Modulation of inositol phospholipid metabolism by polyamines

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At low concentrations of Mg^{2+} , incorporation of ^{32}P from $[\gamma\text{-}^{32}P]ATP$ into phosphatidylinositol 4-phosphate (PIP) and phosphatidylinositol 4,5-bisphosphate (PIP_2) in plasma membranes isolated from human polymorphonuclear leucocytes was enhanced 2–4-fold by the polyamines spermidine and spermine. Polyamines had no effects on inositol phospholipid phosphorylation at high concentrations of Mg^{2+} . At 1 mM- Mg^{2+} , $[^{32}P]PIP_2$ synthesis was maximally enhanced by 2 mM-spermine and 5 mM-spermidine, whereas putrescine only slightly enhanced synthesis. Spermine decreased the EC_{50} (concn. for half-maximal activity) for Mg^{2+} in $[^{32}P]PIP_2$ synthesis from 5 mM to 0.5 mM. Spermine did not modulate the K_m for ATP for $[^{32}P]PIP$ or $[^{32}P]PIP_2$ synthesis. Spermine also decreased the EC_{50} for PI in $[^{32}P]PIP$ synthesis. In contrast, spermine elevated the apparent V_{max} , without affecting the EC_{50} for PIP, for $[^{32}P]PIP_2$ synthesis. Spermine and spermidine also inhibited the hydrolysis of $[^{32}P]PIP_2$ by phosphomonoesterase activity. Therefore polyamines appear to activate inositol phospholipid kinases by eliminating the requirements for super-physiological concentrations of Mg^{2+} . Polyamine-mediated inhibition of polyphosphoinositide hydrolysis would serve to potentiate further their abilities to promote the accumulation of polyphosphoinositides in biological systems.

INTRODUCTION

Although polyphosphoinositides (PIP and PIP_2) are quantitatively minor components of biological membranes, they serve several important functions in cellular regulation (reviewed in refs. [1–3]). Most actively studied are the roles these phospholipids serve as precursors for potent intracellular second messengers, e.g. IP_3 and diacylglycerol, which are produced upon receptor-mediated activation of phospholipase C. Phosphoinositides have also been proposed to serve a variety of other functions, including regulation of membrane-associated enzyme activity [4–6], modulation of membrane fluidity [7], and maintenance of membrane-associated Ca^{2+} pools [8].

Polyphosphoinositides are synthesized by the sequential phosphorylation of the 4- and 5-hydroxyl groups of the inositol moiety of PI by PI kinase and PIP kinase respectively. These kinases appear to be predominantly plasma-membrane-associated, although activity is also present in the cytosol and intracellular organelles [1–3]. The mechanisms by which these inositide kinases are regulated are poorly defined. Both kinases appear to be subject to feedback inhibition by PIP and PIP_2 [9,10]. Relief of this inhibition may partially explain the activation of the lipid kinases upon hormone-stimulated polyphosphoinositide breakdown. Additionally, activation of protein kinase C may lead to stimulation of the inositide kinases [11–13]. These effects may be important for the resynthesis of polyphosphoinositides during the recovery from hormone stimulation. In resting cells, polyphosphoinositides are subject to hydrolysis by

phosphomonoesterases [1]. Therefore, significant kinase activity must also be expressed in the basal metabolic state.

Polyphosphoinositides have been shown to bind bivalent-metal cations, particularly Ca^{2+} [8], and aminoglycosidic antibiotics such as neomycin [14]. It has therefore been proposed that Ca^{2+} serves as the counterion for phosphoinositides in biological systems [8]. However, the cellular polyamines, spermine and spermidine, have been shown to interact strongly with phosphoinositide-containing phospholipid vesicles [15–17]. Since the cellular concentrations of polyamines (0.5–1 mM) are much higher than the concentration of free Ca^{2+} (100–200 nM), it seems likely that polyamines are more important counter-ions for these acidic membrane components than Ca^{2+} may be. We have briefly reported that PIP_2 synthesis in plasma membranes isolated from human PMNs can be enhanced by spermine [18]. Subsequently, Vogel & Hoppe [19] demonstrated that several polycations can enhance PI kinase activity in membranes from A431 cells. In the present study, we have more thoroughly characterized the effects of physiological polyamines on phosphoinositide phosphorylation and dephosphorylation in isolated plasma membranes.

EXPERIMENTAL

Materials

Neomycin sulphate, polyamines, glyceryl-glass controlled-pore beads and phospholipids were obtained

Abbreviations used: p[NH]ppA, 5'-adenylyl imidodiphosphate; IP_3 , *myo*-inositol 1,4,5-trisphosphate; PA, phosphatidic acid; PI, phosphatidylinositol; PIP, phosphatidylinositol 4-phosphate; PIP_2 , phosphatidylinositol 4,5-bisphosphate; PMN, polymorphonuclear leucocyte; EC_{50} , concn. for half-maximal activity.

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from Sigma Chemical Co. [γ - ^{32}P]ATP was purchased from New England Nuclear.

Preparation of plasma membranes from human PMNs

PMNs were isolated from healthy donors as previously described [18]. The cells were lysed by N_2 cavitation and the lysate was centrifuged over a cushion of 43 % (w/v) sucrose [20]. Plasma membranes banding at the interface were removed and recovered by centrifugation at 100000 g for 60 min. Membranes were suspended in 0.25 M-sucrose containing 25 mM-Hepes, pH 7.0, divided into portions and stored at -80°C .

Lipid-phosphorylation assays

Plasma membranes (approx. 25 μg of protein in 25 μl) were preincubated for 5 min at 37°C with the additions described in the text. Phosphorylation was initiated by the addition of an equal volume of mixture containing 50 mM-Hepes, pH 7.0, 200 μM -[γ - ^{32}P]ATP (approx. 2000 c.p.m./pmol) and MgCl_2 at concentrations defined in the text. Reactions were terminated by the addition of 2 ml of chloroform/methanol (1:1, v/v) and lipids were extracted under acidic conditions as previously described [18]. The lipid extracts contained radioactive PA, PIP and PIP_2 [18], and were routinely analysed by affinity chromatography on immobilized neomycin [21]. Radioactivity that did not bind to the column represented [^{32}P]PA, and [^{32}P]PIP and [^{32}P]PIP $_2$ were isolated by sequential elutions [21]. The coefficient of variation for replicate samples within an experiment averaged 4%. Variation of kinase activities in different membrane preparations is greater (up to 30%); however, the effects of the polyamines have been consistently observed in at least 15 preparations. All of the reported experiments have been replicated in at least three different membrane preparations with similar results.

Assay of phosphomonoesterase activity

Plasma membranes were phosphorylated for 5 min as described above before the addition of an equal volume of 50 mM-Hepes, pH 7.0, containing 1 mM-p[NH]ppA and polyamines as described in the text. Incubations were continued for 2–5 min before the ^{32}P -labelled phospholipids were extracted and analysed as described above. Phosphomonoesterase activity was calculated as the decrease in ^{32}P during the hydrolysis, i.e. the 2–5 min incubation.

RESULTS

Effects of polyamines on lipid phosphorylation in PMN plasma membranes

Incubation of PMN plasma membranes with [γ - ^{32}P]ATP and Mg^{2+} leads to the incorporation of ^{32}P into PA, PIP and PIP_2 [18]. In the presence of 1 mM- Mg^{2+} , ^{32}P incorporation into PA was inhibited by spermine and was unaffected by spermidine and putrescine (Fig. 1a). In contrast, spermine, and to a lesser extent spermidine, enhanced the labelling of PIP (Fig. 1b) and PIP_2 (Fig. 1c). After incubation for 1 min, incorporation of ^{32}P into PIP_2 was enhanced 3.5- and 2-fold by the presence of 0.5 mM-spermine and -spermidine respectively. Synthesis of [^{32}P]PIP $_2$ was not significantly affected by 0.5 mM-putrescine. Labelling of PA was depressed by elevation of the Mg^{2+} concentration to 12.5 mM, whereas labelling of PIP and PIP_2 was increased 3- and 4-fold respectively.

Fig. 1. Kinetics of plasma-membrane lipid phosphorylation in the presence of 1 mM- MgCl_2 .

Plasma membranes were incubated for the indicated times in the presence of 1 mM- MgCl_2 and buffer (●) or 0.5 mM-putrescine (○), -spermidine (■) or -spermine (▲) as described in the Experimental section. Reactions were terminated and the phospholipids were extracted and analysed for [^{32}P]PA (a), [^{32}P]PIP (b) and [^{32}P]PIP $_2$ (c). Values represent the mean incorporation of ^{32}P in triplicate samples.

Fig. 2. Concentration-dependence for polyamine-stimulated formation of $[^{32}\text{P}]\text{PIP}_2$

Plasma membranes were phosphorylated for 60 s in the presence of the indicated concentrations of putrescine (●), spermidine (■) or spermine (▲). Contents of $[^{32}\text{P}]\text{PIP}_2$ were then analysed as described in the Experimental section. Values represent the relative rates of $[^{32}\text{P}]\text{PIP}_2$ formation in triplicate samples of a typical experiment.

In the presence of this higher concentration of Mg^{2+} , the polyamines did not affect lipid phosphorylation (results not shown). Synthesis of $[^{32}\text{P}]\text{PIP}_2$ (at 1 mM- Mg^{2+}) was enhanced by approx. 35% and 125% by 0.1 mM- and 2 mM-spermine respectively (Fig. 2), but higher concentrations of spermine inhibited $[^{32}\text{P}]\text{PIP}_2$ formation.

Fig. 3. Effects of polyamines on the Mg^{2+} -dependence of $[^{32}\text{P}]\text{PIP}_2$ formation

Plasma membranes were phosphorylated for 60 s in the presence of the indicated concentrations of Mg^{2+} (●) and 0.5 mM-putrescine (○), -spermidine (■) or -spermine (▲). Contents of $[^{32}\text{P}]\text{PIP}_2$ were then analysed as described in the Experimental section. Values represent the rates of $[^{32}\text{P}]\text{PIP}_2$ formation in triplicate samples of a typical experiment.

Synthesis of $[^{32}\text{P}]\text{PIP}_2$ was maximally enhanced (2.5-fold) by 5 mM-spermidine. High concentrations of putrescine slightly enhanced ^{32}P incorporation into PIP_2 . In the absence of polyamines, synthesis of $[^{32}\text{P}]\text{PIP}_2$ was maximal at 12.5 mM- Mg^{2+} (Fig. 3), similar to requirements reported in other systems [9,22–24]. Addition of 0.5 mM-spermine shifted the Mg^{2+} dose-response curve for $[^{32}\text{P}]\text{PIP}_2$ synthesis markedly to the left, so that significant phosphorylation of PIP occurred at 0.5–1 mM- Mg^{2+} (Fig. 3). A less dramatic shift was induced by 0.5 mM-spermidine, whereas putrescine had no effect on the Mg^{2+} -dependence of PIP kinase.

Effects of spermine on the substrate-dependencies of diacylglycerol kinase, PI kinase and PIP kinase

The mechanisms for spermine-mediated inhibition of $[^{32}\text{P}]\text{PA}$ formation and stimulation of $[^{32}\text{P}]\text{PIP}$ and $[^{32}\text{P}]\text{PIP}_2$ synthesis were examined. Spermine acted as an uncompetitive inhibitor with respect to ATP in the synthesis of $[^{32}\text{P}]\text{PA}$ (Fig. 4a and Table 1). The calculated K_i of 185 μM is within the physiological range of spermine concentration. Spermine acted as a non-competitive activator with respect to ATP in the synthesis of $[^{32}\text{P}]\text{PIP}$ and $[^{32}\text{P}]\text{PIP}_2$ (Figs. 4b and 4c, and Table 1), producing a 1.6- and 2.9-fold elevation of the apparent V_{max} , respectively. The calculated K_{act} values are 720 and 37 μM for PI kinase and PIP kinase respectively.

The effects of spermine on the lipid substrate-dependencies for PI kinase and PIP kinase are demonstrated in Figs. 5(a) and 5(b). Exogenous lipid substrates were added as mixed micelles with 0.1% Triton X-100. Spermine slightly lowered the EC_{50} for PI in the synthesis of $[^{32}\text{P}]\text{PIP}$, but did not alter the apparent V_{max} for the kinase. In contrast, spermine increased V_{max} for PIP kinase, but did not modify its EC_{50} for exogenous substrate.

Effects of polyamines on PIP_2 phosphomonoesterase

Amounts of polyphosphoinositides are regulated by the relative rates of their synthesis and degradation. As PIP phosphomonoesterase has been shown to be sensitive to inhibition by spermine [25], the effects of polyamines on the corresponding activity toward PIP_2 were examined. Incubation of ^{32}P -labelled PMN plasma membranes in the absence of Ca^{2+} resulted in the loss of approx. 4% of the $[^{32}\text{P}]\text{PIP}_2/\text{min}$ (Fig. 6). Addition of spermine and, to a lesser extent, spermidine led to decreases in the rate of this $[^{32}\text{P}]\text{PIP}_2$ hydrolysis. Putrescine did not markedly affect $[^{32}\text{P}]\text{PIP}_2$ hydrolysis. Spermine-mediated inhibition of $[^{32}\text{P}]\text{PIP}_2$ breakdown occurred with an EC_{50} of approx. 0.25 mM (Fig. 7).

DISCUSSION

Phosphoinositide metabolism is considered to be intimately involved in the regulation of many cellular processes. Therefore control of the contents of these lipids is of paramount importance to the cell. Synthesis of polyphosphoinositides occurs through the actions of PI kinase and PIP kinase. Degradation occurs via monoesterases as well as phospholipase C. Since phospholipase C is only significantly active upon hormonal stimulation, the basal contents of polyphosphoinositides are controlled by the relative activities of the kinases and phosphomonoesterases. A consistent feature of reported characterizations of PI kinase and PIP kinase has been

Fig. 4. Effects of spermine on the ATP-dependence of plasma-membrane lipid phosphorylation

Plasma membranes were incubated for 60 s with the indicated concentrations of $[\gamma\text{-}^{32}P]ATP$ (constant specific radioactivity) either in the absence (●) or in the presence (○) of 0.5 mM-spermine. Amounts of $[^{32}P]PA$ (a), $[^{32}P]PIP$ (b) and $[^{32}P]PIP_2$ (c) were then analysed as described in the Experimental section. Values represent the mean incorporation of ^{32}P in triplicate samples. Double-reciprocal plots of the same data are also provided.

demonstrations of their requirements for very high concentrations of Mg^{2+} for the expression of maximal activity [9,22–24]. Therefore, under physiological conditions the cell seems to be able to utilize only a small percentage of its kinase potential. However, it is clear that monoesterase-mediated hydrolysis of polyphospho-

inositides is very active [1,22,26]. Therefore, additional mechanisms for activating the kinases seem to be required if optimal contents of polyphosphoinositides are to be maintained.

We have previously observed increased synthesis of polyphosphoinositides in the presence of spermine when

Table 1. Kinetic parameters for lipid phosphorylation in PMN plasma membranes

Plasma membranes were phosphorylated with various concentrations of [γ - 32 P]ATP as described in Fig. 4 and kinetic constants for ATP were calculated. Values represent means $\pm$ S.E.M. for three experiments; (+/-) indicates changes in constants induced by spermine.

Enzyme	Addition	K_m (μ M)	V_{max} (pmol/mg of protein)
Diacylglycerol kinase	None	400 ± 58	62 ± 6
	Spermine	182 ± 10	26 ± 5
	(+/-)	0.46 ± 0.09	0.43 ± 0.07
PI kinase	None	80 ± 12	7 ± 1
	Spermine	82 ± 12	11 ± 1
	(+/-)	1.02 ± 0.02	1.57 ± 0.22
PIP kinase	None	59 ± 15	31 ± 6
	Spermine	59 ± 15	80 ± 14
	(+/-)	1.00 ± 0	2.94 ± 0.37

PMN plasma membranes were phosphorylated at low concentrations of Mg^{2+} [18]. We have now demonstrated that this highly charged polycation is very effective in stimulating phosphoinositide kinase activity. Spermine shifts the Mg^{2+} -dependence curves of these kinases to the left, so that their maximal activities can be expressed at physiological Mg^{2+} concentrations. Since spermine has been shown to avidly bind to phosphoinositides [15–17], this activation may be due to polyamine binding to the substrate phospholipids, thereby neutralizing their negative charges. This differentially modulates the activities of PI kinase and PIP kinase. For PI kinase, spermine acts as a non-competitive activator with respect to ATP, and enhances the phosphorylation of low concentrations of PI. Therefore, the spermine-PI complex may be of higher affinity to the kinase than is the Mg^{2+} -PI complex. Spermine causes a similar non-competitive activation with respect to ATP for PIP kinase. However, with PIP kinase, spermine enhanced the rate of phosphorylation at all concentrations of PIP tested.

In contrast with its stimulatory effects on phosphoinositide kinases, spermine inhibited the activity of diacylglycerol kinase. This inhibition appears to be due to its action as an uncompetitive inhibitor with respect to ATP. This inhibition was overcome by high concentrations of Mg^{2+} , suggesting that it may be due to competition with Mg^{2+} for binding to ATP. Physiologically, inhibition of diacylglycerol kinase would lead to protection of hormonally produced diacylglycerol from its conversion into PA. Therefore, inhibition of diacylglycerol kinase can lead to potentiation of cellular responses to hormones [27]. In addition, it could be expected to enhance the release of fatty acids, especially arachidonate, through the action of diacylglycerol lipase.

Spermidine and spermine inhibited the hydrolysis of PIP_2 in PMN plasma membranes. Since these incubations were conducted in the absence of Ca^{2+} , this hydrolysis is most likely due to phosphomonoesterase activity. Therefore, similarly to its action on PIP phosphomonoesterase [25], spermine appears to inhibit the activity of PIP_2 phosphomonoesterase. The combined effects of sper-

Fig. 5. Effects of spermine on the lipid-dependence of PI kinase and PIP kinase

Plasma membranes were incubated for 60 s with the indicated concentrations of PI or PIP in 0.1 % Triton X-100. Amounts of [32 P]PIP (a) and [32 P]PIP₂ (b) were then analysed as described in the Experimental section. Values represent the mean incorporation of 32 P in triplicate samples.

mine-induced stimulation of phosphoinositide kinases and inhibition of phosphomonoesterase would lead to, and may in fact be required for, accumulation of polyphosphoinositides in biological membranes. The concentrations of spermine required to elicit these effects are well within the physiological range of this polycation [28].

Individual large bodies of investigation have developed around polyamine metabolism and phosphoinositide metabolism. Our results indicate that these two systems may in fact be closely interactive in the cell. It is

Fig. 6. Effects of polyamines on the kinetics of PIP_2 hydrolysis in plasma membranes

Plasma membranes were phosphorylated for 5 min in the absence of polyamines before the addition of buffer containing p[NH]ppA (●) and 0.5 mM-putrescine (○), -spermidine (■) or -spermine (▲). Incubations were continued for the indicated times before the contents of $[\text{P}^{32}]\text{PIP}_2$ were determined as described in the Experimental section. Values represent the means in triplicate samples.

noteworthy that parallel modulations of cellular polyamine concentrations and phosphoinositide contents occur. For example, cellular transformation leads to, or perhaps results from, increases in both polyamines (reviewed in refs. [28–30]) and polyphosphoinositides [31]. Similarly, phorbol esters induce the expression of polyamine-biosynthetic enzymes (reviewed in ref. [29]), and elevate polyphosphoinositide contents [11–13]. One might speculate that the effects of inhibitors of polyamine synthesis may be partially due to depletion of polyphosphoinositides. This would consequently decrease amounts of their derived second messengers, i.e. diacyl-

Fig. 7. Concentration-dependence of spermine-mediated inhibition of $[\text{P}^{32}]\text{PIP}_2$ hydrolysis

Plasma membranes were phosphorylated for 5 min before the addition of buffer containing p[NH]ppA and the indicated concentration of spermine. Incubations were continued an additional 5 min before the amounts of $[\text{P}^{32}]\text{PIP}_2$ were determined. Values represent the means in triplicate samples.

glycerol and inositol polyphosphates, which regulate a number of cellular processes, including growth, differentiation and proliferation. Therefore, characterization of interactions between polyamine metabolism and polyphosphoinositide metabolism may lead to definition of useful sites of chemotherapeutic modulation.

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