Polyamines inhibit phospholipase C-catalysed polyphosphoinositide hydrolysis

Studies with permeabilized GH₃ cells

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[³H]Inositol-labelled GH₃ rat anterior pituitary tumour cells were permeabilized with digitonin and were incubated at 37 °C in the presence of ATP and Mg²⁺. [³H]Polyphosphoinositide breakdown and [³H]inositol phosphate production were stimulated by hydrolysis-resistant GTP analogues and by Ca²⁺. Of the nucleotides tested, guanosine 5'-[γ-thio]triphosphate (GTPγS) was the most effective stimulus. Activation by GTPγS appeared to be mediated by a guanine nucleotide-binding (G) protein as GTPγS-stimulated [³H]inositol phosphate production was inhibited by other nucleotides with a potency order of GTP = GDP = guanosine 5'-[β-thio]diphosphate > ITP > GMP > UTP = CTP = adenosine 5'-[γ-thio]triphosphate. The stimulatory effects of 10 μM-GTPγS on [³H]inositol phosphate levels were reversed by spermine and spermidine with IC₅₀ values of approx. 0.25 and 2 mM respectively. Putrescine was inhibitory only at higher concentrations. Similarly, GTPγS-induced decreases in [³H]polyphosphoinositide levels were reversed by 2.5 mM-spermine. The inhibitory effects of spermine were not overcome by supramaximal concentrations of GTPγS. In contrast, [³H]inositol phosphate production stimulated by addition of 0.3–0.6 mM-Ca²⁺ to incubation media was only partially inhibited by spermine (5 mM), and spermine was not inhibitory when added Ca²⁺ was increased to 1 mM. These data show that polyamines, particularly spermine, inhibit phospholipase C-catalysed polyphosphoinositide hydrolysis with a marked selectivity towards the stimulatory effects of GTPγS.

INTRODUCTION

The polyamines, spermidine and spermine, and their precursor putrescine, are present in significant quantities in virtually all eukaryotic cells. Although it appears that polyamines are required for normal growth and differentiation, their precise physiological functions and mode of action have yet to be defined (for review see Tabor & Tabor, 1984; Pegg, 1986). Many of the known cellular effects of polyamines are a reflection of their polycationic nature. The interaction of polyamines with nucleic acids and stimulation by polyamines of certain kinase activities can be mimicked by other cations and appear to result from charge neutralization rather than from complex molecular interactions (Tabor & Tabor, 1984; Ahmed *et al.*, 1985; Marton & Morris, 1987).

The interaction of polyamines with biological membranes has a variety of effects (for review see Marton & Morris, 1987), including inhibition of glycoprotein mobility within the plane of the plasma membrane (Schindler *et al.*, 1980), promotion of membrane fusion (Hong *et al.*, 1983) and inhibition of protein kinase C association with membranes (Moruzzi *et al.*, 1987). The primary polyamine binding sites within membranes appear to be acidic phospholipids. Although binding of spermine to phosphatidylserine and phosphatidylinositol (PI) has been demonstrated (Chung *et al.*, 1985), particularly strong interactions between spermine and phosphatidylinositol 4-phosphate (PIP) and phospho-

tidylinositol 4,5-bisphosphate (PIP₂) have been reported (Tadolini & Varani, 1986; Meers *et al.*, 1986).

Binding of polyamines to polyphosphoinositides is of particular interest because of the crucial role that these phospholipids play in transducing receptor activation into intracellular messengers (for review see Nishizuka, 1986; Berridge, 1987). Of prime importance is PIP₂, since its hydrolysis by phospholipase C leads to the formation of the intracellular messengers diacylglycerol and inositol 1,4,5-trisphosphate (IP₃). We have therefore examined the possibility, using GH₃ rat anterior pituitary tumour cells, that polyamines might affect phosphoinositide hydrolysis. GH₃ cells have been widely used in studies concerning phosphoinositide metabolism (Drummond, 1986) and recent studies with permeabilized cells and membrane preparations (Martin *et al.*, 1986; Straub & Gershengorn, 1986) have confirmed indications (Hinkle & Kinsella, 1984; Wojcikiewicz *et al.*, 1986) that a guanine nucleotide-binding (G) protein, termed G_p, is involved in receptor-mediated activation of phospholipase C. We report here that polyamines inhibit G-protein-mediated activation of phosphoinositide hydrolysis in [³H]inositol-labelled permeabilized cells.

MATERIALS AND METHODS

Cell growth and permeabilization

GH₃ cells (passage number 50–90) were routinely maintained in monolayers as described (Wojcikiewicz

Abbreviations used: TRH, thyrotropin-releasing hormone; GTPγS, ATPγS, guanosine and adenosine 5'-[γ-thio]triphosphates; PI, PIP, PIP₂, phosphatidylinositol and its 4-phosphate and 4,5-bisphosphate; IP₁, IP₂, IP₃, inositol monophosphate, 1,4-bisphosphate and 1,4,5-trisphosphate.

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et al., 1986). For permeabilization experiments, cells were subcultured into Linbro 12-well plates (growth area per well 4.5 cm²) at a density of approx. 6×10^5 cells/well in 1.25 ml of culture medium. After 18–24 h, the culture medium was replaced with 1.25 ml/well of fresh culture medium supplemented with 5 μ Ci of [³H]inositol. Two days later, the cells were permeabilized with digitonin essentially as described for other cell types (Mackall *et al.*, 1979; Cascales *et al.*, 1984). The cells were cooled on ice for 10 min and the culture medium was replaced with 0.75 ml/well of ice-cold permeabilization buffer (0.25 M-sucrose, 0.4 mM-dithiothreitol, 10 mM-sodium Hepes, pH 7.4) supplemented with 0.075 mg of digitonin/ml. After incubation for various times on ice (usually 10 min), this buffer was removed and the cells were washed once (incubated for a further 5 min) with 1 ml of fresh permeabilization buffer. For measurement of phosphoinositide hydrolysis the permeabilization buffer was replaced with 0.75 ml of ice-cold incubation medium (50 mM-sodium Hepes, pH 7.4, 40 mM-NaCl, 20 mM-LiCl, 0.5 mM-EDTA, 6 mM-MgCl₂, 2 mM-ATP, 0.3 mg of bovine serum albumin/ml) supplemented, when indicated, with either nucleotides, CaCl₂ or polyamines. HCl was used to reverse any slight changes in pH caused by polyamines. The cells were then transferred to a water bath at 37 °C for 10 min and experimental incubations were terminated by adding 0.75 ml of ice-cold methanol/2 M-HCl (9:1, v/v) to each well.

Analysis of inositol phosphates and phosphoinositides

After termination, the contents of each well were mixed thoroughly with 1 ml of chloroform in polypropylene tubes and then centrifuged (2300 *g* for 10 min). Then 1.1 ml of the upper phase was neutralized with NH₄OH and in most cases analysed using Dowex resin (formate form) as described by Berridge (1983). [³H]inositol phosphates were separated by stepwise elution into three fractions (8 ml each) and were assessed for radioactivity with 10 ml of Budgetsolve (Research Products International). For clarity, data are expressed as '[³H]inositol phosphates', i.e. the sum of radioactivity present in the three fractions.

Certain experimental samples were analysed by h.p.l.c., in which case 1 ml of neutralized upper phase was injected into a Partisil 10 SAX column. A non-linear gradient of 0–1.7 M-ammonium formate adjusted to pH 3.7 with phosphoric acid (B) was used to elute inositol phosphates. The gradient, similar to that described by Irvine *et al.* (1985), was (flow rate 1 ml/min, 30 s fractions collected): 0–10 min, 0 → 15% B; 10–20 min, 15 → 50% B; 20–27 min, 50 → 100% B; 27–30 min, 100% B; 30–40 min, 0% B. [³H]inositol phosphate standards (Amersham Corp.) were eluted as follows; [³H]inositol 1-monophosphate (fraction 21), [³H]inositol 4-monophosphate (fraction 23), [³H]inositol 1,4-bisphosphate (fraction 35) and [³H]inositol 1,4,5-trisphosphate (fraction 54).

[³H]Phosphoinositides were analysed by t.l.c.: 0.5 ml of lower phases were mixed with 5 μ g of PI, PIP and PIP₂, evaporated to dryness and analysed using silica gel HL plates (Analtech) and chloroform/methanol/28% NH₄OH/water (90:90:10:19, by vol.) as solvent. Lipid spots (visualized with iodine vapour) were scraped from the plates, mixed with 0.5 ml of water and assessed for radioactive content in 5 ml of Budgetsolve.

Measurement of free Ca²⁺ concentration (Ca²⁺_{free})

Ca²⁺_{free} was estimated using fura-2 and the methods described by Grynkiewicz *et al.* (1985).

Materials

GH₃ cells were obtained from American Type Culture Collection and media and reagents for cell culture from Flow Laboratories. [³H]inositol (*myo*-[2-³H(N)]inositol, 15 Ci/mmol) was obtained from American Radiolabeled Chemicals; polyamines (free bases), ATP, dithiothreitol, digitonin (lot no. 124F-0262), thyrotropin-releasing hormone (TRH), PI, PIP, PIP₂ and IP₃ were from Sigma Chemical Co.; all nucleotides except ATP were from Boehringer Mannheim Biochemicals; bovine serum albumin was from Armour Pharmaceutical Co. and Dowex AG1-X8 resin (200–400 mesh) was from BioRad.

RESULTS

Permeabilization

Incubation of GH₃ cells for various times with digitonin and subsequent measurement of [³H]inositol phosphate production in the presence of 2 mM-ATP and 6 mM-Mg²⁺ led to (a) a time-dependent decrease in basal and TRH-stimulated [³H]inositol phosphate levels and (b) the appearance of a stimulatory effect of GTP γ S (Fig. 1). The effects of TRH and GTP γ S were additive (Fig. 1). Release of cell contents into the medium (monitored by measurement of lactate dehydrogenase release) was also time-dependent and was maximal after incubation with

Fig. 1. Time-course of digitonin-induced permeabilization

GH₃ cells were cooled, incubated with digitonin (0.075 mg/ml) for times indicated and washed. Cells were then exposed to incubation buffer for 10 min at 37 °C containing either no addition (○), 10 μ M-GTP γ S (■), 1 μ M-TRH (▲) or 10 μ M-GTP γ S and 1 μ M-TRH (◆). The data (total radioactivity present in inositol monophosphate, bisphosphate and trisphosphate fractions) are from one experiment performed in duplicate representative of three independent experiments that gave similar results.

Fig. 2. Inhibition of GTP γ S-stimulated [3 H]inositol phosphate production by nucleotides

Cells were permeabilized with digitonin for 10 min, washed and incubated for 10 min at 37 °C in the absence of stimuli (*) or in the presence of 10 μ M-GTP γ S and various concentrations of GTP (○), GDP (△), GDP β S (◇), GMP (□), ITP (▲), UTP (■), CTP (●) or ATP γ S (◆). The data are the mean of results from two independent experiments in which average GTP γ S-stimulated and basal [3 H]inositol phosphates were 11 690 d.p.m. and 1010 d.p.m. respectively.

0.075 mg of digitonin/ml for 7.5 min (results not shown). These data indicate that permeabilization has occurred to an extent that allows for exchange of substances between the interior and exterior of the cell. In all subsequent experiments cells were incubated with 0.075 mg of digitonin/ml for 10 min.

Characteristics of permeabilized cells

A range of nucleotides were examined for their ability to stimulate [3 H]inositol phosphate formation. At concentrations of 1 mM, the nucleotide effects, expressed as average percentage stimulation of basal levels in two experiments, were GTP γ S 1004 %, guanosine 5'-[β -imido]triphosphate 384 %, ATP γ S 228 %, adenosine 5'-[β -imido]triphosphate 133 %, GTP 116 %. This degree of specificity is characteristic of a G-protein-mediated process (for review see Gilman, 1987) and is consistent with findings obtained from electrically permeabilized GH $_3$ cells (Martin *et al.*, 1986). Additional evidence supporting a role for a G protein in activation of phospholipase C is shown in Fig. 2. The effect of GTP γ S (10 μ M) was reversed by other nucleotides with the rank order of potency GTP = GDP = guanosine 5'-[β -thio]-diphosphate > ITP > GMP > UTP = CTP = ATP γ S. Similar nucleotide specificity has been observed for displacement of GTP γ S binding to purified G proteins involved in regulation of adenylate cyclase (Northup *et al.*, 1982; Bokoch *et al.*, 1984).

Analysis of the water-soluble products of cells stimulated for 10 min with 10 μ M-GTP γ S or with Ca $^{2+}$ showed that IP $_2$ was the predominant radioactive species (Fig. 3a, Table 1). However, inclusion of non-radioactive

Fig. 3. Effects of non-radioactive IP $_3$ on [3 H]inositol phosphate production

Cells were permeabilized and incubated as in Fig. 2 with either (a) no addition (+, no line) or 10 μ M-GTP γ S (solid line), (b) 10 μ M-GTP γ S + 0.2 mM-IP $_3$ or (c) 10 μ M-GTP γ S + 1 mM-IP $_3$. Water-soluble products were analysed by h.p.l.c. Numbers in parentheses next to the peaks of [3 H]IP $_2$ (approx. fraction 35) and [3 H]IP $_3$ (approx. fraction 54) are d.p.m./peak calculated as in Table 1. [3 H]Inositol and [3 H]IP $_1$ peaked in fractions 10 and 21 respectively. The data are from one experiment representative of two independent experiments that gave similar results.

Fig. 4. Effects of polyamines on GTP γ S-stimulated phosphoinositide metabolism

Cells were permeabilized and incubated as in Fig. 2, with 10 μ M-GTP γ S and the concentrations of spermine (●), spermidine (■) or putrescine (▲) indicated. The effects of spermine on unstimulated cells (○, broken line) are also shown. Data presented are mean $\pm$ S.E.M. of four (spermine) or three (spermidine and putrescine) independent experiments.

IP₃ in incubations with GTP γ S reduced the size of the [³H]IP₂ peak and caused an equivalent increase in size of the [³H]IP₃ peak (Figs. 3b and 3c). Levels of [³H]inositol or of [³H]inositol phosphates other than [³H]IP₂ or [³H]IP₃ were unchanged by the addition of non-radioactive IP₃. These data indicate that the majority of [³H]IP₂ (at least 60%) is derived from dephosphorylation of [³H]IP₃ (Fig. 3c). Thus, in GTP γ S-stimulated permeabilized GH₃ cells [³H]PIP₂ hydrolysis appears to be the primary source of [³H]inositol phosphates. It should also be noted that, under all conditions, IP₁ levels were low and essentially invariant (Fig. 3, Table 1) despite the presence of 20 mM-Li⁺ in incubation media to block IP₁ dephosphorylation (Berridge *et al.*, 1982). As the enzyme activity that dephosphorylates IP₂ to IP₁ has been shown, at least in liver, to be mostly soluble (Storey *et al.*, 1984), leakage of this enzyme from the permeabilized cells may have occurred.

Effects of polyamines on phosphoinositide metabolism

The effects of spermine, spermidine and putrescine on [³H]inositol phosphate production are shown in Fig. 4. Although spermine had little effect on basal [³H]inositol phosphate levels (Fig. 4, Table 1), stimulation by GTP γ S (10 μ M) was completely inhibited by both spermine (IC₅₀ of approx. 0.25 mM) and spermidine (IC₅₀ of approx. 2 mM). Putrescine was inhibitory only at concentrations of 10 mM or above. Spermine at 2.5 mM completely inhibited the effects of GTP γ S in the range 0.1–100 μ M (Fig. 5a, Table 1). In an additional experiment (results not shown), 1 mM-spermine was equally effective in inhibiting the stimulatory effects of 10 μ M, 1 mM and 2.5 mM-GTP γ S. Thus, inhibition by spermine could not be reversed in a competitive manner by high levels of GTP γ S.

Addition of Ca²⁺ to the incubation medium also led to a stimulation of [³H]inositol phosphate production (Fig. 5b, Table 1). This effect was partly reversed by 5 mM-

Fig. 5. Inhibition by spermine of GTP γ S- or Ca²⁺-stimulated phosphoinositide metabolism

Cells were permeabilized and incubated as in Fig. 2 with various concentrations of (a) GTP γ S in the absence (○) or presence (●) of 2.5 mM-spermine or (b) added Ca²⁺ in the absence (□) or presence (■) of 5 mM-spermine. The data are mean $\pm$ S.E.M. of three independent experiments.

spermine at levels of added calcium up to 0.6 mM, but not when 1 mM-Ca²⁺ was added to the incubation medium (Fig. 5b, Table 1). Ca²⁺_{free} in incubation medium with no added Ca²⁺ was approx. 600 nM and with 0.3 mM-Ca²⁺ added was approx. 2500 nM. Neither value was markedly altered by the addition of 5 mM-spermine or 10 μ M-GTP γ S. Thus, the effects of spermine and GTP γ S were not due to alteration of Ca²⁺_{free}. Estimates of Ca²⁺_{free} in buffers containing greater than 0.3 mM added Ca²⁺ could not be obtained because of the limitations of the fura-2 assay (Grynkiewicz *et al.*, 1985).

The effects of spermine on [³H]phosphoinositide metabolism and the formation of [³H]inositol phosphates are shown in Table 1. Under basal conditions, phosphoinositide kinase activity was greater than phospholipase C activity as [³H]PIP and [³H]PIP₂ levels increased during the 10 min incubations. In the presence of spermine, larger increases in [³H]PIP levels were observed although [³H]inositol phosphate formation was not significantly altered. These data suggest that spermine activates PI kinase, although effects of spermine on phosphoinositide phosphomonoesterases could also account for the changes seen.

Stimulation of [³H]IP₂ and [³H]IP₃ formation by GTP γ S was accompanied by approx. 25% decreases in [³H]PIP and [³H]PIP₂ levels (Table 1). Both effects were reversed by 2.5 mM-spermine. Similarly, addition of Ca²⁺ to the incubation medium greatly increased [³H]IP₂ and [³H]IP₃ production and decreased [³H]PIP and particularly [³H]PIP₂ levels. Stimulation of [³H]IP₂ and [³H]IP₃ formation by addition of 0.45 mM- or 0.6 mM-Ca²⁺ was partly inhibited by 5 mM-spermine. In contrast, in the presence of 1 mM added Ca²⁺, spermine had no effect on the stimulation of [³H]inositol phosphate production or the decrease in [³H]PIP₂ levels (Table 1, Fig. 5b).

Stimulation of [³H]inositol phosphate production to a similar extent with 10 μ M-GTP γ S or by the addition of 0.45 mM-Ca²⁺ (Fig. 5) generated virtually identical profiles of [³H]inositol phosphates, i.e. under both conditions the ratio of [³H]IP₂: [³H]IP₃ was approx. 5

Table 1. Effects of spermine, GTP γ S and Ca $^{2+}$ on inositol phosphate formation and phosphoinositide metabolism

Permeabilized cells were incubated for 0 min ($t = 0$) or for 10 min with or without additions as indicated. [3 H]Phosphoinositides were analysed by t.l.c. (mean $\pm$ S.E.M. of five independent experiments) and [3 H]inositol phosphates by h.p.l.c. (mean of duplicates from a single experiment). Inositol phosphate data are the sum of radioactivity recovered in fractions 18–24 (IP $_1$), 34–40 (IP $_2$) and 50–56 (IP $_3$). For the lipid data, * ($P < 0.05$) and ** ($P < 0.01$) denote the significance calculated by unpaired t -test of changes from 10 min control incubations. Raw data were multiplied by appropriate factors to yield d.p.m./well. IP $_1$ levels (not shown) were virtually constant under all conditions and varied from the $t = 0$ value (3590 d.p.m./well) by no more than 27%. Similarly, PI levels did not significantly vary from the control value (159 700 $\pm$ 12 600 d.p.m./well) under any condition.

Additions	10 $^{-3} \times$ [3 H]inositol phosphates (d.p.m./well)		10 $^{-3} \times$ [3 H]phosphoinositides (d.p.m./well)	
	IP $_2$	IP $_3$	PIP	PIP $_2$
Control ($t = 0$)	0.41	0.27	3.0 $\pm$ 0.3	0.7 $\pm$ 0.1
Control	0.86	0.49	5.0 $\pm$ 0.5	1.1 $\pm$ 0.1
2.5 mM-spermine	1.21	0.78	6.8 $\pm$ 0.6*	1.4 $\pm$ 0.1
5 mM-spermine	1.16	0.65	7.8 $\pm$ 0.3**	1.2 $\pm$ 0.2
10 μ M-GTP γ S	19.64	4.37	3.9 $\pm$ 0.4*	0.8 $\pm$ 0.1
10 μ M-GTP γ S + 2.5 mM-spermine	1.27	0.87	6.8 $\pm$ 1.0	1.4 $\pm$ 0.1*
0.45 mM-Ca $^{2+}$	24.82	4.58	5.5 $\pm$ 0.5	0.8 $\pm$ 0.1
0.6 mM-Ca $^{2+}$	31.47	4.41	4.2 $\pm$ 0.4	0.7 $\pm$ 0.1*
1 mM-Ca $^{2+}$	22.27	2.01	2.9 $\pm$ 0.4*	0.6 $\pm$ 0.1**
0.45 mM-Ca $^{2+}$ + 5 mM-spermine	4.31	0.93	10.7 $\pm$ 0.9**	1.1 $\pm$ 0.1
0.6 mM-Ca $^{2+}$ + 5 mM-spermine	12.52	2.67	8.5 $\pm$ 0.4**	0.8 $\pm$ 0.1
1 mM-Ca $^{2+}$ + 5 mM-spermine	24.69	2.41	4.4 $\pm$ 0.3	0.6 $\pm$ 0.1**

Fig. 6. Effects of Ca $^{2+}$ on GTP γ S-stimulated phosphoinositide metabolism

Cells were permeabilized and incubated as in Fig. 2 with various concentrations of GTP γ S in (a) the absence or (b) the presence of 5 mM-spermine. Included in incubations were, no added Ca $^{2+}$ (○, ●), 0.3 mM-added Ca $^{2+}$ (□, ■), 0.45 mM-added Ca $^{2+}$ (△, ▲) or 0.6 mM-added Ca $^{2+}$ (◇, ◆). The data are mean $\pm$ S.E.M. of three independent experiments.

(Table 1). These data suggest that both stimuli activate phosphoinositide hydrolysis in a similar manner. However, significant decreases in [3 H]PIP levels were seen with 10 μ M-GTP γ S but not with 0.45 mM added Ca $^{2+}$. This and the fact that [3 H]PIP levels were markedly higher in the presence of 0.45 mM-added Ca $^{2+}$ plus 5 mM-spermine as compared with 5 mM-spermine alone suggest that Ca $^{2+}$ may also increase the rate of PIP formation.

Table 1 also shows that in the presence of stimuli, the increases in radioactivity in [3 H]inositol phosphates were consistently 10–20-fold greater than decreases in radioactivity present in [3 H]phosphoinositides. These data suggest that under stimulated conditions, the polyphosphoinositide pools are continually being replenished by phosphorylation of [3 H]PI. Indeed, when ATP was omitted from the incubation medium, GTP γ S (10 μ M) effects on [3 H]inositol phosphate production were drastically reduced (results not shown).

As the data in Fig. 5(b) and Table 1 indicate that Ca $^{2+}$ still stimulates [3 H]inositol phosphate production in the presence of spermine, the possibility was examined that the inhibitory effects of spermine on GTP γ S-stimulated [3 H]inositol phosphate production might be overcome by addition of Ca $^{2+}$. Fig. 6(a) shows stimulatory effects of GTP γ S in the presence of 0 mM-, 0.3 mM- and 0.45 mM-added Ca $^{2+}$. However, in the presence of 5 mM-spermine, GTP γ S was unable to increase [3 H]inositol phosphate levels even when the effects of spermine were partly reversed by the addition of Ca $^{2+}$.

DISCUSSION

Our studies show that polyamines inhibit GTP γ S- and Ca $^{2+}$ -stimulated polyphosphoinositide hydrolysis in permeabilized GH $_3$ cells. In addition to these effects, polyamines have also been shown to inhibit phospholipase C-catalysed PI hydrolysis using soluble enzymes and substrate (Eichberg *et al.*, 1981; Sagawa *et al.*, 1983), stimulate PI and PIP kinase activities (Cochet & Chambaz, 1986; Lundberg *et al.*, 1986; Vogel & Hoppe, 1986) and inhibit PIP and IP $_3$ dephosphorylation (Smith & Wells, 1984; Seyfred *et al.*, 1984). Although other

explanations are possible, the effects of polyamines on phosphoinositide metabolism in GH_3 cells and in other systems most likely result from the fact that polyamines contain positively charged amino groups that can interact with the negatively charged phosphate groups present in phosphoinositides and inositol phosphates. This would also explain why the order of potency of inhibitory effects in GH_3 cells and in other systems (Eichberg *et al.*, 1981; Moruzzi *et al.*, 1987) is spermine (four amino groups) > spermidine (three amino groups) > putrescine (two amino groups). Similar interactions would also explain why cations such as neomycin have effects equivalent to those of polyamines (Orsulakova *et al.*, 1976; Sagawa *et al.*, 1983; Smith & Wells, 1984; Van Rooijen & Agranoff, 1985).

It is important to note that the present report describes effects of polyamines in cells in which the endogenous phosphoinositides involved in signal transduction have been labelled by metabolic incorporation of [^3H]inositol (for review see Drummond, 1986). Cells were permeabilized with digitonin so that effects of $\text{GTP}\gamma\text{S}$, Ca^{2+} and polyamines on the metabolism of phosphoinositides could be observed. However, digitonin, perhaps because of its detergent properties (see Martin, 1987, for discussion of this point), had inhibitory effects on TRH-stimulated [^3H]inositol phosphate production. In addition, only additivity between the effects of TRH and $\text{GTP}\gamma\text{S}$ was observed. Thus, digitonin treatment, in contrast to electrical permeabilization (Martin *et al.*, 1986), inactivates the TRH response.

The characteristics of $\text{GTP}\gamma\text{S}$ -induced phosphoinositide metabolism in permeabilized GH_3 cells indicated that a G protein, presumably G_p , was involved in activation of phospholipase C. Stimulation by $\text{GTP}\gamma\text{S}$ led to the production of [^3H]IP $_2$ and [^3H]IP $_3$ and a decrease in [^3H]polyphosphoinositide levels. As inhibition of [^3H]IP $_3$ dephosphorylation by the addition of large amounts of non-radioactive IP $_3$ led to the accumulation of [^3H]IP $_3$, the majority of [^3H]inositol phosphates appeared to be derived from phospholipase C-catalysed [^3H]PIP $_2$ hydrolysis. In contrast, an analogous study (Hrbolich *et al.*, 1987) showed that the majority of [^3H]inositol phosphates in stimulated platelets were derived from [^3H]PIP hydrolysis.

The effects of $\text{GTP}\gamma\text{S}$ on [^3H]inositol phosphate production were completely blocked by spermine in a manner that could not be overcome by high concentrations of $\text{GTP}\gamma\text{S}$. These data suggest that spermine does not inhibit binding of $\text{GTP}\gamma\text{S}$ to G_p by simple competition and are consistent with the possibility that polyamines bind to and inhibit the breakdown of PIP $_2$ and perhaps PIP. Despite the fact that addition of Ca^{2+} to the incubation medium appeared to activate phosphoinositide breakdown in a similar manner to $\text{GTP}\gamma\text{S}$, inhibitory effects of spermine were only partial in the range 0.3–0.6 mM added Ca^{2+} and were not seen upon addition of 1 mM- Ca^{2+} . In addition, [^3H]polyphosphoinositide levels were decreased to a similar extent by 1 mM- Ca^{2+} in the absence or presence of 5 mM-spermine. These opposing effects of spermine and Ca^{2+} on phosphoinositide hydrolysis may well result from competition for binding to the polyphosphoinositides. Similar competition has been seen between spermine and Ca^{2+} for binding to phosphatidylserine (Meers *et al.*, 1986) and between Ca^{2+} and aminoglycosides for PIP $_2$ hydrolysis (Van Rooijen & Agranoff, 1985).

All experiments with permeabilized cells were performed in the presence of Mg^{2+} (6 mM) which, like Ca^{2+} and spermine, has a high affinity for isolated PIP $_2$ (Hendrickson, 1969; Tadolini & Varani, 1986). However, the fact that the amount of Ca^{2+} required to overcome the suppressive effects of polyamines on polyphosphoinositide hydrolysis was substantially less than 6 mM indicates that Ca^{2+} interacts more strongly with the polyphosphoinositides in GH_3 cell membranes than does Mg^{2+} . The same conclusion has been drawn from studies of Ca^{2+} binding to erythrocyte membranes (Buckley & Hawthorne, 1972; Kawaguchi & Konishi, 1980).

In spite of the fact that Ca^{2+} and spermine may compete for the same binding site(s), partial reversal of the effects of spermine by Ca^{2+} did not cause the reappearance of a $\text{GTP}\gamma\text{S}$ effect. This indicates that the interaction of spermine with the phosphoinositide/phospholipase C/ G_p system is more complex than simple reversible binding to the polyphosphoinositides and points towards the existence of a second site of interaction, perhaps at the level of G_p . Such an interaction that prevented either binding of $\text{GTP}\gamma\text{S}$ to G_p or coupling of G_p with phospholipase C would account for the apparent irreversibility of spermine-induced inhibition of $\text{GTP}\gamma\text{S}$ effects.

In addition to $\text{GTP}\gamma\text{S}$ - and Ca^{2+} -sensitive phospholipase C activity, the data in Table 1 also show that permeabilized GH_3 cells contain PI kinase activity that appears to be stimulated by spermine under basal conditions. Although other reports have described effects of polyamines on phosphoinositide kinase activities in membranes (Vogel & Hoppe, 1986; Lundberg *et al.*, 1987) and on isolated enzymes (Cochet & Chambaz, 1986; Lundberg *et al.*, 1986), inhibitory effects of spermine on PIP phosphomonoesterase (Smith & Wells, 1984) could also account for the increased levels of [^3H]PIP. Furthermore, the data in Table 1 suggest that in the presence of stimuli, where [^3H]inositol phosphate production was much greater than could be accounted for by decreases in [^3H]polyphosphoinositide levels, the activities of the Mg^{2+} and ATP-dependent phosphoinositide kinases present in GH_3 cells (Martin *et al.*, 1986; Martin, 1987) must have increased in order to replenish the [^3H]polyphosphoinositides being degraded.

This report describes effects of polyamines on Ca^{2+} - and $\text{GTP}\gamma\text{S}$ -stimulated phosphoinositide metabolism in permeabilized cells. The effects of $\text{GTP}\gamma\text{S}$ were blocked completely by polyamines, whereas those of Ca^{2+} were partly inhibited. Substantial amounts of spermine (0.05–2 $\mu\text{mol/g}$ wet wt.) and spermidine (0.1–3 $\mu\text{mol/g}$ wet wt.) are present in eukaryotic tissues (Bachrach, 1973) and cellular polyamine levels vary during growth, differentiation and hormone stimulation (Bachrach, 1973; Feige *et al.*, 1986; Bethell *et al.*, 1982). Whether or not polyamines regulate phosphoinositide hydrolysis in intact cells has yet to be established.

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