Effects of Ca^{2+} on phosphoinositide breakdown in exocrine pancreas

Colin W. TAYLOR,* Janet E. MERRITT, James W. PUTNEY, Jr. and Ronald P. RUBIN

Department of Pharmacology, Medical College of Virginia, Richmond, VA 23298, U.S.A.

Recent studies have established that inositol 1,4,5-trisphosphate [$\text{I}(1,4,5)\text{P}_3$] provides the link between receptor-regulated polyphosphoinositide hydrolysis and mobilization of intracellular Ca^{2+} . Here, we report the effects of Ca^{2+} on inositol trisphosphate (IP_3) formation from phosphatidylinositol biphosphate (PIP_2) catalysed by phospholipase C in intact and electrically permeabilized rat pancreatic acinar cells. (1) In permeabilized cells, the Ca^{2+} -mobilizing agonist caerulein stimulated [^3H] IP_3 formation when the free [Ca^{2+}] was buffered at 140 nM, the cytosolic free [Ca^{2+}] of unstimulated pancreatic acinar cells. When the free [Ca^{2+}] was reduced to less than 10 nM, caerulein did not stimulate [^3H] IP_3 formation. (2) Ca^{2+} in the physiological range stimulated [^3H] IP_3 formation and reduced the amount of [^3H] PIP_2 in permeabilized cells. The effects of Ca^{2+} and the receptor agonist caerulein were additive, but we have not established whether this reflects independent effects on the same or different enzymes. (3) The effect of Ca^{2+} on [^3H] IP_3 formation by permeabilized cells was unaffected by inhibitors of the cyclo-oxygenase and lipoxygenase pathways of arachidonic acid metabolism; nor were the effects of Ca^{2+} mimicked by addition of arachidonic acid. These results suggest that the effects of Ca^{2+} on phospholipase C activity are not a secondary consequence of Ca^{2+} activation of phospholipase A_2 . (4) Changes in free [Ca^{2+}] (< 10 nM–1.2 mM) did not affect the metabolism of exogenous [^3H] $\text{I}(1,4,5)\text{P}_3$ by permeabilized cells. (5) In permeabilized cells, breakdown of exogenous [^3H] IP_3 to [^3H] IP_2 (inositol biphosphate), and formation of [^3H] IP_3 in response to receptor agonists were equally inhibited by 2,3-bisphosphoglyceric acid. This suggests that the [^3H] IP_2 formed in response to receptor agonists is entirely derived from [^3H] IP_3 . (6) In intact cells, [^3H] IP_3 formation was stimulated when ionomycin was used to increase the cytosolic free [Ca^{2+}]. However, a maximal concentration of caerulein elicited ten times as much IP_3 formation as did the highest physiologically relevant [Ca^{2+}]. We conclude that the major effect of receptor agonists on IP_3 formation does not require an elevation of cytosolic free [Ca^{2+}], although the increase in free [Ca^{2+}] that normally follows IP_3 formation may itself have a small stimulatory effect on phospholipase C.

INTRODUCTION

Receptor-stimulated phosphoinositide turnover, first documented in exocrine pancreas (Hokin & Hokin, 1953, 1954), is now known to be a feature of many Ca^{2+} -mobilizing receptors (Michell, 1975). Recent studies, initially from pancreas (Streb *et al.*, 1983) and subsequently from many other tissues (Berridge & Irvine, 1984), have established the link between stimulated phosphoinositide turnover and mobilization of intracellular Ca^{2+} . When activated, Ca^{2+} -mobilizing receptors stimulate phospholipase C, which catalyses a phosphodiesteratic cleavage of PIP_2 , and perhaps PIP , to give diacylglycerol and the water-soluble inositol phosphates IP_3 and IP_2 . $\text{I}(1,4,5)\text{P}_3$ mobilizes Ca^{2+} from intracellular pools, probably from a component of the endoplasmic reticulum, and thus provides a link between receptor-stimulated events at the plasma membrane and the mobilization of intracellular Ca^{2+} .

Although it is now accepted that PIP_2 hydrolysis is usually not a secondary consequence of Ca^{2+} mobiliza-

tion, it is still possible that changes in cytosolic Ca^{2+} could have physiologically relevant effects on phospholipase C. Earlier attempts to investigate these aspects have been compromised because of an inability to establish a direct quantitative relationship between the free cytosolic [Ca^{2+}] and the activity of phospholipase C. In iris smooth muscle, for example, PIP_2 breakdown stimulated by Ca^{2+} ionophore is a secondary effect of Ca^{2+} -stimulated release of noradrenaline, since its effects are antagonized by the α_1 -adrenergic antagonist, prazosin (Akhtar & Abdel-Latif, 1984). Alternatively, direct stimulatory effects of Ca^{2+} on phospholipase A_2 activity resulting in the formation of arachidonic acid may account for the effects of Ca^{2+} on PIP_2 hydrolysis (Lapetina *et al.*, 1978; Rubin *et al.*, 1981). In platelets, for example, arachidonic acid is metabolized by the cyclo-oxygenase pathway to thromboxane A_2 , which is released from the cell, binds to its receptor and stimulates phospholipase C (see Rittenhouse, 1985).

In a study of the effect of Ca^{2+} in leukaemic basophil 2H3 cells, Beavan *et al.* (1984) concluded that, although

Abbreviations used: DMSO, dimethyl sulphoxide; DPG, 2,3-diphospho-D-glyceric acid (pentasodium salt) (more correctly, 2,3-bisphospho-D-glyceric acid); ETYA, 5,8,11,14-eicosatetraenoic acid; GTPyS, guanosine-5'-O-(3-thiotriphosphate) (tetralithium salt); IP, inositol monophosphate; IP_2 , inositol biphosphate; IP_3 , inositol trisphosphate; $\text{I}(1,4,5)\text{P}_3$ etc., inositol 1,4,5-trisphosphate etc.; IP_4 , inositol tetrakisphosphate; PI, phosphatidylinositol; PIP, phosphatidylinositol monophosphate; PIP_2 , phosphatidylinositol biphosphate.

* Present address and address to which correspondence and reprint requests should be sent; AFRC Unit of Insect Neurophysiology and Pharmacology, Department of Zoology, Downing Street, Cambridge CB2 3EJ, U.K.

activation of phospholipase C by cross-linking of the IgE receptor was dependent on extracellular Ca^{2+} , this dependency reflected a requirement for Ca^{2+} at a low-affinity extracellular site and not a direct effect of intracellular Ca^{2+} on phospholipase C. Finally, studies of isolated and partially purified phospholipase C are useful, but may pose problems, since the Ca^{2+} -sensitivity of the enzyme varies enormously according to the conditions under which it is assayed (Irvine *et al.*, 1984; Wilson *et al.*, 1984).

Many of the problems discussed above may be readily overcome if the effects of Ca^{2+} are examined in a permeabilized cell preparation. Under such conditions, the lipid substrates of phospholipase C remain in their native environment. The ionic composition and, most importantly, the free $[\text{Ca}^{2+}]$ may be precisely controlled to mimic the normal intracellular environment. Analysis of stimulation of phospholipase C by receptor agonists is simplified, since the increase in cytosolic $[\text{Ca}^{2+}]$ that normally follows such stimulation is prevented by the presence of EGTA. In the present study, we report the effects of Ca^{2+} and receptor agonists on phospholipase C activity in intact and electrically permeabilized rat pancreatic acinar cells.

MATERIALS AND METHODS

Materials

Collagenase and guanine nucleotides were obtained from Boehringer Mannheim. Peninsula Laboratories (Belmont, CA, U.S.A.) supplied the caerulein. $[\text{^3H}]$ Inositol (10–30 Ci/mmol) was from American Radiolabeled Chemicals (St. Louis, MO, U.S.A.). $[\text{2-}^3\text{H}]\text{I}(1,4,5)\text{P}_3$ and unlabelled $\text{I}(1,4,5)\text{P}_3$ were supplied by Amersham (Arlington Heights, IL, U.S.A.). Essential amino acids were from Gibco. Quin-2 acetoxymethyl ester was from Calbiochem–Behring. Leukotriene B_4 was a gift from Dr. J. Rokach, Merck Frosst, Quebec, Que., Canada; ETYA was a gift from Hoffman–La Roche, Nutley, NJ, U.S.A., and ionomycin was a gift from the Squibb Institute, Princeton, NJ, U.S.A. All other reagents were from Sigma.

Preparation and permeabilization of pancreatic acinar cells

Acinar cells were prepared by collagenase digestion of pancreata from male Sprague–Dawley rats (Merritt *et al.*, 1986). Isolated cells were resuspended in a modified Krebs–Henseleit medium (pH 7.4; O_2/CO_2 , 19:1), supplemented with essential amino acids, glucose (11.5 mM), soybean trypsin inhibitor (0.01%) and bovine serum albumin (0.1% w/v), and containing Ca^{2+} (1.28 mM) and Mg^{2+} (1.18 mM). For experiments with permeabilized cells, the cells were resuspended at a density of 2 mg of cell protein/ml in a Ca^{2+} -free medium whose composition otherwise resembled that of cytosol. This medium had the following composition (mM): KCl, 100; NaCl, 20; NaHCO_3 , 25; MgSO_4 , 5; NaH_2PO_4 , 0.96; EGTA, 1.0; ATP, 1.5; phosphocreatine, 5; creatine kinase, 5 units/ml; bovine serum albumin, 2%; soybean trypsin inhibitor, 0.01%, pH 7.2 at 37 °C; the gas phase was O_2/CO_2 (19:1) [modified from that described by Burgess *et al.* (1983)]. Oligomycin (10 μM), antimycin (10 μM) and 2,4-dinitrophenol (0.5 mM) were added immediately after permeabilization, and the cells incubated for 5 min

before experiments. Inclusion of mitochondrial inhibitors abolishes the responses of residual intact cells and thus ensures that the observed responses are entirely those of permeabilized cells (Merritt *et al.*, 1986). Exogenous GTP was not added, since previous studies (Merritt *et al.*, 1986) showed that, in this permeabilized cell preparation, sufficient GTP is present to support receptor-activated PIP_2 breakdown.

Measurement of $[\text{^3H}]$ inositol phosphates and lipids

Intact cells (50 mg of cell protein/ml) were incubated in the Krebs–Henseleit medium with $[\text{^3H}]$ inositol (100 $\mu\text{Ci/ml}$) for 4–5 h at 37 °C. The cells were washed by centrifugation and resuspended (1 mg of cell protein/ml) in the Krebs–Henseleit medium, for studies of intact cells, and in the cytosol-like medium (2 mg of cell protein/ml) for permeabilization. Incubations (in duplicate) were initiated by addition of 250 μl aliquots of cells to 250 μl of the appropriate medium containing the test substances. Since cells were permeabilized in a Ca^{2+} -free medium, these incubations were initiated by addition of cells to the cytosol-like medium with Ca^{2+} at double its final concentration; with this protocol, changes in free $[\text{Ca}^{2+}]$ could be precisely timed. The free $[\text{Ca}^{2+}]$ of the cytosol-like media was calculated as described by Burgess *et al.* (1983).

Incubations were terminated by addition of 1 ml of cold 4.5% (w/v) HClO_4 , and inositol phosphates were separated by anion-exchange chromatography (Berridge *et al.*, 1983); $[\text{^3H}]$ inositol phosphates were expressed as a percentage of total $[\text{^3H}]$ inositol lipids. Recent reports (Batty *et al.*, 1985; Heslop *et al.*, 1985; Irvine *et al.*, 1985) have shown that the 'IP₃ fraction' eluted from a Dowex column with 1 M-ammonium formate and 0.1 M-formic acid includes not only $\text{I}(1,4,5)\text{P}_3$, but also $\text{I}(1,3,4)\text{P}_3$ and $\text{I}(1,3,4,5)\text{P}_4$. Our measurement of $[\text{^3H}]\text{IP}_3$ therefore includes $[\text{^3H}]\text{IP}_4$ and both isomers of $[\text{^3H}]\text{IP}_3$; in subsequent discussions we therefore refer to this fraction as the 'IP₃ fraction'. However, this measurement still provides a reliable indicator of phospholipase C activity, since $\text{I}(1,4,5)\text{P}_3$, the immediate product of phospholipase C-catalysed PIP_2 hydrolysis, is believed to be the only precursor from which $\text{I}(1,3,4,5)\text{P}_4$ and $\text{I}(1,3,4)\text{P}_3$ can be synthesized (Irvine *et al.*, 1986).

$[\text{^3H}]$ Inositol lipids were separated by t.l.c. (Weiss & Putney, 1981), detected by fluorography and the spots corresponding to $[\text{^3H}]\text{PI}$, $[\text{^3H}]\text{PIP}$ and $[\text{^3H}]\text{PIP}_2$ scraped off and counted for radioactivity.

Metabolism of $[\text{^3H}]\text{I}(1,4,5)\text{P}_3$

Permeabilized cells, at a final cell density equivalent to 1 mg of intact cell protein/ml, were incubated with $[\text{^3H}]\text{I}(1,4,5)\text{P}_3$ (1.0 Ci/mmol) at a final concentration of 10 nM. Incubation conditions and subsequent separation of inositol phosphates were as described above.

Measurement of cytosolic free $[\text{Ca}^{2+}]$ of intact cells

Intact cells (10 mg of cell protein/ml) were incubated for 30 min in Krebs–Henseleit medium containing quin-2 acetoxymethyl ester (100 μM), then washed three times by centrifugation and resuspended in the same medium without quin-2. Intracellular free $[\text{Ca}^{2+}]$ was measured by quin-2 fluorescence as previously described (Merritt & Rubin, 1985).

Fig. 1. Effects of Ca^{2+} and caerulein on formation of $[\text{H}^3]$ inositol phosphates by permeabilized cells

Permeabilized cells were incubated for 6 min at various free $[\text{Ca}^{2+}]$ in the absence (○) or presence (●) of caerulein (100 nM). Results are means $\pm$ S.E.M. for three independent experiments.

RESULTS

Effect of Ca^{2+} on formation of $[\text{H}^3]$ inositol phosphates in permeabilized cells

In permeabilized cells, substantial increases in $[\text{H}^3]\text{IP}_2$ and in the $[\text{H}^3]\text{IP}_3$ fraction were observed in response to both Ca^{2+} (Fig. 1) and caerulein (Merritt *et al.*, 1986); however, their proportions differed according to the stimulus. After 6 min of stimulation with 100 nM-caerulein (free $[\text{Ca}^{2+}] = 140$ nM), the ratio of $[\text{H}^3]\text{IP}_2$ to the $[\text{H}^3]\text{IP}_3$ fraction was about 2.5 (Merritt *et al.*, 1986). By contrast, relatively more $[\text{H}^3]\text{IP}_2$ was produced in response to Ca^{2+} (Fig. 1). Moreover, the higher the free $[\text{Ca}^{2+}]$, the greater the ratio of $[\text{H}^3]\text{IP}_2$ to the $[\text{H}^3]\text{IP}_3$ fraction, such that at 1.2 mM- Ca^{2+} there was more than 10 times as much activity in the $[\text{H}^3]\text{IP}_2$ ($10.1 \pm 2.3\%$; $n = 4$) as in the $[\text{H}^3]\text{IP}_3$ fraction ($0.90 \pm 0.36\%$, $n = 4$). Although both Ca^{2+} and caerulein stimulated formation of $[\text{H}^3]\text{IP}$, the percentage increase was small, since the basal level of $[\text{H}^3]\text{IP}$ was much higher than that of $[\text{H}^3]\text{IP}_2$ and $[\text{H}^3]\text{IP}_3$.

In the absence of added Ca^{2+} (estimated free $[\text{Ca}^{2+}] < 10$ nM, assuming total contaminating $[\text{Ca}^{2+}] < 50$ μM), permeabilized cells did not produce $[\text{H}^3]$ inositol phosphates in response to a near-maximal concentration of caerulein (100 nM). However, when the free $[\text{Ca}^{2+}]$ was buffered at or above the concentration found in unstimulated cells, caerulein stimulated accumulation of

Fig. 2. Effects of caerulein on $[\text{H}^3]\text{IP}_3$ formation at different free $[\text{Ca}^{2+}]$ in permeabilized cells

Permeabilized cells were incubated for 6 min in the presence or absence (C) of caerulein, with the free $[\text{Ca}^{2+}]$ buffered at the concentration shown against such curve. Results are means $\pm$ S.E.M. for three independent experiments.

$[\text{H}^3]$ inositol phosphates. The stimulatory effects of Ca^{2+} and caerulein were additive (Figs. 1 and 2).

Effects of Ca^{2+} on formation of $[\text{H}^3]$ inositol phosphates in intact cells

To study the effects of Ca^{2+} in intact cells, quin-2-loaded cells were treated with ionomycin, and the cytosolic free $[\text{Ca}^{2+}]$ was measured. Under these conditions, where the cytosolic free $[\text{Ca}^{2+}]$ could be increased from 140 nM to 800 nM or more $[\text{H}^3]\text{IP}_3$ formation was stimulated (Fig. 3). In parallel experiments, quin-2 did not affect the formation of $[\text{H}^3]\text{IP}_3$ in response to ionomycin or caerulein. These effects of ionomycin can be directly attributed to its effects on cytosolic $[\text{Ca}^{2+}]$, since in the absence of extracellular Ca^{2+} (100 μM , EGTA) ionomycin had no effect on $[\text{H}^3]\text{IP}_3$ formation. In intact cells, as in permeabilized cells, this effect of Ca^{2+} was additive with caerulein (results not shown).

Effects of arachidonic acid and its metabolites

At concentrations that have been reported to inhibit phospholipase A_2 , preincubation of intact cells with 500 μM -quinacrine (Billah & Lapetina, 1982), 10 μM -4-bromophenacyl bromide (Schrey & Rubin, 1979), or a high concentration (1 mM) of indomethacin (Franson *et al.*, 1980), substantially inhibited caerulein-stimulated phospholipase C activity in pancreatic acinar cells

Fig. 3. Effects of ionomycin on cytosolic free $[Ca^{2+}]$ and $[^3H]$ inositol phosphates in intact cells

The effects of ionomycin on cytosolic free $[Ca^{2+}]$, as measured by Quin 2, are shown in the lower panel. Formation of $[^3H]IP_3$ (●), $[^3H]IP_2$ (○) and $[^3H]IP$ (▲) in response to ionomycin is shown in the upper panels. Results are means $\pm$ S.E.M. for three independent experiments.

(results not shown). These inhibitors cannot therefore be used to differentiate direct effects of Ca^{2+} on phospholipase C from indirect effects mediated by phospholipase A_2 . Instead, we have examined the effects of a product of phospholipase A_2 activation, arachidonic acid, and of its metabolism on phospholipase C activity.

Arachidonate is a substrate for lipoxygenase and cyclo-oxygenase enzymes (Irvine, 1982), these enzymes are inhibited by ETYA and indomethacin respectively. At concentrations that have been reported to be

inhibitory in exocrine pancreas (Rubin *et al.*, 1982; Stenson & Lobos, 1982), even prolonged pretreatment of cells (25 min) with 10 μM -ETYA or 100 μM -indomethacin did not affect caerulein-stimulated phospholipase C activity in intact cells (results not shown), nor did they affect accumulation of $[^3H]$ inositol phosphates in permeabilized cells when the free $[Ca^{2+}]$ was increased to 1 μM (Table 1). Addition of arachidonic acid (5 or 50 $\mu g/ml$) to permeabilized cells, with free $[Ca^{2+}]$ buffered at 140 nM, had no effect on $[^3H]IP_3$ accumulation (results not shown). Leukotriene B_4 , a product of arachidonate metabolism in some cells, binds to a specific cell-surface receptor in neutrophils (Goldman & Goetzl, 1982) and has been reported to activate phospholipase C (Bradford & Rubin, 1985). However, in intact pancreatic acinar cells, leukotriene B_4 (100 nM) did not affect formation of $[^3H]$ inositol phosphates (results not shown).

Finally we investigated whether any water-soluble factor mediates the effects of Ca^{2+} on phospholipase C activity. If such a factor were important, it should be produced by unlabelled permeabilized cells in response to an elevated $[Ca^{2+}]$. We would then expect this factor to be able to stimulate formation of $[^3H]$ inositol phosphates by labelled permeabilized cells when the free $[Ca^{2+}]$ is reduced to 140 nM. Unlabelled permeabilized cells were incubated in medium with no added Ca^{2+} (free $[Ca^{2+}] < 10$ nM) or with the free $[Ca^{2+}]$ buffered at 1230 nM. After 6 min, $[^3H]$ inositol-labelled cells in Ca^{2+} -free medium were simultaneously added with Ca^{2+} , such that the final free $[Ca^{2+}]$ was either 140 nM (total $[Ca^{2+}] = 435 \mu M$) or 1230 nM (total $[Ca^{2+}] = 870 \mu M$). The final density of the unlabelled cells, and so of any soluble factor produced by them, was the same as that used for the experiments shown in Figs. 1 and 2. Formation of $[^3H]$ inositol phosphates by the labelled cells during the 10 s incubation was stimulated when the free $[Ca^{2+}]$ was increased to 1230 nM; however, in medium containing 140 nM-free Ca^{2+} , accumulation of $[^3H]$ inositol phosphates was the same, irrespective of the $[Ca^{2+}]$ to which the unlabelled cells had previously been exposed (Table 2). It is therefore unlikely that Ca^{2+} activation of phospholipase C is mediated by a soluble factor, or, if such a factor exists, it must be very short-lived.

Effects of Ca^{2+} on $[^3H]$ inositol lipids in permeabilized cells

In permeabilized cells, the amount of $[^3H]PIP_2$ was significantly reduced when the free $[Ca^{2+}]$ was 1 μM , and the effect was even more pronounced when the free $[Ca^{2+}]$ was 1.2 mM. $[^3H]PIP$ was unaffected by changes in free $[Ca^{2+}]$ over the physiological range, although it was significantly decreased when the free $[Ca^{2+}]$ was in-

Table 1. Effects of Ca^{2+} on $[^3H]IP_3$ accumulation in permeabilized cells in the presence of ETYA or indomethacin

Permeabilized cells were incubated for 6 min in the cytosol-like medium with the free $[Ca^{2+}]$ buffered at < 10 nM or 1 μM ; the effects of ETYA or indomethacin on $[^3H]IP_3$ formation are shown as the means $\pm$ S.E.M. for three independent experiments.

Treatment	Free $[Ca^{2+}]$. . .	$[^3H]IP_3$ fraction (% of total $[^3H]$ inositol lipids)	
		< 10 nM	1 μM
Control (0.1% DMSO)		0.06 ± 0	0.21 ± 0.08
ETYA (10 μM)		0.07 ± 0.01	0.23 ± 0.07
Indomethacin (100 μM)		0.07 ± 0.01	0.30 ± 0.09

Table 2. A soluble factor does not mediate the effect of Ca^{2+} on phospholipase C activity

Unlabelled permeabilized cells were incubated for 6 min in the cytosol-like medium with the free $[\text{Ca}^{2+}]$ buffered to < 10 nM or 1230 nM. Labelled permeabilized cells were then added, the free $[\text{Ca}^{2+}]$ simultaneously adjusted to 140 nM or 1230 nM, and $[\text{H}^3]$ inositol phosphate formation was measured after a further 10 s. See the text for further details. Results are means $\pm$ S.E.M. for three experiments.

Free $[\text{Ca}^{2+}]$ (nM) Fraction . . .	$[\text{H}^3]$ Inositol phosphates (% of total $[\text{H}^3]$ inositol lipids)		
	$[\text{H}^3]\text{IP}_3$	$[\text{H}^3]\text{IP}_2$	$[\text{H}^3]\text{IP}$
< 10 , then 140	0.07 ± 0.003	0.15 ± 0.01	2.61 ± 0.64
< 10 , then 1230	0.22 ± 0.02	0.42 ± 0.05	2.34 ± 0.03
1230, then 140	0.07 ± 0.003	0.15 ± 0.02	2.57 ± 0.67

creased to 1.2 mM. Changing the free $[\text{Ca}^{2+}]$ from less than 10 nM to 1.2 mM had little effect on the amount of $[\text{H}^3]\text{PI}$ (Table 3).

Effects of Ca^{2+} on $[\text{H}^3]\text{I}(1,4,5)\text{P}_3$ metabolism

When the free $[\text{Ca}^{2+}]$ was buffered at 140 nM, permeabilized cells dephosphorylated added $[\text{H}^3]\text{I}(1,4,5)\text{P}_3$ to $[\text{H}^3]\text{IP}_3$, $[\text{H}^3]\text{IP}$ and $[\text{H}^3]$ inositol (Fig. 4). This breakdown of $[\text{H}^3]\text{IP}_3$ was substantially inhibited by DPG (10 mM). Although the initial breakdown of $[\text{H}^3]\text{IP}_3$ was rapid, there was little further loss between 10 and 30 min, even though about 50% of the activity remained in the IP_3 fraction. However, as discussed above, this IP_3 fraction includes IP_4 and both isomers of IP_3 ; it is therefore possible that some $[\text{H}^3]\text{I}(1,4,5)\text{P}_3$ was converted first into $[\text{H}^3]\text{IP}_4$ and then to $[\text{H}^3]\text{I}(1,3,4)\text{P}_3$ (see Irvine *et al.*, 1986). Such a conversion could explain the relative stability of this IP_3 fraction, since $\text{I}(1,3,4)\text{P}_3$ is dephosphorylated more slowly than is $\text{I}(1,4,5)\text{P}_3$ (Irvine *et al.*, 1985). Neither the rate, nor the eventual extent, of dephosphorylation of $[\text{H}^3]\text{I}(1,4,5)\text{P}_3$ by permeabilized cells was affected by changes in the free $[\text{Ca}^{2+}]$ from less than 10 nM to 1.2 mM (Table 4). These results suggest that, under these conditions, Ca^{2+} affects neither the phosphorylation nor the dephosphorylation of $\text{I}(1,4,5)\text{P}_3$.

Table 3. Effects of free $[\text{Ca}^{2+}]$ on $[\text{H}^3]$ inositol lipids in permeabilized cells

Permeabilized cells were incubated in a cytosol-like medium and after 6 min the $[\text{H}^3]$ inositol lipids were extracted and separated. The ^3H radioactivity of each lipid was expressed as a percentage of the activity recovered from cells incubated in a medium with the free $[\text{Ca}^{2+}]$ buffered at < 10 nM. Results are means $\pm$ S.E.M. for three independent experiments.

Free $[\text{Ca}^{2+}]$	^3H radioactivity (%)		
	$[\text{H}^3]\text{PIP}_2$	$[\text{H}^3]\text{PIP}$	$[\text{H}^3]\text{PI}$
140 nM	104 ± 10	110 ± 10	99 ± 1
1 μM	73 ± 8	109 ± 11	100 ± 1
1.2 mM	34 ± 4	50 ± 6	106 ± 1

Source of $[\text{H}^3]\text{IP}_2$

$[\text{H}^3]\text{IP}_2$ may be formed by either dephosphorylation of $[\text{H}^3]\text{IP}_3$ by a phosphomonoesterase or by phosphodiesteratic cleavage of $[\text{H}^3]\text{PIP}$. When permeabilized cells were incubated for 6 min with exogenous $[\text{H}^3]\text{I}(1,4,5)\text{P}_3$, its dephosphorylation was inhibited by $74 \pm 3\%$ ($n = 3$) in the presence of DPG (10 mM). Prelabelled permeabilized cells synthesize $[\text{H}^3]\text{IP}_3$ and $[\text{H}^3]\text{IP}_2$ in response to either GTP γS alone or in combination with receptor agonists (Merritt *et al.*, 1986). When DPG (10 mM) was included, $[\text{H}^3]\text{IP}_2$ formation was substantially inhibited (Table 5). DPG inhibited $[\text{H}^3]\text{IP}_2$ formation to a similar extent whether the $[\text{H}^3]\text{IP}_2$ was derived from breakdown of exogenous $[\text{H}^3]\text{I}(1,4,5)\text{P}_3$ or after receptor activation of

Fig. 4. Metabolism of exogenous $[\text{H}^3]\text{I}(1,4,5)\text{P}_3$ by permeabilized cells

Changes in the activity of $[\text{H}^3]\text{IP}_3$ fraction ($\blacktriangle$, $\triangle$), $[\text{H}^3]\text{IP}_2$ ($\bullet$, $\circ$) and $[\text{H}^3]\text{IP}$ ($\blacksquare$, $\square$) after addition of $[\text{H}^3]\text{I}(1,4,5)\text{P}_3$ (10 nM) to permeabilized cells in the cytosol-like medium with the free $[\text{Ca}^{2+}]$ buffered at 140 nM. Cells were incubated in the absence (filled symbols) or presence of DPG (10 mM; open symbols). Activities are expressed as a percentage of the initial activity of $[\text{H}^3]\text{I}(1,4,5)\text{P}_3$. At 30 min, the sum of the activities recovered in the $[\text{H}^3]$ inositol phosphates and $[\text{H}^3]$ inositol was $97 \pm 1\%$ of the initial $[\text{H}^3]\text{I}(1,4,5)\text{P}_3$ activity. The activity of $[\text{H}^3]$ inositol is not shown; although it did show a time-dependent increase, even at 30 min this did not exceed 1%. Results are shown as means $\pm$ S.E.M. for three to five independent experiments.

Table 4. Effects of Ca^{2+} on metabolism of exogenous $[^3\text{H}]\text{I}(1,4,5)\text{P}_3$ by permeabilized cells

Permeabilized cells were incubated with $[^3\text{H}]\text{I}(1,4,5)\text{P}_3$ (10 nM) in the cytosol-like medium with the free $[\text{Ca}^{2+}]$ buffered as shown. Results are shown as means $\pm$ S.E.M. for n independent experiments.

Time	Breakdown of [³ H]I(1,4,5)P ₃ (%)					
	Free [Ca ²⁺] . . .	< 10 nM	50 nM	140 nM	800 nM	1.2 mM
30 s		2.0 ± 0.4	3.1 ± 0.3	3.3 ± 0.4	3.3 ± 0.6	4.3 ± 1.4
1 min		6.7 ± 0.2	5.6 ± 0.8	5.7 ± 0.6	6.5 ± 0.6	6.4 ± 1.1
5 min		25.0 ± 3.1	26.3 ± 3.0	29.6 ± 2.3	27.4 ± 1.7	27.1 ± 2.4
10 min		36.1 ± 4.0	39.2 ± 5.3	39.0 ± 2.8	40.6 ± 4.0	45.1 ± 1.2
30 min		47.3 ± 4.1	49.3 ± 5.6	53.4 ± 4.1	47.2 ± 5.1	48.4 ± 4.2
<i>n</i> . . .		3	3	5	3	4

Table 5. Effects of Ca^{2+} , GTP γ S and receptor agonists on $[^3\text{H}]\text{IP}_2$ formation by permeabilized cells

Permeabilized cells (1 mg of cell protein/ml) were incubated for 6 min with or without addition of DPG (10 mM). Under identical conditions, DPG (10 mM) inhibited dephosphorylation of exogenous $[^3\text{H}]\text{IP}_3$ by $74 \pm 3\%$ ($n = 3$). $[^3\text{H}]\text{IP}_2$ formation in response to each stimulus is shown after subtraction of $[^3\text{H}]\text{IP}_2$ formation (0.23 ± 0.04) by cells incubated in a medium containing 140 nM free $[\text{Ca}^{2+}]$. Results are means $\pm$ S.E.M. for five independent experiments. * indicates that inhibition of $[^3\text{H}]\text{IP}_2$ formation by DPG differed significantly ($P < 0.05$; Mann-Whitney U-test) from that expected from studies of the breakdown of exogenous $[^3\text{H}]\text{I}(1,4,5)\text{P}_3$.

Free Ca^{2+}	Other additions	$[^3\text{H}]\text{IP}_2$ (% of total $[^3\text{H}]\text{inositol lipids}$)		Inhibition of $[^3\text{H}]\text{IP}_2$ formation (%)
		Control	+ DPG (10 mM)	
1 μM	None	3.92 ± 0.87	1.29 ± 0.41	69 ± 3
1.2 mM	None	6.87 ± 1.41	4.40 ± 0.86	$35 \pm 4^*$
140 nM	GTP γ S (100 μM)	0.40 ± 0.12	0.16 ± 0.05	62 ± 7
	GTP γ S (10 μM) + Carbachol (100 μM)	0.46 ± 0.12	0.20 ± 0.05	56 ± 16
	GTP γ S (10 μM) + Caerulein (3 nM)	0.49 ± 0.15	0.21 ± 0.06	55 ± 11

phospholipase C in prelabelled cells. These results suggest that the $[^3\text{H}]\text{IP}_2$ formed after receptor activation is derived primarily from $[^3\text{H}]\text{IP}_3$ and not from direct breakdown of $[^3\text{H}]\text{PIP}$. Similarly, the degree of DPG inhibition of $[^3\text{H}]\text{IP}_2$ formation in response to 1 μM -free $[\text{Ca}^{2+}]$ is consistent with $[^3\text{H}]\text{IP}_3$ being the exclusive source of $[^3\text{H}]\text{IP}_2$. However, at much higher (1.2 mM) and physiologically irrelevant free $[\text{Ca}^{2+}]$, it appears likely that some $[^3\text{H}]\text{IP}_2$ may be derived directly from $[^3\text{H}]\text{PIP}$ hydrolysis (Table 5).

DISCUSSION

If $\text{I}(1,4,5)\text{P}_3$ is to provide the link between receptor-stimulated polyphosphoinositide turnover and mobilization of intracellular Ca^{2+} , receptor agonists must be able to activate phospholipase C under the conditions that exist in unstimulated cells. In a cytosol-like medium, with the free $[\text{Ca}^{2+}]$ buffered to that found in unstimulated pancreatic acinar cells, activation of the muscarinic or cholecystokinin receptors stimulated formation of IP_3 (Fig. 2; Streb *et al.*, 1985; Merritt *et al.*, 1986). These results, together with abundant evidence that $\text{I}(1,4,5)\text{P}_3$ mobilizes Ca^{2+} from a component of the endoplasmic reticulum (Streb *et al.*, 1983; Berridge & Irvine, 1984; Taylor & Putney, 1985), firmly support the suggestion

that $\text{I}(1,4,5)\text{P}_3$ is the link between receptors and Ca^{2+} mobilization.

Although an elevation in the free $[\text{Ca}^{2+}]$ is not necessary for phospholipase C activation, the enzyme does appear to be Ca^{2+} -dependent, since in permeabilized pancreatic acinar cells, phospholipase C is unresponsive to caerulein when the free $[\text{Ca}^{2+}]$ is reduced to less than 10 nM (Fig. 2). Studies of purified sheep seminal-vesicle phospholipase C (Wilson *et al.*, 1984), a crude preparation of soluble phospholipase C from brain (Irvine *et al.*, 1984), or the membrane-bound enzymes of red blood cells (Downes & Michell, 1982) or of hepatocytes (Seyfred & Wells, 1984) have demonstrated activation of phospholipase C by submicromolar $[\text{Ca}^{2+}]$ but only at low $[\text{Mg}^{2+}]$ and unphysiologically low ionic strengths. When the ionic strengths and $[\text{Mg}^{2+}]$ were more physiological, phospholipase C activity was stimulated only by unrealistically high $[\text{Ca}^{2+}]$. These results have led most authors to conclude that phospholipase C activity is not normally regulated by Ca^{2+} . However, our results from permeabilized pancreatic acinar cells suggest that Ca^{2+} may play a minor role in regulation of phospholipase C activity. Ca^{2+} in the range of 500 nM to 1 μM [a range that encompasses the cytosolic free $[\text{Ca}^{2+}]$ of maximally stimulated pancreatic acinar cells (Merritt & Rubin, 1985)] stimulated phospholipase C activity when perme-

abilized cells were suspended in a cytosol-like medium. However, none of these studies established whether the Ca^{2+} -regulated phospholipase C is the same enzyme that can be regulated by Ca^{2+} -mobilizing receptors. In exocrine pancreas, the stimulatory effects of Ca^{2+} and caerulein were additive (Fig. 2), a result which may be explained by independent activation of the same or different enzymes.

We have shown that regulation of phospholipase C activity by Ca^{2+} is unlikely to be a consequence of Ca^{2+} -activation of phospholipase A_2 . Briefly, the evidence may be summarized as follows. Neither arachidonic acid nor any soluble factor produced by Ca^{2+} activation of permeabilized cells affected phospholipase C activity. Stimulation of phospholipase C by Ca^{2+} was unaffected by either indomethacin or ETYA, thus ruling out a role for cyclo-oxygenase or lipoxygenase metabolites of arachidonic acid. In exocrine pancreas, the commonly used inhibitors of phospholipase A_2 also substantially inhibited phospholipase C activity. Thus we have been unable to unequivocally eliminate a role for the other products of phospholipase A_2 activity, the lysolipids, in activating phospholipase C.

Another possibility may be that Ca^{2+} -stimulated IP_3 formation merely reflects increased substrate availability without any change in phospholipase C activity. Such a suggestion is unlikely, since the amount of $[\text{^3H}]\text{PIP}_2$ was decreased when the free $[\text{Ca}^{2+}]$ was increased to $1\ \mu\text{M}$ and above; however, it must be conceded that measurement of the total $[\text{^3H}]\text{PIP}_2$ activity need not provide a reliable measure of the relevant phospholipase C-sensitive pool.

In permeabilized pancreatic acinar cells, metabolism of $[\text{^3H}]\text{I}(1,4,5)\text{P}_3$ was unaffected by changes in free $[\text{Ca}^{2+}]$. Similarly in rat hepatocytes, the free $[\text{Ca}^{2+}]$ concentration does not affect dephosphorylation of $\text{I}(1,4,5)\text{P}_3$ (Shears *et al.*, 1985). By contrast, in smooth muscle, dephosphorylation of IP_3 is stimulated by Ca^{2+} (Sasaguri *et al.*, 1985) and a purified $\text{I}(1,4,5)\text{P}_3$ 5-phosphomonoesterase from human platelets is inhibited by Ca^{2+} (Connolly *et al.*, 1985). The reason for these differences is not clear, although it is possible that in purifying an enzyme or working with a broken cell preparation, soluble cofactors, important in mediating the effects of Ca^{2+} , may be lost to various extents. However, in our experiments with pancreatic acinar cells, the effects of Ca^{2+} on $[\text{^3H}]\text{IP}_3$ formation and dephosphorylation were studied under identical conditions. The increased accumulation of IP_3 in response to Ca^{2+} must, therefore, be due to stimulation of its formation rather than to inhibition of its breakdown.

Although these results suggest that Ca^{2+} may directly regulate the activity of phospholipase C, it is important to compare the direct effects of Ca^{2+} and of receptor agonists on phospholipase C activity. Such a comparison is possible only in intact cells, since receptor coupling to phospholipase C is less effective in permeabilized cells (Merritt *et al.*, 1986). That is, in permeabilized cells, the effects of Ca^{2+} relative to receptor agonists may be exaggerated. In intact cells, a maximal concentration of caerulein, which transiently elevated cytosolic free $[\text{Ca}^{2+}]$ to $800\ \text{nM}$, caused a 20-fold increase in IP_3 formation (C. W. Taylor, J. E. Merritt, J. W. Putney, Jr. & R. P. Rubin, unpublished work). However, using ionomycin to increase cytosolic free $[\text{Ca}^{2+}]$ of intact cells to around $800\ \text{nM}$, we observed only a 2-fold increase in IP_3 formation (Fig. 3). We conclude that, in exocrine

pancreas, the major effect of agonists on IP_3 formation does not require an elevation in cytosolic free $[\text{Ca}^{2+}]$. However, the increase in cytosolic free $[\text{Ca}^{2+}]$ that normally follows IP_3 formation may also play a minor role in directly regulating phospholipase C, at least under conditions of maximal stimulation.

This work was supported by grants (nos. AM 28029 and DE 05764) from the National Institutes of Health.

REFERENCES

- Akhtar, R. A. & Abdel-Latif, A. A. (1984) *Biochem. J.* **224**, 291–300
- Batty, I. R., Nahorski, S. R. & Irvine, R. F. (1985) *Biochem. J.* **232**, 211–215
- Beavan, M. A., Moore, J. P., Smith, B. A., Hesketh, R. & Metcalfe, J. C. (1984) *J. Biol. Chem.* **259**, 7137–7142
- 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
- Billah, M. M. & Lapetina, E. G. (1982) *J. Biol. Chem.* **257**, 5196–5200
- Bradford, P. B. & Rubin, R. P. (1985) *Mol. Pharmacol.* **27**, 74–78
- Burgess, G. M., McKinney, J. S., Fabiato, A., Leslie, B. A. & Putney, J. W., Jr. (1983) *J. Biol. Chem.* **258**, 15336–15345
- Connolly, T. M., Bross, T. E. & Majerus, P. W. (1985) *J. Biol. Chem.* **260**, 7868–7874
- Downes, C. P. & Michell, R. H. (1982) *Biochem. J.* **202**, 53–58
- Franson, R. C., Eisen, D., Jesse, R. & Lanni, C. (1980) *Biochem. J.* **186**, 633–636
- Goldman, D. W. & Goetzl, E. J. (1982) *J. Immunol.* **129**, 1600–1604
- Heslop, J. P., Irvine, R. F., Tashjian, A. H. & Berridge, M. J. (1985) *J. Exp. Biol.* **119**, 395–402
- Hokin, M. R. & Hokin, L. E. (1953) *J. Biol. Chem.* **203**, 967–977
- Hokin, M. R. & Hokin, L. E. (1954) *J. Biol. Chem.* **209**, 549–558
- Irvine, R. F. (1982) *Biochem. J.* **204**, 3–16
- Irvine, R. F., Letcher, A. J. & Dawson, R. M. C. (1984) *Biochem. J.* **218**, 177–185
- 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
- Lapetina, E. G., Chandrabose, K. A. & Cuatrecasas, P. (1978) *Proc. Natl. Acad. Sci. U.S.A.* **75**, 818–822
- 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
- Michell, R. H. (1975) *Biochim. Biophys. Acta* **415**, 81–147
- Rittenhouse, S. E. (1985) in *Inositol and Phosphoinositides* (Bleasdale, J. E., Eichberg, J. & Hauser, G., eds.), pp. 459–473, Humana Press, Clifton, NJ
- Rubin, R. P., Sink, L. E. & Freer, R. J. (1981) *Biochem. J.* **194**, 497–505
- Rubin, R. P., Kelly, K. L., Halenda, S. P. & Laychock, S. G. (1982) *Prostaglandins* **24**, 179–193
- Sasaguri, T., Hirata, M. & Kuriyama, H. (1985) *Biochem. J.* **231**, 497–503
- Schrey, M. P. & Rubin, R. P. (1979) *J. Biol. Chem.* **254**, 11234–11241
- Seyfred, M. A. & Wells, W. W. (1984) *J. Biol. Chem.* **259**, 7666–7672
- Shears, S. B., Storey, D. T., Michell, R. H. & Kirk, C. J. (1985) *Biochem. Soc. Trans.* **13**, 944

- Stenson, W. F. & Lobos, E. (1982) *Am. J. Physiol.* **242**, G493–G497
- Streb, H., Irvine, R. F., Berridge, M. J. & Schulz, I. (1983) *Nature (London)* **306**, 67–69
- Streb, H., Heslop, J. P., Irvine, R. F., Schulz, I. & Berridge, M. J. (1985) *J. Biol. Chem.* **260**, 7309–7315
- 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
- Wilson, D. B., Bross, T. E., Hofmann, S. L. & Majerus, P. W. (1984) *J. Biol. Chem.* **259**, 11718–11724

Received 30 January 1986/19 May 1986; accepted 3 June 1986