, Ins(2,4,5)P_3. These data have led us to propose that Ins(1,3,4,5)P_4 regulates Ca^{2+} entry into cells, but we could only speculate on the underlying mechanism (1). Here we have extended those studies to examine another naturally-occurring inositol phosphate, Ins(1,3,4,5,6)P_5, and have also examined more closely the requirement for co-injection of an $InsP_3$.

MATERIALS AND METHODS

Ionomycin and Ins(1,3,4,5,6)P_5 were obtained from Calbiochem; the latter was purified to 99.9% purity by ionophoresis (2). Other inositol

Abbreviations: $InsP_3$, $InsP_3$, $InsP_4$ and $InsP_5$ Inositol bis-, tris-, tetrakis-, and pentakis-phosphates respectively, with isomeric numbering as appropriate.

0006-291X/87 $1.50

phosphates were prepared as in ref. 1. Microinjection of eggs of Lytechinus variegatus was as in ref. 1, and the quantification of egg activation is also described in that paper. Note that all experiments described in this paper (except those in Fig. 1) were carried out between September and November, 1986, before the occurence of the change in the physiology of eggs of Lytechinus variegatus which we have attributed to a possible difference in egg maturity (R.F. Irvine and R.M. Moor submitted for publication).

Ins(1,3,4,5)$\underline{P}_4$ phosphatase was assayed as in Batty et al. (3), and the products were examined in detail by hplc (4) while routine separation of inositol phosphates for the assays was as in ref. 5. [^{3}H]Ins(1,3,4,5)$\underline{P}_4$ (sp. act 1Ci/mmole) was obtained from Amersham (U.K.). [^{3}H]Ins(1,3,4,5,6)$\underline{P}_5$ (sp. act approx. 10 mCi/mmole), prepared from turkey erythrocytes labelled with [^{3}H]myo-inositol, was a generous gift from Dr. L. Stephens of Smith, Kline and French Research, Welwyn, U.K.

RESULTS AND DISCUSSION

Ins(1,3,4,5,6)$\underline{P}_5$

As Ins(1,3,4,5)$\underline{P}_4$ was much more active than a random mixture of Ins$\underline{P}_4$ isomers in this particular bioassay (1), we thought it would be interesting to examine Ins(1,3,4,5,6)$\underline{P}_5$, especially since Ins$\underline{P}_5$ (of unknown configuration, but probably the 1,3,4,5,6 isomer) has been found in several mammalian tissues (6-9). On its own Ins(1,3,4,5,6)$\underline{P}_5$ was inactive at millimolar concentrations, but when co-injected with 50 μM Ins(2,4,5)$\underline{P}_3$ (see ref. 1) it activated eggs of Lytechninus variegatus at micromolar levels (1% cell volume). This activation assay is only semi-quantitative (1), so several direct comparison experiments were performed to compare Ins(1,3,4,5,6)$\underline{P}_5$ with Ins(1,3,4,5,6)$\underline{P}_4$ at the same, or slightly different, doses. Table 1 summarises three such experiments. At 20 n molar, Ins(1,3,4,5)$\underline{P}_4$ and Ins(1,3,4,5,6)$\underline{P}_5$ were indistinguishable in that they activated about half the eggs, but at 0.1 μmolar Ins(1,3,4,5)$\underline{P}_4$ was unequivocally more active than 20 n molar Ins(1,3,4,5,6)$\underline{P}_5$. We conclude that in this assay the two compounds are of very similar efficacy.

Many cells however, possess an active Ins(1,3,4,5)$\underline{P}_4$-5-phosphatase(3, 10, 11). If sea urchin eggs have such an activity, then this could exaggerate the potency of Ins(1,3,4,5,6)$\underline{P}_5$ if that compound were resistant to the enzyme. We therefore prepared homogenates of eggs of Lytechinus variegatus in Tris maleate buffer pH 7.0, 0.25 M sucrose, and examined their phosphatase activity. Ins(1,3,4,5)$\underline{P}_4$ was hydrolysed (Fig. 1) and Ins$\underline{P}$, Ins$\underline{P}_2$, and Ins$\underline{P}_3$ were all detectable within 20 mins. When the Ins$\underline{P}_3$ formed was examined by hplc (ref 4) a single Ins$\underline{P}_3$ with chromatographic properties identical to Ins(1,3,4)$\underline{P}_3$ was found (results not shown). This allows us the conclusion that eggs of Lytechinus variegatus have an active Ins(1,3,4,5)$\underline{P}_4$-5-phosphatase. By calculating the approximate dilution of the egg cytosol in the homogenate, and assuming first order rate kinetics, we estimate that the half-life of Ins(1,3,4,5)$\underline{P}_4$ at low concentrations

Table 1. Activation of eggs of Lytechinus variegatus by Ins(1,3,4,5)$\underline{P}_4$ and Ins(1,3,4,5,6)$\underline{P}_5$ when co-injected with Ins(2,4,5)$\underline{P}_3$

	Ins(1,3,4,5)$\underline{P}_4$	Ins(1,3,4,5,6)$\underline{P}_5$
Expt. 1	1 μM 9/10	1 μM 9/12
Expt. 2	0.02 μM 6/12	0.02 μM 8/14
Expt. 3	0.1 μM 13/13	0.02 μM 5/14

Results record three separate experiments in which direct comparison of Ins(1,3,4,5)$\underline{P}_4$ and Ins(1,3,4,5,6)$\underline{P}_5$ injections were made, at the same concentrations (Expts. 1 and 2) or different concentrations (Expt 3). Numbers represent the number of eggs raising a full fertilization membrane out of the total number of eggs microinjected. Volumes injected, 23 pl (Expt. 1), 6 pl (Expt. 2), 7 pl (Expt. 3).

(less than 0.1 μM see ref. 11) is probably less than one minute in an intact egg; we emphasize however, that this is only an approximate number.

At a similar concentration (see Methods - we do not know the exact specific activity of the [^{3}H]Ins$\underline{P}_5$, but the concentration in these hydrolase experiments is probably below the micromolar concentrations injected to activate the eggs), Ins(1,3,4,5,6)$\underline{P}_5$ was not detectably hydrolysed (Fig. 1). We cannot from these data prove that Ins(1,3,4,5)$\underline{P}_4$-5-phosphatase does not hydrolyse Ins(1,3,4,5,6)$\underline{P}_5$, but we can conclude that at low concentrations this inositol phosphate is a poor substrate for the enzyme and that its half-life in these eggs is therefore probably longer than that of Ins(1,3,4,5)$\underline{P}_4$. Therefore, given that a microinjected inositol phosphate has to remain in the cell for at least 15 sec. for full activation of the fertilization membrane (see ref. 12), it is probable that Ins(1,3,4,5)$\underline{P}_4$ is a better agonist than Ins(1,3,4,5,6)$\underline{P}_5$ because the action of Ins$\underline{P}_5$ is almost certainly extended by its resistance to phosphatase digestion.

Whatever its relative efficacy, the suggestion that Ins(1,3,4,5,6)$\underline{P}_5$ may be a partial agonist has important implications given its high resting levels in some tissues (e.g. refs. 6-9); either it is there to activate constitutively the process normally controlled by Ins(1,3,4,5)$\underline{P}_4$ (i.e. under our interpretation, it hands over the control of Ca^{2+} entry to Ins(1,4,5)$\underline{P}_3$, see ref. 1 and below), or we suggest a more likely interpretation is, that endogenous Ins(1,3,4,5,6)$\underline{P}_5$ does not have access to

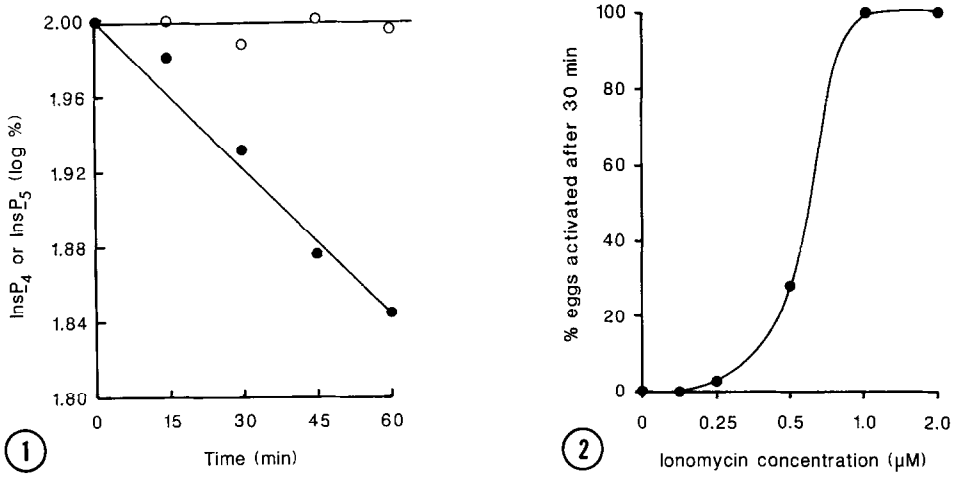

Fig. 1. Hydrolysis of inositol phosphates by homogenates of eggs of Lytechinus variegatus

Results are typical of three separate experiments, and plot the hydrolysis of [^{3}H]Ins(1,3,4,5) (-●-) as judged by the formation of total hydrolysis products. (-o-), parallel incubations using [^{3}H]Ins(1,3,4,5,6)$\underline{P}_5$. For details see text.

Fig. 2. Activation of eggs of Lytechinus variegatus by ionomycin

Eggs were incubated in batches in artificial sea water (ref. 1) containing increasing concentrations of ionomycin, and activated eggs (those with raised fertilization envelopes) counted after 30 min. These data are the combined results of two separate experiments, and similar results were obtained in two other experiments.

Ins(1,3,4,5)$\underline{P}_4$'s site of action and so it is not likely to be freely soluble in the cytosol. If it were, then the controlled and rapid metabolism of Ins(1,3,4,5)$\underline{P}_4$ in the presence of such high resting Ins(1,3,4,5,6)$\underline{P}_5$ levels (6-9) makes little biological sense.

Effect of Ca^{2+} versus Ins(2,4,5)P_3 injections

We were not able to tell previously whether the sensitization of eggs to Ins(1,3,4,5)$\underline{P}_4$ caused by Ins(1,4,5)$\underline{P}_3$ co-injection was due to the Ins(2,4,5)$\underline{P}_3$ or to the Ca^{2+} that is mobilised (1), though we did argue from earlier experiments of Putney (13 and see also ref. 14) that it is not likely that Ins(1,3,4,5)$\underline{P}_4$ requires a high resting level of Ca^{2+} to work. Nevertheless, we have conducted an extensive series of experiments to investigate this further, by asking the question: can we sensitize the eggs to Ins(1,3,4,5)$\underline{P}_4$ (on its own) by raising the Ca^{2+} in them with an ionophore?

Batches of eggs were titrated with increasing ionomycin concentrations to raise fertilization envelopes. Fig. 2 shows the combined results of two

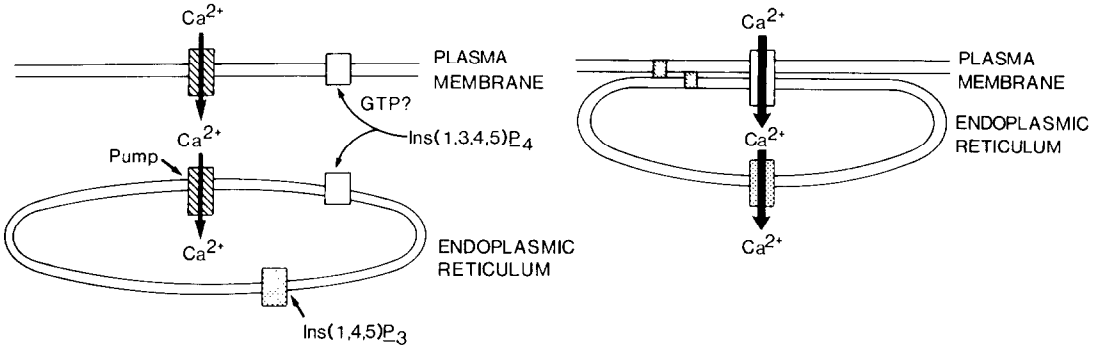

Fig. 3. Proposed mechanism by which Ins(1,3,4,5)P_4 could stimulate Ca^{2+} influx by a process requiring Ins(1,4,5)P_3

Before Ins(1,3,4,5)P_4 action, the Ca^{2+} enters the e.r. by an indirect route of unknown mechanism, but one that certainly involves a Ca^{2+} pump in the E.R. (see ref. 19). We propose that Ins(1,3,4,5)P_4 then causes a coupling of the two membranes, so that Ca^{2+} entry into the E.R. is direct through a channel analogous to that formed in a gap junction. Evidence from the laboratories of Dawson (ref. 21) and Gill (D. Gill pers. commun.) has suggested a GTP-mediated linking of Ca^{2+} pools in the cell, and thus this Figure also contains a suggestion of the possibility that GTP may participate in the Ins(1,3,4,5)P_4-induced process. Given that some of these effects of GTP involve intracellular membranes, it may be that the concept illustrated above, where InsP_4 couples membranes together, may also extend to membranes other than the plasma membrane and the endoplasmic reticulum.

such experiments where the eggs were very similar in each batch, but in every experiment a titration with ionomycin was done afresh to find the correct threshold dose. In the experiments in Fig. 1, 0.3 μM ionomycin would activate only 30% of the eggs whereas 1 μM would activate them all over 30 min. At 2 uM ionomycin, all eggs raised envelopes in less than 10 mins [N.B. If EGTA was substituted for Ca^{2+} in the sea water, 2 μM ionomycin still raised envelopes as found by e.g. Steinhardt and Eppel (15) and Schmidt et al. (16); the envelopes were less distinct, but clearly visible, confirming that we are able to see envelopes if they are raised in the absence of extracellular Ca^{2+}].

The eggs of interest in these experiments are those that at 0.3 uM ionomycin have not activated. We found that for at least an hour these eggs would activate if (a) the ionomycin was raised to 1 μM (activation was then full in < 5 mins) (b) 1 % cell volume of 50 μM Ins(2,4,5)P_3 and 1 μM Ins(1,3,4,5)P_4 (see ref. 1) were injected (c) in one experiment, simply raising the Ca^{2+} in the sea water from 10 mM to 30 mM activated 30% of the eggs (controls with the 30 mM Ca^{2+} but no ionomycin did not activate). We interpret these experiments as showing us that those eggs not activated

immediately by sub-threshold ionomycin, have a raised Ca^{2+} level which is very close to that which will activate them. In three separate experiments we injected 31 such eggs with 1% cell volume of 100 μM Ins(1,3,4,5)$\underline{P}_4$ (hplc pure) and not one egg showed any signs of raising a fertilization envelope.

From these experiments we infer that we cannot sensitize sea urchin eggs to Ins(1,3,4,5)$\underline{P}_4$ by raising their Ca^{2+}, and thus it must be the presence of an Ins$\underline{P}_3$ that Ins(1,3,4,5)$\underline{P}_4$ requires, to exert its effect; taken in conjunction with our earlier data (ref. 1) we believe that the simplest interpretation is that Ins(1,4,5)$\underline{P}_3$ and Ins(1,3,4,5)$\underline{P}_4$ are together regulating Ca^{2+} homoeostasis.

There are a number of ways this could happen, but one that we find attractive is the suggestion (ref. 1) that Ins(1,3,4,5)$\underline{P}_4$ is modulating the well-documented mechanism by which Ca^{2+} can enter the Ins(1,4,5)$\underline{P}_3$-sensitive Ca^{2+} pool from outside the cell (17, 18 and see ref. 19 for discussion). Fig. 3 shows a schematic representation of our proposal in which Ins(1,3,4,5)$\underline{P}_4$ joins the plasma membrane and endoplasmic reticulum functionally in a manner analogous to a gap juction (which can carry Ca^{2+}, see ref. 20). We regard Fig. 3 as a possible explanation for our clear experimental demonstration of an absolute requirement for an Ins$\underline{P}_3$ to be present, in order that Ins(1,3,4,5)$\underline{P}_4$ can exert its biological effect.

Acknowledgements

We are very grateful to Dr. Len Stephens for his gift of [^{3}H]Ins(1,3,4,5,6)$\underline{P}_5$, and to Andrew Letcher and David Lander for their help in the preparation of the inositol phosphates.

REFERENCES

1. Irvine, R.F. and Moor, R.M. (1986). Biochem. J. 240, 917-920.
2. Seiffert, U.B. and Agranoff, B.W. (1985). Biochim. Biophys. Acta. 98, 574-581.
3. Batty, I.R., Nahorski, S.R. and Irvine, R.F. (1985). Biochem. J. 232, 211-215.
4. Irvine, R.F., Anggard, E.E., Letcher, A.J. and Downes, C.P. (1985). Biochem. J. 229, 505-511.
5. Wreggett, K.A. and Irvine, R.F. (1987). Biochem. J. (in press).
6. Helslop, J.P., Irvine, R.F., Tashjian, A.H. and Berridge, M.J. (1985). J. Exp. Biol. 119, 395-401.
7. Morgan, R.U., Chang, J.P. and Katt, K.J. (1987). J. Biol. chem. 262, 1166-1171.
8. Tilly, B.C., van Paridon, P.A., Verlaan, I., Wirtz, K.W.A., de Laat S.W. and Moolemaar, W.H. (1987). Biochem. J. 244, 129-135.
9. Jackson, T.R., Hallam, T.J., Downes, C.P. and Hanley, M.P. (1987). EMBO J. 6, 49-54.
10. Biden, T.J. and Wollheim, C.B. (1986). J. Biol. Chem. 261, 11931-11934.
11. Connolly, T.M., Bansal, V.S., Bross, T.E., Irvine, R.F. and Majerus, P.W. (1987). J. Biol. Chem. 262, 2141-2149.

12. Swann, K. and Whitaker, M. (1986). J. Cell. Biol. 103, 2333-2342.
13. Putney, J.W. Jnr. (1977). J. Physiol. (London) 268, 139-149.
14. Pozzan, T., diVirgilio, F., Vicentini, L.M. and Meldolesi, J. (1986). Biocehm. J. 234, 547-553.
15. Steinhardt, R.A. and Epel, D. (1974). Proc. Nutn. Acad. Sci. 71, 1915-1919.
16. Schmidt, T., Patton, C. and Epel, D. (1982). Develop. Biol. 90, 284-290.
17. Casteels, R. and Droogmans, G. (1981). J. Physiol. (London). 317, 263-269.
18. Poggioli, J. and Putney, J.W. Jnr. (1982). Pflügers Archiv. 392, 239-243.
19. Putney, J.W. Jnr. (1986). Cell Calcium. 7, 1-12.
20. Dunlap, K., Takeda, T. and Brehm, P. (1987). Nature 325, 60-62.
21. Dawson, A.P., Hills, G. and Comerford, J.G. (1987). Biochem. J. 244, 87-92.