Evidence that chlorpromazine and prostaglandin E_1 but not neomycin interfere with the inositol phospholipid metabolism in intact human platelets

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Human platelets that had been prelabelled with [^{32}P]P_i were stimulated with trombin in the presence or absence of neomycin, prostaglandin E_1 (PGE_1) or chlorpromazine. The content of [^{32}P]P_i in phosphatidylinositol 4-phosphate, phosphatidylinositol 4,5-bisphosphate and phosphatidic acid (PA) were determined. The data demonstrate that PGE_1 and chlorpromazine but not neomycin interfere with the tight metabolic relationship that exists between the inositol phospholipids and PA in thrombin-stimulated platelets [(1989) Biochem. J. 263, 621–624]. Our results therefore indicate that neomycin does not inhibit signal transduction in intact platelets at the level of the inositol phospholipid metabolism.

Neomycin; Chlorpromazine; Prostaglandin E_1; Signal transduction; Inositol phospholipid; Thrombin; Platelet

1. INTRODUCTION

Receptor-mediated increase in the turnover of the inositol phospholipids is recognized as a major transmembrane signal-processing mechanism (reviewed in [1]). The activation of the phosphoinositide-specific phosphodiesterase (PLC) leads to the formation of inositol 1,4,5-trisphosphate and diacylglycerol which mobilizes intracellular Ca^{2+} and activates protein kinase C, respectively.

Human platelets constitute a suitable model system to study stimulus–response coupling in general and agonist-induced enhancement of polyphosphoinositide (PPI) metabolism in particular. In a recent article [2] we demonstrated that receptor-mediated activation of the PPI cycle in platelets is under tight metabolic control. The relationship between the degree of PLC activation (measured as [^{32}P]PA accumulation) and the ^{32}P-radioactivity in PIP and PIP_2 therefore represents a tool to see whether or not a platelet inhibitor interacts with the specific steps in the inositol phospholipid metabolism [2]. Neomycin, chlorpromazine and prostaglandin E_1 (PGE_1) are commonly used inhibitors of platelet activation which are all believed to interfere with the platelet signal processing mechanisms [3–5]. Although under continuous debate, neomycin and chlorpromazine have been thought primarily to interfere with the inositol phospholipid metabolism [3,4,6–8] whereas PGE_1 has been demonstrated to inhibit platelet activation through elevation of the cAMP level [5].

In this study we use the tight metabolic relationship between the polyphosphoinositides and PA to evaluate whether neomycin, chlorpromazine and PGE_1 specifically interfere with the thrombin-induced changes in inositol phospholipid metabolism.

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Abbreviations: PA, phosphatidic acid; PIP, phosphatidylinositol 4-phosphate; PIP_2, phosphatidylinositol 4,5-bisphosphate; PGE_1, prostaglandin E_1; PPI, polyphosphoinositide

2. MATERIALS AND METHODS

2.1. *Materials*

Neomycin sulfate, PGE_1, theophylline, and chlorpromazine were obtained from Sigma, ^{32}P-orthophosphate (carrier-free) from Amersham and thrombin (bovine) from Hoffmann-La Roche. The sulfate of the neomycin sulfate preparation was exchanged with chloride as described in [9].

2.2. *Platelet isolation, labelling and incubation*

Platelet-rich plasma was prepared from ACD-anticoagulated human blood as previously described [10], and incubated with [^{32}P]P_i (0.1 mCi/ml, 60 min, 37°C). The cells were subsequently gel-filtered into a nominally Ca^{2+}- and phosphate-free Tyrode's solution [10]. The final platelet concentration was standardized at 3.5×10^8/ml. The experiments were performed at 37°C and neomycin, chlorpromazine or PGE_1 plus theophylline were added 90 s before the cells were exposed to thrombin. To increase the inhibitory effect of PGE_1, the phosphodiesterase inhibitor theophylline (0.5 mM, final concentration) was always added together with the prostaglandin.

2.3. *Phospholipid extraction and chromatography*

After 90 s of thrombin stimulation, 0.5 ml of platelet suspension was withdrawn and mixed with 2 ml of chloroform/methanol/conc. HCl (20:40:1, by vol.; 0°C). The lipids were extracted as previously

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described and subsequently separated by thin-layer chromatography in chloroform/methanol/20% methylamine (60:36:10, by vol.) [10]. The inositol lipids were visualized by overnight radioautography and scraped off the plates for determination of radioactivity by liquid scintillation counting.

3. RESULTS AND DISCUSSION

In a previous communication [2] we demonstrated that the steady state levels of PIP and PIP_2 correspond tightly to the degree of activation of the PPI-specific phosphodiesterase (measured as appearance of [^{32}P]PA) irrespective of the agonist used. Hence, inhibitors of platelet activation which act on specific steps in the metabolism of the inositol phospholipids should be expected to interfere with this metabolic relationship between PA, PIP and PIP_2. In contrast, platelet inhibitors which directly or indirectly affect the PLC activation (e.g. receptor occupancy, autocrine stimulation) should not interfere with the tight metabolic relationship between PA and the inositol phospholipids. This was shown for the specific thrombin antagonist hirudin and the cyclooxygenase inhibitor aspirin [2].

In this communication, the effects of the platelet inhibitors PGE_1, chlorpromazine and neomycin on thrombin-induced changes in inositol phospholipid metabolism were tested. The data are plotted as [^{32}P]PIP or [^{32}P]PIP_2 vs [^{32}P]PA (degree of PLC activation), and the effects of increasing concentrations of thrombin alone were always tested vs thrombin plus inhibitor.

The addition of PGE_1 together with thrombin clearly decreased both the formation of PA as well as the levels of PIP and PIP_2 when compared to thrombin alone (Fig. 1). The [^{32}P]PIP/[^{32}P]PA and [^{32}P]PIP_2/[^{32}P]PA relationships therefore became clearly different from the relationships obtained with thrombin alone as well as for thrombin plus hirudin [2].

Chlorpromazine has previously been shown to affect the levels of PIP and PIP_2 in unstimulated platelets [3]. This is in accordance with the present data that demonstrate a 100% increase in PIP and a 20% increase in PIP_2 in the unstimulated cells (Fig. 2). The addition of thrombin together with chlorpromazine revealed that the drug markedly enhanced the thrombin-induced formation of [^{32}P]PA and [^{32}P]PIP, whereas the formation of [^{32}P]PIP_2 was increased at low degrees of stimulation and markedly decreased when the cells were stimulated to [^{32}P]PA levels of 500% or above. The [^{32}P]PIP/[^{32}P]PA and [^{32}P]PIP_2/[^{32}P]PA relationships induced by thrombin plus chlorpromazine were therefore considerably different from the relationships obtained with thrombin alone.

The effects of neomycin are demonstrated in Fig. 3. In contrast to the two other platelet inhibitors, the addition of neomycin together with thrombin only induced a shift to the left of the [^{32}P]PIP/[^{32}P]PA and [^{32}P]PIP_2/[^{32}P]PA relationships as if only a lower concentration of thrombin were used. When the cells were incubated with increasing concentrations of neomycin before exposure to the highest thrombin concentration, the [^{32}P]PIP/[^{32}P]PA and [^{32}P]PIP_2/[^{32}P]PA relationships remained unaltered when compared to the relationships obtained with thrombin alone (not shown). Hence, the effect of neomycin was similar to the effect of the thrombin antagonist hirudin [2].

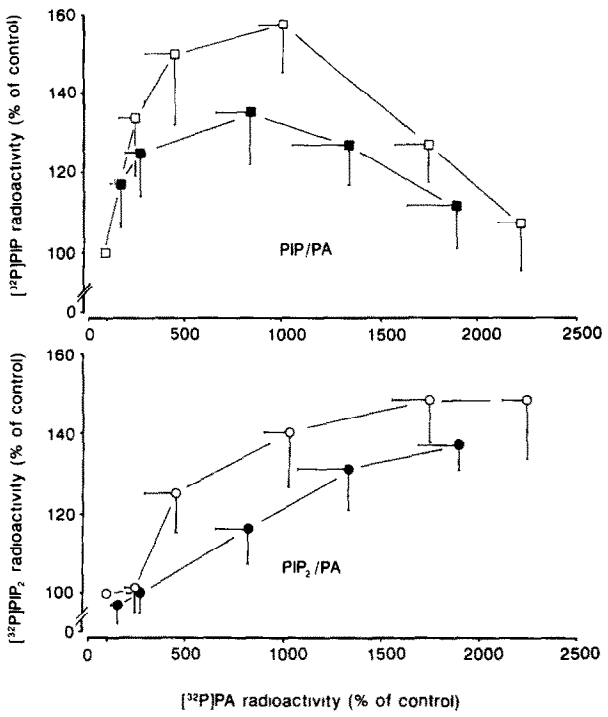

Fig. 1. Relationship between [^{32}P]PIP_2, [^{32}P]PIP and [^{32}P]PA in thrombin-stimulated platelets in the absence and presence of PGE_1 plus theophylline. [^{32}P]P_i-prelabelled platelets were stimulated with increasing concentrations of thrombin (0.03, 0.05, 0.1, 0.3 and 0.5 U/ml) with (closed symbols) or without (open symbols) PGE_1 plus theophylline (25 nM and 0.5 mM final concentrations, respectively). The data represent the [^{32}P]PIP_2/[^{32}P]PA and [^{32}P]PIP/[^{32}P]PA relationships obtained for each dose of thrombin and are expressed as means ± SD of 4 independent determinations.

The present data indicate that PGE_1 and chlorpromazine but not neomycin interfere with specific steps in the metabolism of the inositol phospholipids upon thrombin stimulation of platelets. PGE_1 is generally believed to inhibit platelet activation by increasing the level of intracellular cAMP thereby activating cAMP-dependent protein kinase which has been shown to inhibit the intracellular calcium mobilization [5]. However, there is also evidence that PGE_1 affects the kinases and phosphatases involved in the metabolism of the inositol phospholipids [11,12] as is also supported by this study. The data also support a previous report which has shown profound effects of

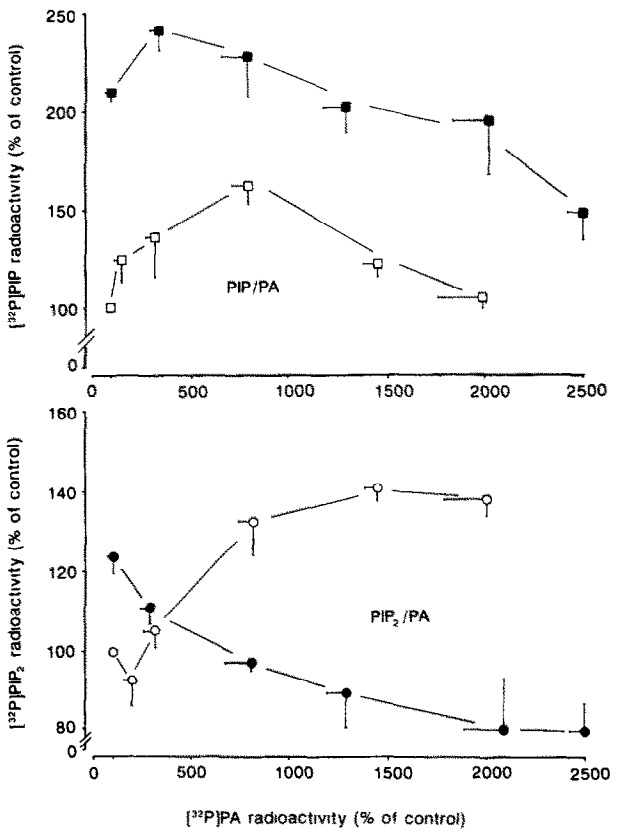

Fig. 2. Relationship between [^{32}P]PIP_2, [^{32}P]PIP and [^{32}P]PA in thrombin-stimulated platelets in the absence (open symbols) and presence (closed symbols) of chlorpromazine (30 μM, final concentration). The experiment was performed as explained in the legends to Fig. 1 and the data are expressed as means ± SD of 4 independent determinations.

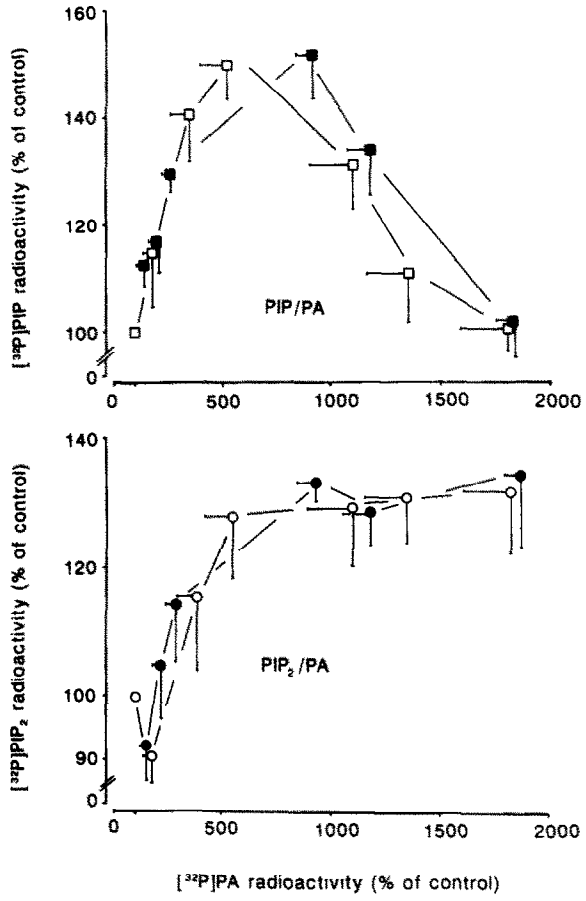

Fig. 3. Relationship between [^{32}P]PIP_2, [^{32}P]PIP and [^{32}P]PA in thrombin-stimulated platelets in the absence (open symbols) and presence (closed symbols) of neomycin (2 mM, final concentration). The experiment was performed as explained in the legends to Fig. 1 and the data are expressed as means ± SD of 4 independent determinations. The thrombin concentrations used were 0.03, 0.05, 0.08, 0.1, 0.3 and 0.5 U/ml (final concentrations).

chlorpromazine on inositol phospholipid metabolism in resting as well as thrombin-stimulated platelets [3]. The mechanism of the inhibitory action of the drug on platelet responses is presently unknown.

Neomycin has been shown to bind to the inositol phospholipids [6] and it has generally been assumed that it inhibits cellular activation by interfering with the signal transduction mechanism at the level of these phospholipids [4,7,8]. However, recent data from our laboratory [9] as well as others [13] have indicated that the drug interferes with the signal transduction at an earlier stage. The drug was demonstrated to affect the GTPase in human platelet membranes [13], but it is still an open question whether the highly polar molecule can penetrate the intact plasma membrane. The present data show that the drug affects the relationship between the inositol phospholipids in a similar way to the change induced by simply decreasing the strength of the stimulus. Such results were also obtained by the addition of the specific thrombin-antagonist hirudin to thrombin-stimulated cells [2]. Our results therefore strongly suggest that neomycin does not interfere with specific steps in the inositol phospholipid metabolism, but acts to inhibit (directly or indirectly) signal transduction in platelets at a level prior to the PLC activation. From other cells it has been demonstrated that neomycin inhibits the binding of Herpes Simplex virus 1 to its cellular receptor [14]. Moreover, the inositol phospholipids which indeed can be influenced by the drug [6], have been demonstrated to take part in the structure of some surface proteins recognized as receptors [15]. We therefore suggest that neomycin interferes with the thrombin–receptor interaction in intact human platelets. Since neomycin also inhibits platelet responses induced by collagen and platelet activating factor (PAF) [4], it is possible that the receptors for thrombin, collagen and PAF all share the same susceptibility for the antibiotic.

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