Phosphoinositide hydrolysis by guanosine 5'-[γ -thio]triphosphate-activated phospholipase C of turkey erythrocyte membranes

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Phosphatidylinositol (PtdIns), phosphatidylinositol 4-phosphate (PtdIns4P) and phosphatidylinositol 4,5-bisphosphate [PtdIns(4,5) P_2] of turkey erythrocytes were labelled by using either [32 P] P_i or [3 H]inositol. Although there was little basal release of inositol phosphates from membranes purified from labelled cells, in the presence of guanosine 5'-[γ -thio]triphosphate (GTP[S]) the rate of accumulation of inositol bis-, tris- and tetrakis-phosphate (Ins P_2 , Ins P_3 and Ins P_4) was increased 20–50-fold. The enhanced rate of accumulation of 3 H-labelled inositol phosphates was linear for up to 20 min; owing to decreases in 32 P specific radioactivity of phosphoinositides during incubation of membranes with unlabelled ATP, the accumulation of 32 P-labelled inositol phosphates was linear for only 5 min. In the absence of ATP and a nucleotide-regenerating system, no Ins P_4 was formed, and the overall inositol phosphate response to GTP[S] was decreased. Analyses of phosphoinositides during incubation with ATP indicated that interconversions of PtdIns to PtdIns4P and PtdIns4P to PtdIns(4,5) P_2 occurred to maintain PtdIns(4,5) P_2 concentrations; GTP[S]-induced inositol phosphate formation was accompanied by a corresponding decrease in 32 P- and 3 H-labelled PtdIns, PtdIns4P and PtdIns(4,5) P_2 . In the absence of ATP, only GTP[S]-induced decreases in PtdIns(4,5) P_2 occurred. Since inositol monophosphate was not formed under any condition, PtdIns is not a substrate for the phospholipase C. The production of Ins P_2 was decreased markedly, but not blocked, under conditions where Ins(1,4,5) P_3 5-phosphomonoesterase activity in the preparation was inhibited. Thus the predominant substrate of the GTP[S]-activated phospholipase C of turkey erythrocyte membranes is PtdIns(4,5) P_2 . Ins(1,4,5) P_3 was the major product of this reaction; only a small amount of Ins(1:2-cyclic, 4,5) P_3 was released. The effects of ATP on inositol phosphate formation apparently involve the contributions of two phenomena. First, the P_2 -receptor agonist 2-methylthioadenosine triphosphate (2MeSATP) greatly increased inositol phosphate formation and decreased [3 H]PtdIns4P and [3 H]PtdIns(4,5) P_2 in the presence of a low (0.1 μ M) concentration of GTP[S]. ATP over the concentration range 0–100 μ M produced effects in the presence of 0.1 μ M-GTP[S] essentially identical with those observed with 2MeSATP, suggesting that the effects of low concentrations of ATP are also explained by a stimulation of P_2 -receptors. Higher concentrations of ATP also increase inositol phosphate formation, apparently by supporting the synthesis of substrate phospholipids. NaF, which is a non-specific activator of guanine nucleotide regulatory proteins, increased inositol phosphate formation in a concentration-dependent manner. Ca^{2+} in the range 10 nM–1 μ M slightly increased the accumulation of inositol phosphates. GTP[S] had only a small effect on inositol phosphate formation at 10 nM- Ca^{2+} ; the effects of GTP[S] were markedly enhanced with increasing concentrations of Ca^{2+} between 10 nM and 1 μ M, with a 1.5–2-fold increase in GTP[S]-stimulated inositol phosphate accumulation occurring over the physiologically relevant range, i.e. 0.1–1 μ M.

INTRODUCTION

As with hormonal regulation of adenylate cyclase and phototransduction in the retina (Gilman, 1984), a G-protein apparently is necessary for communication between hormone-occupied cell-surface receptors and phospholipase C (Cockcroft & Gomperts, 1985; Litosch *et al.*, 1985; Smith *et al.*, 1985; Gonzales & Crews, 1985; Straub & Gershengorn, 1986; Martin *et al.*, 1986;

Merritt *et al.*, 1986; Hepler & Harden, 1986; Uhing *et al.*, 1986). Full understanding of the events leading to hormonally mediated phosphoinositide hydrolysis, Ca^{2+} mobilization and activation of protein kinase C requires identification of this G-protein(s). To this end, we previously suggested that turkey erythrocytes provide a potentially useful model for study of the regulation of phospholipase C in that, compared with mammalian erythrocyte membrane preparations, the enzyme of

Abbreviations used: Ins P_2 , inositol bisphosphate; Ins P_3 , inositol trisphosphate (isomeric form unspecified); Ins(1,3,4) P_3 , inositol 1,3,4-trisphosphate; Ins(1,4,5) P_3 , inositol 1,4,5-trisphosphate; Ins P_4 , inositol 1,3,4,5-tetrakisphosphate; PtdIns, phosphatidylinositol; PtdIns4P, phosphatidylinositol 4-monophosphate; PtdIns(4,5) P_2 , phosphatidylinositol 4,5-bisphosphate; G-protein, guanine nucleotide regulatory protein; GTP[S], guanosine 5'-[γ -thio]triphosphate; p[NH]ppG, guanosine 5'-[β -imino]triphosphate; 2MeSATP, 2-methylthioadenosine triphosphate.

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turkey erythrocyte membranes is retained in a state that is remarkably responsive to activators of G-proteins (Harden *et al.*, 1987). In the present report we describe in detail the GTP[S]-induced changes in phosphoinositide concentrations and inositol phosphate production in radiolabelled turkey erythrocyte membranes. In addition, data are presented that indicate that $\text{PtdIns}(4,5)\text{P}_2$ is the principal substrate hydrolysed by this activated phospholipase C.

MATERIALS AND METHODS

Radiolabelling of turkey erythrocyte phosphoinositides

Turkey erythrocytes were labelled with ^{32}P exactly as previously described (Harden *et al.*, 1987). Turkey blood normally contained up to 2% leucocytes, but, as heparin tends to cause aggregation of the white cells, the washed erythrocyte preparations contained very few (<0.2%) intact viable leucocytes (results not shown). Briefly, washed erythrocytes were incubated with 1–2 mCi of $^{32}\text{P}[\text{P}_i]$ for 2 h in an incubation buffer consisting of 5 mM-sodium pyruvate, 5 mM-inosine, 1 mM-adenine, 10 mM-glucose, 57.5 mM-trisodium citrate and 50 mM-Hepes, pH 7.2. Membranes were prepared as previously described (Harden *et al.*, 1987) after lysis of erythrocytes in 5 mM-sodium phosphate buffer (pH 7.4) containing 5 mM- MgCl_2 and 1 mM-EGTA.

Modifications of the previously described (Harden *et al.*, 1987) ^3H inositol-labelling procedure were used. ^3H inositol (usually 0.5 mCi) in ethanol was dried under N_2 and resuspended in 12 ml of Dulbecco's modified Eagle's medium and 4 ml of chicken serum containing penicillin and streptomycin. This solution was passed through a 0.2 μm -pore filter and combined with 4 ml of packed erythrocytes that had been washed as previously described (Harden *et al.*, 1987). The cell suspension was placed in a stirred flask designed for micro-carrier beads, and the flask was placed on a magnetic stirrer operating at 55–60 rev./min. The flask was maintained at 37 °C in an humidified atmosphere of CO_2 /air (1:19) for 20–48 h. Most incubations were for approx. 24 h, since some cell death began to occur with longer times of incubation. Cells were lysed and membranes prepared as previously described in detail (Harden *et al.*, 1987).

Assay of phospholipase C activity

A 50 μl sample of ^3H inositol- or ^{32}P -labelled turkey erythrocyte membranes was added to an assay buffer, usually consisting of the following final concentrations of added constituents: 0.424 mM- CaCl_2 , 5.91 mM- MgSO_4 , 2 mM-EGTA, 115 mM-KCl, 5 mM- KH_2PO_4 , 5 mM- Na_2ATP , 10 mM-phosphocreatine, creatine kinase (1 unit/assay) and 10 mM-Hepes, pH 7.0. Under these conditions free Ca^{2+} concentration measured by fura2 fluorescence was 250 nM, and the calculated free Mg^{2+} concentration was 1 mM. Final assay volume was 200 μl . Incubations were at 37 °C and the reactions were usually stopped by addition of 800 μl of ice-cold 6.25% (v/v) HClO_4 . The acid-inactivated samples were centrifuged at 2000 g for 5 min and the resultant pellets kept for determination of phospholipids. The supernatants were transferred to polypropylene tubes, 20 μl of 100 mM-EDTA and 1.0 ml of tri-n-octylamine/1,1,2-trichlorotrifluoroethane (1:1, v/v) were added, and the samples were vortex-mixed vigorously for 10 s. After centrifugation at 2000 g for 5 min, 800 μl of the aqueous upper

phase was transferred to a Dowex AG1-X8 (200–400 mesh) column (0.6 ml bed volume) with 10 ml of water. Then 10 ml of 0.2 M-ammonium formate/0.1 M-formic acid was added, and the eluate was usually discarded. InsP_2 was eluted with 10 ml of 0.4 M-ammonium formate/0.1 M-formic acid, InsP_3 was eluted with 12 ml of 0.8 M-ammonium formate/0.1 M-formic acid, and InsP_4 was eluted with 8 ml of 1.2 M-ammonium formate/0.1 M-formic acid. The elution pattern of inositol phosphates was confirmed by using individual radioactive InsP_2 , InsP_3 and InsP_4 standards. Columns were used up to five times after regeneration with 5 ml of 2.0 M-ammonium formate/0.1 M-formic acid and resuspension and washing of the resin with 10 ml of water. ^{32}P was quantified by Čerenkov counting at approx. 55% efficiency, and ^{32}P data are presented as c.p.m. ^3H was quantified by scintillation counting in a solution consisting of 15 g of 2,5-diphenyloxazole, 0.375 g of 1,4-bis-(5-phenyloxazol-2-yl)benzene, 2 litres of xylene and 2 litres of Triton X-100. ^3H data are presented as d.p.m. With the membrane preparation and conditions described above, approx. 200–250 assays were carried out with each ^{32}P - or ^3H -labelled membrane preparation.

Assay of phosphoinositides

^{32}P - and ^3H -labelled phosphoinositides were quantified exactly as previously described (Harden *et al.*, 1987). Briefly, this method involves deacylation of phosphoinositides by exposure to NaOH, followed by separation of glycerophosphoinositol, glycerophosphoinositol 4-phosphate and glycerophosphoinositol 4,5-bisphosphate by Dowex chromatography. The identity of radioactive PtdIns , $\text{PtdIns}4\text{P}$ and $\text{PtdIns}(4,5)\text{P}_2$ was confirmed by two-dimensional t.l.c.

H.p.l.c. analysis of ^{32}P -labelled compounds

A Partisil 10 SAX h.p.l.c. column was used to analyse radiolabelled compounds as previously described (Hawkins *et al.*, 1986).

Preparation of $^{32}\text{P}[\text{Ins}(1,4,5)\text{P}_3]$ and $^{32}\text{P}[\text{Ins}(1:2\text{-cyclic},4,5)\text{P}_3]$

Fresh human erythrocytes were centrifuged at 1100 g for 5 min, the plasma was aspirated, and the cells were resuspended in 150 mM- NaCl /1.5 mM-Hepes (pH 7.2). Centrifugation was repeated once with resuspension of cells in 150 mM- NaCl /1.5 mM-Hepes (pH 7.2), and a second time with resuspension of the erythrocytes in 5 mM-sodium pyruvate/5 mM-inosine/1 mM-adenine/10 mM-glucose/57.5 mM-trisodium citrate/50 mM-Hepes, pH 7.2. After a final centrifugation, 1.2 ml of packed cells was added to 0.8 ml of the citrate buffer containing approx. 5 mCi of $^{32}\text{P}[\text{P}_i]$. The cells were incubated with shaking in a 37 °C water bath for 2 h. The labelled cells were lysed on ice for 15 min in 15 vol. of 20 mM-Tris (pH 7.1)/1 mM-EDTA. Washed membranes were prepared by six centrifugations (17000 g for 10 min at 4 °C) and resuspensions in 20 mM-Tris (pH 7.1)/1 mM-EDTA. A final centrifugation was carried out at 39000 g for 10 min and the resultant supernatant discarded. The packed ^{32}P -labelled membranes (1.2 ml) were added to 1.2 ml of 2.75 mM- Ca^{2+} /36.7 mM-sodium acetate (pH 4.9). The pH of the resultant suspension was approx. 5.5. After incubation at 37 °C for 45 min, 66 μl of 0.1 M-EDTA (pH 7.0) was added, and the sample centrifuged at 39000 g for 10 min. Approx. 1.5 ml of

supernatant containing approx. 7×10^6 d.p.m. of ^{32}P was collected.

$[^3\text{H}]\text{Ins}(1,4,5)\text{P}_3$ (15×10^3 d.p.m.) and $[^3\text{H}]\text{Ins}(1,4)\text{P}_2$ (15×10^3 d.p.m.) were added to 850 μl of supernatant, and the sample was injected on a 25 cm Partisil 10-SAX h.p.l.c. column. Inositol phosphates were eluted with a gradient running from water to 3.5 M-ammonium formate adjusted to pH 3.7 with H_3PO_4 (Hawkins *et al.*, 1987); fractions (0.375 ml) were collected every 0.3 min. Approx. 22% of the radioactivity was eluted as a peak (fractions 82 and 83) with a retention time predicted for that of $\text{Ins}(1:2\text{-cyclic},4,5)\text{P}_3$ (Hawkins *et al.*, 1987). A peak (fractions 88–90) of ^{32}P also co-eluted with standard $[^3\text{H}]\text{Ins}(1,4,5)\text{P}_3$. Confirmation of the identity of the fractions supposedly consisting of $\text{Ins}(1:2\text{-cyclic},4,5)\text{P}_3$ was accomplished by acid hydrolysis; 30 μl of the pooled h.p.l.c. fractions (fractions 82 and 83) was added to 70 μl of water and 100 μl of 10% HClO_4 . After incubation at room temperature for 10 min, the acid was removed by extraction with 200 μl of tri-*n*-octylamine/1,1,2-trichlorotrifluoroethane (1:1, v/v). Then 133 μl of the aqueous upper phase was combined with 15×10^3 d.p.m. of $[^3\text{H}]\text{Ins}(1,4,5)\text{P}_3$ and 15×10^3 d.p.m. of $[^3\text{H}]\text{Ins}(1,4)\text{P}_2$, diluted to 1.0 ml with water, and 850 μl of the sample was injected and eluted from h.p.l.c. as described above. Acid hydrolysis resulted in complete conversion of the supposed cyclic inositol phosphate into other ^{32}P -containing species. Approx. 80% of the resultant radioactive species co-eluted with authentic $[^3\text{H}]\text{Ins}(1,4,5)\text{P}_3$, and the remainder was eluted as a trailing peak behind $\text{Ins}(1,4,5)\text{P}_3$ in the position expected for $\text{Ins}(2,4,5)\text{P}_3$ (Hawkins *et al.*, 1987).

Assays designed to determine the possible production of $\text{Ins}(1:2\text{-cyclic},4,5)\text{P}_3$ by GTP[S]-stimulated turkey erythrocyte membranes were terminated under neutral conditions by the addition of ice-cold 2 mM-EDTA, pH 7.0, directly to the reaction tube, followed by centrifugation at 2000 *g* for 5 min. The supernatant was collected and combined with inositol phosphate standards before h.p.l.c. analysis.

Materials

Materials were obtained from the previously specified sources (Harden *et al.*, 1987).

RESULTS

GTP[S]-stimulated inositol phosphate formation by turkey erythrocyte membranes

Small but reproducible increases in $[^{32}\text{P}]\text{InsP}_2$, $[^{32}\text{P}]\text{InsP}_3$ and $[^{32}\text{P}]\text{InsP}_4$ release from ^{32}P -labelled membranes occurred in the presence of ATP. Addition of a maximally effective concentration of GTP[S] markedly accelerated the release of each inositol phosphate (Fig. 1). Accumulations of $[^{32}\text{P}]\text{InsP}_2$ and $[^{32}\text{P}]\text{InsP}_4$ were essentially linear for 10 min; accumulation of $[^{32}\text{P}]\text{InsP}_3$ was linear for approx. 5 min. $[^{32}\text{P}]\text{InsP}_4$ reached a maximum within 15 min; $[^{32}\text{P}]\text{InsP}_2$ and $[^{32}\text{P}]\text{InsP}_3$ continued to increase over a 30 min period. In the absence of added ATP and an ATP-regenerating system, the inositol phosphate response was markedly changed. First, basal amounts of radioactivity eluted from Dowex columns in the three fractions decreased with time. Previous h.p.l.c. analysis has shown that the background radioactivity observed with ^{32}P -labelled turkey erythrocyte membranes predominantly consists of non-inositol-

Fig. 1. Time course of GTP[S]-stimulated increase in inositol $[^{32}\text{P}]$ phosphate accumulation

^{32}P -labelled turkey erythrocyte membranes were prepared and incubations carried out in the absence ($\circ$, $\triangle$) or presence ($\bullet$, $\blacktriangle$) of 50 μM -GTP[S] as described in the Materials and methods section. Incubations were with (the three panels on the right) or without (the three panels on the left) ATP and an ATP-regenerating system (see the Materials and methods section). Data are presented for fractions containing $[^{32}\text{P}]\text{InsP}_2$ (top two panels), $[^{32}\text{P}]\text{InsP}_3$ (middle two panels) and $[^{32}\text{P}]\text{InsP}_4$ (bottom two panels) purified by Dowex chromatography. The data are means of triplicate assays which varied by less than 10% of the mean, and are representative of results obtained in four similar experiments.

containing compounds (Harden *et al.*, 1987). Thus the decrease, in the absence of ATP, of ^{32}P -labelled compounds identified by Dowex chromatography probably represents decreases in the amount of ^{32}P -labelled ADP, ATP and GTP in the preparation. In the absence of ATP, the effect of GTP[S] also was markedly altered. $[^{32}\text{P}]\text{InsP}_4$ was not increased by GTP[S], and the increase in $[^{32}\text{P}]\text{InsP}_3$ was much less than in the presence of ATP. The GTP[S]-induced increase in $[^{32}\text{P}]\text{InsP}_2$ was approximately the same as in the presence of ATP.

Although ^{32}P -labelled turkey erythrocyte membranes provide an inexpensive and reliable preparation in which to examine G-protein-mediated regulation of phospholipase C, there are several disadvantages to this approach. First, there is a background radioactivity in ^{32}P -labelled membranes that primarily consists of non-inositol-containing compounds (Harden *et al.*, 1987).

Fig. 2. Time course of GTP[S]-stimulated increase in $[^3\text{H}]$ -inositol phosphate accumulation

$[^3\text{H}]$ Inositol-labelled turkey erythrocyte membranes were prepared and incubations carried out in the absence ($\circ$, $\triangle$) or presence ($\bullet$, $\blacktriangle$) of $50 \mu\text{M}$ -GTP[S] as described in the Materials and methods section. Incubations were with (the three panels on the right) or without (the three panels on the left) ATP and an ATP-regenerating system (see the Materials and methods section). Data are presented for fractions containing $[^3\text{H}]\text{InsP}_2$ (top two panels), $[^3\text{H}]\text{InsP}_3$ (middle two panels) and $[^3\text{H}]\text{InsP}_4$ (bottom two panels) purified by Dowex chromatography. The data are means of triplicate assays which varied by less than 10% of the mean, and are representative of results obtained in three similar experiments.

Thus the absolute magnitude of the response to GTP[S] is decreased accordingly when Dowex chromatography is used to purify inositol phosphates. Of greater concern, however, is the problem of the specific radioactivity of the $[^3\text{P}]\text{Ins}(1,4,5)\text{P}_3$ produced by phosphoinositide hydrolysis. Assuming that $\text{PtdIns}(4,5)\text{P}_2$ is the major substrate for the phospholipase C (see below), then any new substrate formed and subsequently hydrolysed during incubation of membranes with ATP will result in release of $[^3\text{P}]\text{Ins}(1,4,5)\text{P}_3$ of lower specific radioactivity than that initially released. In light of these properties of ^{32}P -labelled membranes, we also have examined in detail the response of $[^3\text{H}]$ inositol-labelled turkey erythrocyte membranes to GTP[S]. Both the problem of background radioactivity contributed by non-inositol-containing compounds and changes in specific radioactivity during membrane incubation with ATP are circumvented with $[^3\text{H}]$ inositol labelling.

As illustrated in Fig. 2, there is a very small, but

Fig. 3. Time course of GTP[S]-stimulated inositol phosphate formation by turkey erythrocyte membranes

$[^3\text{H}]$ Inositol-labelled turkey erythrocyte membranes were incubated with ($\bullet$) or without ($\circ$) $50 \mu\text{M}$ -GTP[S] under the conditions described in the Materials and methods section. The accumulation of total ^3H -labelled inositol phosphates was measured with time. The data are means of triplicate determinations and are representative of results obtained with six separate preparations of $[^3\text{H}]$ inositol-labelled membranes.

readily measurable, basal accumulation of $[^3\text{H}]\text{InsP}_2$, $[^3\text{H}]\text{InsP}_3$ and $[^3\text{H}]\text{InsP}_4$ during incubation of $[^3\text{H}]$ inositol-labelled membranes with ATP. Addition of a maximally effective concentration of GTP[S] resulted in rapid accumulations of $[^3\text{H}]\text{InsP}_2$, $[^3\text{H}]\text{InsP}_3$ and $[^3\text{H}]\text{InsP}_4$ that were maintained at an essentially linear rate for up to 20 min. GTP[S]-stimulated increases in inositol phosphate formation ranged from 20- to 50-fold during a 15 min challenge. As illustrated in Fig. 3, activation of the turkey erythrocyte phospholipase C displays a property observed with activation of adenylate cyclase by stable GTP analogues in the same membrane preparation (Schramm & Rodbell, 1975; Tolkovsky *et al.*, 1982), i.e. maximal effects of GTP[S] were only observed after a lag of 1–2 min. As with ^{32}P -labelled membranes, the response to GTP[S] was modified by lack of ATP and a nucleotide-regenerating system (Fig. 2). GTP[S] had no effect on $[^3\text{H}]\text{InsP}_4$ amounts in the absence of ATP, the accumulation of $[^3\text{H}]\text{InsP}_3$ was markedly decreased, and $[^3\text{H}]\text{InsP}_2$ increased, but not to the extent observed in the presence of ATP.

Relative formation of $[^3\text{H}]\text{Ins}(1,4,5)\text{P}_3$ and $[^3\text{H}]\text{Ins}(1,3,4)\text{P}_3$ during stimulation with GTP[S]

Interpretation of data obtained by measuring InsP_3 accumulation by Dowex chromatography is complicated in that both $\text{Ins}(1,4,5)\text{P}_3$ and $\text{Ins}(1,3,4)\text{P}_3$ are eluted in the same fraction. Thus, h.p.l.c. analysis was carried out to quantify the relative accumulation of the two InsP_3 isomers during stimulation of $[^3\text{H}]$ inositol-labelled membranes with GTP[S]. As illustrated in the h.p.l.c.

Fig. 4. H.p.l.c. analysis of [^3H]inositol phosphates released from GTP[S]-stimulated turkey erythrocyte membranes

[^3H]Inositol-labelled membranes were prepared as described in the Materials and methods section. After a 5 min incubation in the presence (a) or absence (b) of $50\text{ }\mu\text{M}$ -GTP[S], the reaction was stopped by addition of $800\text{ }\mu\text{l}$ of ice-cold 2 mM -EDTA, pH 7.0. After centrifugation at 2000 g for 5 min, the supernatant was collected and 0.7 ml was combined with [^{32}P]Ins(1:2-cyclic,4,5) P_3 and [^{32}P]Ins(1,4,5) P_3 standards prepared as described in the Materials and methods section. The samples were injected on a Partisil 10 SAX h.p.l.c. column eluted with an ammonium formate/phosphate gradient and fractions collected every 0.3 min. Data are presented as d.p.m. of [^3H]inositol compounds ($\bullet$) and c.p.m. of [^{32}P]labelled compounds ($\circ$) eluted per fraction. Elution times for authentic Ins(1,4) P_2 , Ins(1:2-cyclic,4,5) P_3 , Ins(1,3,4) P_3 , Ins(1,4,5) P_3 and Ins(1,3,4,5) P_4 standards are indicated in (a).

tracing depicted in Fig. 4, incubation of membranes for 5 min with GTP[S] resulted in marked increases in [^3H]Ins P_2 , [^3H]Ins(1,4,5) P_3 , [^3H]Ins(1,3,4) P_3 and [^3H]Ins(1,3,4,5) P_4 . The accumulation of Ins(1,4,5) P_3 reached a maximum within 5 min of challenge with GTP[S] and had declined by 20 min of incubation (Fig. 5). In contrast, [^3H]Ins(1,3,4) P_3 continued to increase throughout the 20 min incubation with GTP[S].

GTP[S]-induced formation of Ins(1:2-cyclic,4,5) P_3

Although Ins(1,4,5) P_3 is assumed to be the principal inositol phosphate produced by phospholipase C attack on PtdIns(4,5) P_2 , assays *in vitro* have demonstrated that Ins(1:2-cyclic,4,5) P_3 also may be formed (Dawson *et al.*, 1971; Wilson *et al.*, 1985a,c; Sekar *et al.*, 1987). Ins(1:2-cyclic,4,5) P_3 is not formed in parotid glands acutely stimulated with carbachol (Hawkins *et al.*, 1987), but has

been reported to accumulate during prolonged stimulation of mouse pancreatic minilobules with caerulein (Sekar *et al.*, 1987) and during stimulation of platelets with thrombin (Ishii *et al.*, 1986; Majerus *et al.*, 1986). Experiments were carried out to determine if the cyclic inositol phosphate is a product of the turkey erythrocyte enzyme. [^{32}P]Ins(1:2-cyclic,4,5) P_3 standard was prepared from human erythrocytes as described in the Materials and methods section. As shown in Fig. 4, h.p.l.c. analysis of [^3H]inositol phosphates, after termination of phosphoinositide hydrolysis under neutral conditions, indicated that very little ^3H radioactivity co-migrated with [^{32}P]Ins(1:2-cyclic,4,5) P_3 standard after stimulation of membranes with GTP[S] for 5 min. Utilization of an h.p.l.c. elution gradient (N.-S. Wong & R. H. Michell, personal communication) that completely resolves Ins(1:2-cyclic,4,5) P_3 from Ins(1,3,4) P_3 and Ins(1,4,5) P_3

Fig. 5. Time course of GTP[S]-stimulated accumulation of $[^3\text{H}]\text{Ins}(1,4,5)\text{P}_3$ and $[^3\text{H}]\text{Ins}(1,3,4)\text{P}_3$

$[^3\text{H}]\text{Inositol}$ -labelled turkey erythrocyte membranes were incubated for the indicated times with $50\ \mu\text{M}$ -GTP[S] under the conditions described in the Materials and methods section. Accumulation of $[^3\text{H}]\text{Ins}(1,3,4)\text{P}_3$ (a; $\blacktriangle$) and $[^3\text{H}]\text{Ins}(1,4,5)\text{P}_3$ (b; $\blacksquare$) was assessed by h.p.l.c. analysis as described in the Materials and methods section and depicted in Fig. 4. The data are means of duplicate determinations.

confirmed this result. That is, after a 5 min stimulation with $50\ \mu\text{M}$ -GTP[S], 15879 d.p.m. were recovered in InsP_2 and 252 d.p.m. in the $\text{Ins}(1:2\text{-cyclic},4,5)\text{P}_3$ peak in samples from reactions terminated under neutral conditions. Termination of the reaction with acid resulted in 17405 d.p.m. recovered in InsP_2 and only 33 d.p.m. recovered in the elution position of $\text{Ins}(1:2\text{-cyclic},4,5)\text{P}_3$. If we assume that $\text{Ins}(1:2\text{-cyclic},4,5)\text{P}_3$ was not significantly metabolized under these conditions, the cyclic compound only accounts for 1% of the radioactivity accumulating in inositol phosphates during 5 min of stimulation with GTP[S]. More detailed studies of the factors that influence the proportion of cyclic and non-cyclic products of this reaction will be required to understand fully the significance of these results.

Analysis of phosphoinositides in ^{32}P - and ^3H -labelled turkey erythrocyte membranes

As illustrated in Fig. 6, $[^{32}\text{P}]\text{PtdIns}$, $[^{32}\text{P}]\text{PtdIns}4\text{P}$ and $[^{32}\text{P}]\text{PtdIns}(4,5)\text{P}_2$ decreased slowly during a 30 min incubation in the presence of ATP. Addition of GTP[S] caused a rapid and marked decrease in radioactivity in all three phosphoinositides. The decrease in radioactivity associated with lipids could be accounted for by radioactivity appearing in inositol phosphates. In the absence of ATP, radioactivity recovered in PtdIns did not change during a 30 min incubation in the absence of GTP[S]; a time-dependent decrease in $[^{32}\text{P}]\text{PtdIns}4\text{P}$ occurred, and an even larger decrease occurred in the amount of $[^{32}\text{P}]\text{PtdIns}(4,5)\text{P}_2$. Whereas GTP[S] had no

Fig. 6. Quantification of ^{32}P -labelled phosphoinositides during incubation of turkey erythrocyte membranes in the absence or presence of ATP and GTP[S]

The phosphoinositide pool of turkey erythrocyte membranes was labelled with ^{32}P , and incubations were carried out with ($\blacktriangle$, $\bullet$) or without ($\triangle$, $\circ$) $50\ \mu\text{M}$ -GTP[S] as described in the Materials and methods section. Incubations were also without (left panels) ATP, phosphocreatine and creatine kinase or with (right panels) ATP and the regenerating system at the concentrations described in the Materials and methods section. $[^{32}\text{P}]\text{PtdIns}$ (top panels), $[^{32}\text{P}]\text{PtdIns}4\text{P}$ (middle panels), and $[^{32}\text{P}]\text{PtdIns}(4,5)\text{P}_2$ (bottom panels) were determined as described in the Materials and methods section. The data are means of triplicate determinations and are representative of results obtained in four experiments.

effect on $[^{32}\text{P}]\text{PtdIns}$ and $[^{32}\text{P}]\text{PtdIns}4\text{P}$ amounts in the absence of ATP, a marked decrease in $[^{32}\text{P}]\text{PtdIns}(4,5)\text{P}_2$ was observed (Fig. 6).

For the reasons discussed above, analysis of phosphoinositides labelled with ^{32}P presents several problems in interpretation. Thus it was also important to assess PtdIns, PtdIns4P and PtdIns(4,5) P_2 amounts measured after incorporation of $[^3\text{H}]\text{inositol}$. During incubation with ATP, $[^3\text{H}]\text{PtdIns}$ decreased, and $[^3\text{H}]\text{PtdIns}4\text{P}$ and $[^3\text{H}]\text{PtdIns}(4,5)\text{P}_2$ increased (Fig. 7). The changes in $[^3\text{H}]\text{PtdIns}$, $[^3\text{H}]\text{PtdIns}4\text{P}$ and $[^3\text{H}]\text{PtdIns}(4,5)\text{P}_2$ during a 30 min incubation with ATP were -9.3 ± 1.6 , $+175.4 \pm 11.4$ and $+132.6 \pm 6.7\%$ (means $\pm$ S.E.M. for five experiments carried out in triplicate) respectively. Addition of GTP[S] resulted in a decrease in radioactivity in all three phosphoinositides.

Fig. 7. Quantification of ^3H -labelled phosphoinositides during incubation of turkey erythrocyte membranes in the absence or presence of ATP and GTP[S]

The phosphoinositide pool of turkey erythrocyte membranes was labelled with ^3H inositol and incubations carried out with ($\blacktriangle$, $\bullet$) or without ($\triangle$, $\circ$) $50\ \mu\text{M}$ -GTP[S] as described in the Materials and methods section. Incubations were also without (left panels) ATP, phosphocreatine and creatine kinase or with (right panels) ATP and the regenerating system at the concentrations described in the Materials and methods section. ^3H PtdIns (top panels), ^3H PtdIns4P (middle panels) and ^3H PtdIns(4,5) P_2 (bottom panels) were determined as described in the Materials and methods section. The data are means of triplicate determinations and are representative of results obtained in four experiments.

In contrast, in the absence of ATP there was an increase in the amount of radioactivity associated with PtdIns, and decreases in ^3H PtdIns4P and ^3H PtdIns(4,5) P_2 . Addition of GTP[S] in the absence of ATP resulted in no change in ^3H PtdIns and ^3H PtdIns4P amounts, and a decrease in ^3H PtdIns(4,5) P_2 .

Phosphoinositide specificity of the GTP[S]-activated phospholipase C of turkey erythrocyte membranes

Since no inositol monophosphate is formed upon activation of phospholipase C in turkey erythrocyte membranes (Harden *et al.*, 1987), endogenously labelled PtdIns is not a substrate for this enzyme. However, whether this phospholipase C hydrolyses both PtdIns4P and PtdIns(4,5) P_2 or only PtdIns(4,5) P_2 has not been established. That is, Ins P_2 formed in response to GTP[S] could originate from the phospholipase C-catalysed

breakdown of PtdIns4P or from the action of Ins(1,4,5) P_3 5-phosphomonoesterase on Ins(1,4,5) P_3 formed as a consequence of PtdIns(4,5) P_2 breakdown. Analysis of lipid amounts during GTP[S]-mediated stimulation of phosphoinositide hydrolysis in the absence of ATP (Figs. 6 and 7) suggests that the predominant substrate is PtdIns(4,5) P_2 , but by no means unequivocally proves this point. To address this question more directly, experiments were carried out with the goal of inhibiting Ins(1,4,5) P_3 5-phosphomonoesterase such that any Ins P_2 formed would occur as a result of hydrolysis of PtdIns4P. ^3H -labelled membranes were incubated with or without $10\ \text{mM}$ -Ins(1,4,5) P_3 and GTP[S] for 5 min. Exogenous ^{32}P Ins(1,4,5) P_3 also was included to assess the effectiveness of inhibition of Ins(1,4,5) P_3 metabolism. H.p.l.c. analysis (Table 1) indicated that, in the absence of added non-radioactive compound, over 50 % of the ^{32}P Ins(1,4,5) P_3 was converted into metabolic products. In the presence of $10\ \text{mM}$ -Ins(1,4,5) P_3 , GTP[S]-stimulated formation of total inositol phosphates was decreased by 37 % and the metabolism of exogenous ^{32}P Ins(1,4,5) P_3 was completely prevented. Although GTP[S]-stimulated accumulation of ^3H Ins P_4 or ^3H Ins(1,3,4) P_3 also did not occur during incubation in the presence of $10\ \text{mM}$ -Ins(1,4,5) P_3 , some formation of ^3H Ins P_2 took place. This Ins P_2 accumulation, which amounted to 12 % of the total ^3H inositol phosphate formed, indicates that ^3H PtdIns(4,5) P_2 is the principal substrate for the turkey erythrocyte phospholipase C under the conditions of these assays, but some GTP[S]-stimulated hydrolysis of ^3H PtdIns4P also occurs.

Evidence for receptor-mediated effects of ATP

One conclusion from the results obtained with GTP[S] incubations in the absence and in the presence of ATP is that ATP is needed to support a pool of substrate for the activated phospholipase C. Although this is clearly a contributing factor, it apparently does not fully explain the effects of ATP. That is, we recently have obtained preliminary results indicating that ADP and ATP stimulate an increase in inositol phosphate formation in intact turkey erythrocytes, suggesting that a P_2 -purinergic receptor couples to phospholipase C in these cells (C. P. Berrie, P. T. Hawkins, C. P. Downes & T. K. Harden, unpublished work). Thus it was important to establish whether this putative receptor could be measured by using the membrane preparation. Membranes were incubated with a sub-maximal concentration of GTP[S] ($0.1\ \mu\text{M}$) and the indicated concentrations of ATP or the P_2 -receptor agonist, 2MeSATP, for 5 min. ATP caused a biphasic increase in inositol phosphate formation (Fig. 8). The approximate $K_{0.5}$ for stimulation by lower concentrations of ATP was $3\ \mu\text{M}$. At concentrations greater than $300\ \mu\text{M}$, a second component of activation was observed. The P_2 -purinergic receptor agonist 2MeSATP also increased inositol phosphate formation, with a $K_{0.5}$ value of approx. $10\ \text{nM}$ (Fig. 8). The maximal effect observed with 2MeSATP was similar to that observed with $100\ \mu\text{M}$ -ATP. The response to 2MeSATP apparently followed simple mass-action kinetics, with no second component of stimulation at higher concentrations. The effects of ATP and 2MeSATP on ^3H -phosphoinositide amounts also were examined under the same conditions as for the inositol phosphate analysis. 2MeSATP caused a concentration-dependent decrease in ^3H PtdIns4P and ^3H PtdIns(4,5) P_2 , with $K_{0.5}$ values

Table 1. Effect of a high concentration of Ins(1,4,5) P_3 on the GTP[S]-stimulated accumulation of [3 H]Ins P_2 and the metabolism of exogenous [32 P]Ins(1,4,5) P_3

A tracer amount of [32 P]Ins(1,4,5) P_3 was added to [3 H]inositol-labelled membranes and the samples were incubated with 50 μ M-GTP[S] for 5 min in the absence or the presence of 10 mM non-radioactive Ins(1,4,5) P_3 . H.p.l.c. analyses were carried out as described in the Materials and methods section and Fig. 4 legend. 32 P and 3 H radioactivity appearing in the positions expected for [32 P]P $_i$, Ins P_2 , Ins(1,3,4) P_3 , Ins(1,4,5) P_3 and Ins(1,3,4,5) P_4 were quantified by scintillation counting, and data representative of results from two separate determinations are presented.

Conditions	[32 P]P $_i$	Radioactivity							
		Ins P_2		Ins(1,3,4) P_3		Ins(1,4,5) P_3		Ins(1,3,4,5) P_4	
		3 H	32 P	3 H	32 P	3 H	32 P	3 H	32 P
Zero time	70	102	—	—	—	93	2158	—	—
5 min, 50 μ M-GTP[S] + 10 mM Ins(1,4,5) P_3	81	1086	—	—	—	7639	2155	—	—
5 min, 50 μ M-GTP[S]	284	4496	66	1144	54	4968	838	3244	688

Fig. 8. Concentration-dependent stimulation of inositol phosphate formation by 2MeSATP and ATP

3 H-labelled turkey erythrocyte membranes were incubated with 0.1 μ M-GTP[S] under the conditions described in the Materials and methods section in the absence of ATP and an ATP-regenerating system. Incubations were for 5 min with the indicated added concentrations of 2MeSATP (a) or ATP (b). The data are means of triplicate determinations and are representative of results obtained in five separate experiments.

identical with that for stimulation of inositol phosphate formation (Fig. 9). In contrast, ATP caused a concentration-dependent decrease in [3 H]PtdIns4 P and [3 H]PtdIns(4,5) P_2 at concentrations up to 100 μ M (Fig. 9). Above this concentration ATP caused a small increase in [3 H]PtdIns(4,5) P_2 and a large increase in [3 H]PtdIns4 P . The increase in labelled phosphoinositides at high concentrations coincides with the second component of the ATP-induced increase in inositol phosphate

Fig. 9. Concentration-dependent modification of [3 H]PtdIns4 P and [3 H]PtdIns(4,5) P_2 amounts by 2MeSATP and ATP

[3 H]PtdIns4 P (top two panels) and [3 H]PtdIns(4,5) P_2 (bottom two panels) levels were measured under the conditions described in Fig. 8 for 2MeSATP (left panels) and ATP (right panels). The data are plotted as percentages of [3 H]PtdIns4 P or [3 H]PtdIns(4,5) P_2 amounts measured in the absence of added 2MeSATP or ATP. The data are from a single experiment carried out in triplicate assays and are representative of results from three experiments.

formation. In the absence of any added guanine nucleotide, 2MeSATP had no effect on phosphoinositide amounts or inositol phosphate formation at any concentration tested. In the absence of added guanine nucleotide, ATP had no effect on phosphoinositide amounts at concentrations less than 300 μ M, increased [3 H]PtdIns(4,5) P_2 slightly at concentrations above 300 μ M, and markedly increased [3 H]PtdIns4 P at concentrations greater than 300 μ M (J. L. Boyer & T. K. Harden, unpublished work). These results are consistent with the idea that 2MeSATP and low concentrations of

ATP (1–100 μM) stimulate inositol phosphate formation through activation of a purinergic receptor, with higher concentrations of ATP additionally acting to support the synthesis of substrate phospholipids.

Activation of phospholipase C by activators of G-proteins

We have reported previously the relative effects of GTP[S], p[NH]ppG, GTP, GDP and GDP[S] on inositol phosphate formation by ^{32}P -labelled turkey erythrocyte membranes (Harden *et al.*, 1987). Similar results were obtained with ^3H -inositol-labelled membranes. NaF is a well-known activator of G-proteins (Gilman, 1984). As illustrated in Fig. 10, NaF caused a concentration-dependent increase in inositol phosphate formation by ^3H -inositol-labelled turkey erythrocyte membranes. The highest concentration of NaF tested produced an effect that was approximately equal to that of a maximally effective concentration of p[NH]ppG and 70% of that produced by a maximal concentration of GTP[S].

Ca^{2+} -dependence of GTP[S]-stimulated ^3H -inositol phosphate formation by turkey erythrocyte membranes

We previously reported (Harden *et al.*, 1987) that the phospholipase C of turkey erythrocyte membranes, in contrast with the enzyme of membranes from a number of mammalian erythrocytes (Allan & Michell, 1978; Downes & Michell, 1981; Harden *et al.*, 1987) is comparatively unresponsive to supraphysiological concentrations of Ca^{2+} . The influence of Ca^{2+} on enzyme activity has now been examined over a wide range of concentrations. The effects of Ca^{2+} alone, or in

Fig. 11. Ca^{2+} -dependence of GTP[S]-stimulated inositol phosphate formation

Turkey erythrocyte membranes labelled with ^3H -inositol were prepared and incubated in the presence of 5 mM-ATP and an ATP-regenerating system and with the indicated concentrations of Ca^{2+} alone (○), or Ca^{2+} with 0.4 μM -GTP[S] (▲) or 40 μM -GTP[S] (●). The data are the means of triplicate determinations and are representative of results obtained in five experiments.

Fig. 10. NaF-stimulated inositol phosphate formation by turkey erythrocyte membranes

Turkey erythrocyte membranes labelled with ^3H -inositol were prepared and incubated with the indicated concentrations of NaF, with 100 μM -p[NH]ppG, or with 50 μM -GTP[S] as described in the Materials and methods section. The incubations with NaF also contained 2.5 μM - AlCl_3 . The data are means of triplicate determinations.

combination with ATP and a concentration of GTP[S] that either produces approx. 50% (0.4 μM) of the effect of a maximal concentration of stable GTP analogue or produces a maximal effect (40 μM), were examined. In the absence of GTP[S], no remarkable effect of Ca^{2+} was observed at any concentration tested (Fig. 11). An approximate doubling of inositol phosphate formation occurred between 0.1 and 1 μM - Ca^{2+} . At concentrations of Ca^{2+} greater than 10 μM , inositol phosphate formation decreased, with accumulation at 1 mM- Ca^{2+} only approximately half of that observed at 10 μM - Ca^{2+} . The effects of GTP[S] were very small at Ca^{2+} concentrations below 20 nM. With increasing concentrations of Ca^{2+} the effect of GTP[S] increased. In the physiological range of Ca^{2+} concentrations, e.g. 0.1–1 μM , there was a 1.5–2.0-fold increase in the response to GTP[S]. Maximal responsiveness to GTP[S] was observed at concentrations of Ca^{2+} of approx. 1 μM . The response to GTP[S] decreased at concentrations of Ca^{2+} greater than 10 μM . Little apparent change occurred in the GTP[S] activation curve in that the effect of 0.4 μM -GTP[S] relative to that of 40 μM -GTP[S] was similar at all Ca^{2+} concentrations tested.

DISCUSSION

A principal goal of this study was to establish the substrate-specificity of a membrane-associated guanine nucleotide-stimulated phospholipase C. The results indicate that, although the major substrate for the

activated enzyme is $\text{PtdIns}(4,5)\text{P}_2$, some hydrolysis of $\text{PtdIns}4\text{P}$ also occurs. Since the decrease in radioactivity in PtdIns , $\text{PtdIns}4\text{P}$ and $\text{PtdIns}(4,5)\text{P}_2$ can be completely accounted for by radioactivity appearing in InsP_2 , InsP_3 and InsP_4 , i.e. no inositol monophosphate is formed, there is very clearly no breakdown of endogenously labelled PtdIns . The involvement of $\text{PtdIns}4\text{P}$ as a substrate for the GTP[S]-activated enzyme proved more difficult to establish. Although the large increases in InsP_2 occurring during incubation with GTP[S] could simply result from $\text{Ins}(1,4,5)\text{P}_3$ metabolism, owing to the high amounts of $\text{Ins}(1,4,5)\text{P}_3$ 5-phosphomonoesterase activity present in the membrane preparation, it was not possible, based on kinetic analyses of inositol phosphate accumulation, to establish the origin of the InsP_2 . Support for the inclusion of $\text{PtdIns}4\text{P}$ as a substrate also could not be provided by analysis of phosphoinositide amounts. In the absence of ATP, no GTP[S]-induced decrease in [^3H] $\text{PtdIns}4\text{P}$ occurred, and the GTP[S]-induced increase in radioactivity in inositol phosphates could be completely accounted for by a corresponding decrease in radiolabel in $\text{PtdIns}(4,5)\text{P}_2$. However, these data do not rule out the possibility that InsP_2 is formed after $\text{PtdIns}4\text{P}$ hydrolysis, with a corresponding replacement of hydrolysed $\text{PtdIns}4\text{P}$ by phosphomonoesterase-catalysed conversion of $\text{PtdIns}(4,5)\text{P}_2$ into $\text{PtdIns}4\text{P}$. As such, more direct experiments were carried out with the goal of inhibiting $\text{Ins}(1,4,5)\text{P}_3$ 5-phosphomonoesterase-catalysed conversion of $\text{Ins}(1,4,5)\text{P}_3$ into InsP_2 . This approach proved difficult, owing to the high activity (and high K_m) of $\text{Ins}(1,4,5)\text{P}_3$ 5-phosphomonoesterase and because of the existence of two distinct pathways of $\text{Ins}(1,4,5)\text{P}_3$ metabolism in the turkey erythrocyte membrane preparation (Morris *et al.*, 1987). For example, addition of 1 mM- $\text{Ins}(1,4,5)\text{P}_3$ only inhibited conversion of exogenous [^{32}P] $\text{Ins}(1,4,5)\text{P}_3$ into [^{32}P] InsP_2 by 80–90% during a 5 min incubation with membranes. By increasing the concentration of $\text{Ins}(1,4,5)\text{P}_3$ to 10 mM, no measurable metabolism of exogenous [^{32}P] $\text{Ins}(1,4,5)\text{P}_3$ was observed. Under these conditions, GTP[S]-stimulated increases in [^3H] InsP_2 also were markedly decreased but not prevented. Estimates based on h.p.l.c. analysis indicate that 85–90% of the ^3H -labelled inositol phosphates formed during 5 min in the presence of GTP[S] originate from the hydrolysis of $\text{PtdIns}(4,5)\text{P}_2$; the remainder appear as a consequence of the hydrolysis of $\text{PtdIns}4\text{P}$. Similar conclusions have been drawn by Cockcroft & Taylor (1987) in studies of fluoride- and GTP[S]-stimulated phospholipase C of rat hepatocyte membranes. The specificity of GTP[S]-stimulated turkey erythrocyte phospholipase C also agrees with that originally predicted from experiments with parotid-gland slices (Downes & Wusteman, 1983) and dispersed acinar cells (Aub & Putney, 1984), where the prolonged accumulation of inositol monophosphate could be fully accounted for by the rate of hydrolysis of inositol bisphosphate. However, this conclusion contrasts with reports that PtdIns is directly hydrolysed in thrombin-stimulated platelets (Wilson *et al.*, 1985b; Verhoeven *et al.*, 1986; Majerus *et al.*, 1986) and thyroliberin (TRH)-stimulated GH_3 pituitary cells (Imai & Gershengorn, 1986; but see Drummond & Raeburn, 1984).

Another notable property of the turkey erythrocyte membrane phospholipase C is the persistence of response to GTP[S]. Total inositol phosphate formation occurs

linearly for up to 20 min under the conditions used. Since $\text{PtdIns}(4,5)\text{P}_2$ is the principal substrate for enzyme action and more inositol phosphate is formed during this time than was initially present in $\text{PtdIns}(4,5)\text{P}_2$, PtdIns kinase and $\text{PtdIns}4\text{P}$ kinase activities are well preserved in the membrane preparation under standard assay conditions. An approximate doubling of ^3H in $\text{PtdIns}4\text{P}$ and a 20–50% increase in ^3H in $\text{PtdIns}(4,5)\text{P}_2$ occurred during 30 min of incubation of membranes with ATP, with a corresponding decrease occurring in ^3H in PtdIns . Whether the flux of PtdIns to $\text{PtdIns}4\text{P}$ and $\text{PtdIns}4\text{P}$ to $\text{PtdIns}(4,5)\text{P}_2$ under resting conditions is sufficient to account for the maintenance of $\text{PtdIns}4\text{P}$ and $\text{PtdIns}(4,5)\text{P}_2$ at near-starting amounts of radioactivity during incubation with GTP[S] cannot be unambiguously established by the data.

Data presented here suggest that there is an important additional effect of ATP, i.e. the turkey erythrocyte phospholipase C is activated by a purinergic receptor. This is revealed most clearly by a concentration-dependent stimulation of inositol phosphate formation during incubation of membranes with a purinergic receptor agonist, 2MeSATP, that would not support phosphoinositide kinase activity. The fact that low concentrations (0–100 μM) of ATP generated a concentration/effect curve essentially superimposable with that for 2MeSATP for stimulation of inositol phosphate formation suggests that, under the conditions of these assays and at lower concentrations of ATP, the effects of ATP principally involve receptor stimulation. At concentrations of ATP in excess of 300 μM , both support of substrate synthesis and receptor stimulation apparently occur, and thus the data obtained under conditions like those used in Figs. 1–4 would involve effects of ATP at both levels. Complete quantitative delineation of the relative contributions of different effects of ATP will require analysis of the turkey erythrocyte phosphoinositide signalling system under a widely ranging set of conditions.

The kinetics of accumulation of InsP_2 , InsP_3 and InsP_4 indicate that the membrane preparation is highly enriched in $\text{Ins}(1,4,5)\text{P}_3$ 3-kinase, $\text{Ins}(1,4,5)\text{P}_3$ 5-phosphomonoesterase and $\text{Ins}(1,3,4,5)\text{P}_4$ 5-phosphomonoesterase activities. Although the capacity to degrade $\text{Ins}(1,3,4)\text{P}_3$ has not been examined directly, experiments measuring the metabolism of endogenously produced (e.g. Fig. 5) or exogenously provided $\text{Ins}(1,4,5)\text{P}_3$ suggest that the 1,3,4-isomer is degraded little, if at all, by the preparation. Although the data have been discussed as if the inositol phosphates that accumulate are metabolic products of $\text{Ins}(1,4,5)\text{P}_3$, the experiments were, for the most part, carried out under conditions that would not differentiate between cyclic and non-cyclic inositol phosphates. Soluble phospholipase C from several sources has been shown to catalyse the formation of both $\text{Ins}(1,4,5)\text{P}_3$ and $\text{Ins}(1:2\text{-cyclic},4,5)\text{P}_3$ from $\text{PtdIns}(4,5)\text{P}_2$ (Dawson *et al.*, 1971; Wilson *et al.*, 1985a,c). Accordingly, it was important to establish whether $\text{Ins}(1:2\text{-cyclic},4,5)\text{P}_3$ was a prominent product (1988), but contrasts with the conclusions drawn from stimulated turkey erythrocyte membranes. As illustrated in Fig. 4, some cyclic product is apparently formed by the membrane preparation. However, relative to the total amount of inositol phosphates produced, the amount of cyclic compound that accumulates is very small. This is in accordance with results obtained with rat parotid gland

(Hawkins *et al.*, 1987) and WRK-1 cells (Wong *et al.*, 1988), but contrasts with the conclusions drawn from studies on human platelets (Wilson *et al.*, 1985a,c; Majerus *et al.*, 1986). Prolonged stimulation of mouse pancreatic minilobules with caerulein also results in significant accumulation of the cyclic compound (Sekar *et al.*, 1987).

The properties of regulation of the turkey erythrocyte phospholipase C suggest that it is highly analogous to a hormonally regulated counterpart in mammalian cells. For example, its activation by GTP analogues and NaF is reminiscent of responses observed with cell-free preparations from mammalian cells. Moreover, preliminary data with 2MeSATP and ATP suggest that the turkey erythrocyte enzyme is regulated in intact cells and membranes by a purinergic receptor. One possible difference concerns the observed effects of Ca^{2+} . Note that the precise site of action of Ca^{2+} in these experiments has not been defined: in addition to phospholipase C and its regulatory G-protein, inositol phospholipid kinases and phosphomonoesterases also are potential targets for regulation (see, e.g., Raval & Allan, 1985). At very low concentrations of Ca^{2+} the response to GTP[S] was markedly decreased, and over the physiological range of Ca^{2+} , i.e. 0.1–1 μM , there was a 1.5–2-fold increase in stimulation by GTP[S]. Most notably, Ca^{2+} itself caused very little stimulation of inositol phosphate formation, even at very high concentrations. This is in marked contrast with several mammalian membrane phospholipase C activities that have been studied. These express sharply stimulated activities at supraphysiological Ca^{2+} concentrations and/or 'guanine nucleotide dependent' increases in sensitivity of the enzyme to Ca^{2+} (Martin *et al.*, 1986; Uhing *et al.*, 1986; Cockcroft, 1986; Smith *et al.*, 1986). One possibility is that the guanine nucleotide-stimulated component of these membrane systems is poorly preserved upon cell lysis and membrane preparation, and therefore the Ca^{2+} -stimulated activity becomes a much more prominent proportion of the measured cell-free response. Alternatively, the Ca^{2+} -activated enzyme may not be the same as that regulated by guanine nucleotides. A case in point is the phospholipase C of human erythrocyte membranes. This enzyme is markedly stimulated by high concentrations of Ca^{2+} (Allan & Michell, 1978; Downes & Michell, 1981; Harden *et al.*, 1987) and is insensitive to GTP[S] or NaF (Harden *et al.*, 1987). Whether this activity simply represents another form of phospholipase C or whether these properties, which markedly diverge from those of the enzyme in turkey erythrocyte membranes, are a consequence of absence of the appropriate G-protein, will be useful questions to answer.

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