Inositol 1:2-cyclic,4,5-trisphosphate is only a weak agonist at inositol 1,4,5-trisphosphate receptors

Alan L. WILLCOCKS*, James STRUPISH*, Robin F. IRVINE† and Stefan R. NAHORSKI*

*Department of Pharmacology and Experimental Therapeutics, University of Leicester, P.O. Box 138, Medical Sciences Building, University Road, Leicester LE1 9HN, and †Department of Biochemistry, AFRC Institute of Animal Physiology and Genetics Research, Babraham Hall, Cambridge CB2 4AT, U.K.

The activity of inositol 2,4,5-trisphosphate and inositol 1:2-cyclic,4,5-trisphosphate relative to inositol 1,4,5-trisphosphate was examined by two assays; firstly, in a binding assay using rat cerebellar membranes, and secondly, in a Ca^{2+} -mobilization assay using permeabilized Swiss 3T3 cells. In both assays the first two phosphates have a potency at least an order of magnitude less than inositol 1,4,5-trisphosphate. The possible reasons for differences between these results and previous data are discussed, as are the implications for any putative physiological role for the cyclic trisphosphate.

INTRODUCTION

There is now considerable evidence that the hydrolysis of phosphatidylinositol 4,5-bisphosphate is catalysed by a phosphoinositidase C controlled by receptors and guanine nucleotide-binding proteins, with the resulting production of the second messengers $\text{Ins}(1,4,5)\text{P}_3$ and 1,2-diacylglycerol (reviewed in refs. [1,2]). The first of these is believed to interact with intracellular receptors associated with the endoplasmic reticulum, or perhaps with specialized calciosomes [3], and as a consequence it is thought to release Ca^{2+} by the opening of channels in those structures. There have been several reports of specific binding sites of $\text{Ins}(1,4,5)\text{P}_3$ in cellular preparations, though, in at least some cases, the relative potencies of inositol phosphates differ substantially from those observed in Ca^{2+} -release assays (e.g. [4–8]). In previous studies we have examined in some detail the specific InsP_3 -binding sites found in cerebellar particulate preparations, and we have been able to demonstrate the strict stereospecificity and positional specificity for $\text{Ins}(1,4,5)\text{P}_3$ [9]. More recently, we have also observed an identical specificity for Ca^{2+} release from permeabilized GH_3 and Swiss 3T3 cells [10]. However, the possibility still remained that this agreement was only circumstantial, and that the binding site could, in part, reflect interaction with enzymes associated with the metabolism of $\text{Ins}(1,4,5)\text{P}_3$. It seems unlikely that the 5'-phosphatase is a binding target, in view of the relatively low affinity this enzyme has for its substrate [4,11,12], but the possibility of a membrane-bound $\text{Ins}(1,4,5)\text{P}_3$ 3-kinase [13,14] as a binding site could not be wholly eliminated.

In the present study we have examined the affinity of two trisphosphates, $\text{Ins}(2,4,5)\text{P}_3$ and $\text{Ins}(1:2\text{-cyclic},4,5)\text{P}_3$, for the cerebellar binding sites. These trisphosphates are both relatively poor substrates for the kinase and phosphatase. $\text{Ins}(1:2\text{-cyclic},4,5)\text{P}_3$, however, has been reported to be equipotent to $\text{Ins}(1,4,5)\text{P}_3$ in releasing Ca^{2+} from *Limulus* photoreceptor cells [15], platelets [15] and Swiss 3T3 cells [16]. For this reason we had hoped

that $\text{Ins}(1:2\text{-cyclic},4,5)\text{P}_3$ might serve as a diagnostic indicator for whether the cerebellar binding site resembles most closely the receptor for InsP_3 -induced Ca^{2+} release as opposed to InsP_3 kinase or phosphatase. As a direct comparison we also therefore examined its potency in a Ca^{2+} -mobilization assay.

MATERIALS AND METHODS

Inositol phosphates

$\text{Ins}(1:2\text{-cyclic},4,5)\text{P}_3$ was prepared exactly as described by Irvine *et al.* [16], but with modifications in the resulting procedure after purification by h.p.l.c., in order to minimize non-enzymic hydrolysis of the $\text{Ins}(1:2\text{-cyclic},4,5)\text{P}_3$ to $\text{Ins}(1,4,5)\text{P}_3$. After elution from the h.p.l.c. column [16], the radioactivity in fractions in the InsP_3 region was determined by Čerenkov counting to locate the $[\text{32P}]\text{Ins}(1,4,5)\text{P}_3$ included as an internal marker. Fractions containing $\text{Ins}(1:2\text{-cyclic},4,5)\text{P}_3$ [17,18] were then diluted 7-fold, and pumped (with a peristaltic pump) through a Accell QMA Sep-pak ([19]; Waters Chromatography, batch no. P5269A1). P_1 was eluted with 20 ml of 0.15 M-ammonium formate [19], and the $\text{Ins}(1:2\text{-cyclic},4,5)\text{P}_3$ was then eluted with 10 ml of 1 M-triethylamine bicarbonate, pH 7.0, and was freeze-dried. It was stored in portions desiccated at -80°C until use. The purity was not checked by ionophoresis, but it is at least 95 % as judged by the experiments described below. $\text{Ins}(1,4,5)\text{P}_3$ and $\text{Ins}(2,4,5)\text{P}_3$ were prepared as in refs. [16] and [20] respectively.

Binding assay

This was exactly as described by Willcocks *et al.* [9], by using rat cerebellar membranes, $[\text{3H}]\text{Ins}(1,4,5)\text{P}_3$ and centrifugation to separate free and bound ligand.

$^{45}\text{Ca}^{2+}$ -mobilization assay

This was performed exactly as described by Strupish *et al.* [10], by using saponin-permeabilized Swiss 3T3 cells in suspension, and employing centrifugation through a

Abbreviations used: InsP , InsP_2 , InsP_3 , *myo*-inositol mono-, bis- and tris-phosphates respectively, with isomeric numbering as appropriate (all numbering for the D-configuration); $\text{Ins}(1:2\text{-cyclic},4,5)\text{P}_3$, D-*myo*-inositol 1:2-cyclic,4,5-trisphosphate; $\text{GroPIns}(4,5)\text{P}_2$, glycerophosphoinositol 4,5-bisphosphate.

silicone-oil mixture to separate the cells from buffer. $^{45}\text{Ca}^{2+}$ release was assayed after a 1 min exposure to inositol phosphates.

RESULTS

Fig. 1 shows the relative potencies (direct comparisons in a series of experiments) of $\text{Ins}(1:2\text{-cyclic},4,5)\text{P}_3$, $\text{Ins}(2,4,5)\text{P}_3$ and $\text{Ins}(1,4,5)\text{P}_3$ competing for the binding of $[^3\text{H}]\text{Ins}(1,4,5)\text{P}_3$ to rat cerebellar membranes. Also

Fig. 1. Displacement of $[^3\text{H}]\text{Ins}(1,4,5)\text{P}_3$ from cerebellar membranes by inositol phosphates

$[^3\text{H}]\text{Ins}(1,4,5)\text{P}_3$ was used at 15 nM. The data shown are means $\pm$ S.E.M. for three separate experiments. $\square$, $\text{Ins}(1,4,5)\text{P}_3$; $\blacklozenge$, $\text{Ins}(1:2\text{-cyclic},4,5)\text{P}_3$; $\blacksquare$, $\text{Ins}(1:2\text{-cyclic},4,5)\text{P}_3$ which has been acid-treated (see the text); $\blacktriangle$, $\text{Ins}(2,4,5)\text{P}_3$.

Table 1. Relative potencies of inositol phosphates in binding to cerebellar membranes and in mobilizing Ca^{2+} from permeabilized Swiss 3T3 cells

IC_{50} and EC_{50} values (respectively concentrations producing 50% inhibition of specific $[^3\text{H}]\text{Ins}(1,4,5)\text{P}_3$ (15 nM) binding to cerebellar membranes, or producing 50% of maximal response in $^{45}\text{Ca}^{2+}$ -release assays) were obtained by analysing data by computer-assisted curve fitting. Values are means $\pm$ S.E.M. ($n = 3$).

	Cerebellum binding (IC_{50})	Swiss 3T3 cells (EC_{50})
$\text{Ins}(1:2\text{-cyclic},4,5)\text{P}_3$	$2.4 (\pm 0.14) \times 10^{-6} \text{ M}$	$3.56 (\pm 0.06) \times 10^{-6} \text{ M}$
+ Acid	$9.9 (\pm 0.06) \times 10^{-8} \text{ M}$	$2.90 (\pm 0.01) \times 10^{-7} \text{ M}$
$\text{Ins}(2,4,5)\text{P}_3$	$8.6 (\pm 0.03) \times 10^{-7} \text{ M}$	$2.8 (\pm 0.44) \times 10^{-6} \text{ M}$
$\text{GroPIns}(4,5)\text{P}_2$	$3 \times 10^{-6} \text{ M}^*$	$2.2 (\pm 0.14) \times 10^{-6} \text{ M}$
$\text{Ins}(1,4,5)\text{P}_3$	$5.5 (\pm 0.24) \times 10^{-8} \text{ M}$	$2.1 (\pm 0.1) \times 10^{-7} \text{ M}$

* $n = 1$.

Fig. 2. Mobilization of Ca^{2+} from permeabilized Swiss 3T3 cells

Data shown are means $\pm$ S.E.M. of three separate experiments. $^{45}\text{Ca}^{2+}$ was loaded at 100–300 nM, and the percentage released represents the fraction of $^{45}\text{Ca}^{2+}$ mobilized from this high-affinity component (see ref. [10]). Symbols are the same as for Fig. 1.

plotted ($\blacksquare$) is the potency of an $\text{Ins}(1:2\text{-cyclic},4,5)\text{P}_3$ sample which was treated at room temperature with 0.2 M-HCl for 60 min, followed by neutralization with NaOH. It is apparent that this sample is now very similar to $\text{Ins}(1,4,5)\text{P}_3$ in this assay. These results are summarized in the ' IC_{50} ' column of Table 1, which also includes data for $\text{GroPIns}(4,5)\text{P}_2$, an inositol phosphate which has a potency similar to that of $\text{Ins}(1:2\text{-cyclic},4,5)\text{P}_3$.

The ' EC_{50} ' column of Table 1, and Fig. 2, show similar comparative studies using the Ca^{2+} -mobilization assay in Swiss 3T3 cells [10]. The potencies for $\text{Ins}(1,4,5)\text{P}_3$, $\text{Ins}(2,4,5)\text{P}_3$ and $\text{GroPIns}(4,5)\text{P}_2$ are similar to those obtained previously by a different assay (but using the same cells [20]). However, the potency of $\text{Ins}(1:2\text{-cyclic},4,5)\text{P}_3$ is about an order of magnitude less than that of $\text{Ins}(1,4,5)\text{P}_3$, i.e. a result very similar to that in the binding assay (Fig. 1).

It is important to note that an acid-treatment regime identical with that used with $\text{Ins}(1:2\text{-cyclic},4,5)\text{P}_3$ (above) had no discernible effect on the potency of either $\text{Ins}(1,4,5)\text{P}_3$ or $\text{GroPIns}(4,5)\text{P}_2$ (results not shown). We did not assay the purity of the $\text{Ins}(1:2\text{-cyclic},4,5)\text{P}_3$ in terms of contamination with $\text{Ins}(1,4,5)\text{P}_3$ by ionophoresis, but this acid-induced shift gives us confidence that the preparation must be of the order of 95% (or more) pure. Of course, our data do not provide any firm evidence whether the pure compound possesses weak activity, or whether its ability to release Ca^{2+} resides entirely in a slight contamination with $\text{Ins}(1,4,5)\text{P}_3$. This question is extremely difficult to answer satisfactorily, given the possibility that $\text{Ins}(1,4,5)\text{P}_3$ could be formed during the actual assays used to test for biological activity. The potencies documented above must therefore be taken as maximum potencies, with the proviso that $\text{Ins}(1:2\text{-cyclic},4,5)\text{P}_3$ may in fact be even less potent than shown here.

DISCUSSION

The original aim of this study was to study $\text{Ins}(1,4,5)P_3$, $\text{Ins}(2,4,5)P_3$ and $\text{Ins}(1:2\text{-cyclic},4,5)P_3$ in a binding assay using cerebellar membranes, in comparison with a Ca^{2+} -mobilization assay in Swiss 3T3 cells, so that we could get some idea of whether the two 'receptors' in these systems are functionally related. However, our hope that $\text{Ins}(1:2\text{-cyclic},4,5)P_3$ might serve as a diagnostic tool in this respect was frustrated, in that in our hands it is not a strong Ca^{2+} mobilizer.

The apparent ineffectiveness of $\text{Ins}(1:2\text{-cyclic},4,5)P_3$ in mobilizing Ca^{2+} in these experiments is surprising, in view of several reports that it has a Ca^{2+} -mobilizing potency similar to that of $\text{Ins}(1,4,5)P_3$ (e.g. [15,16]), and this requires some explanation. The high potency of $\text{Ins}(1:2\text{-cyclic},4,5)P_3$ in acute activation of *Limulus* photoreceptors could perhaps be explained partly by its resistance to dephosphorylation of phosphorylation [11,21], but this possibility was ruled out, at least in the experiments on anchored permeabilized Swiss 3T3 cells by inclusion of an inhibitor of $\text{Ins}(1,4,5)P_3$ phosphatase [16]. Thus a ready explanation for its ability to mobilize Ca^{2+} in those cells and in permeabilized platelets [15] is not immediately apparent.

The purity of the $\text{Ins}(1:2\text{-cyclic},4,5)P_3$ used previously [16] was estimated to be about 90% as judged by ionophoresis, but various other factors, such as subsequent decomposition of $\text{Ins}(1:2\text{-cyclic},4,5)P_3$ in solution, pH [22], or differences in ATP concentrations in permeabilized-cell experiments (ATP being a possible competitor for the $\text{Ins}(1,4,5)P_3$ receptors [9]), may have influenced the apparent potency. These factors become especially important when it is noted that, because of the limited availability of material, no comparison was made previously of $\text{Ins}(1:2\text{-cyclic},4,5)P_3$ and $\text{Ins}(1,4,5)P_3$ directly, nor were the acid-treated controls used as they were in the present experiments [16]. For these reasons we consider that the data in Fig. 2 and Table 1 are a much more reliable indicator (with the proviso noted above) of the true relative potency of these two compounds. Finally, the only other Ca^{2+} -mobilization assay *in vitro* that directly compares $\text{Ins}(1,4,5)P_3$ and $\text{Ins}(1:2\text{-cyclic},4,5)P_3$ is in permeabilized platelets [15], and there the complex curve for $\text{Ins}(1,4,5)P_3$ -induced Ca^{2+} release makes interpretation, and therefore comparison, difficult.

The low potency of $\text{Ins}(1:2\text{-cyclic},4,5)P_3$ reported here in Swiss 3T3 cells is not unique to those cells. The same batch of $\text{Ins}(1:2\text{-cyclic},4,5)P_3$ used here was also tested on rat liver microsomes (P. Cullen & A. P. Dawson, personal communication). It gave similar results, in that it showed at least a 20-fold lower potency in releasing Ca^{2+} as compared with $\text{Ins}(1,4,5)P_3$, and after acid treatment (see above) showed a very similar potency. Furthermore, J. W. Putney (personal communication) also tested the same $\text{Ins}(1:2\text{-cyclic},4,5)P_3$ batch and found that it was less potent than $\text{Ins}(1,4,5)P_3$ in rat permeabilized parotid cells. Meyer *et al.* [23] have recently reported a low potency of $\text{Ins}(1:2\text{-cyclic},4,5)P_3$ in permeabilized RBL cells when compared directly with $\text{Ins}(1,4,5)P_3$. Finally, in experiments involving microinjection into intact sea-urchin eggs, Crossley *et al.* [24] observed that $\text{Ins}(1:2\text{-cyclic},4,5)P_3$ was 10-fold less potent than $\text{Ins}(1,4,5)P_3$ (and that particular batch of $\text{Ins}(1:2\text{-cyclic},4,5)P_3$ was desalted by previously used

methods [16] which we know cause the formation of at least 8% contamination with $\text{Ins}(1,4,5)P_3$ [11]).

In conclusion, our results have two implications, one practical and one physiological. Although it seems likely on the basis of the present and past data [9,10] that the binding and Ca^{2+} -release sites are closely related, it will be difficult to distinguish definitively between various sites which could bind $\text{Ins}(1,4,5)P_3$ on the basis of comparative potencies of $\text{Ins}(1:2\text{-cyclic},4,5)P_3$ and $\text{Ins}(2,4,5)P_3$, not only because there is some degree of variation between systems, but also because $\text{Ins}(1:2\text{-cyclic},4,5)P_3$ is not as good an agonist in mobilizing Ca^{2+} as has been hitherto supposed. The latter observation leads in turn to the physiological implications of these results, in that, although at early times after stimulation $\text{Ins}(1:2\text{-cyclic},4,5)P_3$ is only found at low concentrations [12,25,26], after prolonged stimulation it does increase (e.g. [27–29]), and it has therefore been suggested as possibly having a function as a 'long-term' $\text{Ins}(1,4,5)P_3$ analogue (e.g. [30]). $\text{Ins}(1:2\text{-cyclic},4,5)P_3$ has not yet been reported to accumulate to concentrations exceeding those of $\text{Ins}(1,4,5)P_3$, and from that one might conclude from the present data that the contribution it makes to Ca^{2+} mobilization might not be significant. It is true, however, that both $\text{Ins}(1,4,5)P_3$ and $\text{Ins}(1:2\text{-cyclic},4,5)P_3$ might accumulate to concentrations considerably in excess of those required to saturate the intracellular $\text{Ins}(1,4,5)P_3$ receptor (although as yet there are no firm experimental results on this); if that were so, then, after removal of $\text{Ins}(1,4,5)P_3$ after receptor blocking, the much-slower-decreasing $\text{Ins}(1:2\text{-cyclic},4,5)P_3$ might make a significant contribution to Ca^{2+} mobilization (see [29]). These circumstances are more likely to prevail at pharmacological rather than physiological agonist concentrations, however, and overall our data suggest that some caution should be used in assessing the physiological significance of $\text{Ins}(1:2\text{-cyclic},4,5)P_3$.

We thank Andy Letcher and David Lander for their help in preparing the inositol phosphates, and Keith Wreggett for helpful discussions. This work was supported by the Wellcome Trust, and [^3H] $\text{Ins}(1,4,5)P_3$ was kindly provided by NEN (DuPont).

REFERENCES

- Berridge, M. J. & Irvine, R. F. (1984) *Nature* (London) **312**, 315–321
- Nishizuka, Y. (1984) *Nature* (London) **308**, 693–698
- Volpe, P., Krause, K.-H., Hashimoto, S., Zorzato, F., Pozzan, T., Meldolesi, J. & Lew, D. P. (1988) *Proc. Natl. Acad. Sci. U.S.A.* **85**, 1091–1095
- Baukal, A. J., Guillemette, G., Rubin, R., Spät, A. & Catt, K. (1985) *Biochem. Biophys. Res. Commun.* **133**, 532–538
- Spät, A., Fabiato, A. & Rubin, R. P. (1986) *Biochem. J.* **233**, 929–932
- Spät, A., Bradford, P. G., McKinney, J. S., Rubin, R. P. & Putney, J. W., Jr. (1986) *Nature* (London) **319**, 514–516
- Guillemette, G., Balla, T., Baukal, A. J., Spät, A. & Catt, K. J. (1987) *J. Biol. Chem.* **262**, 1010–1015
- Worley, P. F., Baraban, J. M., Colvin, J. S. & Snyder, S. H. (1987) *Nature* (London) **325**, 159–161
- Willcocks, A. L., Cooke, A. M., Potter, B. V. L. & Nahorski, S. R. (1987) *Biochem. Biophys. Res. Commun.* **146**, 1071–1078
- Strupish, J., Cooke, A. M., Potter, B. V. L., Gigg, R. & Nahorski, S. R. (1988) *Biochem. J.* **253**, 901–905

11. Connolly, T. M., Bansal, V. S., Bross, T. E., Irvine, R. F. & Majerus, P. W. (1987) *J. Biol. Chem.* **262**, 2146–2149
12. Hawkins, P. T., Berrie, C. P., Morris, A. J. & Downes, C. P. (1987) *Biochem. J.* **243**, 211–218
13. Morris, A. J., Downes, C. P., Harden, T. K. & Michell, R. H. (1987) *Biochem. J.* **248**, 489–493
14. Irvine, R. F., Moor, R. M., Pollock, W. K., Smith, P. M. & Wreggett, K. A. (1988) *Philos. Trans. R. Soc. London B* **320**, 281–298
15. Wilson, D. B., Connolly, T. M., Bross, T. E., Majerus, P. W., Sherman, W. R., Tyler, A. N., Rubin, L. J. & Brown, J. E. (1985) *J. Biol. Chem.* **260**, 13496–13501
16. Irvine, R. F., Letcher, A. J., Lander, D. J. & Berridge, M. J. (1986) *Biochem. J.* **240**, 301–304
17. Irvine, R. F., Anggard, E. E., Letcher, A. J. & Downes, C. P. (1985) *Biochem. J.* **229**, 505–511
18. Irvine, R. F., Letcher, A. J., Heslop, J. P. & Berridge, M. J. (1986) *Nature (London)* **320**, 631–634
19. Wreggett, K. A. & Irvine, R. F. (1987) *Biochem. J.* **245**, 655–660
20. Irvine, R. F., Brown, K. D. & Berridge, M. J. (1984) *Biochem. J.* **221**, 269–272
21. Connolly, T. M., Wilson, D. B., Bross, T. E. & Majerus, P. W. (1986) *J. Biol. Chem.* **261**, 122–126
22. Worley, P. F., Baraban, J. M., Suttapone, S., Wilson, V. S. & Snyder, S. H. (1987) *J. Biol. Chem.* **262**, 12132–12136
23. Meyer, T., Hultowka, D. & Stryer, L. (1988) *Science* **240**, 653–656
24. Crossley, I., Swann, K., Chambers, E. & Whitaker, M. (1988) *Biochem. J.* **252**, 257–262
25. Wong, N. S., Barker, C. J., Shears, S. B., Kirk, C. J. & Michell, R. H. (1988) *Biochem. J.* **252**, 1–5
26. Tarver, A. P., King, W. C. & Rittenhouse, S. F. (1987) *J. Biol. Chem.* **262**, 17268–17271
27. Dixon, J. F. & Hokin, L. E. (1987) *Biochem. Biophys. Res. Commun.* **144**, 1208–1213
28. Sekar, M. C., Dixon, J. E. & Hokin, L. E. (1987) *J. Biol. Chem.* **262**, 340–344
29. Hughes, A. R., Takemura, H. & Putney, J. W., Jr. (1988) *J. Biol. Chem.* **263**, 10314–10319
30. Majerus, P. W., Connolly, T. M., Deckmyn, H., Ross, T. S., Bross, T. E., Ishii, H., Bansal, V. S. & Wilson, D. B. (1986) *Science* **234**, 1519–1526

Received 27 July 1988/19 October 1988; accepted 2 November 1988