Effect of pertussis toxin and neomycin on G-protein-regulated polyphosphoinositide phosphodiesterase

A comparison between HL60 membranes and permeabilized HL60 cells

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The promyelocytic HL60 cell can be differentiated with dimethyl sulphoxide or dibutyl cyclic AMP leading to the appearance of fMetLeuPhe receptors on the cell surface. G-protein-stimulated polyphosphoinositide phosphodiesterase (PPI-pde) activity was assessed in membranes prepared from both differentiated and non-differentiated HL60 cells. Both the extent of the response and the rank order of potency of the GTP analogues to stimulate PPI-pde activation {guanosine 5'-[γ -thio]triphosphate (GTP[S]) > guanosine 5'-[$\beta\gamma$ -imido]triphosphate (p[NH]ppG) > guanosine 5'-[$\beta\gamma$ -methylene]triphosphate (p[CH₂]ppG) remains unchanged after differentiation with dimethyl sulphoxide. In comparison, differentiation by dibutyl cyclic AMP leads to diminution of PPI-pde activity when stimulated by GTP[S] or fluoride, but not by millimolar concentrations of Ca²⁺. GTP[S]-stimulated PPI-pde in membranes is sensitive to the presence of Ca²⁺ (pCa 8–5). Pertussis-toxin pretreatment of intact HL60 cells leads to inhibition of both the secretory response and the formation of inositol phosphates when stimulated by fMetLeuPhe. In contrast, pertussis-toxin pretreatment has no effect on either GTP[S]- or fluoride-stimulated PPI-pde. Neomycin in a concentration-dependent manner inhibits both GTP[S] plus Ca²⁺ (pCa 5)-stimulated secretion and PPI-pde activation in streptolysin-O-permeabilized cells. The extent of PPI-pde activation in membranes compared with streptolysin-O-permeabilized cells reveals that the membrane preparation does not possess all the components that make up the inositide signalling system.

INTRODUCTION

Ca²⁺-mobilizing receptors activate polyphosphoinositide phosphodiesterase (PPI-pde), generating two intracellular second messengers, IP₃ (inositol trisphosphate) and diacylglycerol [1,2]. There is accumulating evidence that activation of membrane-bound PPI-pde is coupled to receptors via a putative guanine nucleotide binding protein, G_p [3–5]. PPI-pde activation in many cell types can be stimulated by non-hydrolysable analogues of GTP and by fluoride, indicating the common features shared with other G-protein-mediated signalling systems [6,7].

In order to understand the individual components that make up the inositide signalling system, we have studied the effect of two potential inhibitors of PPI-pde activity. Pretreatment with pertussis toxin blocks agonist-mediated stimulation of PPI-pde activity in some types of cells, but not in others [3,4]. The effects of pertussis toxin on G_i, G_o and transducin are known to cause ADP-ribosylation of a cysteine residue of the G-protein, leading to the uncoupling of the receptor from the G-protein while leaving responses to GTP[S] or fluoride intact [8–11]. We would thus predict that pertussis-toxin pretreatment would inhibit receptor-stimulated PPI-pde without affecting PPI-pde stimulation by fluoride or GTP[S]. Contrary to the above prediction, it has been reported that GTP[S]-stimulated PPI-pde activity in rabbit neutrophils [12] and fluoride-stimulated PPI-pde

activity in hamster fibroblasts [13] can be inhibited by pertussis-toxin pretreatment. We have examined the effect of pertussis-toxin pretreatment of HL60 cells on PPI-pde activity, and find that only the receptor-stimulated PPI-pde activity is blocked similarly to the other G-protein-mediated signalling systems.

Neomycin has been frequently used as an inhibitor of PPI-pde activity stimulated either directly by millimolar concentrations of Ca²⁺ or indirectly by guanine nucleotides [14–17]. This effect of neomycin has been exploited to study the relevance of the inositide signalling pathway in cellular processes [17–19]. In a recent study on streptolysin-O-permeabilized mast cells, we used neomycin to dissociate GTP[S]-stimulated PPI-pde activity from GTP[S]-stimulated secretion, which led us to conclude that the secretory response is under the control of a second G-protein, G_s [17]. Since secretion from HL60 cells also appears to be under the control of a second G-protein [20], we have analysed the effects of neomycin on PPI-pde activity and on exocytosis.

METHODS AND MATERIALS

Materials

Pertussis toxin was obtained from Porton Products, CAMR, Porton Down, Salisbury, Wilts., U.K., and bovine serum albumin (Pathocyte IV) from ICN Bio-

Abbreviations used: PPI-pde, polyphosphoinositide phosphodiesterase; DMSO, dimethyl sulphoxide; G-protein, guanine nucleotide regulatory protein; GTP[S], guanosine 5'-[γ -thio]triphosphate; p[NH]ppG, guanosine 5'-[$\beta\gamma$ -imido]triphosphate; p[CH₂]ppG, guanosine 5'-[$\beta\gamma$ -methylene]triphosphate; IP₁, inositol monophosphate; IP₂, inositol bisphosphate; IP₃, inositol trisphosphate.

medicals, High Wycombe, Bucks., U.K. The sources of all other materials were as described previously [20].

Methods

Culturing and labelling of HL60 cells. HL60 cells were maintained in culture and labelled with [3 H]inositol as described previously [20], except that Medium 199 was routinely supplemented with bovine serum albumin (3.5 mg/ml).

Differentiation of HL60 cells. HL60 cells were differentiated with either DMSO [21,22] or dibutyryl cyclic AMP [23] towards a neutrophil-like cell. The cells were incubated with 1.3% (v/v) DMSO for 3 days and then transferred to the labelling medium with 1% DMSO for a further 2 days. When dibutyryl cyclic AMP was used as the differentiating agent, it was added at 300 μ M for 40 h to the labelling medium. In both cases the appearance of receptors for fMetLeuPhe were monitored by measurement of fMetLeuPhe-induced secretion of β -glucuronidase in intact cells.

Preparation and activation of membranes. Membranes were prepared at 4 °C. The cells (2×10^8 – 3×10^8) were washed three times in a buffered salt solution (pH 7.2), which contained 137 mM-NaCl, 2.7 mM-KCl and 20 mM-Hepes, and finally suspended in buffer (pH 7.2) consisting of 250 mM-sucrose, 20 mM-Hepes and 100 μ M-EGTA. The cells were disrupted by sonication for 10 s with an MSE probe, and the homogenate was centrifuged at 600 *g* for 5 min to remove any intact cells. The resulting homogenate was diluted with buffer (20 mM-Pipes/150 mM-KCl, pH 6.8; termed 'Pipes/K buffer') to 40 ml and centrifuged at 35000 rev./min (86500 *g*_{av.}) for 20 min to pellet the membranes.

The pellet was resuspended in the Pipes/K buffer at a protein concentration of 1–2 mg/ml. The Pipes/K buffer was supplemented with MgATP (2 mM final concn.), Ca^{2+} buffered with EGTA at the required pCa (as indicated in the Figures), MgCl_2 (2 mM final concn.), LiCl (10 mM final concn.) and 2,3-bisphosphoglycerate (2 mM final concn.). Ca^{2+} was buffered with 3 mM-EGTA as described previously [24]. The concentration of the supplements was twice the final concentration at this stage. Portions (100 μ l) of membranes were dispensed into glass tubes which contained an equal volume of buffer supplemented with the various additions as indicated in the individual Figures. The membranes were incubated for 10 min at 37 °C. In experiments where the Ca^{2+} concentration was varied (Fig. 2), the Ca^{2+} buffer was not added to the membranes, but was added directly to the tubes.

When fluoride or millimolar Ca^{2+} was used as an activator, Ca^{2+} -EGTA buffers were excluded, because activation by fluoride is dependent on the presence of Al^{3+} [25,26], which would be chelated by EGTA. In this case the concentration of free Ca^{2+} was approx. 1–5 μ M, owing to the presence of contaminating Ca^{2+} [27].

Incubations were terminated by addition of chloroform/methanol (1:2, v/v), and the aqueous top phase was used for the analysis of inositol phosphates by ion-exchange chromatography on Dowex columns as described previously [17]. It was observed that most of the changes were mainly confined to IP_3 , and to a lesser extent to IP_2 (see Fig. 2), with very little change in IP_1 . In many experiments the IP_2 and IP_3 fractions were

collected together unless specified otherwise. The bottom chloroform phase, which contained the lipids, was dried down, and total radioactivity (d.p.m.) used per incubation was determined where required.

Cell permeabilization and stimulation. Cells were permeabilized with streptolysin O as described previously [20]. In brief, cells were permeabilized with 0.4 i.u. of streptolysin O/ml in a buffered salt solution (pH 6.8) which contained 137 mM-NaCl, 2.7 mM-KCl, 20 mM-Pipes, 5.6 mM-glucose, 1 mg of albumin/ml, Ca^{2+} buffered with 3 mM-EGTA (pCa 7 or pCa 5 as indicated in Figure legends), 2 mM-MgATP, 2 mM-MgCl₂ and 10 mM-LiCl. Other additions, such as guanine nucleotides, were made as indicated in the Figure legends. After 10 min at 37 °C, the cells were sedimented at 600 *g* for 5 min, and 50 μ l of the supernatant was retained for the measurement of β -glucuronidase secretion as described previously [28]. The rest of the incubation was quenched with chloroform/methanol and processed as above. In permeabilized cells increases in all three inositol phosphates are observed [20] and were monitored either individually or collected together as indicated in the Figure legends. For the experiment illustrated in Fig. 4, the incubations were terminated after 30 min.

Measurement of secretion and inositol phosphate formation stimulated by fMetLeuPhe in intact HL60 cells. Differentiated HL60 cells were washed three times and finally resuspended in a buffered salt solution (pH 7.4) containing 20 mM-Hepes, 137 mM-NaCl, 2.7 mM-KCl, 1 mM- CaCl_2 , 10 mM-LiCl, 5.6 mM-glucose and 1 mg of albumin/ml. The cells were pretreated with cytochalasin B [28] and transferred into tubes containing fMetLeuPhe for 10 min at 37 °C. Secretion and inositol phosphate formation were measured as described above.

Pretreatment with pertussis toxin. Pretreatment of cells with pertussis toxin (500 ng/ml) was carried out in Medium 199 with the cells concentrated to 10^7 /ml for 2 h at 37 °C.

Expression of data. All determinations were carried out in duplicate. In some experiments the increase in inositol phosphates is expressed as a function of the total radioactivity (d.p.m.) incorporated in the inositol lipids. This allowed us to calculate the percentage hydrolysis of the total inositol lipids in individual experiments, so that comparison could be made between membranes and permeabilized cells.

RESULTS AND DISCUSSION

Comparison of PPI-pde activity in differentiated and non-differentiated HL60 cells

The HL60 cell is a promyelocytic cell line which can be terminally differentiated into a neutrophil-like cell by inducers such as DMSO and dibutyryl cyclic AMP. Upon differentiation, receptors for agonists such as fMetLeuPhe are expressed [22,23]. We have recently demonstrated that the undifferentiated HL60 cell contains both PPI-pde activity and an associated G-protein [20]. Here we compare the activity of PPI-pde (assessed by measurement of IP_3 formation) stimulated

Fig. 1. Effect of GTP analogues on IP_3 production in membranes prepared from undifferentiated and differentiated HL60 cells

HL60 cells were differentiated with DMSO for 5 days. Membranes were prepared from 10^8 cells (control or differentiated) and incubated with the GTP analogues GTP[S], p[NH]ppG and p[CH₂]ppG (each 100 μM) in the presence of pCa 7 for 10 min. The amount of radioactivity (d.p.m.) in the inositol lipids present in the individual incubations from undifferentiated and differentiated cells was the same.

by three different analogues of GTP in membranes prepared from DMSO-differentiated and undifferentiated cells. The concentration of DMSO used here gives maximum induction of maturation without inhibition of cell growth [21,29], allowing us to compare control cells with differentiated cells directly. Addition of 100 μM -GTP[S], -p[NH]ppG or -p[CH₂]ppG stimulates the formation of IP_3 , the product of hydrolysis of phosphatidylinositol bisphosphate (Fig. 1).

The potency of the three analogues to activate PPI-pde (GTP[S] > p[NH]ppG > p[CH₂]ppG) is similar to their potency in activating other G-protein-stimulated functions such as adenylate cyclase, cyclic GMP phosphodiesterase or exocytotic secretion [20,30,31]. The degree of PPI-pde activation for both the DMSO-differentiated and the non-differentiated cell is similar (based either on cell number or on d.p.m. incorporated into inositol lipids).

When dibutyryl cyclic AMP is used as a differentiating agent, the cells stop proliferating [23]. Therefore, for comparison of control cells with the dibutyryl cyclic AMP-differentiated cells, we have expressed the radioactivity (d.p.m.) in inositol phosphates as a percentage of the total inositol lipids. Table 1 shows that there is a decrease in the response to either GTP[S] or

Table 1. Comparison of PPI-pde activity in membranes prepared from undifferentiated and dibutyryl cyclic AMP-differentiated HL60 cells

Membranes prepared from undifferentiated and dibutyryl cyclic AMP-differentiated cells were stimulated with either GTP[S] in the presence of pCa 7 or fluoride or Ca^{2+} (0.5 mM) for 10 min. The results are summarized from 24 experiments. The mean basal contents of inositol phosphates present in the controls [expressed as a percentage of radioactivity (d.p.m.) incorporated in the total inositol lipids] was 0.11 ± 0.04 ($n = 13$) in differentiated cells and 0.29 ± 0.10 ($n = 11$) in undifferentiated cells. The stimulated increase in inositol phosphates observed in individual experiments was expressed as a percentage of d.p.m. incorporated in the total inositol lipids. Results are presented as means $\pm$ s.d. for the numbers of independent experiments show in parentheses.

	Inositol lipid hydrolysed (%)	
	Differentiated	Undifferentiated
GTP[S] (100 μM)	0.34 ± 0.15 (12)	0.72 ± 0.27 (12)
Fluoride (50 mM)	0.79 ± 0.28 (3)	1.25 ± 0.21 (11)
Ca^{2+} (0.5 mM)	0.31 ± 0.21 (3)	0.37 ± 0.16 (9)

fluoride in differentiated HL60 cells compared with non-differentiated cells. PPI-pde activity can also be activated directly by millimolar concentrations of Ca^{2+} [32], and we have utilized this response to assess the effect of differentiation on the catalytic activity itself. Table 1 shows that the response to millimolar concentrations of Ca^{2+} is hardly affected. We have consistently observed that millimolar concentrations of Ca^{2+} are less active in stimulating production of IP_2 plus IP_3 compared with GTP[S] or fluoride (see Table 1 and Fig. 6a).

Differentiation of HL60 cells with DMSO or dibutyryl cyclic AMP leads to an increase in the major pertussis-toxin substrate as assessed by immunochemical analysis. The increase is only apparent when the results are expressed as μg of HL60-membrane protein, but not when expressed on a cell-equivalent basis [33,34]. This difference is most likely due to the decrease in size that occurs upon differentiation [21,29]. The major pertussis-toxin substrate (40 kDa) of neutrophils and HL60 cells is structurally different from all previously described pertussis-toxin substrates [35,36]. Since receptor-stimulated PPI-pde activation is pertussis-toxin-sensitive in these cells [37,38; also see below], the pertussis-toxin substrate in these cells may be the putative G_p .

At a functional level, the process of differentiation with DMSO or dibutyryl cyclic AMP leads to no change or a 50% decrease respectively in PPI-pde activity. Thus the increase in the G-protein observed on a protein basis does not appear to be reflected on a functional level. The effect of differentiation with dibutyryl cyclic AMP only decreases PPI-pde activation stimulated by GTP[S] or fluoride, but not by Ca^{2+} , indicating that the catalytic activity itself is not altered during differentiation. The undifferentiated cell is therefore a useful model with which to study the interaction of the G-protein with the enzyme, PPI-pde.

Ca^{2+} -dependence of PPI-pde activation

In many cell types, including neutrophils and HL60

Fig. 2. Ca^{2+} -dependence of PPI-pde activity in the presence and the absence of GTP[S] in HL60 membranes

Membranes prepared from undifferentiated HL60 cells were incubated for 10 min without (○) or with (●) GTP[S] ($100 \mu\text{M}$) in the presence of different concentrations of Ca^{2+} . Ca^{2+} was buffered with 3 mM-EGTA in the range pCa 8–5. (a) IP₂; (b) IP₃.

cells, Ca^{2+} is necessary to observe maximal activation by receptor-directed agonists [4]. We have therefore assessed the Ca^{2+} -dependence of the HL60 membrane PPI-pde activity. Ca^{2+} alone can stimulate PPI-pde activity when increased from 100 nM to $10 \mu\text{M}$, the physiologically relevant range, but this increase is relatively small (Fig. 2). In the experiment presented in Fig. 2, the radioactivity (d.p.m.) in IP₃ at pCa 7 is 203, compared with 284 at pCa 5. Ca^{2+} only becomes very effective when its concentration is raised above $100 \mu\text{M}$ (Fig. 2). In the presence of GTP[S], the Ca^{2+} requirement is shifted to lower concentrations, such that maximal IP₃ production is observed at pCa 5. The stimulation of PPI-pde activity by Ca^{2+} is analogous to the activation of adenylate cyclase by forskolin, in that both can activate the catalytic unit without the intervention of the G-protein [39–41]. However, the affinity for forskolin is increased by agents that stimulate G_s [41], similarly to the increase in Ca^{2+} affinity when G_p is activated.

The Ca^{2+} -sensitivity of the G-protein-regulated PPI-pde observed in membranes has also been demonstrated in permeabilized and intact HL60 cells [20,42]. It is most acute in intact cells, since depletion of intracellular concentrations of Ca^{2+} to 30 nM completely inhibits the stimulation of IP₃ formation by fMetLeuPhe [42]. On the other hand, complete inhibition of PPI-pde activity is only seen in membranes in the presence of 10 mM-EGTA (Fig. 2). The different Ca^{2+} -sensitivities observed

in cells compared with membranes may be related to separate activities of PPI-pde (see below).

Three distinct effects of Ca^{2+} can be discerned. (1) Ca^{2+} alone can stimulate PPI-pde activity to a minor extent when its concentration is increased in the physiologically relevant range, i.e. 100 nM raised to $10 \mu\text{M}$, and to a greater extent at millimolar concentrations. (2) Ca^{2+} is required in the nanomolar range for activation by GTP[S]. (3) Ca^{2+} potentiates the GTP[S]-stimulated PPI-pde activity when its concentration is increased in the physiologically relevant range. One consequence of Ca^{2+} -induced potentiation is that in intact cells the receptor-mediated increase in cytosolic Ca^{2+} will activate the production of IP₃. This amplification of the Ca^{2+} signal may provide the fine control for the generation of Ca^{2+} transients [43].

Effect of pertussis toxin on G-protein-regulated PPI-pde

Pertussis-toxin pretreatment of HL60 cells leads to inhibition of both the functional response (i.e. secretion) and inositol phosphate production when differentiated HL60 cells are stimulated with fMetLeuPhe (Figs. 3a and 3b). Similar inhibition of fMetLeuPhe-stimulated inositol phosphate formation is observed in streptolysin-O-permeabilized cells [4]. In contrast, stimulation of PPI-pde by GTP[S] either in membranes or in permeabilized HL60 cells is not inhibited when the cells are pretreated with pertussis toxin (Fig. 4).

Fluoride (as a complex with aluminium) is a universal activator of G-proteins, and here we demonstrate that fluoride can also activate PPI-pde activity in HL60 membranes (Fig. 5). It is also active in permeabilized cells [44] and intact HL60 cells (results not shown). Maximal activation is observed at about 50 mM-fluoride. The dependence on fluoride is not changed when $30 \mu\text{M-AlCl}_3$ is added, indicating that sufficient contaminating Al^{3+} is already present (results not shown). Compared with GTP[S], fluoride is a more potent activator of PPI-pde (see Fig. 6a and Table 1). Stimulation by fluoride is also unaffected by pertussis-toxin pretreatment.

Pertussis toxin has been shown to ADP-ribosylate a cysteine residue which is the fourth amino acid from the C-terminus on three G-proteins whose sequences are known, G_i , G_o and transducin [7,9]. The effect of ADP-ribosylation on these G-proteins leads to the uncoupling of the receptor from the G-protein without impairing the ability of fluoride or GTP[S] to activate the G-proteins [8,10,11]. We show here that the activation of PPI-pde by the receptor is blocked in pertussis-toxin-pretreated cells (Fig. 3), but this does not affect the activation of PPI-pde when stimulated directly by GTP[S] (Fig. 4) or fluoride (Fig. 5). This situation is analogous to the characteristics known for the other G-proteins, and also implies that G_p may possess the same cysteine residue.

Although G_p in neutrophils and HL60 cells is pertussis-sensitive, in many other cell types receptor-coupled PPI-pde activity is not inhibited by pertussis toxin [3,4]. This would suggest that G_p is heterogeneous. However, it remains possible that G_p is the same protein in all cells, but, owing to post-translational modifications, the cysteine residue is not available for ADP-ribosylation in those cells which are known to be pertussis-toxin insensitive. This idea is supported by the observation that this cysteine residue, which is also present on the *ras* protein, is the site of palmitoylation for anchoring the protein to the membrane [45,46].

Fig. 3. Effect of pertussis-toxin pretreatment on fMetLeuPhe-induced (a) secretion and (b) inositol phosphate formation in intact HL60 cells

Differentiated HL60 cells were preincubated with pertussis toxin for 2 h and then stimulated with fMetLeuPhe for 10 min. The radioactivity present in the inositol lipids in the individual incubations was 66000 d.p.m. $\circ$, Control cells; $\bullet$, cells pretreated with pertussis toxin.

Fig. 4. Comparison of GTP[S]-stimulated PPI-pde activity in membranes and streptolysin-O-permeabilized HL60 cells prepared from control and pertussis-toxin-treated cells

HL60 cells were pretreated with pertussis toxin, and the activity of PPI-pde stimulated with GTP[S] was determined in membranes and in streptolysin-O-permeabilized HL60 cells. The Ca^{2+} concentration was buffered at pCa 7 with 3 mM-EGTA. Only increases in IP_3 and IP_2 are observed in membrane preparations, whereas in permeabilized cells increases in all three inositol phosphates are observed. $\circ$, Control cells; $\bullet$, cells pretreated with pertussis toxin. (a) IP_3 plus IP_2 and (b) IP_1 in streptolysin-O-permeabilized cells, (c) IP_3 plus IP_2 in membranes, expressed as percentages of radioactivity (c.p.m.) present in the total inositol lipids, giving a measure of the percentage of the inositol lipid hydrolysed.

Effect of neomycin on PPI-pde activity and on secretion

Neomycin has been widely used as an inhibitor of PPI-pde activity, and the mechanism of neomycin-mediated inhibition is understood to depend on its ability to bind

phosphatidylinositol bisphosphate and, to a lesser extent, phosphatidylinositol monophosphate [14,17,47]. This would mean that neomycin should inhibit PPI-pde activity regardless of its mode of activation. Fig. 6(a) illustrates the inhibition of IP_3 production by neomycin

in membranes stimulated with millimolar concentrations of Ca^{2+} , GTP[S] or fluoride. Neomycin also suppresses the basal content of IP_3 ; 1 mM-neomycin causes near-

Fig. 5. Effect of fluoride in HL60 membranes prepared from control (●) or pertussis-toxin-pretreated (○) cells

HL60 cells were pretreated with pertussis toxin and membranes derived from them were activated with fluoride for 10 min.

complete inhibition of PPI-pde activity in membranes when Ca^{2+} (500 μM) or GTP[S] is used as an activator. However, 1 mM-neomycin only partially inhibits the response caused by fluoride, and complete inhibition is observed at 3 mM (Fig. 6a). This would imply that neomycin is not acting solely at the level of substrate sequestration.

In order to test whether we could use neomycin to block PPI-pde activity selectively while leaving secretion intact as in mast cells [17], we have measured the concentration-dependence of neomycin-mediated inhibition for both processes in permeabilized HL60 cells. Neomycin inhibits the two processes with a similar dependence, with secretion being slightly less sensitive than IP_3 production (Fig. 6b). The mechanism by which neomycin inhibits secretion in permeabilized HL60 cells is not clear, but it is unlikely to be due to inhibition of IP_3 formation, since secretion (in the presence of pCa 5) can be independent of PPI-pde activity [20]. Since the effects of neomycin are not confined to inhibition of PPI-pde activity, any inhibition by neomycin of a particular process cannot always be attributed to inhibition of PPI-pde activity.

In our study on mast cells, neomycin blocked IP_3 production at 300 μM , while leaving secretion intact. Neomycin will block secretion in mast cells when its concentration is raised to millimolar (B. D. Gomperts, unpublished work). Thus the secretory mechanisms of the two cell types are not fundamentally different in their response to neomycin. In HL60 cells, the concentration-dependences for neomycin inhibition of secretion and IP_3 production happen to be similar, whereas in mast cells there is a region where secretion is relatively unaffected while PPI-pde activity is totally blocked.

Fig. 6. Effect of neomycin (a) on PPI-pde activation in membranes stimulated with fluoride, GTP[S] or Ca^{2+} and (b) on PPI-pde activation and secretion in streptolysin-O-permeabilized HL60 cells

(a) Membranes prepared from HL60 cells were stimulated with 100 mM-fluoride (□), 100 μM -GTP[S] (○), 0.5 mM- Ca^{2+} (■) or no addition, (●) for 10 min in the presence of increasing concentrations of neomycin. (b) Streptolysin-O-permeabilized HL60 cells were stimulated with GTP[S] (25 μM) in the presence of neomycin. Both secretion and IP_3 production were measured. The results are presented as a percentage of the response obtained in the absence of neomycin for both secretion (●) and IP_3 production (○). The Ca^{2+} concentration was maintained at pCa 5.

Comparison of the extent of PPI-pde stimulation observed in membranes and in permeabilized cells

We have observed that the degree of stimulation of PPI-pde activity in membranes compared with permeabilized undifferentiated HL60 cells is different. This became apparent when we expressed our data as percentage hydrolysis (i.e. d.p.m. observed in inositol phosphates as a percentage of the total radioactivity incorporated in the inositol lipids). Fig. 4 illustrates the results from an experiment where we have compared membranes with permeabilized cells. The percentage hydrolysis stimulated with GTP[S] in permeabilized cells is approx. 10-fold greater than in membrane preparations (Fig. 4). We have also summarized our results for all our experiments, and find that the mean maximum percentage hydrolysis stimulated by GTP[S] at pCa 7 obtained in membranes is 0.72 ± 0.27 (mean $\pm$ S.D., $n = 12$), in contrast with 3.56 ± 0.97 ($n = 13$) in permeabilized cells.

It is well established that there is inositol-lipid-hydrolysing activity in the cytosol which consists of multiple forms of enzymes based on molecular mass and comparison of the catalytic and immunologic properties [48–52]. The presence of this activity in permeabilized cells may account for the differences that we observe. Although permeabilization with streptolysin O generates lesions such that lactate dehydrogenase is released to the extracellular medium [20], it is possible that the cytosolic inositol-lipid-hydrolysing activity is still retained in these permeabilized cells, i.e. that this activity may not be truly cytosolic. The observation that the Ca^{2+} -sensitivity of the PPI-pde activity in membranes is different from that seen in permeabilized cells further supports the notion that multiple activities are involved [20]. In preliminary studies, we have been able to restore the GTP[S]-stimulated extent of hydrolysis in membranes to that observed in permeabilized cells by addition of cytosol prepared from pig lymphocytes to HL60 membranes [4]. Thus, in order to study the regulation of IP_3 production, it may be necessary not only to study the membrane-bound form of PPI-pde activity but also the cytosolic activity. It is clear that the permeabilized cell preparation is a more complete model for studying the inositol signalling system, rather than utilizing membranes.

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