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The roles of phospholipase D and a GTP-binding protein in guanosine 5′-[γ-thio]triphosphate-stimulated hydrolysis of phosphatidylcholine in rat liver plasma membranes

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1. Guanosine 5′-[γ-thio]triphosphate (GTP[S]) stimulated by 50% the rate of release of [^{3}H]choline and [^{3}H]phosphorylcholine in rat liver plasma membranes labelled with [^{3}H]choline. About 70% of the radioactivity released in the presence of GTP[S] was [^{3}H]choline and 30% was [^{3}H]phosphorylcholine. 2. The hydrolysis of phosphorylcholine to choline and the conversion of choline to phosphorylcholine did not contribute to the formation of [^{3}H]choline and [^{3}H]phosphorylcholine respectively. 3. The release of [^{3}H]choline from membranes was inhibited by low concentrations of SDS or Triton X-100. Considerably higher concentrations of the detergents were required to inhibit the release of [^{3}H]phosphorylcholine. 4. Guanosine 5′-[βγ-imido]triphosphate and guanosine 5′-[αβ-methylene]triphosphate, but not adenosine 5′-[γ-thio]-triphosphate, stimulated [^{3}H]choline release to the same extent as did GTP[S]. The GTP[S]-stimulated [^{3}H]choline release was inhibited by guanosine 5′-[β-thio]diphosphate, GDP and GTP but not by GMP. 5. It is concluded that, in rat liver plasma membranes, (*a*) GTP[S]-stimulated hydrolysis of phosphatidylcholine is catalysed predominantly by phospholipase D with some contribution from phospholipase C, and (*b*) the stimulation of phosphatidylcholine hydrolysis by GTP[s] occurs via a GTP-binding regulatory protein.

INTRODUCTION

Vasopressin and a number of other agonists increase the concentration of diacylglycerol in hepatocytes (Takenawa *et al.*, 1982; Thomas *et al.*, 1983; Hughes *et al.*, 1984; Bocckino *et al.*, 1985). It has been proposed that in these cells (Thomas *et al.*, 1983; Hughes *et al.*, 1984; Bocckino *et al.*, 1985), as in other cell types (Kaibuchi *et al.*, 1983; and reviewed in Kikkawa & Nishizuka, 1986), this increase in diacylglycerol concentration activates protein kinase C which, in turn, is responsible for some of the cellular responses to the agonists (Thomas *et al.*, 1983; Hughes *et al.*, 1984; Bocckino *et al.*, 1985). Experiments with intact hepatocytes have shown that the increase in diacylglycerol induced by agonists is derived predominantly from phosphatidylcholine and possibly from other non-inositol phospholipids, with a relatively small contribution from phosphoinositides (Hughes *et al.*, 1984; Bocckino *et al.*, 1985; Irving & Exton, 1987; Pickford *et al.*, 1987; Polverino & Barritt, 1988; Augert *et al.*, 1989).

In rat liver plasma membranes, guanosine 5′-[γ-thio]triphosphate (GTP[S]) stimulates the hydrolysis of phosphatidylcholine to yield phosphatidic acid and diacylglycerol. The formation of these lipids has been shown to involve both phospholipases C and D (Irving & Exton, 1987; Bocckino *et al.*, 1987*a*). However, the relative contributions of each of these enzymes to phosphatidylcholine hydrolysis is uncertain. Furthermore, although there is some evidence for a role of a GTP-binding regulatory protein (G-protein) in the hydrolysis of phosphatidylcholine catalysed by phospholipase D (Bocckino *et al.*, 1987*a*), this evidence is not conclusive. The aims of the present experiments were to determine the relative contributions of phospholipases C and D to the GTP[S]-induced stimulation of phosphatidylcholine hydrolysis in rat liver plasma membranes, and to evaluate the role of a G-protein in the action of GTP[S]. The results show that the predominant pathway of hydrolysis of phosphatidylcholine in rat liver plasma membranes is catalysed by phospholipase D and provide evidence which indicates that the stimulation by GTP[S] occurs through the action of a G-protein.

EXPERIMENTAL

Preparation of plasma membranes labelled with [^{3}H]choline

[*methyl*-^{3}H]Choline (100 or 200 μCi per rat) was administered intraperitoneally to fed male Wistar rats (250–300 g) 18 h before the animals were killed (Irving & Exton, 1987). Plasma membrane vesicles were prepared using the Percoll method of Prpic *et al.* (1984). The amounts of radioactivity associated with the [^{3}H]choline-labelled membranes were 22000 ± 1000 ($n = 9$) and 53000 ± 4000 d.p.m./mg of protein ($n = 17$) for preparations isolated from rats given 100 and 200 μCi respectively. The membrane protein concentration was measured by the method of Lowry *et al.* (1951).

Incubation of plasma membranes and the isolation and separation of [^{3}H]choline and its water-soluble metabolites

Membranes were resuspended (final protein concentration of about 2 mg/ml) in 50 mM-Tris/HCl, pH 7.5, 1 mM-EGTA, 0.1 mg of leupeptin/ml and 0.1 mg of antipain/ml (Irving & Exton, 1987). The incubations (final volume 2.5 ml) were conducted in plastic scintillation pots at 30 °C and consisted of 25 mM-Tris/HCl, pH 7.5, 1.0 mM-EGTA, 0.05 mg of leupeptin/ml, 0.05 mg of antipain/ml, 0.4 mM-$CaCl_2$ [free Ca^{2+} calculated to be 0.1 μM (Burgess *et al.*, 1983)], 10 mM-$MgCl_2$ and membranes (1.0 mg of protein/ml). At the times indicated,

Abbreviations used: GTP[S], guanosine 5′-[γ-thio]triphosphate; p[NH]ppG, guanosine 5′-[βγ-imido]triphosphate; pp[CH_2]pG, guanosine 5′-[αβ-methylene]triphosphate; GDP[S], guanosine 5′-[β-thio]diphosphate; ATP[S], adenosine 5′-[γ-thio]triphosphate; G-protein, GTP-binding regulatory protein.

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samples of incubation medium (0.4 ml) were taken and placed in glass tubes which contained 3 ml of chloroform/methanol (1:2, v/v). The samples were mixed vigorously, allowed to stand at room temperature for 1 h, and then centrifuged at 1000 *g* for 15 min at room temperature. The upper aqueous layer was placed into a tube which contained 3 ml of chloroform and 1.5 ml of water. The samples were vigorously mixed and centrifuged at 1000 *g* for 2 min, and the upper phase was collected with a Pasteur pipette.

Separation of [^{3}H]choline and its water-soluble metabolites was performed on AG1-X8 resin converted from the chloride to the formate form by treatment with NaOH and formic acid. Columns of about 2.0 ml of resin in disposable 5 ml pipette tips plugged with glass wool were prepared. The columns were washed exhaustively with distilled water (usually about 30 column volumes), until a consistent eluate pH of 4.5 was obtained. The eluate (fraction 1) obtained following the addition of a sample of the water-soluble membrane extract to the column contained [^{3}H]choline. The remaining [^{3}H]choline was eluted with 5 × 2 ml of water (fractions 2–6). [^{3}H]Phosphorylcholine was then eluted with 5 × 2 ml of 50 mM-NH_4HCO_2 (fractions 7–11). Each fraction eluted was mixed with 10 ml of ACS II scintillation cocktail (Amersham Australia, Sydney, New South Wales, Australia) and the amount of radioactivity was determined using a Mark III (Searle Analytic Inc.) or a Beckman LS 5000TD scintillation counter, each programmed for d.p.m. calculations. The method employed for the separation of choline from phosphorylcholine was verified using [*methyl*-^{3}H]choline and [U-^{14}C]phosphorylcholine.

The separation of ^{3}H-labelled water-soluble metabolites of choline by t.l.c. was performed using silica gel 60 plates (E. Merck, Darmstadt, Germany) and 0.5% NaCl/methanol/conc. NH_4OH(50:50:1, by vol.) as described by Yavin (1976). The total amount of phosphatidylcholine in rat liver plasma membranes was determined by measurement of phospholipid phosphorus (Bartlett, 1959) after separation of phosphatidylcholine by t.l.c. in which the solvent system (prepared from re-distilled reagents) consisted of chloroform/ethanol/triethylamine/water (15:17:17.5:4.25, by vol) (Touchstone *et al.*, 1980).

Materials

AG1-X8 (100–200 mesh) was obtained from Bio-Rad Laboratories, Richmond, CA, U.S.A.; [*methyl*-^{3}H]choline and [U-^{14}C]phosphorylcholine from Dupont Ltd., Melbourne, Victoria, Australia; leupeptin and antipain from the Peptide Institute, Osaka, Japan; $AlCl_3$ from BDH Chemicals Australia Pty. Ltd., Adelaide, S.A., Australia; and antibody Y13-259 from Oncogene Science, Manhasset, NY, U.S.A. The trilithium salt of guanosine 5′-[β-thio]diphosphate (GDP[S]), and the tetralithium salts of GTP[S], guanosine 5′-[$\beta\gamma$-imido]triphosphate (p[NH]ppG), guanosine 5′-([α,β-methylene]triphosphate (pp[CH_2]pG), and adenosine 5′-[γ-thio]triphosphate (ATP[S]) were obtained from Boehringer Mannheim Australia Pty. Ltd., North Ryde, N.S.W., Australia. The sodium salts of GMP, AMP, GDP, GTP, and *p*-nitrophenyl phosphate (disodium salt), NaF, SDS and Triton X-100 were obtained from Sigma Chemical Co., St. Louis, MO, U.S.A. All other reagents were of the highest grade available.

RESULTS

Relative contributions of phospholipases C and D

The incubation of membranes labelled with [^{3}H]choline at 30 °C resulted in a linear increase in total ^{3}H-labelled water-soluble metabolites measured as a function of time (Fig. 1*a*).

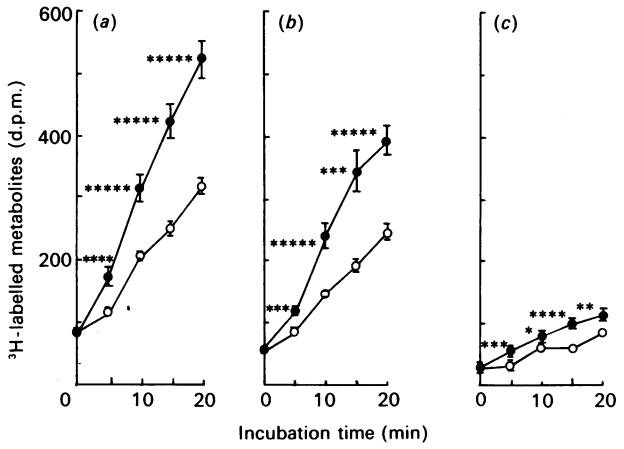

Fig. 1. Effect of GTP[S] on time courses for the release of total ^{3}H-labelled metabolites, [^{3}H]choline and [^{3}H]phosphorylcholine from membranes labelled with [^{3}H]choline

Plasma membranes labelled with [^{3}H]choline were incubated in the absence (○) or presence (●) of 100 μM-GTP[S]. At the times indicated, samples were removed, and the water-soluble radioactivity was extracted and fractionated as described in the Experimental section. The data are (*a*) total ^{3}H-labelled metabolites, (*b*) [^{3}H]choline and (*c*) [^{3}H]phosphorylcholine. The data are the means ± S.E.M. of experiments conducted with eight separate preparations of plasma membranes. The degrees of significance (Student's *t* test for paired samples) for GTP[S]-stimulated activity compared with the control are: *$P < 0.05$, **$P < 0.02$, ***$P < 0.01$, ****$P < 0.002$ and *****$P < 0.001$.

About 70% of the radioactivity released was [^{3}H]choline (Fig. 1*b*) and 30% was [^{3}H]phosphorylcholine (Fig. 1*c*). The amount of phosphatidylcholine in the plasma membrane fraction was estimated to be 115 nmol/mg of membrane protein (mean of two determinations). The amount of radioactivity incorporated into phosphatidylcholine was 8000 d.p.m./mg of membrane protein (mean of two determinations). From these values and from the data of Fig. 1 it was calculated that in the absence of GTP[S] the rates of conversion of phosphatidylcholine to choline and phosphorylcholine were 0.66 ± 0.03 and 0.21 ± 0.01 ($n = 8$) nmol/min per mg of protein respectively.

GTP[S] (100 μM) increased by about 60% the rates of formation of total ^{3}H-labelled metabolites (Fig. 1*a*), [^{3}H]choline (Fig. 1*b*) and [^{3}H]phosphorylcholine (Fig. 1*c*). A similar effect of GTP[S] on total ^{3}H-labelled metabolites was observed when water-soluble metabolites were extracted with trichloroacetic acid (results not shown; cf. Irving & Exton, 1987). The distribution of radioactivity among metabolites of choline released from [^{3}H]choline-labelled membranes treated with GTP[S] for 15 min was confirmed using t.l.c. The relative amounts of radioactivity present in choline, phosphorylcholine, glycerophosphorylcholine and CDP-choline were 68 ± 2, 27 ± 2, 5 ± 2 and 0.4 ± 0.4% (means ± S.E.M., $n = 7$) of the total amount of radioactivity present in water-soluble metabolites extracted from the membrane incubations. The effect of GTP[S] on the formation of [^{3}H]choline and [^{3}H]phosphorylcholine was concentration-dependent, with half-maximal responses obtained at about 1 μM- and about 10 μM-GTP[S] respectively (Table 1, and results not shown).

The possibility that the formation of [^{3}H]choline in membranes labelled with [^{3}H]choline could be accounted for by a phosphomonoesterase (e.g. alkaline phosphatase) which catalyses the hydrolysis of phosphorylcholine to choline was tested by incubating unlabelled membranes at 30 °C with 1 μM-[U-^{14}C]phosphorylcholine. This resulted in the time-dependent

Table 1. Stimulation of [^{3}H]choline release by purine ribonucleoside triphosphates

Plasma membrane labelled with [^{3}H]choline were incubated in the presence of the indicated purine ribonucleoside triphosphate. After 15 min, samples were removed, the water-soluble radioactivity was extracted and fractionated as described in the Experimental section, and the amount of radioactivity present in [^{3}H]choline was measured. For each incubation conducted in the presence of a purine ribonucleoside triphosphate, the d.p.m. in [^{3}H]choline are expressed as a percentage of the d.p.m. released in a corresponding control incubation to which water was added in place of a purine ribonucleoside triphosphate. The amount of radioactivity released in control incubations was 710 ± 50 d.p.m./mg of membrane protein ($n = 17$). The data are the means $\pm$ S.E.M. of the numbers of experiments shown in parentheses. The degrees of significance (Student's t test for paired samples) for incubations conducted in the presence of a purine ribonucleoside triphosphate compared with control incubations are: $*P < 0.02$; $**P < 0.01$ and $***P < 0.001$.

Purine ribonucleoside triphosphate	Concentration (μM)	Amount of [^{3}H]choline released (% of control)
GTP[S]	1	124 ± 4 (3)**
	2	134 ± 2 (3)**
	10	142 ± 3 (13)***
	100	147 ± 4 (6)***
p[NH]ppG	10	126 ± 4 (3)*
	100	138 ± 4 (6)***
pp[CH_2]pG	10	111 ± 1 (3)**
	100	130 ± 3 (3)***
ATP[S]	10	80 ± 4 (3)*
	100	78 ± 4 (3)

formation of [^{14}C]choline (Fig. 2*a*) and a corresponding decrease in [^{14}C]phosphorylcholine (Fig. 2*b*). Of the [^{14}C]phosphorylcholine, 40% was converted to [^{14}C]choline in 20 min. On the basis of the specific radioactivity of the [^{14}C]phosphorylcholine, it was calculated that under the conditions of this experiment the rate of conversion of phosphorylcholine to choline was 0.02 ± 0.003 ($n = 7$) nmol/min per mg of membrane protein. This conversion was inhibited by *p*-nitrophenyl phosphate, with 100 μM giving a complete inhibition (Figs. 2*a* and 2*b*). Surprisingly, the breakdown of [U-^{14}C]phosphorylcholine was also inhibited by 100 μM-GTP[S] (Figs. 2*c* and 2*d*). Since GTP[S] was present as the Li^+ salt, the effect of 400 μM-LiCl was tested. This had no effect on the breakdown of [U-^{14}C]phosphorylcholine (results not shown).

When membranes labelled with [^{3}H]choline were incubated at 30 °C in the presence of 100 μM-*p*-nitrophenyl phosphate, the effect of GTP[S] on the rates of [^{3}H]choline and [^{3}H]phosphorylcholine production was not substantially altered. Moreover, *p*-nitrophenyl phosphate had no substantial effect on the production of [^{3}H]choline and [^{3}H]phosphorylcholine in the absence of GTP[S] (results not shown).

The possibility that, in membranes labelled with [^{3}H]choline and treated with GTP[S], [^{3}H]phosphorylcholine is formed from [^{3}H]choline through the action of choline kinase (Macara, 1989) was also tested. The incubation of membranes in the presence of 1 or 10 mM unlabelled choline had no effect on the rate of accumulation of [^{3}H]choline (results not shown).

The properties of the GTP[S]-stimulated phospholipases C and D which hydrolyse phosphatidylcholine were further investigated by testing the ability of detergents to inhibit the release of choline and phosphorylcholine from phosphatidylcholine. The treatment of membranes for 15 min with low concentrations of either SDS or Triton X-100 caused a marked inhibition of [^{3}H]choline release in the presence and absence of GTP[S] and

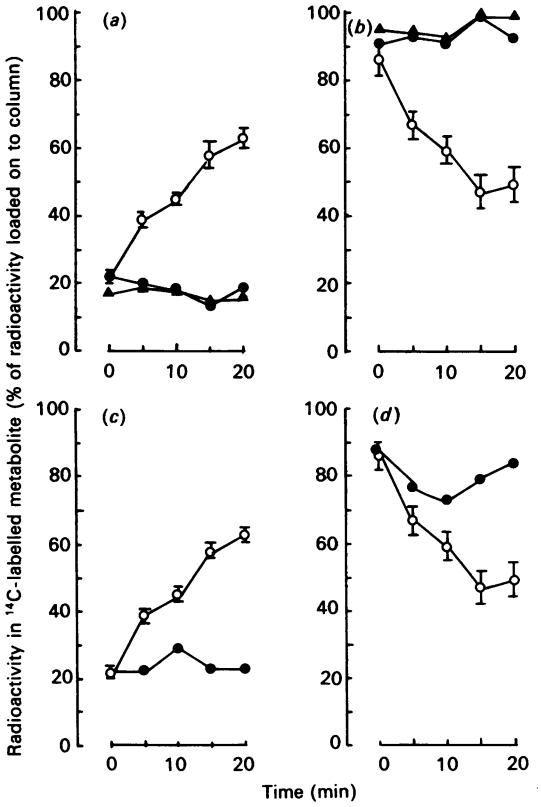

Fig. 2. Inhibition by *p*-nitrophenyl phosphate and GTP[S] of the hydrolysis of [^{14}C]phosphorylcholine by unlabelled plasma membranes

Unlabelled plasma membranes were incubated in the presence of 1 μM-[U-^{14}C]phosphorylcholine (containing 20 nCi of [U-^{14}C]phosphorylcholine) in the absence of further additions (○) or in the presence of 100 μM (▲) or 1 mM (●) *p*-nitrophenyl phosphate (*a*, *b*) and in the absence of further additions (○) or in the presence of 100 μM-GTP[S] (●) (*c*, *d*). At the times indicated samples were removed, the water-soluble radioactivity was extracted and [^{14}C]choline (*a*, *c*) and [^{14}C]phosphorylcholine (*b*, *d*) were fractionated as described in the Experimental section. The data are the means $\pm$ S.E.M. of eight (control) experiments, or the means of two experiments.

inhibited the ability of GTP[S] to stimulate [^{3}H]choline release (Figs. 3*a* and 3*c*). Half-maximal effects of SDS and Triton X-100 were observed at about 0.005% (w/v) and 0.02% (w/v) respectively. By contrast, the release of [^{3}H]phosphorylcholine and the ability of GTP[S] to stimulate [^{3}H]phosphorylcholine release were considerably more resistant to the actions of the detergents (Figs. 3*b* and 3*d*). The concentration of SDS which gave half-maximal inhibition of phosphorylcholine release was about 0.02% (Fig. 3*b*), whereas Triton X-100 had little effect on the release of phosphorylcholine (Fig. 3*d*). Results similar to those shown in Fig. 3 were obtained when the membranes were incubated for 15 min in the presence of detergent, washed, resuspended and incubated for 15 min in the presence or absence of GTP[S] for measurement of the release of [^{3}H]choline and [^{3}H]phosphorylcholine (results not shown).

Effects of purine ribonucleotide polyphosphates on [^{3}H]choline release

p[NH]ppG and pp[CH_2]pG both caused a significant stimulation of [^{3}H]choline release, comparable with that induced by GTP[S], whereas [^{3}H]choline release was inhibited by ATP[S] (Table 1). In the presence of 2 mM-GDP[S], neither 1 or 2 μM-GTP[S] caused a significant stimulation of [^{3}H]choline release,

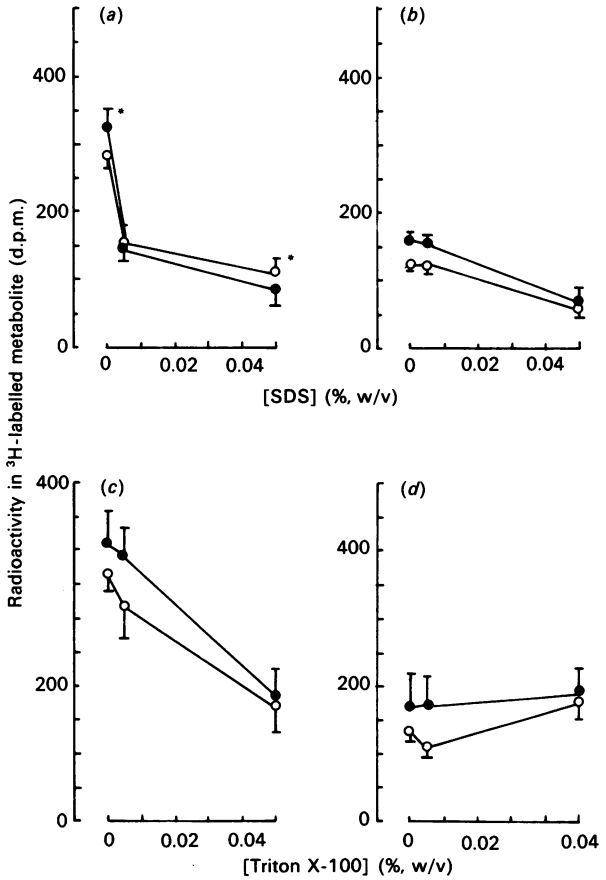

Fig. 3. Effects of SDS (*a*, *b*) and Triton X-100 (*c*, *d*) on the release of [³H]choline and [³H]phosphorylcholine from plasma membranes labelled with [³H]choline incubated in the presence or absence of GTP[S]

Plasma membranes labelled with [³H]choline were incubated in the absence or in the presence of the indicated concentrations of SDS (*a*, *b*) or Triton X-100 (*c*, *d*), and in the absence (○) or presence (●) of GTP[S]. After 15 min samples were removed, the water-soluble radioactivity was extracted and [³H]choline (*a*, *c*) and [³H]phosphorylcholine (*b*, *d*) were measured. The results are the means ± S.E.M. of six (*a*, *b*) and three (*c*, *d*) experiments. The degree of significance (Student's *t* test for paired samples) is $*P < 0.05$.

whereas the stimulation caused by 10 μM-GTP[S] was substantially decreased (Table 2). GDP inhibited the release of [³H]choline observed in the absence of GTP[S] in a concentration-dependent manner (Table 2). In the presence of 5 mM-GDP, GTP[S] was unable to stimulate [³H]choline release (Table 2). Similar results were obtained with GTP in place of GDP[S] (Table 2). Since GTP and GDP are rapidly metabolized to GMP by liver membrane preparations (Salamon & Rodbell, 1975), the effects of GMP were also examined. GMP inhibited [³H]choline release in the absence of GTP[S] but did not inhibit the stimulation caused by GTP[S] (Table 2). Likewise, AMP also had no effect (Table 2).

NaF, or a combination of NaF and $AlCl_3$, caused a 30% inhibition of the release of each of [³H]choline and [³H]phosphorylcholine measured in the absence of GTP[S] (results not shown) (cf. Bocckino *et al.*, 1987*a*). The incubation of [³H]choline-labelled membranes with antibody Y13-259 (67 μg of antibody/100 μg of membrane protein), an inhibitor of the function of the *ras* proteins (Mulcahy *et al.*, 1985; Kung *et al.*, 1986), had no effect on the rate of release of [³H]choline or [³H]phosphorylcholine, in either the presence or the absence of GTP[S] (results not shown).

Table 2. Ability of purine ribonucleoside polyphosphates to inhibit the stimulation of [³H]choline release by GTP[S]

Experiments were performed and the data were treated as described in the legend to Table 1. When used, GDP[S] was added 5 min before GTP[S]. The data are the means ± S.E.M. of the numbers of experiments shown in parentheses or the means ± range of 2 experiments. The data for the addition of GTP[S] alone are the same data as presented in Table 1. The degrees of significance (Student's *t* test for paired samples) for incubations conducted in the presence of purine ribonucleotide polyphosphates compared with control incubations are: $*P < 0.02$, $**P < 0.01$, and $***P < 0.001$.

Concentration of GTP[S] (μM)	Other additions	Amount of [³H]choline released (% of control)
1	None	124 ± 4 (3)**
2	None	134 ± 2 (3)**
10	None	142 ± 3 (13)***
0	2 mM-GDP[S]	96 ± 13 (3)
1	2 mM-GDP[S]	103 ± 9 (3)
2	2 mM-GDP[S]	108 ± 8 (3)
10	2 mM-GDP[S]	112 ± 3 (3)*
0	1 mM-GDP	75 ± 4 (2)
0	2 mM-GDP	69 ± 7 (2)
0	5 mM-GDP	55 ± 6 (4)**
10	1 mM-GDP	117 ± 17 (2)
10	2 mM-GDP	105 ± 9 (2)
10	5 mM-GDP	60 ± 5 (4)**
0	5 mM-GTP	62 ± 11 (2)
10	5 mM-GTP	66 ± 6 (2)
0	5 mM-GMP	69 ± 0 (2)
10	5 mM-GMP	131 ± 8 (2)
0	5 mM-AMP	65 ± 3 (2)
10	5 mM-AMP	117 ± 10 (2)

DISCUSSION

The results obtained show that the increase caused by GTP[S] in the release of radioactivity from membranes labelled with [³H]choline is composed predominantly of [³H]choline release (85%), with about 15% due to the release of [³H]phosphorylcholine. Although the membranes contain an enzyme activity which can hydrolyse phosphorylcholine to choline, the experiments conducted with *p*-nitrophenyl phosphate have shown that this reaction does not account for the formation of [³H]choline. Moreover, the observation that the inclusion of unlabelled choline in the incubation medium does not diminish the amount of [³H]phosphorylcholine formed indicates that choline kinase is not responsible for the formation of [³H]phosphorylcholine, as proposed for intact NIH-3T3 cells (Macara, 1989). On the basis of these observations, it is concluded that the predominant pathway of phosphatidylcholine hydrolysis in rat liver plasma membranes is through phospholipase D (cf. Bocckino *et al.*, 1987*a*,*b*) with a small contribution from phospholipase C. A pathway of phosphatidylcholine hydrolysis which involves phospholipase D has also been identified in a number of other cell types (Cabot *et al.*, 1988; Rubin, 1988; Pai *et al.*, 1988; Billah *et al.*, 1989*a*,*b*; Liscovitch, 1989; Kiss & Anderson, 1989; Qian & Drewes, 1990). The results of the present experiments conducted with SDS and Triton X-100 indicate that, in the hepatocyte plasma membrane, the environment of phospholipase D, or the catalytic activity of the enzyme itself, is considerably more susceptible to disruption by these detergents than is the environment or the catalytic activity of phospholipase C.

The conclusion that [^{3}H]phosphorylcholine is not substantially hydrolysed to [^{3}H]choline under the conditions employed in the present experiments differs from the conclusion reached by Irving & Exton (1987). This difference may be due to a difference in the amount of alkaline phosphatase associated with the different plasma membrane preparations. The surprising observation that GTP[S] inhibits the hydrolysis of phosphorylcholine to choline may indicate that GTP[S] directly inhibits the enzyme responsible for this interconversion or that an inhibitory G-protein is coupled to alkaline phosphatase.

Three experimental observations indicate that GTP[S] stimulates phospholipase D by binding to a G-protein. Firstly, [^{3}H]choline release is stimulated by p[NH]ppG and pp[CH_2]pG as well as by GTP[S] but is not stimulated by ATP[S]. Secondly, the concentration of GTP[S] which gave half-maximal stimulation of the formation of [^{3}H]choline is in the same range as the concentrations reported to activate the G-protein which is coupled to phosphatidylinositol-specific phospholipase C (Cockcroft & Gomperts, 1985; Wallace & Fain, 1985; Hepler & Harden, 1986; Uhing *et al.*, 1986; Cockcroft & Taylor, 1987; Taylor & Exton, 1987). Thirdly, GTP[S]-stimulated [^{3}H]choline release was inhibited by GDP[S] and GDP, but not by GMP. The inhibition of GTP[S]-stimulated choline release by GTP is likely to be due to the rapid hydrolysis of GTP to GDP (Salamon *et al.*, 1975). The results obtained with antibody Y13-259 indicate that a *ras* protein is unlikely to be involved in the activation of phospholipase D.

It would be predicted that NaF and AlF_4^- should also stimulate phospholipase D activity, since AlF_4^- is known to activate oligomeric G-proteins (Sternweis & Gilman, 1982; Bigay *et al.*, 1987). However, activation of phospholipase D was not observed. It is likely that the failure of AlF_4^- to mimic the effects of GTP[S] on the formation of [^{3}H]choline and [^{3}H]phosphorylcholine is due to the inhibition by AlF_4^- of the activities of phospholipases C and D, since both AlF_4^- and NaF inhibited the basal release of [^{3}H]choline and [^{3}H]phosphorylcholine (cf. Bocckino *et al.*, 1987*a*). Although the results obtained with purine ribonucleoside polyphosphates provide evidence which indicates that a G-protein couples the action of GTP[S] to phospholipase D, alternative possibilities cannot be completely excluded in view of the results obtained with AlF_4^- and the inhibition of basal phospholipase D activity by GMP and AMP.

The conclusion that a G-protein mediates the activation of phospholipase D in hepatocyte plasma membranes is similar to conclusions reached by Bocckino *et al.* (1987*a*,*b*) for hepatocytes and by Pai *et al.* (1988) and Tettenborn & Mueller (1988) for HL-60 granulocytes. The action of a G-protein may be one of three types of mechanism by which agonists can activate phospholipase D. The other two are phosphorylation by protein kinase C (Tettenborn & Mueller, 1987; Cabot *et al.*, 1988; Augert *et al.*, 1989; Ben-Av & Liscovitch, 1989; Billah *et al.*, 1989*b*; Liscovitch, 1989) and allosteric activation by Ca^{2+} (Augert *et al.*, 1989; Billah *et al.*, 1989*b*).

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