Isomers of inositol trisphosphate in exocrine pancreas

Janet E. MERRITT,* Colin W. TAYLOR, Ronald P. RUBIN† and James W. PUTNEY, Jr.
Department of Pharmacology, Medical College of Virginia, Box 524, Richmond, VA 23298-0001, U.S.A.

In rat pancreatic acinar cells, the Ca^{2+} -mobilizing receptor-agonist, caerulein, at both maximal and submaximal concentrations, stimulated a rapid, transient, increase in [^3H]inositol 1,4,5-trisphosphate [(1,4,5) IP_3], followed by a slower, sustained, increase in [^3H]inositol 1,3,4-trisphosphate [(1,3,4) IP_3]. Neither activation of protein kinase C by phorbol dibutyrate nor prevention of the caerulein-stimulated elevation of cytosolic [Ca^{2+}] significantly affected the pattern of formation of the two isomers of IP_3 . Although carbachol evoked an increase in cytosolic [Ca^{2+}], it did not significantly stimulate [^3H](1,4,5) IP_3 accumulation, but did promote [^3H](1,3,4) IP_3 accumulation. Moreover, both carbachol and caerulein maintained hormone-sensitive intracellular Ca^{2+} pools in a Ca^{2+} -depleted state after [^3H](1,4,5) IP_3 had returned to basal concentrations. One interpretation of these findings is that total cellular concentrations of [^3H](1,4,5) IP_3 may not accurately reflect the concentration of this putative mediator in biologically relevant compartments.

INTRODUCTION

In pancreas, as in many other tissues, an early response to activation of Ca^{2+} -mobilizing receptors is phospholipase C-catalysed hydrolysis of $\text{PI}(4,5)\text{P}_2$ to (1,4,5) IP_3 and diacylglycerol (Berridge, 1984). Each of these products functions as an intracellular messenger; diacylglycerol activates protein kinase C (Nishizuka, 1984) and (1,4,5) IP_3 mobilizes Ca^{2+} from an intracellular pool (Streb *et al.*, 1983; Berridge & Irvine, 1984). Further studies, initially of parotid gland (Irvine *et al.*, 1984, 1985) and subsequently of other tissues (Burgess *et al.*, 1985; Batty *et al.*, 1985; Heslop *et al.*, 1985), have shown that a second isomer of IP_3 , (1,3,4) IP_3 , is also formed in response to receptor activation. Here we report the effects, in pancreatic acinar cells, of caerulein (cholecystikinin receptor-agonist) and carbachol (muscarinic receptor-agonist) on the formation of each of these isomers of IP_3 . In addition, we have investigated whether any of the products of phospholipase C activation may themselves control the formation of each isomer.

MATERIALS AND METHODS

Caerulein was obtained from Peninsula Laboratories (Belmont, CA, U.S.A.) and [^3H]inositol (10–30 Ci/mmol) was from American Radiolabeled Chemicals Inc. (St. Louis, MO, U.S.A.). Nylon HPLC filters (0.45 μm pore size) were purchased from Micron Separations Inc. (Honeye Falls, NY, U.S.A.). All other reagents were obtained from Sigma.

Pancreatic acinar cells from male Sprague–Dawley rats were prepared by collagenase digestion (Merritt *et al.*, 1986). Cells (50 mg of cell protein/ml) in a modified Krebs–Henseleit medium (Merritt *et al.*, 1986) were incubated with [^3H]inositol (100 $\mu\text{Ci}/\text{ml}$) for 4–5 h at 37 °C. Cells were washed by centrifugation, resuspended (2 mg of cell protein/ml) and incubated with the appropriate additions for the times specified. Incubations

were terminated by addition of 18% (w/v) HClO_4 (final concn. 3%). The pH of the supernatants was adjusted to pH 8–9 by addition of a mixture of KOH (3 M) and sodium tetraborate (54 mM). Inositol phosphates were separated by anion-exchange chromatography (Berridge *et al.*, 1983); a sample of the [^3H] IP_3 fraction was counted for radioactivity, and total [^3H] IP_3 was expressed as a percentage of total [^3H]inositol lipids. Total [^3H]inositol lipids were extracted as described by Weiss & Putney (1981). The remainder of the [^3H] IP_3 fraction was passed over a Dowex 50 column (H^+ form), mannitol (4 mg) was added to the eluate, and the samples were frozen and freeze-dried (Burgess *et al.*, 1985; Irvine *et al.*, 1985).

For h.p.l.c. analysis, samples were resuspended in 1 ml of distilled water containing ATP (30 $\mu\text{g}/\text{ml}$) and [^{32}P](1,4,5) IP_3 (about 200 c.p.m.; prepared from erythrocyte ghosts as described by Downes *et al.*, 1982). The samples were filtered and isomers of IP_3 were separated by h.p.l.c. (Whatman Partisil 10 SAX anion-exchange column), with a curvilinear gradient (0–1 M-ammonium formate adjusted to pH 3.7 with H_3PO_4) (Irvine *et al.*, 1985; Burgess *et al.*, 1985). The A_{259} was monitored to detect ATP, a marker for (1,3,4) IP_3 in this system. In all experiments, the total [^3H] IP_3 radioactivity recovered from the h.p.l.c. column exceeded 200 c.p.m. From the ratio of [^3H](1,4,5) IP_3 to [^3H](1,3,4) IP_3 recovered from h.p.l.c. and the total amount of [^3H] IP_3 (calculated as described above), the amounts of each isomer of [^3H] IP_3 were calculated as a percentage of total [^3H]inositol lipids.

RESULTS AND DISCUSSION

A maximal concentration of caerulein (100 nM) stimulated a rapid, but transient, increase in [^3H](1,4,5) IP_3 formation, followed by a slower, but sustained, increase in [^3H](1,3,4) IP_3 (Fig. 1). The same

Abbreviations used: IP_3 , inositol trisphosphate; IP_4 , inositol tetrakisphosphate; $\text{PI}(4,5)\text{P}_2$, phosphatidylinositol 4,5-bisphosphate; PDBu, 4 β -phorbol 12,13-dibutyrate.

* Present address: Smith, Kline and French Research Ltd., The Frythe, Welwyn, Herts. AL6 9AR, U.K.

† To whom reprint requests should be addressed.

Fig. 1. Accumulation of $[^3\text{H}](1,4,5)\text{IP}_3$ and $[^3\text{H}](1,3,4)\text{IP}_3$ in response to a maximal concentration of caerulein

Time courses of accumulation of $[^3\text{H}](1,4,5)\text{IP}_3$ (●) and $[^3\text{H}](1,3,4)\text{IP}_3$ (○) in pancreatic acinar cells stimulated with a maximal concentration of caerulein (100 nM) are shown as the means $\pm$ S.E.M. for three independent experiments, with duplicate determinations in each. Results for the first 80 s are shown on a larger scale in the inset.

Fig. 2. Effects of maximal and submaximal concentrations of caerulein on the accumulation of $[^3\text{H}](1,4,5)\text{IP}_3$ and $[^3\text{H}](1,3,4)\text{IP}_3$

Cells were stimulated with a maximal (100 nM; Δ , $\blacktriangle$) or a submaximal (0.3 nM; $\circ$, $\bullet$) concentration of caerulein. Accumulations of $[^3\text{H}](1,4,5)\text{IP}_3$ (●, $\blacktriangle$) and $[^3\text{H}](1,3,4)\text{IP}_3$ (○, Δ) are shown as means $\pm$ S.E.M. for three independent experiments, with duplicate determinations in each.

pattern of stimulation was observed in cells stimulated with a submaximal concentration of caerulein (0.3 nM) (Fig. 2). In previous studies of parotid gland stimulated with carbachol (Irvine *et al.*, 1985), and of hepatocytes stimulated with angiotensin II (Burgess *et al.*, 1985), a

Fig. 3. Accumulation of $[^3\text{H}](1,4,5)\text{IP}_3$ and $[^3\text{H}](1,3,4)\text{IP}_3$ in cells stimulated with carbachol

Cells were stimulated with carbachol (1 mM), and accumulation of $[^3\text{H}](1,4,5)\text{IP}_3$ (●) and $[^3\text{H}](1,3,4)\text{IP}_3$ (○) was measured. Results are shown as means $\pm$ S.E.M. for duplicate determinations from three independent experiments.

transient increase in $[^3\text{H}](1,4,5)\text{IP}_3$ also preceded a sustained increase in $[^3\text{H}](1,3,4)\text{IP}_3$.

The source of $(1,3,4)\text{IP}_3$ has until recently been unclear, since phosphatidylinositol 3,4-bisphosphate, from which $(1,3,4)\text{IP}_3$ could be directly formed by phosphodiesteratic cleavage, appears not to be present in those cells that have been examined (Irvine *et al.*, 1985). A $(1,4,5)\text{IP}_3$ kinase has now been described in *Xenopus laevis* oocytes and brain homogenates that phosphorylates $(1,4,5)\text{IP}_3$ to $(1,3,4,5)\text{IP}_4$, and this can then be dephosphorylated by a 5-phosphomonoesterase to $(1,3,4)\text{IP}_3$ (Irvine *et al.*, 1986). At present, there is no direct evidence to allow the importance of this pathway to be assessed in cells stimulated with receptor-agonists. Nevertheless, the time course of formation of $(1,4,5)\text{IP}_3$ and $(1,3,4)\text{IP}_3$ (Fig. 1) and reports of agonist-stimulated $(1,3,4,5)\text{IP}_4$ formation (Batty *et al.*, 1985; Heslop *et al.*, 1985; J. E. Merritt, C. W. Taylor, R. P. Rubin & J. W. Putney, Jr., unpublished work on exocrine pancreas) are entirely consistent with the proposal that $(1,3,4,5)\text{IP}_4$, derived from $(1,4,5)\text{IP}_3$, is the source of $(1,3,4)\text{IP}_3$. However, increased availability of $(1,4,5)\text{IP}_3$ may not be the only factor controlling synthesis of $(1,3,4)\text{IP}_3$. In caerulein-stimulated pancreatic acinar cells, for example, $[^3\text{H}](1,4,5)\text{IP}_3$ returns to the resting concentration while $[^3\text{H}](1,3,4)\text{IP}_3$ continues to accumulate (Fig. 1); and when cells were stimulated with carbachol there was substantial formation of $[^3\text{H}](1,3,4)\text{IP}_3$, but no significant increase in $[^3\text{H}](1,4,5)\text{IP}_3$ (Fig. 3). However, it must be conceded that carbachol was less effective than caerulein in stimulating formation of total $[^3\text{H}]\text{IP}_3$, which may explain the lack of effect of carbachol on $[^3\text{H}](1,4,5)\text{IP}_3$ formation.

We next investigated whether the lag preceding formation of $[^3\text{H}](1,3,4)\text{IP}_3$ reflects a time during which products of the initial cleavage of $\text{PI}(4,5)\text{P}_2$ activate protein kinase C or mobilize intracellular Ca^{2+} and perhaps thereby regulate $[^3\text{H}](1,3,4)\text{IP}_3$ formation. Acti-

Fig. 4. Effect of PDBu on accumulation of the two isomers of $[^3\text{H}]\text{IP}_3$ in response to caerulein

Cells were pretreated with PDBu ($10\ \mu\text{M}$; $\circ$) or with an equal amount of the solvent (0.1% dimethyl sulphoxide; $\bullet$) for 30 min. Subsequent incubations included dimethyl sulphoxide or PDBu with a maximal concentration of caerulein ($100\ \text{nM}$). Accumulations of $[^3\text{H}](1,3,4)\text{IP}_3$ and $[^3\text{H}](1,4,5)\text{IP}_3$ are shown in (a) and (b) respectively. Results are shown as means of duplicate determinations from a single experiment; a second experiment gave the same results. The insets show results over a longer time.

vation of protein kinase C by diacylglycerol can be mimicked by phorbol diesters (Nishizuka, 1984). Preincubation (30–60 min) and subsequent incubation (6 min) of pancreatic acinar cells with a phorbol diester, PDBu ($10\ \mu\text{M}$), a treatment that we have previously shown to stimulate amylase secretion by pancreatic acini (Merritt & Rubin, 1985), did not affect $[^3\text{H}]\text{IP}_3$ formation in response to maximal or submaximal concentrations of carbachol ($1\ \text{mM}$, $100\ \mu\text{M}$) or caerulein ($100\ \text{nM}$, $1\ \text{nM}$) (results not shown). By contrast, in liver, phorbol diesters desensitize the α_1 -adrenoceptor, perhaps a consequence of loss of surface receptors (Lynch *et al.*, 1985b) and a decrease in receptor affinity for agonists (Corvera *et al.*, 1986). In astrocytoma cells, the muscarinic-cholinergic receptor is desensitized by phorbol diesters, but changes in the receptor do not fully account for the desensitization, suggesting that there may also be changes in the coupling protein or in phospholipase C (Orellana *et al.*, 1985). Our results from pancreatic acinar cells clearly demonstrate that desensitization by phorbol

Fig. 5. Effects of cytosolic Ca^{2+} on accumulation of the two isomers of $[^3\text{H}]\text{IP}_3$ in response to caerulein

Cells were pretreated with carbachol in the absence or presence of extracellular Ca^{2+} , and the muscarinic receptor was then blocked with atropine. As described in the text, this protocol allows the responses to be studied in the absence ($\circ$) or presence ($\bullet$) of a caerulein-stimulated increase in cytosolic $[\text{Ca}^{2+}]$. Accumulation of $[^3\text{H}](1,3,4)\text{IP}_3$ (a) and $[^3\text{H}](1,4,5)\text{IP}_3$ (b) is shown as the mean $\pm$ S.E.M. for three independent experiments, each performed in duplicate. The insets show results over a longer time.

diesters is not a universal feature of Ca^{2+} -mobilizing receptors.

In pancreatic acinar cells, caerulein-stimulated formation of $[^3\text{H}](1,4,5)\text{IP}_3$ or $[^3\text{H}](1,3,4)\text{IP}_3$ was unaffected by pretreatment (30 min) and subsequent incubation with PDBu ($10\ \mu\text{M}$) (Fig. 4). This result implies that protein kinase C does not regulate formation or the metabolism of either isomer of IP_3 .

We next determined whether the other limb of the phosphoinositide-signalling pathway (that which leads to an increase in cytosolic $[\text{Ca}^{2+}]$) could play a role in regulating formation of the isomers of IP_3 . Pancreatic acinar cells were incubated for 15 min with carbachol ($1\ \text{mM}$) in the presence of CaCl_2 ($1.28\ \text{mM}$) or in its

absence (with 100 μM -EGTA); under these conditions, [^3H]IP₃ formation was stimulated, and then during a further incubation of 15 min with atropine (1 μM) the amount of [^3H]IP₃ returned to the resting value (results not shown). This protocol allows hormone-sensitive intracellular Ca²⁺ pools to be totally emptied such that, in the absence of CaCl₂, when the muscarinic-cholinergic receptor is blocked with atropine and the cells are then stimulated with caerulein, there is no increase in Ca²⁺ efflux (Putney *et al.*, 1983). Prevention of the increase in cytosolic [Ca²⁺] in this way had little effect on the rates of formation of either isomer of IP₃ in response to subsequent addition of caerulein (100 nM), although the initial rate of formation of [^3H](1,4,5)IP₃ was slightly lower and the final concentration of [^3H](1,3,4)IP₃ slightly decreased (Fig. 5). These results suggest that the increase in cytosolic [Ca²⁺] that would usually follow formation of (1,4,5)IP₃ is not important in regulating the formation of either isomer of IP₃.

Comparison of Figs. 4 and 5 reveals that, although pretreatment of cells with carbachol had no effect on initial rates of [^3H]IP₃ formation, it substantially decreased the final amount of [^3H](1,3,4)IP₃ accumulated. We cannot fully explain this result, though it is possible that prolonged pretreatment with carbachol decreases either the specific radioactivity or the size of the hormone-sensitive PI(4,5)P₂ pool.

Maximal concentrations of carbachol or caerulein that produce comparable increases in intracellular [Ca²⁺] in pancreatic acinar cells (Merritt & Rubin, 1985) have very different effects on the concentration of [^3H](1,4,5)IP₃ (Figs. 1 and 3). After stimulation with either agonist, [^3H](1,4,5)IP₃ returned to resting values within 30 s, yet the hormone-sensitive intracellular Ca²⁺ pool, assumed to be regulated by (1,4,5)IP₃, remained empty for as long as receptor-agonist is present (Putney *et al.*, 1983). One possible explanation of this apparent paradox is that, during stimulation, the specific radioactivity of [^3H](1,4,5)IP₃ decreases and masks a sustained elevation in the cytosolic concentration of (1,4,5)IP₃. If this occurred, we should expect that, after stimulation with carbachol, blockade of the muscarinic receptor should cause the [^3H](1,4,5)IP₃ radioactivity to fall to below that observed before stimulation. To test this possibility we measured [^3H](1,4,5)IP₃ before stimulation, after stimulation with carbachol (1 mM) for 70 s, and in cells first stimulated with carbachol (1 mM) for 70 s and then treated with atropine (1 μM) for 5 min. After each of these treatments, the [^3H](1,4,5)IP₃ radioactivity was the same, but atropine caused the amount of [^3H](1,3,4)IP₃ to fall close to baseline (results not shown). Unless, throughout the entire time course, changes in the specific radioactivity of [^3H](1,4,5)IP₃ exactly counter-balance changes in its mass, the simplest explanation of the results is that neither the mass nor the specific radioactivity changes during this time.

In conclusion, although there is convincing evidence derived from a number of diverse systems that (1,4,5)IP₃ serves as the intracellular messenger to mobilize Ca²⁺, the present study reveals that, in the absence of a detectable increase in the concentration of [^3H](1,4,5)IP₃, hormone-sensitive intracellular Ca²⁺ pools remain empty in the presence of carbachol or caerulein. Indeed, with carbachol intracellular pools can be emptied and kept empty without any detectable increase in [^3H](1,4,5)IP₃. At present we cannot fully explain this apparent

paradox. It is noteworthy that, in many tissues stimulated with receptor-agonists that activate adenylate cyclase, a similar paradox exists. In the parotid gland, for example, cyclic AMP-dependent protein kinase and the responses regulated by that kinase can be stimulated in the absence of a measurable increase in cellular cyclic AMP content (Spearman & Butcher, 1982). The most likely explanation of this result is that receptor-agonists increase cyclic AMP in a cytosolic compartment that is sufficiently small as to contribute insignificantly to the measurement of total cellular cyclic AMP. A similar compartmentalization may underlie the lack of correlation between total cellular [^3H](1,4,5)IP₃ concentrations and the behaviour of hormone-sensitive intracellular Ca²⁺ pools. Indeed, we have previously reported that only a fraction of the endoplasmic reticulum pool, perhaps a fraction that is closely associated with the plasma membrane, is responsive to (1,4,5)IP₃ (Taylor & Putney, 1985; Putney, 1986), and in hepatocytes a very small and submaximal increase of IP₃ is sufficient to elevate cytosolic Ca²⁺ maximally, although the isomers of IP₃ were not resolved in that study (Lynch *et al.*, 1985a). We conclude therefore that total cellular concentrations of [^3H](1,4,5)IP₃ may not accurately reflect the concentration of this putative second messenger in biologically relevant compartments. Another possibility is that IP₄, which is also formed in the pancreas after receptor activation (J. E. Merritt, C. W. Taylor, R. P. Rubin & J. W. Putney, Jr., unpublished work), may play a role in regulation of intracellular Ca²⁺ pools or in regulating Ca²⁺ fluxes at the plasma membrane. Further studies, however, will be needed to distinguish between these or other possibilities.

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REFERENCES

- Batty, I. R., Nahorski, S. R. & Irvine, R. F. (1985) *Biochem. J.* **232**, 211–215
- Berridge, M. J. (1984) *Biochem. J.* **220**, 345–360
- Berridge, M. J. & Irvine, R. F. (1984) *Nature (London)* **312**, 315–321
- Berridge, M. J., Dawson, R. M. C., Downes, C. P., Heslop, J. P. & Irvine, R. F. (1983) *Biochem. J.* **212**, 473–482
- Burgess, G. M., McKinney, J. S., Irvine, R. F. & Putney, J. W., Jr. (1985) *Biochem. J.* **232**, 237–243
- Corvera, S., Schwarz, K. R., Graham, R. M. & Garcia-Sainz, J. A. (1986) *J. Biol. Chem.* **261**, 520–526
- Downes, C. P., Mussat, M. C. & Michell, R. H. (1982) *Biochem. J.* **203**, 169–177
- Heslop, J. P., Irvine, R. F., Tashjian, A. & Berridge, M. J. (1985) *J. Exp. Biol.* **119**, 395–402
- Irvine, R. F., Letcher, A. J., Lander, D. J. & Downes, C. P. (1984) *Biochem. J.* **223**, 237–243
- Irvine, R. F., Anggard, E. A., Letcher, A. J. & Downes, C. P. (1985) *Biochem. J.* **229**, 505–511
- Irvine, R. F., Letcher, A. J., Heslop, J. P. & Berridge, M. J. (1986) *Nature (London)* **320**, 631–634
- Lynch, C. J., Blackmore, P. F., Charest, R. & Exton, J. H. (1985a) *Mol. Pharmacol.* **28**, 93–99
- Lynch, C. J., Charest, R., Bocckino, S. B., Exton, J. H. & Blackmore, R. F. (1985b) *J. Biol. Chem.* **260**, 2844–2851
- Merritt, J. E. & Rubin, R. P. (1985) *Biochem. J.* **230**, 151–159
- Merritt, J. E., Taylor, C. W., Rubin, R. P. & Putney, J. W., Jr. (1986) *Biochem. J.* **236**, 337–343
- Nishizuka, Y. (1984) *Nature (London)* **308**, 693–698
- Orellana, S. A., Solski, P. A. & Brown, J. H. (1985) *J. Biol. Chem.* **260**, 5236–5239

- Putney, J. W., Jr. (1986) *Cell Calcium* **7**, 1–12
- Putney, J. W., Jr., Burgess, G. M., Halenda, S. P., McKinney, J. S. & Rubin, R. P. (1983) *Biochem. J.* **212**, 483–488
- Spearman, T. N. & Butcher, F. R. (1982) *Mol. Pharmacol.* **21**, 121–127
- Streb, H., Irvine, R. F., Berridge, M. J. & Schulz, I. (1983) *Nature (London)* **306**, 67–69
- Taylor, C. W. & Putney, J. W., Jr. (1985) *Biochem. J.* **232**, 435–438
- Weiss, S. J. & Putney, J. W., Jr. (1981) *Biochem. J.* **194**, 463–468

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