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Neomycin inhibits agonist-stimulated polyphosphoinositide metabolism and responses in human platelets

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SUMMARY: The effect of neomycin on agonist-induced changes in polyphosphoinositide metabolism was studied in human platelets. Neomycin induced no changes in the ^{32}P-labeled phospholipids of unstimulated cells. Between 1 and 5 mM of neomycin the initial thrombin-induced decrease in [^{32}P]phosphatidylinositol-4,5-bis-phosphate and production of [^{32}P]phosphatidic acid were gradually inhibited. In contrast, the production of [^{32}P]phosphatidylinositol-4-phosphate was increased. The thrombin-induced decrease in mass of phosphatidylinositol was completely inhibited at 5 mM of neomycin. Collagen- and PAF aceter-induced changes in platelet phosphoinositide metabolism as well as aggregation and dense granule secretion induced by all three agonists were inhibited by neomycin. The results indicate that neomycin inhibit platelet resonses by selective interference with the interconversions and hydrolysis of polyphosphoinositides upon thrombin stimulation.

Stimulation of platelets with thrombin induces an initial degradation of PIP_2 to IP_3 and DG (1). Subsequently, increased turnover in the polyphosphoinositide cycle results in the resynthesis of PIP and PIP_2, and the production of PA. In parallel PI rapidly disappears (2) through either phosphorylation or direct phosphodiesteratic hydrolysis. IP_3 has been shown to mobilize Ca^{2+} from intracellular stores (3,4) which in turn activates a PI-specific phosphodiesterase, thus explaining the thrombin-induced disappearance of large amounts of PI (5). Neomycin, an aminoglycoside antibiotic, interacts with the metabolism of the polyphosphoinositides, particularly PIP_2, in many cells (6,7). In this study we examined the

Abbreviations: PI, Phosphatidylinositol; PIP, Phosphatidylinositol-4-phosphate; PIP_2, Phosphatidylinositol-4,5-bisphosphate; PA, Phosphatidic acid; DG, Diacylglycerol; IP_3, Inositol-1,4,5-trisphosphate.

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effect of neomycin on polyphosphoinositide metabolism in thrombin-stimulated human platelets, a widely used model system for stimulus-response coupling.

MATERIALS AND METHODS

Platelet isolation, pulse-labelling and incubation. Platelet-rich plasma (PRP) was prepared by differential centrifugation from human blood and incubated with ^{32}P-orthophosphate (0.1 mCi/ml, carrier-free, Amersham, Code PBS-11) for 60 min at 37°C as described previously (8). The platelets were thereafter transferred into a Ca^{2+}- and phosphate-free Tyrode's solution (pH 7.3) containing 5 mM glucose and 0.2% (w/v) bovine serum albumin (fraction V, Miles Laboratories) by gel-filtration (9,10). The gel-filtered platelets were standardized at a concentration of 3.5×10^8 cells/ml and then incubated at 37°C. At 90 sec of incubation, neomycin sulfate (N-1876 Sigma, 100 mM in water, pH 7.3) was added. After another 90 sec, the platelets were stimulated with 0.5 U/ml of thrombin (La Roche, bovine), 2 µg/ml of collagen (Hormon-chemie Munchen, GMBH) or 500 nM of PAF (1-O-hexadecyl-2-acetyl-sn-glyceryl-3-phosphorylcholine, Sigma). When collagen or PAF was used as agonist, the platelets were stirred at 900 rpm, and for PAF, 1 mg/ml of fibrinogen (Grade L, Kabi Vitrum) was added together with the agonist. Samples were taken at 10, 90 and 180 sec of stimulation to establish the maximal decrease in PIP_2, the resynthesis of phosphoinositides and the maximal decrease in mass of PI, respectively. Since one molecule of neomycin is associated with 3 molecules of sulfate, sodium sulfate (Merck, art. 6649) was used as control at molarities 3 times higher than the corresponding neomycin concentration.

Phospholipid extraction and chromatography. Samples from the incubation mixture were collected in 4 vol of chloroform/methanol/conc. HCl (20:40:1) and the phospholipids were extracted as previously described (11). Control experiments showed that the extraction of PIP_2 was decreased by 5% in the presence of neomycin, for which proper corrections were made. The extraction of the other phospholipids was not affected. Separation and quantitation of phospholipid radioactivity was done by the methylamine system as previously descibed (8).

Mass determination of PI. PI was scraped off the thin-layer chromatography plates and digested for 30 min at 180°C in 100 µl of 70% perchloric acid. Inorganic phosphate was measured with the malachite-green method according to Vickers et al (12).

Platelet responses. Aggregation of unlabeled, gel-filtered platelets prepared as described above was measured in a Payton dual channel aggregation module (Payton Assoc. Inc.). Dense granule secretion was measured after 60 sec of stimulation as the extracellular appearance of ATP plus ADP. Both methods are previously described in detail by Steen and Holmsen (13).

RESULTS

Addition of neomycin (1-5 mM) to unstimulated platelets did not affect the ^{32}P-labeling of the phospholipids within 3 min of incubation. In contrast, the changes in polyphosphoinositide metabolism following thrombin addition were markedly affected by the presence of neomycin. The initial decrease in [^{32}P]PIP_2 was gradually inhibited by increasing concentrations of neomycin (Fig. 1). Complete inhibition was observed at 5 mM of neomycin. Time course experiments confirmed that this inhibition was real and not due to an

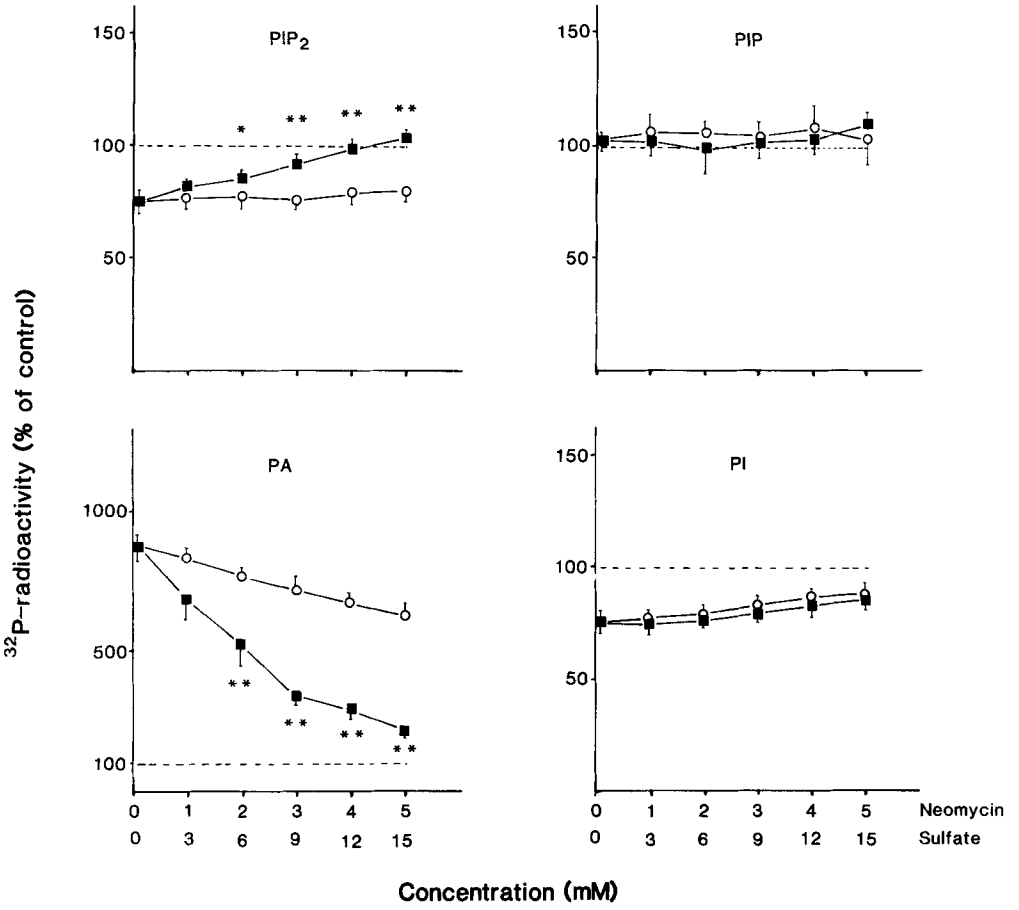

Figure 1. Effect of neomycin on the initial thrombin-induced changes in the phospholipids.
Human gel-filtered platelets were incubated at 37°C. Neomycin (■) or sulfate (○) was added after 90 sec and the platelets were stimulated with 0.5 U/ml of thrombin after another 90 sec. The incubations were stopped after 10 sec of stimulation. The data are presented as percent of control (broken line) and are means of 4 separate experiments. The 100% values represent 2502, 1740, 502 and 65 cpm/0.5ml for PIP_2, PIP, PI and PA, respectively. Data obtained in the presence of neomycin were tested to the sulfate controls by paired Student's t-test. * and ** represent $p<0.05$ and $p<0.025$, respectively.

acceleration or retardation of the PIP_2-breakdown (results not shown). The production of $[^{32}P]PA$ gradually decreased with increasing concentrations of neomycin and in parallel, the thrombin-induced decrease in $[^{32}P]PI$ at 10 sec of stimulation was slightly inhibited. However, this effect on $[^{32}P]PI$ was also found for sulfate controls. $[^{32}P]PIP$ was not significantly affected in the presence or absence of the antibiotic.

The thrombin-induced resynthesis of $[^{32}P]PIP_2$ was partially inhibited by increasing concentrations of neomycin (Fig. 2). However, the

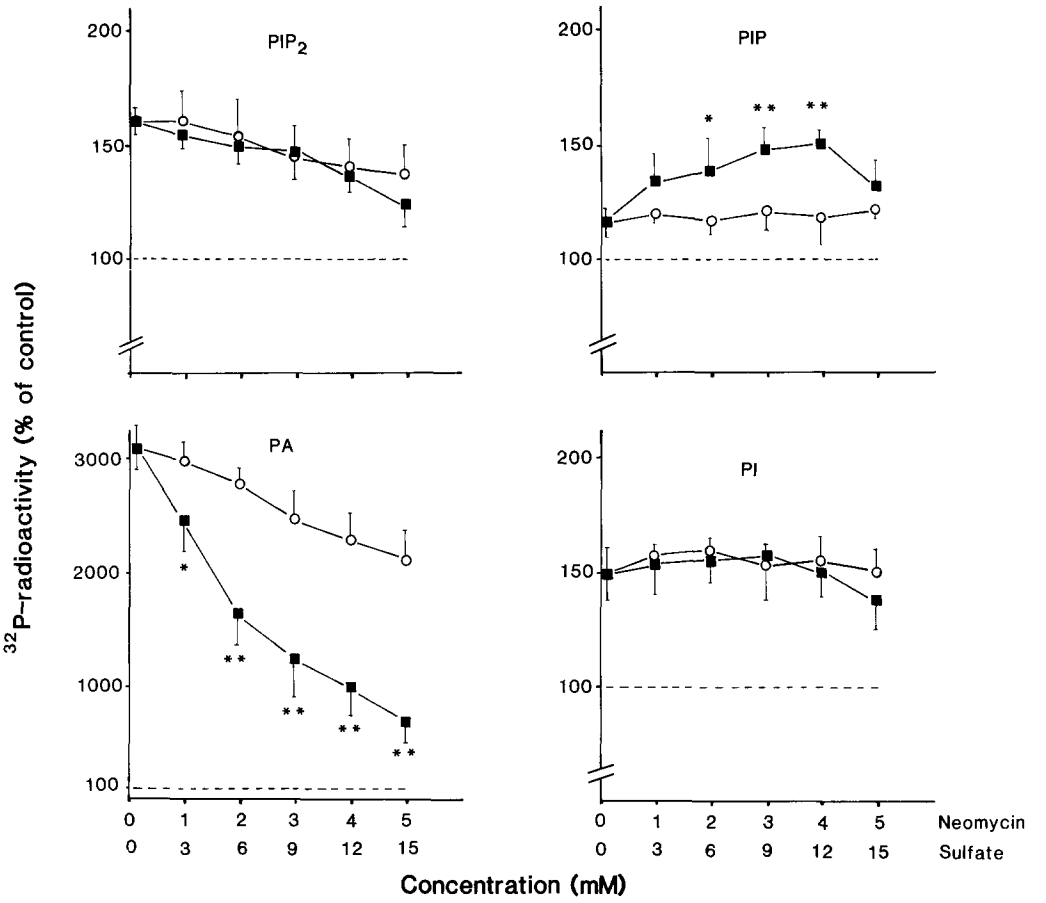

Figure 2. Effect of neomycin on the thrombin-induced resynthesis of phospholipids.
Experiments were performed as described in detail in the legend to fig. 1, except that samples were collected at 90 sec after the addition of thrombin.

sulfate controls showed parallel inhibition. Neomycin specifically increased the thrombin-induced formation of [^{32}P]PIP. Between 1 and 4 mM of the

TABLE 1

Effect of neomycin on the thrombin-induced disappearance of PI

Addition	Mass of PI ($nmol/10^{11}$ cells)	n	p
0.9% NaCl + Thrombin	1347 ± 199	7	-
15 mM Sulfate + Thrombin	1437 ± 184	7	N.S.
2 mM Neomycin + Thrombin	1488 ± 227	3	N.S.
5 mM Neomycin + Thrombin	1857 ± 148	7	<0.01
0.9% NaCl + 0.9% NaCl	1801 ± 116	7	<0.01

Gel-filtered human platelets were incubated at 37°C. After 90 sec, neomycin, sulfate or saline in equal volumes were added and after another 90 sec, the platelets were stimulated with 0.5 U/ml of thrombin. The incubations were stopped after 180 sec of stimulation and the extracts were analyzed for mass of PI. The data were tested statistically by paired Student's t-test to the mass of PI after thrombin-treatment alone. N.S. is non-significant, p>0.05.

TABLE 2

Effect of neomycin on PAF and collagen induced changes in phosphoinositide turnover

Addition	PIP_2	PIP	PI	PA
12 mM Sulfate + PAF	134	152	147	501
4 mM Neomycin + PAF	101	112	120	275
12 mM Sulfate + Collagen	135	176	178	667
4 mM Neomycin + Collagen	98	90	125	415

Gel-filtered platelets were incubated as described in Table 1 and stimulated with 500 nM of PAF or 2 ug/ml of collagen. After 90 sec of stimulation, extracts were analyzed for ^{32}P-radioactivity in phosphoinositides and PA. Data are expressed as per cent of control and represent mean of duplicate experiments which varied less than 10%. The 100% values represent 4924, 2850, 895 and 153 cpm/0.5 ml for PIP_2, PIP, PI and PA, respectively.

antibiotic, [^{32}P]PIP was 15-25% higher than in its absence. The production of [^{32}P]PA was strongly inhibited by neomycin, and at 2 mM, [^{32}P]PA was reduced to almost 50% of the sulfate controls. For [^{32}P]PI, no effect of neomycin was seen. In contrast, neomycin inhibited the thrombin-induced disappearance of PI (Table 1). At 2 mM, the decrease in mass of PI was reduced by 25% while it was totally abolished with 5 mM.

In contrast to thrombin, neither collagen nor PAF induced an initial

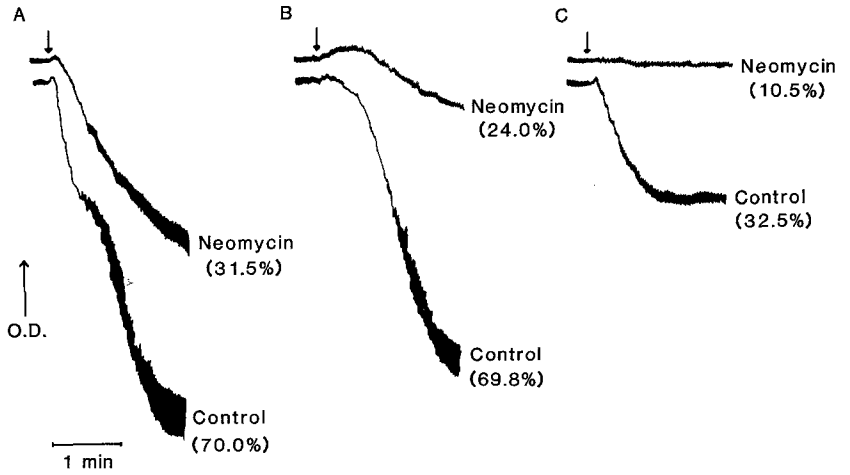

Figure 3. Effect of neomycin on agonist induced aggregation and dense granule secretion.
Gel-filtered platelets (0.5 ml) were stirred at 900 rpm. 4 mM of neomycin or 12 mM of sulfate in controls was added 90 sec before the addition of 0.5 U/ml of thrombin (A), 2 ug/ml of collagen (B) and 500 nM of PAF (C) (the addition of the agonist is indicated by the arrow). Secretion of ATP plus ADP was measured after 60 sec of stimulation and is indicated in parentheses as per cent of total cellular ATP plus ADP. Secretion is expressed as mean of duplicate experiments.

decrease in $[^{32}P]PIP_2$. This is in accordance with MacIntyre et al (14). However, both agonists induced increased labeling of the phosphoinositides after 90 sec of stimulation (Table 2). In the presence of 4 mM of neomycin, this effect was clearly inhibited and the formation of $[^{32}P]PA$ substantially reduced.

The effect of neomycin on agonist-induced platelet aggregation and dense granule secretion is shown in fig 3. In the presence of 4 mM of the antibiotic both aggregation and secretion were conciderably inhibited as compared to sulfate controls.

DISCUSSION

Our results show that neomycin inhibits the initial thrombin-induced breakdown of PIP_2 and production of PA. At 4 mM of neomycin, the disappearance of PIP_2 is abolished, most likely reflecting inhibition of hydrolysis and thus reduced IP_3 formation. Consequently, the thrombin-induced calcium liberation is severely affected. At the same time, the production of PA is markedly inhibited, indicating a reduced production of DG. Thus the two major pathways leading to platelet activation are affected which is supported by the inhibition of aggregation and secretion (Fig. 3) Studies on the PIP kinase and the polyphosphoinositide monoesterases (6,7,15) as well as on the phosphodiesterase (16) in other cells, support the thesis that neomycin exerts its action through direct binding to the substrate rather than to the enzymes (6,17). At low concentrations it is thought to bind PIP_2 preferentially over PIP, and to lack affinity for PI (7,15,16).

However, the results in the present study indicate that thrombin still activates the polyPI-cycle in the presence of neomycin. This is reflected by the increase in PIP found after 90 sec of stimulation, and by the unaltered resynthesis of both PI and PIP_2. The increase in PIP is probably due to the continuous conversion of PI to PIP. As the latter becomes bound to neomycin, it cannot be further converted to PIP_2 and hence accumulates. It could also result from an increased formation of PIP from PIP_2, but this is unlikely since the thrombin-induced increase in $[^{32}P]PIP_2$ was

unaffected by neomycin. Hence, there is still a considerable resynthesis of PIP_2 at concentrations of neomycin where the thrombin-induced decrease in PIP_2 is substantially reduced. This indicates that PIP_2 is bound more efficiently than PIP which is in accordance with previous reports (7,17).

Mass studies show that the thrombin-induced disappearance of PI is inhibited by neomycin. It has been proposed that PI is hydrolysed by the direct action of a calcium-sensitive phosphodiesterase (5). Since PIP_2 degradation, and hence IP_3 formation is inhibited by neomycin, the subsequent calcium mobilization and activation of the phosphodiesterase are blocked by the presence of the antibiotic. In contrast to the mass, the thrombin-induced changes in $[^{32}P]$PI are hardly affected by neomycin. This is unexpected since at the same concentrations, the production of PA is markedly reduced. Thrombin-induced changes in $[^{32}P]$PI are the result of both resynthesis and degradation. Resynthesized $[^{32}P]$PI derives from $[^{32}P]$PA which is shown to be in equilibrium with γ-ATP (11). Degraded $[^{32}P]$PI has a much lower specific radioactivity (11), and measured thrombin-induced changes in $[^{32}P]$PI result mainly from resynthesis. It is therefore likely that neomycin leaves the thrombin-induced resynthesis of PI unaltered.

It is remarkable that neomycin not affects the labeling of polyphosphoinositides in unstimulated cells, wherease it fully inhibits the initial changes after thrombin stimulation. In permeabilized cells and membrane extracts, the half effective concentration of neomycin is from 10 to 50 times lower than in intact cells (17-19). This indicates that neomycin only passes the membrane to a little degree. Upon stimulation, it is therefore possible that the permeability of the platelet to neomycin is increased.

Prentki et al (19) showed that neomycin complexes with several polyphosphorylated compounds such as IP_3 and ATP. In addition, the IP_3-induced Ca^{2+} release from non-mitochondrial pools of permeabilized cells was inhibited. This inhibition was explained by the general ability of the antibiotic to bind polyvalent anions like IP_3. However, others have shown that neomycin not affects the $[^{32}P]P_i$ incorporation into ATP in

intact cells (7). In addition, our results show direct inhibition of the thrombin-induced PIP_2 hydrolysis together with increased labelling of PIP in the presence of neomycin. These results could hardly be explained by complexing of the antibiotic to IP_3 and ATP under our experimental conditions.

Studies on [^{3}H]inositol labeled platelets have indicated that among various agonists, only thrombin-induced responses and [^{3}H]inositol phosphate formation were inhibited by the presence of neomycin (20). This sharply contrasts with our results in which the antibiotic clearly affected both responses and phosphoinositide metabolism induced by PAF and collagen. The inhibitory effect is therefore not restricted to thrombin stimulation. However, the concentration of PAF and collagen used by Siess and Lapetina (20) was respectively 2 and 12 times higher as compared to our experiments whereas the concentration of neomycin was only 2 mM. When we increased the concentration of PAF and collagen to 1 μM and 4 μg/ml respectively (results not shown), the inhibitory effect of 4 mM of neomycin on the agonist induced aggregation became substantially reduced.

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