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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′-[γ-thio]triphosphate (GTP[S]) stimulated the formation of inositol phosphates (Ins*P*s) in digitonin-permeabilized chromaffin cells prelabelled with [^{3}H]inositol. The production of Ins*P*s was potentiated by ATP. Guanosine 5′-[β-thio]diphosphate (GDP[S]) caused a GTP-reversible shift to higher concentrations in the Ca^{2+}-concentration–response curve for the release of Ins*P*s 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 Ins*P*s were derived from phosphatidylinositol 4-phosphate (PtdIns*P*) or 4,5-bisphosphate (PtdInsP_2), the amount of Ins*P*s produced could be several times the total amount of PtdIns*P* and PtdInsP_2 in the cells and was largely accounted for by a decrease in PtdIns. The levels of labelled PtdIns*P* 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 Ins*P*s. ATP and its γ-thio and βγ-imido analogues stimulated the formation of Ins*P*s 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 Ins*P*s by phospholipase C, and (2) ATP markedly potentiates the stimulated formation of Ins*P*s, 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 Ins*P*s.

INTRODUCTION

The interactions of many hormones, neurotransmitters, and growth factors with their appropriate receptors lead to the production of inositol phosphates (Ins*P*s) and diacylglycerol. These substances serve as second messengers which link receptors to cellular responses. InsP_3 and InsP_4 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 some tissues, an increase in intracellular Ca^{2+} levels may represent another mechanism by which phospholipase C can be activated (Eberhard & Holz, 1988). The production of Ins*P*s in bovine adrenal-medullary chromaffin cells is stimulated by depolarization with increased K^+ (Eberhard & Holz, 1987; Sasakawa *et al.*, 1987), nicotinic and muscarinic agonists (Eberhard & Holz, 1987; Nakaki *et al.*, 1988), P_2-purinergic agonists (Sasakawa *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 Ins*P*s 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_2 and phosphatidylinositol 4-phosphate (PtdIns*P*) 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′-[γ-thio]triphosphate (GTP[S]) decreased the net phosphorylation of PtdInsP_2 and PtdIns*P* 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 inositide-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 Ins*P*s 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 medullae, purified by differential plating (Waymire *et al.*, 1983) and cultured as monolayers in 6.4 mm-diameter collagen-coated

Abbreviations used: p[NH]ppA, adenosine 5′-[βγ-imido]triphosphate; ATP[S], adenosine 5′-[γ-thio]triphosphate; GDP[S], guanosine 5′-[β-thio]diphosphate; GTP[S], guanosine 5′-[γ-thio]triphosphate; Ins*P*s, inositol phosphates; KGEP, buffer containing potassium glutamate, EGTA and Pipes; PtdIns, PtdIns*P* and PtdInsP_2, phosphatidylinositol, its 4-phosphate and 4,5-bisphosphate; PSS, physiological salt solution.

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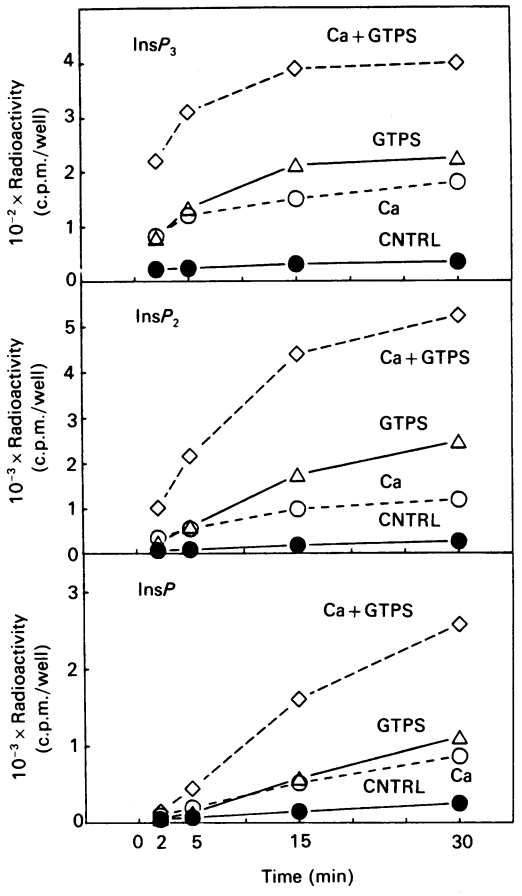

Fig. 1. Time course of Ca^{2+}- and GTP[S]-stimulated release of Ins*P*s

Inositol-prelabelled cells were permeabilized for 4 min with KGEP containing 20 μM-digitonin and 2 mM-MgATP. The permeabilization medium was removed, and fresh KGEP without digitonin containing 2 mM-MgATP and no addition (CNTRL), 10 μM free Ca^{2+}, 10 μM-GTP[S], or Ca^{2+} together with GTP[S] (Ca+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.

wells (150000 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 μCi/ml) in Eagle's minimal essential medium containing 11 μM-*myo*-inositol (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 μM-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 μl 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 °C for up to 2 weeks before Ins*P*s analysis. Immediately after removing the KGEP from the cells, 100 μl of ice-cold methanol/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 μl 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 °C for up to 1 week before separation by t.l.c.

Assay of phosphoinositides

Lipids were extracted by mixing 400 μl of chloroform/methanol (2:1, v/v) with the methanolic samples, which were then washed with 250 μl of 10 mM-EDTA in 1 M-HCl (vigorous vortex-mixing followed by centrifugation to separate phases). Then 200 μl of the lower organic phase was transferrd to another tube. The upper phase and interfacer were washed with 200 μl of chloroform and 200 μl of the resulting lower phase was then pooled with the first organic sample. The organic samples (400 μl) were washed with 400 μl 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 and redissolved in chloroform/methanol (2:1, v/v) before storage at −20 °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., ^{32}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, by vol.) (modified from Jolles *et al.*, 1981), or spotted on silica-gel HL plates (Analtech, Newark, DE, U.S.A.) and developed in chloroform/methanol/water/conc. NH_3 (44:44:7:5, by vol.) (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*P*; 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 ^{32}P markers on the t.l.c. plates were detected by autoradiography, scraped, sonicated in 250 μl of water and counted for ^{3}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]P_i 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 × 1.0 ml washes of 5 mM-sodium tetraborate/60 mM-sodium formate. These washes were usually discarded. InsP_1 was eluted with 3 × 1.0 ml of 0.2 M-ammonium formate/0.1 M-formic acid; the columns were then washed with 3 × 1.0 ml of the same buffer. InsP_2 were eluted with 7 × 1.0 ml of 0.4 M-ammonium formate/0.1 M-formic acid, fol-

lowed by 3 × 3.0 ml washes with the same buffer. InsP_3 was eluted with 4 × 1.0 ml of 1.0 M-ammonium formate/0.1 M-formic acid. Radioactivity in the eluates was determined by scintillation counting as described above. The InsP_3 fraction also contains InsP_4 (Batty *et al.*, 1985; Heslop *et al.*, 1985; Irvine *et al.*, 1985). Since the only known source of InsP_4 is Ins(1,4,5)P_3 and therefore PtdInsP_2, 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, Ins*P*s released into the medium were entirely from permeabilized cells, since in cells not treated with digitonin (intact cells) Ins*P*s produced in response to depolarization-induced Ca^{2+} 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 $(S.E.M._1^2 + S.E.M._2^2)^{\frac{1}{2}}$.

Materials

All reagents were obtained from standard commercial sources. *myo*-[2-^{3}H]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 [^{3}H]inositol. [γ-^{32}P]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

Ca^{2+} and GTP[S] stimulate the release of Ins*P*s

Ca^{2+} (10 μM) and a maximally effective concentration of GTP[S] (10 μM) each stimulated the release of [^{3}H]InsP_3, [^{3}H]InsP_2 and [^{3}H]InsP_1 from [^{3}H]inositol-prelabelled digitonin-permeabilized chromaffin cells (Fig. 1). The two agents had similar effects. Most of the radioactivity released was in the InsP_2 and InsP_1 fractions. A maximal concentration of the inositol-5-phosphatase inhibitor 2,3-bisphosphoglycerate (10 mM) caused a 3-fold enhancement of Ca^{2+}-dependent InsP_3 release, and a 5-fold enhancement of GTP[S]-dependent InsP_3 release, with concomitant decreases in InsP_2 and InsP_1 (results not shown). Thus a significant portion of the InsP_2 and InsP_1 produced was probably derived from InsP_3. However, the experiments did not rule out direct hydrolysis of PtdIns*P* or PtdIns.

Guanine nucleotides modulate Ca^{2+}-dependent phospholipase C activity

The effects of guanine nucleotides on the release of Ins*P*s stimulated by various concentrations of Ca^{2+} were examined. The response to Ca^{2+} alone was half-maximal at about 10 μM-Ca^{2+} and maximal at 100–300 μM-Ca^{2+} (Fig. 2*a*). Guanosine 5′-

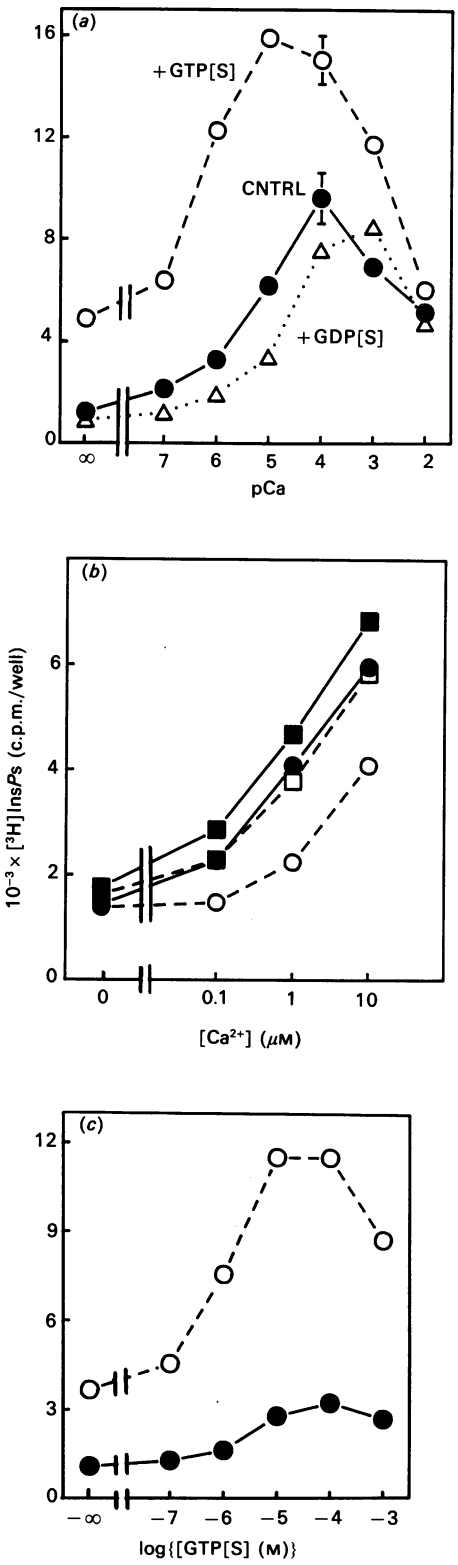

Fig. 2. Interactions of guanine nucleotides and Ca^{2+} on the release of Ins*P*s

Inositol-prelabelled cells were incubated 15 min in KGEP containing 20 μM-digitonin, 2 m-MgATP, and (*a*) various concentrations of free Ca^{2+} in the absence (CNTRL) or presence of 10 μM-GTP[S] or 500 μM-GDP[S], (*b*) various concentrations of Ca^{2+} 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-Ca^{2+}. Total Ins*P*s 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 Ins*P*s in intact cells

Inositol-prelabelled cells were incubated 30 min with PSS containing 10 mM-LiCl, 1.5 mM-$MgCl_2$, and either no further additions (No addn.) or 1 mM-ATP, -ATP[S], -p[CH_2]ppA (adenosine 5′-[$\beta\gamma$-methylene]triphosphate) or -p[NH]ppA. The incubation solution was then removed and the cells were permeabilized for 10 min with KGEP containing 20 μM-digitonin. Total Ins*P*s released into the permeabilization medium were determined as described in the Materials and methods section. Values in parentheses are percentages of the radioactivity present in 'No addn.'.

Addition	Ins*P*s (c.p.m./well)
No addn.	1867±103 (100)
ATP	3406±38 (182)
ATP[S]	4000±210 (214)
p[CH_2]ppA	2282±146 (122)
p[NH]ppA	4642±81 (249)

[β-thio]diphosphate (GDP[S]) (500 mM) caused a 5–10-fold decrease in the Ca^{2+}-sensitivity, which was reversed by 1 mM-GTP (Fig. 2*b*). Thus residual GTP in the cell probably contributes to the sensitivity to Ca^{2+} of the production of Ins*P*s. 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 Ins*P*s in the absence of added Ca^{2+} (Fig. 2*a*). 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 Ins*P*s in response to GTP[S] is at least partially dependent on a small amount of Ca^{2+} ($\ll 0.1$ μM), as has been reported in other permeabilized cell types (Martin *et al.*, 1986; Vallar *et al.*, 1987; Stutchfield & Cockcroft, 1988). The effects of Ca^{2+} and GTP[S] together were greater then additive (Figs. 1, 2*a* and 2*c*). GTP[S] decreased the Ca^{2+} concentration required for a maximal release of Ins*P*s from 100 μM to 10 μM (Fig. 2*a*). 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. 2*c*). Thus Ca^{2+} increased the magnitude, but not the sensitivity, of the response to GTP[S]. GDPS (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 Ins*P*s

Extracellular ATP can activate phospolipase-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 Ins*P*s in response to Ca^{2+} and GTP[S] in digitonin-permeabilized chromaffin cells, we examined the effects of ATP and non-hydrolysable ATP analogues on the formation of Ins*P*s 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′-[γ-thio]triphosphate (ATP[S]) or adenosine 5′-[$\beta\gamma$-imido]triphosphate (p[NH]ppA) (1 mM each) promotes the accumulation of Ins*P*s, which is consistent with previous reports (Sasakawa *et al.*, 1989; Allsup & Boarder, 1990). Adenosine 5′-[$\beta\gamma$-methylene]triphosphate was ineffective. In contrast, Table 2 shows that in digitonin-permeabilized cells neither ATP nor p[NH]ppA stimulated the release of Ins*P*s. 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 Ins*P*s 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 2. Interactions of Ca^{2+}, GTP[S], ATP, p[NH]ppA and methacholine in digitonin-permeabilized cells

Inositol-prelabelled cells were permeabilized for 4 min with NaGEP (139 mM-sodium glutamate, 5 mM-EGTA, 20 mM-Pipes, pH 6.6) containing 2 mM-$MgCl_2$ and 20 μM-digitonin. The permeabilization medium was removed, and NaGEP containing 0.1 μM free Ca^{2+}, 2 mM-$MgCl_2$, 10 mM-LiCl and either no further additions (No. addn.) or 1 mM-ATP, 1 mM-p[NH]ppA, 30 μM-GTP[S], 300 μM-methacholine, and/or 10 μM additional free Ca^{2+} was added as indicated. The amount of Ins*P*s released into the medium after 5 min was determined as described in the Materials and methods section and is shown in the Table. Values in parentheses are percentages of the radioactivity present in 'No addn.'.

Addition	Ins*P*s (c.p.m.)
No addn.	315±17 (100)
ATP	305±16 (97)
p[NH]ppA	362±17 (115)
Methacholine	416±8 (132)
GTP[S]	928±25 (294)
GTP[S]+ATP	1752±64 (556)
GTP[S]+p[NH]ppA	866±29 (275)
GTP[S]+methacholine	931±24 (296)
GTP[S]+methacholine+ATP	1837±109 (583)
Ca^{2+}	739±30 (235)
Ca^{2+}+ATP	1186±46 (377)
Ca^{2+}+p[NH]ppA	722±25 (229)

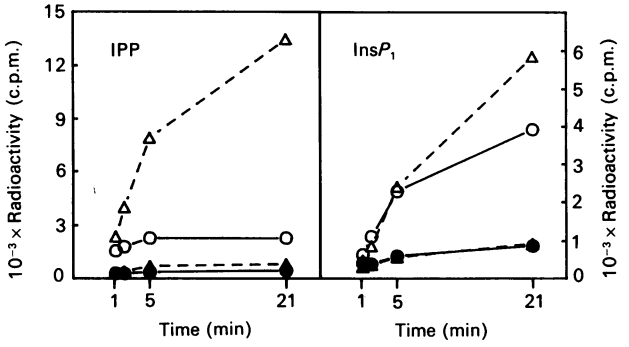

Fig. 3. Effect of ATP on the time course of stimulated Ins*P*s release

Inositol-prelabelled cells were permeabilized for 4 min with KGEP containing 20 μM-digitonin and either 2 mM-MgATP (△, ▲; broken lines) or 2 mM-$MgCl_2$ (○, ●; continuous lines). The permeabilizing medium was removed and the cells were then incubated with KGEP containing either 2 mM-MgATP or 2 mM-$MgCl_2$ in either the absence (●, ▲) or the presence (○, △) of 10 μM-Ca^{2+}+10 μM-GTP[S]. At the indicated times the medium was removed and assayed for inositol polyphosphates (IPP) and $InsP_1$. The Figure shows the combined results of two experiments performed with different cell-culture preparations.

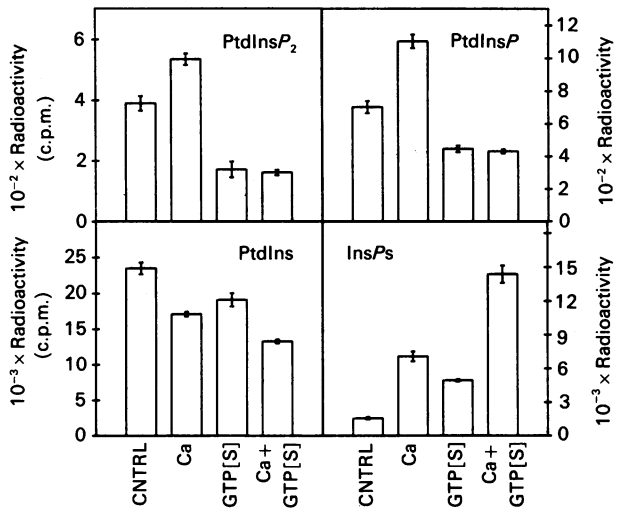

Fig. 4. Effect of GTP[S] and Ca^{2+} on phosphoinositide levels and release of Ins*P*s

Inositol-prelabelled cells were incubated 15 min with KGEP containing 20 μM-digitonin, 2 mM-MgATP, and no addition (CNTRL), 10 μM-Ca^{2+} (Ca), 10 μM-GTP[S] or Ca^{2+} together with GTP[S] (Ca + GTP[S]). The incubation medium was then removed, and total Ins*P*s released into medium were assayed as described in the Materials and methods section. Cellular lipids were separated by t.l.c. and quantified as described in the Materials and methods section. The production of Ins*P*s was accounted for mainly by decreases in PtdIns. With Ca^{2+}, the sum of the Ins*P*s produced and increases in PtdIns*P* and PtdInsP_2 totalled 5829 ± 432 c.p.m. [(5284 ± 427) + (400 ± 55) + (145 ± 31) c.p.m.]; PtdIns decreased by 6372 ± 884 c.p.m. With GTP[S], the difference in the Ins*P*s produced and the decreases in PtdIns*P* and PtdInsP_2 was 2752 ± 139 c.p.m. [(3227 ± 128) − (257 ± 42) − (218 ± 35) c.p.m.]; PtdIns decreased by 4357 ± 1242 c.p.m. With Ca^{2+} and GTP[S] together, the difference in the Ins*P*s produced and the decreases in PtdIns*P* and PtdInsP_2 was 11795 ± 740 c.p.m. [(12295 ± 739) − (272 ± 39) − (228 ± 26) c.p.m.]; PtdIns decreased by 10192 ± 862 c.p.m.

Table 3. Effect of Ca^{2+} and ATP in the preincubation on subsequent GTP[S]-induced release of Ins*P*s

Inositol-prelabelled cells were permeabilized for 4 min with KGEP containing 20 μM-digitonin and 2 mM-$MgCl_2$. The permeabilizing medium was removed, and the cells were then incubated (first incubation) for 5 min in KGEP containing either 2 mM-$MgCl_2$ or 2 mM-MgATP, in the absence or presence of 10 μM-Ca^{2+}. The medium was removed and the cells were incubated (second incubation) in a Ca^{2+}-free KGEP containing 2 mM-$MgCl_2$ (0 ATP) with or without 10 μM-GTP[S]. After 15 min, the release of total Ins*P*s into the test solution was determined. 'ΔGTP[S]' is the GTP[S]-dependent release of Ins*P*s.

First incubation		Radioactivity released in second incubation (c.p.m.)		
Ca^{2+}	ATP	−GTPS	+GTPS	ΔGTPS
−	−	148 ± 4	535 ± 13	387 ± 14
+	−	275 ± 13	1167 ± 32	892 ± 35
−	+	230 ± 14	1063 ± 23	833 ± 27
+	+	592 ± 18	3192 ± 119	2600 ± 120

alone, and only slightly enhanced the response to Ca^{2+} or GTP[S]. 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 Ins*P*s 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 Ins*P*s 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]PtdIns*P* and [^{3}H]PtdInsP_2 in the cells (Fig. 4). The production of Ins*P*s 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 polyphosphoinosides 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 Ins*P*s 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]PtdIns*P* and [^{3}H]PtdInsP_2. Similar changes in the levels of ^{32}P-labelled polyphosphoinositides were observed in permeabilized cells incubated with [γ-^{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]PtdIns*P* 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] (Δ GTP[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 Ins*P*s by Ca^{2+}, guanine nucleotides and ATP in digitonin-permeabilized chromaffin cells. Our main conclusions are: (1) a GTP-binding protein participates in the Ca^{2+}-induced production of Ins*P*s by phospholipase C; and (2) ATP markedly potentiates the stimulated formation of Ins*P*s, 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^{2+} and GTP[S] on polyphosphoinositide synthesis probably contribute to the synergistic action of Ca^{2+} and GTP[S] on the generation of Ins*P*s.

In digitonin-permeabilized chromaffin cells, guanine nucleotides regulate the relationship between Ca^{2+} concentration and the production of inositol phosphates. GDP[S] caused a rightward shift (Figs. 2*a* and 2*b*) and GTP[S] caused a leftward shift (10–30-fold, Fig. 2*a*) in the Ca^{2+}-concentration–response curve. Thus the ability of stimuli which increase intracellular Ca^{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^{2+}. The interaction between GTP[S] and Ca^{2+} caused not only a leftward shift in the Ca^{2+}-concentration–response curve but also supra-additive increases in the maximal responses in the Ca^{2+}- and GTP[S]-concentration–response curves (Figs. 2*a* and 2*c*). These effects are consistent with the ability of Ca^{2+} to increase the synthesis of the polyphosphoinositides (Eberhard & Holz, 1991) as discussed below.

The production of Ins*P*s involves the hydrolysis not only of pre-existing lipids but also of newly formed PtdIns*P* and PtdInsP_2, as shown in the present study: (*a*) the amounts of labelled InsP_2 and InsP_3 produced by stimulation with Ca^{2+}, GTP[S] or both agents together could be several times the total cellular amounts of labelled PtdIns*P* and PtdInsP_2; (*b*) the increases in InsP_2 and InsP_3 were accounted for by decreases in PtdIns; (*c*) InsP_2 and InsP_3 formation was markedly potentiated by ATP in a manner consistent with an ATP requirement for PtdIns and PtdIns*P* 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^{2+} increases polyphosphoinositide synthesis to a greater extent than does GTP[S] (Eberhard & Holz, 1991). Ca^{2+} influx in intact cells and medium containing micromolar Ca^{2+} in permeabilized cells increased the levels of labelled PtdIns*P* and PtdInsP_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^{2+}-induced increments in PtdIns*P* and PtdInsP_2 (Fig. 4). Most importantly, preincubation of permeabilized cells with the combination of Ca^{2+} and ATP caused a pronounced amplification of the amounts of inositol phosphates produced upon subsequent addition of GTP[S] (Table 3). Thus PtdInsP_2 and PtdIns*P* synthesized through a Ca^{2+}-dependent mechanism may by subject to rapid hydrolysis by phospholipase C activated by GTP[S]. Indeed, the Ca^{2+} concentration (about 10 μM) at which a maximal Ins*P* response was obtained in the presence of GTP[S] (Fig. 2*a*) is the same as that which produces the greatest accumulations of PtdIns*P* and PtdInsP_2 in the absence of GTP[S] (Eberhard & Holz, 1991).

It has been reported that micromolar Ca^{2+} inhibits the PtdIns kinase activity of purified chromaffin-granule membranes (Husebye & Flatmark, 1988). We were unable to detect an effect of micromolar Ca^{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^{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_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^{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^{2+}-induced increase in phospholipase C substrates, and (*b*) modulation of the Ca^{2+}-sensitivity of phospholipase C by guanine nucleotides.

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REFERENCES

Allsup, D. J. & Boarder, M. R. (1990) Mol. Pharmacol. **38**, 84–91
Batty, I. R., Nahorski, S. R. & Irvine, R. F. (1985) Biochem. J. **232**, 211–215
Berridge, M. J. (1987) Annu. Rev. Biochem. **56**, 159–193
Berridge, M. J., Dawson, R. M. C., Downes, C. P., Heslop, J. P. & Irvine, R. F. (1983) Biochem. J. **212**, 473–482
Best, L. (1986) Biochem. J. **238**, 773–779
Boyer, J. L., Downes, C. P. & Harden, T. K. (1989) J. Biol. Chem. **264**, 884–890
Chang, D., Hsieh, P. S. & Dawson, D. C. (1988) Comput. Biol. Med. **18**, 351–366
Downes, C. P., Hawkins, P. T. & Stephens, L. (1989) in Inositol Lipids in Cell Signalling (Michell, R. H., Drummond, A. H. & Downes, C. P., eds.), pp. 3–38, Academic Press, New York
Dunn, L. A. & Holz, R. W. (1983) J. Biol. Chem. **258**, 4989–4993
Eberhard, D. A. & Holz, R. W. (1987) J. Neurochem. **49**, 1634–1643
Eberhard, D. A. & Holz, R. W. (1988) Trends Neurosci. **11**, 517–520
Eberhard, D. A. & Holz, R. W. (1991) Cell. Mol. Neurobiol. **11**, 357–370
Eberhard, D. A., Cooper, C. L., Low, M. G. & Holz, R. W. (1990) Biochem. J. **268**, 15–25
Fain, J. N., Wallace, M. A. & Wojcikiewicz, R. J. (1988) FASEB J. **2**, 2569–2574
Fisher, S. K., Holz, R. W. & Agranoff, B. W. (1981) J. Neurochem. **37**, 491–497
Fisher, S. K., Domask, L. M. & Roland, R. M. (1989) Mol. Pharmacol. **35**, 195–204
Heslop, J. P., Irvine, R. F., Tashjian, A. H., Jr. & Berridge, M. J. (1985) J. Exp. Biol. **119**, 395–401
Holz, R. W., Senter, R. A. & Frye, R. A. (1982) J. Neurochem. **39**, 635–646
Holz, R. W., Bittner, M. A., Peppers, S. C., Senter, R. A. & Eberhard, D. A. (1989) J. Biol. Chem. **264**, 5412–5419
Husebye, E. S. & Flatmark, T. (1988) Biochem. Pharmacol. **37**, 4149–4156
Irvine, R. F., Anggard, E. E., Letcher, A. J. & Downes, C. P. (1985) Biochem. J. **229**, 505–511
Jolles, J., Schrama, L. H. & Gispen, W. H. (1981) Biochim. Biophys. Acta **666**, 90–98
Martin, T. F. J., Lucas, D. O., Baijjalieh, S. M. & Kowalchyk, J. A. (1986) J. Biol. Chem. **261**, 2918–2927
Mitchell, K. T., Ferrell, J. E., Jr. & Huestis, W. H. (1986) Anal. Biochem. **158**, 447–453
Nakaki, T., Sasakawa, N., Yamamoto, S. & Kato, R. (1988) Biochem. J. **251**, 397–403
Plevin, R. & Boarder, M. R. (1988) J. Neurochem. **51**, 634–641
Portzehl, H., Caldwell, P. C. & Reugg, J. C. (1964) Biochim. Biophys. Acta **79**, 581–591
Sasakawa, N., Nakaki, T., Yamamoto, S. & Kato, R. (1987) FEBS Lett. **223**, 413–416

Sasakawa, N., Nakaki, T., Yamamoto, S. & Kato, R. (1989) J. Neurochem. **52**, 441–447
Stutchfield, J. & Cockcroft, S. (1988) Biochem. J. **250**, 375–382
Taylor, C. W., Merritt, J. E., Putney, J. W., Jr. & Rubin, R. P. (1986) Biochem. J. **238**, 765–772
Vallar, L., Biden, T. J. & Wollheim, C. B. (1987) J. Biol. Chem. **262**, 5049–5056
Waymire, J. C., Bennett, W. F., Boehme, R., Hanteins, L., Gilmer-Waymire, K. & Haycock, J. (1983) J. Neurosci. Methods **7**, 329–351
Whitaker, M. (1985) FEBS Lett. **189**, 137–140

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