Inositol 1,3,4,5-tetrakisphosphate causes release of Ca^{2+} from permeabilized mouse lymphoma L1210 cells by its conversion into inositol 1,4,5-trisphosphate

Peter J. CULLEN,*§ Robin F. IRVINE,† Bjørn K. DRØBAK‡ and Alan P. DAWSON*

*School of Biological Sciences, University of East Anglia, Norwich NR4 7TJ, U.K., †AFRC Institute of Animal Physiology and Genetics Research, Babraham, Cambridge CB2 4AT, U.K., and ‡Department of Cell Biology, John Innes Institute, Colney Lane, Norwich NR4 7UH, U.K.

Addition of $\text{Ins}(1,3,4,5)\text{P}_4$ at micromolar concentrations causes release of Ca^{2+} from electroporated L1210 cells, but not from digitonin-permeabilized cells. This was shown to be due to its conversion into $\text{Ins}(1,4,5)\text{P}_3$, because only the electroporated cells convert $\text{Ins}(1,3,4,5)\text{P}_4$ into $\text{Ins}(1,4,5)\text{P}_3$. Thus electroporation appears to activate or expose an $\text{Ins}(1,3,4,5)\text{P}_4$ 3-phosphatase.

INTRODUCTION

A role for $\text{Ins}(1,4,5)\text{P}_3$ in controlling the release of Ca^{2+} from a non-mitochondrial intracellular store is now well established [1,2]. Recently, work has been directed towards a possible second-messenger role for the phosphorylation product of $\text{Ins}(1,4,5)\text{P}_3$ metabolism, $\text{Ins}(1,3,4,5)\text{P}_4$. The initial observations by Irvine & Moor [3] showed that the injection of sub-micromolar concentrations of $\text{Ins}(1,3,4,5)\text{P}_4$ into the eggs of the sea urchin *Lytechinus variegatus* resulted in the raising of a fertilization envelope provided that a Ca^{2+} -mobilizing inositol phosphate was present. A synergy of $\text{Ins}(1,4,5)\text{P}_3$ and $\text{Ins}(1,3,4,5)\text{P}_4$ has been observed in internally perfused lacrimal cells [4]. However, application of $\text{Ins}(1,3,4,5)\text{P}_4$ by itself by microinjection [3], by cell perfusion [4], or to permeabilized cells [5] has been shown not to mobilize Ca^{2+} .

Until recently it was thought that the only route for removal of $\text{Ins}(1,3,4,5)\text{P}_4$ was by its phosphorylation by a specific 5-phosphatase, yielding $\text{Ins}(1,3,4)\text{P}_3$ [6]. However, it now appears that, in human erythrocytes [7] and in pig brain cytosol [8], a 3-phosphatase exists which can convert $\text{Ins}(1,3,4,5)\text{P}_4$ back into $\text{Ins}(1,4,5)\text{P}_3$. Were this to happen to a significant degree in any experimental system in which Ca^{2+} homeostasis was being studied, one might expect to see an apparent Ca^{2+} mobilization induced by $\text{Ins}(1,3,4,5)\text{P}_4$.

Here we report that the addition of micromolar concentrations of $\text{Ins}(1,3,4,5)\text{P}_4$ to permeabilized cells of the mouse lymphoma cell line L1210 results in the release of substantial amounts of stored Ca^{2+} , and that this release is almost certainly caused by the rapid conversion of $\text{Ins}(1,3,4,5)\text{P}_4$ into $\text{Ins}(1,4,5)\text{P}_3$. We have found that this effect is dependent on the way in which the cells are permeabilized.

MATERIALS AND METHODS

Ca^{2+} -sensitive electrode membranes containing the neutral ionophore ETH1001 were prepared by the method described by Clapper & Lee [9]. Phosphocreatine

and creatine kinase were purchased from Sigma, Poole, Dorset, U.K. ATP, GTP and dithiothreitol were from BCL, Lewes, Sussex, U.K.

Ca^{2+} uptake and release were routinely measured with a Ca^{2+} -sensitive electrode. The assay medium consisted of 100 mM-KCl, 10 mM-Hepes/KOH, pH 7.0, 1 mM-dithiothreitol, 3 mM- MgCl_2 , 2.5 mM-ATP, 10 μg of creatine kinase/ml, 10 mM-phosphocreatine, 50 μM -GTP and 5 mM-succinate (K^+ salt) in a final volume of 1 ml. The endogenous $[\text{Ca}^{2+}]$ was such that the initial pCa was approx. 5.1, making further Ca^{2+} addition unnecessary. The cells routinely lowered the free $[\text{Ca}^{2+}]$ in the medium to sub-micromolar values at steady state. Experiments were carried out at 37 °C.

Cells were grown in Dulbecco's Modified Eagle's Medium supplemented with 10% (v/v) newborn-calf serum, 50 μg of streptomycin/l, 50 i.u. of penicillin/l and 2 mM-L-glutamine. A 1 litre portion of medium was inoculated with a stock solution of cells, which were left to grow at 37 °C until a cell density of approx. 1×10^6 cells/ml, as measured on a Coulter electronic cell counter, was reached (usually 6–7 days incubation period). At 24 h before the cells were to be used, they were concentrated by centrifugation at 2000 g for 45 min. The resulting pellet was resuspended in 300 ml of fresh Dulbecco's medium and re-incubated at 37 °C overnight.

For experimental work, 30 ml of the concentrated cells was centrifuged at 2000 g for 10 min in a bench centrifuge at 4 °C. The pellet was resuspended in 30 ml of ice-cold 0.9% (w/v) NaCl/10 mM-Hepes/KOH, pH 7.0, and respun as above. Then 1 ml of 100 mM-KCl/10 mM-Hepes/KOH, pH 7.0, was used to resuspend the final pellet. The remaining constituents of the Ca^{2+} -uptake medium (see above) were added to this cell suspension (1×10^8 cells/ml).

Electroporation was carried out with a Bio-Rad Gene Pulser. Cells at a concentrations of 1×10^8 cells/ml were suspended in the assay medium described above and placed into the electroporation cuvette. Five pulses of 0.8 kV with a capacitance of 25 μF was the standard permeabilization procedure. Permeabilization by digi-

§ To whom reprint requests should be addressed.

Fig. 1. Effect of $\text{Ins}(1,3,4,5)\text{P}_4$ on Ca^{2+} efflux in electroporated cells

Incubation conditions were as described in the Materials and methods section. (a) Response to $2\ \mu\text{M}$ - $\text{Ins}(1,3,4,5)\text{P}_4$ and the subsequent response to $1\ \mu\text{M}$ - $\text{Ins}(1,4,5)\text{P}_3$ after recovery from the $\text{Ins}(1,3,4,5)\text{P}_4$ addition. (b) and (c) Effects of sequential additions of $2\ \mu\text{M}$ - $\text{Ins}(1,3,4,5)\text{P}_4$ and $1\ \mu\text{M}$ - $\text{Ins}(1,4,5)\text{P}_3$ on Ca^{2+} release without waiting for recovery between the additions. Traces shown are typical of at least five independent experiments for each condition.

Fig. 2. Lack of effect of $\text{Ins}(1,3,4,5)\text{P}_4$ on digitonin-permeabilized cells

The Figure shows a Ca^{2+} -electrode trace under conditions identical with that of Fig. 1, except that the cells were permeabilized by $10\ \mu\text{g}$ of digitonin in the incubation, not by electroporation. $\text{Ins}(1,3,4,5)\text{P}_4$ ($2\ \mu\text{M}$) and $\text{Ins}(1,4,5)\text{P}_3$ ($1\ \mu\text{M}$) were added as shown. The trace is typical of at least ten independent experiments.

tonin was carried out by addition of $10\ \mu\text{g}$ of digitonin (in $1\ \mu\text{l}$ of dimethyl sulphoxide) to the assay medium described above.

For measurement of $\text{Ins}(1,3,4,5)\text{P}_4$ degradation, the cells were allowed to accumulate Ca^{2+} for approx. 15 min before $2\ \mu\text{M}$ - $\text{Ins}(1,3,4,5)\text{P}_4$ spiked with $[^3\text{H}]\text{Ins}(1,3,4,5)\text{P}_4$ (20000 d.p.m.) was added to the incubation medium; 30 s later, $700\ \mu\text{l}$ samples were transferred to Microfuge tubes containing $300\ \mu\text{l}$ of ice-cold trichloroacetic acid (10%, w/v). After 15 min on ice, samples were centrifuged ($12000\ g$ for 2 min) and the supernatants collected. Trichloroacetic acid was removed by washing

Fig. 3. H.p.l.c. analysis of the inositol phosphates produced during degradation of added $\text{Ins}(1,3,4,5)\text{P}_4$

The arrows A, B and C mark respectively the elution times of $\text{Ins}(1,3,4)\text{P}_3$, $\text{Ins}(1,4,5)\text{P}_3$ and $\text{Ins}(1,3,4,5)\text{P}_4$. (a) Electroporated cells; (b) digitonin-permeabilized cells; (c) electroporated cells, followed by digitonin ($10\ \mu\text{g}$) added after 10 min. In all cases, $\text{Ins}(1,3,4,5)\text{P}_4$ was added 15 min after the start of the incubation. Conditions of the incubations were as in Figs. 1 and 2, and the h.p.l.c. method is described in the Materials and methods section. Determinations were carried out in duplicate. Similar results were obtained in two other independent experiments.

with water-saturated diethyl ether. Samples were neutralized with NH₃ before being freeze-dried and stored at -20 °C until analysis.

After re-constitution of the samples in water and spiking with [³²P]Ins(1,4,5)P₃, the inositol triphosphates were separated by h.p.l.c. on a Partisil 10 SAX column by isocratic elution with 0.55 mM-sodium phosphate, pH 3.7, followed by a switch to 1 M-phosphate to elute Ins(1,3,4,5)P₄ (K. A. Wreggett & R. F. Irvine, unpublished work). The [³²P]Ins(1,4,5)P₃ spike and ³H-labelled compounds were measured by liquid-scintillation spectrometry.

RESULTS AND DISCUSSION

As shown in Fig. 1, the addition of 2 µM-Ins(1,3,4,5)P₄ to an electroporated suspension of L1210 cells results in a substantial release of Ca²⁺ from internal stores. The Ca²⁺ released appears to originate from the Ins(1,4,5)P₃-sensitive pool, since the addition of 1 µM-Ins(1,4,5)P₃ after Ins(1,3,4,5)P₄ results in a much diminished Ins(1,4,5)P₃-induced Ca²⁺ release. Furthermore, if the Ins(1,4,5)P₃-sensitive pool is depleted of Ca²⁺ by adding a maximal dose of either Ins(1,4,5)P₃ or Ins(2,4,5)P₃ (the latter was used in some experiments to ensure that no Ins(1,3,4,5)P₄ was produced [3]), then the Ins(1,3,4,5)P₄-induced Ca²⁺ release is abolished (see Fig. 1b).

Fig. 2 illustrates the effect of Ins(1,3,4,5)P₄ addition on Ca²⁺ release from cells permeabilized by digitonin. The addition of 2 µM-Ins(1,3,4,5)P₄ did not induce any substantial Ca²⁺ release, even though the effect of Ins(1,4,5)P₃ is similar in size to that for electroporated cells. In experiments in which digitonin was added 10 min after electroporation of the cells, Ins(1,3,4,5)P₄ brought about Ca²⁺ release similar to that observed in Fig. 1 (results not shown).

Since Doughney *et al.* [7] and Hoer *et al.* [8] have recently shown that Ins(1,3,4,5)P₄ can be converted back into Ins(1,4,5)P₃ by a 3-phosphatase, we decided to investigate the metabolism of [³H]Ins(1,3,4,5)P₄ under the various conditions described above. By h.p.l.c. techniques we observed that in electroporated cells approx. 3% of the [³H]Ins(1,3,4,5)P₄ was apparently in the form of [³H]Ins(1,4,5)P₃ after 30 s, by comparison with an internal [³²P]Ins(1,4,5)P₃ standard (Fig. 3a). This inositol triphosphate was not rigorously identified as the 1,4,5-isomer, although it is almost certainly not the 3,4,5-isomer, which we find to be eluted behind the [³²P]Ins(1,4,5)P₃ standard. Although we cannot exclude the presence of Ins(1,3,5)P₃, it is unlikely that this latter species would cause Ca²⁺ efflux. The dephosphorylation was not, however, observed in cells which had been permeabilized by digitonin (Fig. 3b). Interestingly, in cells initially permeabilized by electroporation, followed later by addition of digitonin, [³H]Ins(1,3,4,5)P₄ was still converted into [³H]Ins(1,4,5)P₃ (Fig. 3c).

It therefore appears that the Ca²⁺ efflux observed upon

addition of Ins(1,3,4,5)P₄ can probably be explained by the production of Ins(1,4,5)P₃. Although the concentration of Ins(1,4,5)P₃ produced is rather low (approx. 60 nM), it is likely to be present for a considerable length of time as a steady-state concentration, and is thus equivalent to infusion of low Ins(1,4,5)P₃ concentrations. Continuous infusion of Ins(1,4,5)P₃ has been shown to be a very effective way of unloading intracellular Ca²⁺ stores [10].

The way in which the method of permeabilization affects this conversion appears to be complex. Digitonin does not directly inhibit the 3-phosphatase, because conversion of [³H]Ins(1,3,4,5)P₄ into [³H]Ins(1,4,5)P₃ still occurs when digitonin is added to electroporated cells. It therefore seems most likely that electroporation in some way leads to activation of the 3-phosphatase activity. Although we have not explored this directly, we have found that, if digitonin-permeabilized cells are subjected to electroporation, Ins(1,3,4,5)P₄ releases Ca²⁺ (results not shown).

In conclusion, whatever the mechanism of activation or the physiological significance of this 3-dephosphorylation, our results suggest that caution should be used in interpreting experiments where Ins(1,3,4,5)P₄ shows biological activity. Also, it must not be overlooked that in our present experiments Ins(1,3,4,5)P₄ itself may be having an effect on Ca²⁺ movements in electroporated cells, but that this is being masked by the much larger Ca²⁺ efflux induced by Ins(1,4,5)P₃ after its production from Ins(1,3,4,5)P₄.

We thank Derek Fulton and Andy Letcher for their technical expertise and Trish Hawley for assistance with the tissue cultures. P.J.C. acknowledges support from the S.E.R.C., and B.K.D. acknowledges support from A.F.R.C. via a grant-in-aid to the John Innes Institute.

REFERENCES

1. Berridge, M. J. (1987) *Annu. Rev. Biochem.* **56**, 159–193
2. Putney, J. W. (1987) *Am. J. Physiol.* **252**, G149–G157
3. Irvine, R. F. & Moor, R. M. (1986) *Biochem. J.* **240**, 917–920
4. Morris, A. P., Gallacher, D. V., Irvine, R. F. & Peterson, O. H. (1987) *Nature (London)* **330**, 653–655
5. Irvine, R. F., Letcher, A. J., Lander, D. J. & Berridge, M. J. (1986) *Biochem. J.* **240**, 301–304
6. Batty, I. R., Nahorski, S. R. & Irvine, R. F. (1985) *Biochem. J.* **232**, 211–215
7. Doughney, C., McPherson, M. A. & Dormer, R. L. (1988) *Biochem. J.* **251**, 927–929
8. Hoer, D., Kwiatkowski, A., Seib, C., Rosenthal, W., Schultz, G. & Oberdisse, E. (1988) *Biochem. Biophys. Res. Commun.* **154**, 668–675
9. Clapper, D. L. & Lee, H. C. (1985) *J. Biol. Chem.* **260**, 13947–13954
10. Prentki, M., Corkey, B. E. & Matschinsky, F. M. (1985) *J. Biol. Chem.* **260**, 9185–9190