Regulation of the formation of inositol phosphates by calcium, guanine nucleotides and ATP in digitonin-permeabilized bovine adrenal chromaffin cells

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Both micromolar Ca\(^{2+}\) and guanosine 5′-[\(\gamma\)-thio]triphosphate (GTP[S]) stimulated the formation of inositol phosphates (InsPs) in digitonin-permeabilized chromaffin cells prelabelled with [\(^{3}H\)]inositol. The production of InsPs was potentiated by ATP. Guanosine 5′-[\(\beta\)-thio]diphosphate (GDP[S]) caused a GTP-reversible shift to higher concentrations in the \(Ca^{2+}\)-concentration–response curve for the release of InsPs without changing the maximal response. GTP[S] caused a shift to lower concentrations of \(Ca^{2+}\) and also increased the maximal response. The effects of GTP[S] and \(Ca^{2+}\) were synergistic. Although as much as \(80\%\) of the InsPs were derived from phosphatidylinositol 4-phosphate (PtdInsP) or 4,5-bisphosphate (PtdInsP\(_2\)), the amount of InsPs produced could be several times the total amount of PtdInsP and PtdInsP\(_2\) in the cells and was largely accounted for by a decrease in PtdIns. The levels of labelled PtdInsP and PtdInsP\(_2\) increased on stimulation with \(Ca^{2+}\), but decreased on stimulation with GTP[S] or the combination of \(Ca^{2+}\) and GTP[S]. Preincubation with \(Ca^{2+}\) and ATP amplified the subsequent GTP[S]-induced production of InsPs. ATP and its \(\gamma\)-thio and \(\beta\)-imido analogues stimulated the formation of InsPs in intact cells. However, only ATP potentiated the responses to \(Ca^{2+}\) and GTP[S] in permeable cells. Our main conclusions are: (1) a GTP-binding protein participates in the \(Ca^{2+}\)-induced production of InsPs by phospholipase C, and (2) ATP markedly potentiates the stimulated formation of InsPs, an effect with arises from its role in polyphosphoinositide synthesis and does not involve purinergic receptor activation in permeabilized cells. The data also suggest that the different effects of \(Ca^{2+}\) and GTP[S] on polyphosphoinositide synthesis probably contribute to the synergistic action of \(Ca^{2+}\) and GTP[S] on the generation of InsPs.

INTRODUCTION

The interactions of many hormones, neurotransmitters, and growth factors with their appropriate receptors lead to the production of inositol phosphates (InsPs) and diacylglycerol. These substances serve as second messengers which link receptors to cellular responses. InsP\(_1\) and InsP\(_2\) increase intracellular \(Ca^{2+}\), and diacylglycerol activates protein kinase C (for review see Berridge, 1987). The molecular mechanisms involved in the coupling of stimuli to the inositol phosphate response have been intensively investigated. There is compelling evidence that receptors communicate through a GTP-binding protein to a phospholipase C which acts preferentially on phosphatidylinositol 4,5-bisphosphate (PtdInsP\(_2\)) (for review see Fain et al., 1988).

In bovine adrenal medullary chromaffin cells the formation of InsPs is stimulated by depolarization with increased K\(^+\) (Eberhard & Holz, 1987; Sasaki & et al., 1987), nocotinic and muscarinic agonists (Eberhard & Holz, 1987; Nakaki et al., 1988), P\(_{2}\)-purinergic agonists (Sasaki & et al., 1989; Allsup & Boarder, 1990) and other hormones (e.g. Plevin & Boarder, 1988). These studies showed the responses to nicotinic stimulation and elevated K\(^+\) to be dependent on \(Ca^{2+}\) influx, whereas the responses to muscarinic and P\(_{2}\)-purinergic agonists are relatively independent of extracellular \(Ca^{2+}\), and therefore may be mediated by a G-protein. In permeabilized chromaffin cells, the formation of InsPs is stimulated by micromolar \(Ca^{2+}\) (Whitaker, 1985; Eberhard & Holz, 1987) as well as by activators of GTP-binding proteins (Eberhard et al., 1990).

Stimulus–response coupling in the PtdIns pathway involves regulation of phosphoinositide synthesis as well as hydrolysis. The mechanisms involved in regulating synthesis are still being defined, but appear to be both dependent and independent of inositol phospholipid hydrolysis (for review see Downes et al., 1989). In a recent study, we found that \(Ca^{2+}\) promoted the accumulation of labelled PtdInsP\(_1\) and phosphatidylinositol 4-phosphate (PtdInsP\(_2\)) in both intact and permeabilized chromaffin cells (Eberhard & Holz, 1991). This effect was accompanied, but not caused, by phospholipase C activation. Unlike \(Ca^{2+}\), the GTP analogue guanosine 5′-[\(\gamma\)-thio]triphosphate (GTP[S]) decreased the net phosphorylation of PtdInsP\(_1\) and PtdInsP\(_2\) in permeable cells.

In the present study we extended these observations by examining the interaction of \(Ca^{2+}\)- and guanine-nucleotide-dependent mechanisms controlling the formation of inositol-derived second messengers in digitonin-permeabilized chromaffin cells. We found that guanine nucleotides modulate \(Ca^{2+}\)-dependent phospholipase C activity. In addition, the data suggest that synergistic interaction between \(Ca^{2+}\) and guanine nucleotides in the formation of InsPs probably results in part from a \(Ca^{2+}\)-stimulated increase in polyphosphoinositide synthesis.

MATERIALS AND METHODS

Chromaffin cells were isolated by dissociation of bovine adrenal medulla, purified by differential plating (Waymire et al., 1983) and cultured as monolayers in 6.4 mm-diameter collagen-coated

Abbreviations used: p[NH]3ppA, adenosine 5′-[\(\gamma\)-thio]triphosphate; ATP[S], adenosine 5′-[\(\gamma\)-thio]triphosphate; GDP[S], guanosine 5′-[\(\beta\)-thio]diphosphate; GTP[S], guanosine 5′-[\(\gamma\)-thio]triphosphate; InsPs, inositol phosphates; KGEF, buffer containing potassium glutamate, EGTA and Pipes; PtdIns, PtdInsP\(_1\) and PtdInsP\(_2\), phosphatidylinositol, its 4-phosphate and 4,5-bisphosphate; PSS, physiological salt solution.

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Fig. 1. Time course of Ca\(^{2+}\)- and GTP\(S\)-stimulated release of Ins\(P\)_s

Inositol-prelabelled cells were permeabilized for 4 min with KGEP containing 20 \(\mu\)M-diginton and 2 mM-MgATP. The permeabilization medium was removed, and fresh KGEP without digitonin containing 2 mM-MgATP and no addition (CNTRL), 10 \(\mu\)M free Ca\(^{2+}\), 10 \(\mu\)M-GTP\(S\), or Ca\(^{2+}\) together with GTP\(S\) (Ca\(^{2+}\)+GTP\(S\)) was added. After incubation for the indicated times, the medium was removed and Ins\(P\)_s released into the medium were assayed as described in the Materials and methods section.

![Diagram](image)

wells (150,000 cells/well) as previously described (Holz et al., 1982). The cultures consisted of at least 90% chromaffin cells and contained virtually no visually detectable fibroblasts or endothelial cells.

Experiments were performed 4–12 days after culture preparation. Cellular inositol-containing lipids were labelled by incubation for 36–60 h with myo-[2-\(^3\)H]inositol (20 \(\mu\)Ci/ml) in Eagle’s minimal essential medium containing 11 \(\mu\)M-myoinositol (Whittaker M. A. Bioproducts, Walkersville, MD, U.S.A.) supplemented with 10% (v/v) dialysed fetal-bovine serum (GIBCO Laboratories, Grand Island, NY, U.S.A.), glutamine, penicillin and streptomycin. Inositol incorporation into lipid was maximal after about 48 h of labelling. Immediately before starting an experiment, the labelling medium was removed and the cells were washed for 15 min in physiological salt solution (PSS), containing 145 mM-NaCl, 5.6 mM-KCl, 2.2 mM-CaCl\(_2\), 0.5 mM-MgCl\(_2\), 5.6 mM-glucose, 0.5 mM-ascorbic acid and 15 mM-Hepes, pH 7.4. Experiments were initiated by removing the PSS wash and permeabilizing the cells as described by Dunn & Holz (1983) with 20 \(\mu\)M-digitonin in a solution (KGEP) containing 139 mM-potassium glutamate, 20 mM-Pipes (pH 6.6), 5 mM-EGTA and various amounts of Ca\(^{2+}\) to give free Ca\(^{2+}\) concentrations calculated by the computer program of Chang et al. (1988) by using the constants of Portzehl et al. (1964). Other components of the KGEP solutions are detailed in the Figure legends; 50–100 \(\mu\)l of KGEP was used per well. Experiments were terminated by quantitatively transferring the KGEP to tubes containing 1.0 ml of ice-cold water. The samples were stored at \(-20^\circ\)C for up to 2 weeks before Ins\(P\)_s analysis. Immediately after removing the KGEP from the cells, 100 \(\mu\)l of ice-cold methanol/\(v/v\) conc. HCl (100:1, \(v/v\)) was added to the culture wells. The wells were scraped and the contents transferred to tubes on ice. The wells were washed with 100 \(\mu\)l of methanol, which was pooled with the first methanolic solution. Lipids were extracted from the samples on the same day as the experiment. Lipid extracts were stored at \(-20^\circ\)C for up to 1 week before separation by t.l.c.

Assay of phosphoinositides

Lipids were extracted by mixing 400 \(\mu\)l of chloroform/methanol (2:1, \(v/v\)) with the methanolic samples, which were then washed with 250 \(\mu\)l of 10 mM-EDTA in 1 M-HCl (vigorous vortex-mixing followed by centrifugation to separate phases). Then 200 \(\mu\)l of the lower organic phase was transferred to another tube. The upper phase and interfacers were washed with 200 \(\mu\)l of chloroform and 200 \(\mu\)l of the resulting lower phase was then pooled with the first organic sample. The organic samples (400 \(\mu\)l) were washed with 400 \(\mu\)l of 10 mM-EDTA in 1 M-HCl/methanol (1:1, \(v/v\)). The entire lower phase was transferred to a new tube for subsequent lipid analysis. The samples were dried under \(N_2\), redissolved in chloroform/methanol (2:1, \(v/v\)) before storage at \(-20^\circ\)C. After each wash in the extraction procedure, the total volumes of the organic phases were measured to determine recoveries.

For separation of phosphoinositides by t.l.c., \(^{33}\)P-labelled lipids were added to the samples as internal standards. The samples were then either spotted on heat-activated oxalate-impregnated silica-gel 60 plates (E. Merck, Darmstadt, Germany) and developed in chloroform/acetone/methanol/acetic acid/ water (40:15:15:12.8, \(v/v\)) to separate Ins\(P\)_s, Ins\(P\)_2, lyso-PtdIns, Ins\(P\)_3 and PtdIns, or spotted on silica-gel HL plates (Analytech, Newark, DE, U.S.A.) and developed in chloroform/methanol/water/conc. NH\(_3\) (44:44:7:5, \(v/v\)) (adapted from Mitchell et al., 1986). The former system provided good separations of PtdIns from phosphatidic acid but poor resolution of lyso-PtdIns from PtdIns; the latter system separated lyso-PtdIns and PtdIns\(P\) at the expense of the separation of PtdIns and phosphatidic acid, and was used in the experiment shown in Fig. 4. The \(^{33}\)P markers on the t.l.c. plates were detected by autoradiography, scraped, sonicated in 250 \(\mu\)l of water and counted for \(^{33}\)H and \(^{32}\)P radioactivity in 4 ml of Universol ES. The \(^{32}\)P-labelled lipids were prepared by incubating chromaffin cells for 30 min with \(^{32}\)P-PIP, in PSS, followed by lipid extraction; the short labelling period allowed \(^{32}\)P to be preferentially incorporated into the rapidly cycling phosphoinositides and phosphatidate (Fisher et al., 1981).

Assay of Ins\(P\)_s

Ins\(P\)_s released into the incubation media were separated by anion-exchange chromatography as described by Berridge et al. (1983). The samples were applied to Dowex AG1–X8 columns (formate form, 0.3 ml bed volume). Free inositol and glycerophosphoinositol were eluted with 8 \(\times\) 1.0 ml washes of 5 mM-sodium tetraborate/60 mM-sodium formate. These washes were usually discarded. Ins\(P\)_s was eluted with 3 \(\times\) 1.0 ml of 0.2 mM-ammonium formate/0.1 mM-fumaric acid; the columns were then washed with 3 \(\times\) 1.0 ml of the same buffer. Ins\(P\)_3 was eluted with 7 \(\times\) 1.0 ml of 0.4 mM-ammonium formate/0.1 mM-fumaric acid, fol-
Regulation of formation of inositol phosphates in chromaffin cells

Polar myo-[2-3H]Inositol of means well+S.E.M.; cell culture preparations. The chromaffin InsP, and maximal at 100-300 μM-Ca2+. Since the only known source of InsP2 is Ins(1,4,5)P2 and therefore PtdInsP2, for our purposes it was not necessary to separate these species, nor did the conclusions drawn from this study require that the individual isomers of the various inositol phosphates be separated and identified.

Previous work has demonstrated that virtually all cells are permeabilized by digitonin in the monolayer cultures of chromaffin cells (Dunn & Holz, 1983). In the present study, InsPs released into the medium were entirely from permeabilized cells, since in cells not treated with digitonin (intact cells) InsPs produced in response to depolarization-induced Ca2+ influx were completely retained by the cells.

Data analysis

The Figures and Tables show data from individual experiments representative of similar experiments performed in two or more cell culture preparations. Data shown are average c.p.m./well±S.E.M.; there were four wells/group. Error bars smaller than the point symbols are omitted. Differences between the means of groups were tested for significance by Student's t test. The S.E.M. associated with the difference between the means of two groups was calculated by (S.E.M.1+ S.E.M.2)2.

Materials

All reagents were obtained from standard commercial sources. myo-[2-3H]inositol (1 mCi/ml, 10-30 Ci/mmol) was from American Radiolabelled Chemicals (St. Louis, MO, U.S.A.). Polar contaminants were removed before use by mixing a few mg of Dowex AG1-X8 with aqueous [3H]inositol. [γ-32P]ATP was from Amersham Corp. (Arlington Heights, IL, U.S.A.). Guanine nucleotides (Li salts) were from Boehringer Mannheim Biochemicals (Indianapolis, IN, U.S.A.).

RESULTS

Ca2+ and GTP[S] stimulate the release of InsPs

Ca2+ (10 μM) and a maximally effective concentration of GTP[S] (10 μM) each stimulated the release of [3H]InsP2, [3H]InsP3 and [3H]InsP4 from [3H]inositol-prelabelled digitonin-permeabilized chromaffin cells (Fig. 1). The two agents had similar effects. Most of the radioactivity released was in the InsP2 and InsP3 fractions. A maximal concentration of the inositol-5-phosphatase inhibitor 2,3-bisphosphoglycerate (10 mM) caused a 3-fold enhancement of Ca2+-dependent InsP2 release, and a 5-fold enhancement of GTP[S]-dependent InsP3 release, with concomitant decreases in InsP2 and InsP3 (results not shown). Thus a significant portion of the InsP3 and InsP2 produced was probably derived from InsP2. However, the experiments did not rule out direct hydrolysis of PtdInsP2 or Ptdlns.

Guanylate nucleotides modulate Ca2+-dependent phospholipase C activity

The effects of guanylate nucleotides on the release of InsPs stimulated by various concentrations of Ca2+ were examined. The response to Ca2+ alone was half-maximal at about 10 μM-Ca2+ and maximal at 100-300 μM-Ca2+ (Fig. 2a). Guanosine 5'-

Fig. 2. Interactions of guanine nucleotides and Ca2+ on the release of InsPs

Inositol-prelabelled cells were incubated 15 min in KGE containing 20 μM-digoxin, 2 mM-MgATP, and (a) various concentrations of free Ca2+ in the absence (CNTRL) or presence of 10 μM-GTP[S] or 500 μM-GDP[S], (b) various concentrations of Ca2+ in the absence (•, no addn.) or presence of 500 μM-GDP[S] (○), 1 mM-GTP (■), or GDP[S] and GTP together (□, GDP[S]+GTP), or (c) various concentrations of GTP[S] in the absence (■) or presence (○) of 10 μM-Ca2+. Total InsPs released into the medium were assayed as described in the Materials and methods section.
Table 1. Effect of ATP and ATP analogues on accumulation of InsPs in intact cells

<table>
<thead>
<tr>
<th>Addition</th>
<th>InsPs (c.p.m./well)</th>
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<tbody>
<tr>
<td>No addn.</td>
<td>1867 ± 103 (100)</td>
</tr>
<tr>
<td>ATP</td>
<td>3406 ± 38 (182)</td>
</tr>
<tr>
<td>ATP[S]</td>
<td>4000 ± 210 (214)</td>
</tr>
<tr>
<td>p[CH]ppA</td>
<td>2282 ± 146 (122)</td>
</tr>
<tr>
<td>p[NH]ppA</td>
<td>4642 ± 81 (249)</td>
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</tbody>
</table>

[β-thio]diphosphate (GDP[S] (500 μM) caused a 5–10-fold decrease in the Ca^{2+}-sensitivity, which was reversed by 1 mM-GTP (Fig. 2b). Thus residual GTP in the cell probably contributes to the sensitivity to Ca^{2+} of the production of InsPs. GTP alone had small and variable effects on Ca^{2+}-induced release, perhaps because the amount of endogenous GTP was sufficient to satisfy the requirements of the process. GTP[S] (10 μM) stimulated the release of InsPs in the absence of added Ca^{2+} (Fig. 2a). Increasing the concentration of EGTA in the incubation medium from 5 mM to 15 mM inhibited by 40% the effect of GTP[S] in the absence of added Ca^{2+} (results not shown). Therefore the production of InsPs in response to GTP[S] is at least partially dependent on a small amount of Ca^{2+} (~0.1 μM), as has been reported in other permeabilized cell types (Martin et al., 1986; Vallat et al., 1987; Stutchfield & Cockcroft, 1988). The effects of Ca^{2+} and GTP[S] together were greater then additive (Figs. 1, 2a and 2c). GTP[S] decreased the Ca^{2+} concentration required for a maximal release of InsPs from 100 μM to 10 μM (Fig. 2a). The synergy between Ca^{2+} and GTP[S] was most evident in InsP_{2} release and was seen in both the initial rate and maximal amount of release (Fig. 1).

Ca^{2+} (10 μM) caused a synergistic response with GTP[S] at all concentrations without changing the half-maximally effective concentration (Fig. 2c). Thus Ca^{2+} increased the magnitude, but not the sensitivity, of the response to GTP[S]. GDP(S) (300 μM) shifted the concentration of GTP[S] that half-maximally stimulated phospholipase C activity from 1 μM to 10 μM without decreasing the maximal response (results not shown).

Roles of ATP and ATP analogues in the formation of InsPs

Extracellular ATP can activate phospholipase-C-linked purinergic receptors in the plasma membrane (Boyer et al., 1989). Intracellular ATP is required for synthesis of the polyphosphoinositide phospholipase C substrates. In order to evaluate the importance of each of these mechanisms in modulating the production of InsPs in response to Ca^{2+} and GTP[S] in digitonin-permeabilized chromaffin cells, we examined the effects of ATP and non-hydrolyzable ATP analogues on the formation of InsPs in intact and permeabilized chromaffin cells.

The following results indicate that the predominant effect of ATP in permeabilized cells is the maintenance of the polyphosphoinositides. Table 1 shows that incubation of intact cells with ATP, adenosine 5'-[β-thio]triphosphate (ATP[S]) or

adenosine 5'-[β-imido]triphosphate (p[NH]ppA) (1 mM each) promotes the accumulation of InsPs, which is consistent with previous reports (Sasakawa et al., 1989; Allsup & Boarder, 1990). Adenosine 5'-[β-methylene]triphosphate was ineffective. In contrast, Table 2 shows that in digitonin-permeabilized cells neither ATP nor p[NH]ppA stimulated the release of InsPs. The muscarinic agonists methacholine (Table 2) and muscarine (results not shown) were similarly without effect in digitonin-permeabilized cells, although they stimulate the production of InsPs in intact cells. However, the response to either Ca^{2+} or GTP[S] was potentiated by ATP, but not by p[NH]ppA or methacholine. In other experiments, ATP[S] was without effect.
Table 3. Effect of Ca\(^{2+}\) and ATP in the preincubation on subsequent GTP[S]-induced release of InsPs

<table>
<thead>
<tr>
<th>First incubation</th>
<th>Radioactivity released in second incubation (c.p.m.)</th>
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<tbody>
<tr>
<td>Ca(^{2+})</td>
<td>-GTPS</td>
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<td>----</td>
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<td>-</td>
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These findings indicate that in the digitonin-permeabilized chromaffin cell the purinergic and muscarinic receptors are uncoupled from phospholipase C and that the potentiation by ATP of the responses to Ca\(^{2+}\) and GTP[S] requires the hydrolysis of the terminal phosphate of ATP.

The effect of ATP on the time course of stimulated production of InsPs also suggests that ATP functions primarily to sustain the polyphosphoinositide levels rather than to activate phospholipase C through a receptor-mediated mechanism. ATP (1 mM) had little effect on the stimulated release of InsPs at early times, but permitted the sustained release at later times, when resynthesis of the polyphosphoinositides is likely to be most important (Fig. 3).

To our knowledge no studies have been published demonstrating active phospholipase C-linked receptors in permeabilized chromaffin cells or chromaffin-cell membranes. It is possible that digitonin permeabilization somehow decouples the receptors from phospholipase C. However, the guanine-nucleotide-dependent coupling of muscarinic receptors to phospholipase C is preserved in digitonin-permeabilized SK-N-SH neuroblastoma cells (Fisher et al., 1989), whereas a decrease in the efficiency of receptor coupling to phospholipase C was noted in electroporated pancreatic acinar cells (Taylor et al., 1986) as well as in digitonin-permeabilized pancreatic islets (Best, 1986).

Effects of Ca\(^{2+}\) on polyphosphoinositide synthesis may enhance the response to GTP[S]

The stimulated release of [\(^{3}H\)]inositol phosphates was several-fold greater than the total amount of [\(^{3}H\)]PtdInsP and [\(^{3}H\)]PtdInsP\(_2\) in the cells (Fig. 4). The production of InsPs is largely accounted for by a decrease in PtdIns (see Fig. 4 legend for quantification). Therefore, phospholipase C activation by GTP[S], as well as by Ca\(^{2+}\), is accompanied by an increase in synthesis of the polyphosphoinositides from PtdIns. These observations suggest that polyphosphoinositide synthesis is regulated by mechanisms involving Ca\(^{2+}\) and guanine nucleotides. The availability of substrate for phospholipase C is likely to be a factor determining the amounts of InsPs generated by activation of phospholipase C.

The effects of Ca\(^{2+}\) and GTP[S] on labelled polyphosphoinositide levels could not be predicted simply by the amounts of inositol phosphates generated by these agents. Ca\(^{2+}\) increased, whereas GTP[S] decreased, [\(^{3}H\)]PtdInsP and [\(^{3}H\)]PtdInsP\(_2\). Similar changes in the levels of [\(^{32}P\)]P-labelled polyphosphoinositides were observed in permeabilized cells incubated with [\(^{32}P\)]ATP (Eberhard & Holz, 1991), which indicates that the effects occur on newly synthesized lipids and are independent of the labelling method. In the presence of GTP[S], the effect of Ca\(^{2+}\) on [\(^{3}H\)]PtdInsP and [\(^{3}H\)]PtdInsP\(_2\) was diminished or abolished (Fig. 4), whereas the amounts of [\(^{3}H\)]inositol phosphates produced were greater than additive. Therefore Ca\(^{2+}\) may have increased the synthesis of labelled polyphosphoinositides which were susceptible to subsequent hydrolysis by GTP[S]-activated phospholipase C. The following experiment is consistent with this notion. Cells were permeabilized in the absence of ATP (or Ca\(^{2+}\)) to deplete them of ATP (Holz et al., 1989). They were then incubated with Ca\(^{2+}\) and ATP either alone or in combination, and finally incubated without Ca\(^{2+}\) or ATP in the absence or presence of GTP[S] (Table 3). The response to GTP[S] (ΔGTP[S]) was approximately doubled when either Ca\(^{2+}\) or ATP was included in the preincubation. The combination of Ca\(^{2+}\) and ATP caused a nearly 7-fold increase in the GTP[S] response. Thus the stimulatory effects of Ca\(^{2+}\) and ATP on subsequent GTP[S]-induced generation of inositol phosphates are amplified by each other. The data are consistent with Ca\(^{2+}\) acting through stimulation of ATP-dependent synthesis of the polyphosphoinositides.
DISCUSSION

We have investigated the regulation of the production of InsPs by Ca\textsuperscript{2+}, guanine nucleotides and ATP in digitonin-permeabilized chromaffin cells. Our main conclusions are: (1) a GTP-binding protein participates in the Ca\textsuperscript{2+}-induced production of InsPs by phospholipase C; and (2) ATP markedly potentiates the stimulated formation of InsPs, an effect which arises from its role in polyphosphoinositide synthesis and does not involve purinergic-receptor activation in permeabilized cells. The data also suggest that the different effects of Ca\textsuperscript{2+} and GTP[S] on polyphosphoinositide synthesis probably contribute to the synergistic action of Ca\textsuperscript{2+} and GTP[S] on the generation of InsPs.

In digitonin-permeabilized chromaffin cells, guanine nucleotides regulate the relationship between Ca\textsuperscript{2+} concentration and the production of inositol phosphates. GdPS[S] caused a rightward shift (Figs. 2a and 2b) and GTP[S] caused a leftward shift (10–30-fold, Fig. 2a) in the Ca\textsuperscript{2+}-concentration–response curve. Thus the ability of stimuli which increase intracellular Ca\textsuperscript{2+} to activate phospholipase C in chromaffin cells may be modulated by a GTP-binding protein. Conversely, the activation of phospholipase C by receptors coupled to GTP-binding proteins in intact cells may be modulated by intracellular Ca\textsuperscript{2+}. The interaction between GTP[S] and Ca\textsuperscript{2+} caused not only a leftward shift in the Ca\textsuperscript{2+}-concentration–response curve but also supra-additive increases in the maximal responses in the Ca\textsuperscript{2+}- and GTP[S]-concentration–response curves (Figs. 2a and 2c). These effects are consistent with the ability of Ca\textsuperscript{2+} to increase the synthesis of the polyphosphoinositides (Eberhard & Holz, 1991) as discussed below.

The production of InsPs involves the hydrolysis not only of pre-existing lipids but also of newly formed PtdInsP and PtdInsP\textsubscript{2}, as shown in the present study: (a) the amounts of labelled InsP\textsubscript{2} and InsP\textsubscript{3} produced by stimulation with Ca\textsuperscript{2+}, GTP[S] or both agents together could be several times the total cellular amounts of labelled PtdInsP and PtdInsP\textsubscript{2}; (b) the increases in InsP\textsubscript{2} and InsP\textsubscript{3} were accounted for by decreases in PtdIns; (c) InsP\textsubscript{2} and InsP\textsubscript{3} formation was markedly potentiated by ATP in a manner consistent with an ATP requirement for PtdIns and PtdInsP kinases rather than for purinergic-receptor activation. Regulation of the lipid kinases or phosphomonoesterases involved in polyphosphoinositide metabolism may therefore profoundly influence the amounts of second messengers produced when phospholipase C is activated.

Ca\textsuperscript{2+} increases polyphosphoinositide synthesis to a greater extent than does GTP[S] (Eberhard & Holz, 1991). Ca\textsuperscript{2+} influx in intact cells and medium containing micromolar Ca\textsuperscript{2+} in permeabilized cells increased the levels of labelled PtdInsP and PtdInsP\textsubscript{2}. In contrast, muscarinic agonist in intact cells and GTP[S] in permeabilized cells did not increase the levels of these lipids. In the present study, GTP[S] caused a loss of the Ca\textsuperscript{2+}-induced increments in PtdInsP and PtdInsP\textsubscript{2} (Fig. 4). Most importantly, preincubation of permeabilized cells with the combination of Ca\textsuperscript{2+} and ATP caused a pronounced amplification of the amounts of inositol phosphates produced upon subsequent addition of GTP[S] (Table 3). Thus PtdInsP\textsubscript{2} and PtdInsP\textsubscript{3} synthesized through a Ca\textsuperscript{2+}-dependent mechanism may by subject to rapid hydrolysis by phospholipase C activated by GTP[S]. Indeed, the Ca\textsuperscript{2+} concentration (about 10 μM) at which a maximal InsP\textsubscript{2} response was obtained in the presence of GTP[S] (Fig. 2a) is the same as that which produces the greatest accumulations of PtdInsP and PtdInsP\textsubscript{2} in the absence of GTP[S] (Eberhard & Holz, 1991).

It has been reported that micromolar Ca\textsuperscript{2+} inhibits the PtdIns kinase activity of purified chromaffin-granule membranes (Husebye & Flatmark, 1988). We were unable to detect an effect of micromolar Ca\textsuperscript{2+} on PtdIns kinase activity in chromaffin-cell homogenates. It is possible that the most of the PtdIns kinase in chromaffin cells is not on the chromaffin-granule membrane.

Synergistic interactions between GTP[S] and micromolar and sub-micromolar concentrations of Ca\textsuperscript{2+} have been demonstrated in other permeabilized cell preparations (Martin et al., 1986; Vallar et al., 1987; Stutchfield & Cockcroft, 1988; Fisher et al., 1989). The detailed study of GH\textsubscript{3} pituitary cells by Martin et al. (1986) found that the synergy consisted of two components: a supra-additive increase in maximal response, which was ATP-dependent, and an increased Ca\textsuperscript{2+}-sensitivity, which did not require ATP. The results of the present study are consistent with these previous findings and suggest that synergy may arise at two points: (a) a Ca\textsuperscript{2+}-induced increase in phospholipase C substrates, and (b) modulation of the Ca\textsuperscript{2+}-sensitivity of phospholipase C by guanine nucleotides.

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Regulation of formation of inositol phosphates in chromaffin cells


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