Neomycin inhibits inositol phosphate formation in human platelets stimulated by thrombin but not other agonists

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Neomycin (0.1–1 mM) added to human platelet-rich plasma or washed platelets prelabeled with [^{3}H]inositol inhibits aggregation, ATP secretion (ID_{50} 0.2 mM) and formation of [^{3}H]inositol mono-, bis- and trisphosphate (ID_{50} 0.6–0.8 mM) in response to thrombin (0.25 U/ml). The production of inositol phosphates in response to other platelet agonists (vasopressin, platelet activating factor, prostaglandin endoperoxide analogs and collagen) is not inhibited by neomycin, even at a concentration of 2 mM. At this concentration neomycin reduces the secretion of ATP stimulated by these agents (by up to 50%). The results indicate that neomycin has multiple effects on platelets that are unrelated to a specific inhibition of inositol phospholipid degradation by phospholipase C. Low concentrations (0.1–1 mM) of neomycin might selectively inhibit the interaction of thrombin with the platelet surface, and high concentrations (>2 mM) might unspecifically reduce platelet secretion in response to various platelet agonists.

Neomycin *Platelet aggregation* *Platelet release reaction* *Inositol phosphates* *Thrombin* *Phospholipase C*

1. INTRODUCTION

Neomycin, an ototoxic and nephrotoxic polycationic drug, has been shown to interact with anionic phospholipids such as the polyphosphoinositides [1–3]. Neomycin binds to phosphatidylinositol 4,5-bisphosphate [4,5], the degradation of which by phospholipase C leads to the formation of the second messengers: 1,2-diacylglycerol and inositol (Ins) 1,4,5-trisphosphate (P_3) [6,7]. Neomycin decreases ^{32}P-labeling and the mass of phosphatidylinositol 4,5-bisphosphate in various biological systems [2,3,8] and inhibits renal phosphatidylinositol-specific phospholipase C at low concentrations (ID_{50} 0.03 mM) [9]. These effects of neomycin have been used to study the relevance of the inositol phospholipid metabolism for the mechanism of signal transduction in various cells. For example, neomycin has been found to block Ca^{2+}-dependent histamine secretion from mast cells [10], excitation-contraction coupling in skeletal muscle [11], carbachol-induced amylase secretion in permeabilized pancreatic acini [12] and thrombin-induced cell proliferation of hamster fibroblasts [13]. However, concurrent determinations of inositol phospholipid degradation were carried out in only a few studies [12,13], and in none of them was more than one agonist used. Recently, neomycin was also shown to bind specifically to Ins 1,4,5-P_3, thus inhibiting Ca^{2+}-mobilization [14]. This questioned the usefulness of neomycin as a specific inhibitor for the degradation of the inositol phospholipids.

Inositol phospholipid hydrolysis by phospholipase C is observed in platelets in response to many stimuli [15]. We have studied the effect of neomycin on inositol phospholipid metabolism and platelet function, and found that low concentrations of neomycin inhibited selectively the action of thrombin on platelets, but not that of other agonists.

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2. MATERIALS AND METHODS

2.1. *Materials*

Neomycin sulfate was obtained from Sigma (St. Louis, MO) and was dissolved in H_2O as a 200 mM stock solution. [^{3}H]Inositol was obtained from Amersham and was purified before use [16]. γ-Thrombin was a gift from Dr J.W. Fenton (Albany, NY). Acetylsalicylic acid was from Sigma and was dissolved as a 1 M stock solution in ethanol. All other materials were obtained as described [16,17].

2.2. *Methods*

Citrated (0.38%) platelet-rich plasma was obtained from healthy human volunteers. Platelet aggregation and ATP secretion were measured with a Lumi-Aggregometer® (Chrono-Log, Havertown, PA). Neomycin was added 1 min before the platelet agonist to 0.44 ml of platelet-rich plasma containing 0.04 ml of the Luciferase/Luciferin reagent.

The production of [^{3}H]inositol phosphates in stimulated platelets was determined as described [18,19]. Platelet-rich plasma from 200 ml of citrated blood was incubated with aspirin (1 mM) for 15 min at 37°C and centrifuged (800 × g for 10 min) after addition of prostacyclin (50 ng/ml). The platelets were resuspended in 3 ml of a buffer containing Hepes (20 mM), NaCl (138 mM), $NaH_2PO_4 \cdot H_2O$ (3.3 mM), KCl (2.9 mM), EGTA (1 mM), $MgCl_2$ (1 mM), apyrase (6 units ADPase/ml), glucose (5 mM), prostacyclin (500 ng/ml) and [^{3}H]inositol (0.6 mCi/ml). The platelet suspension was incubated at 37°C for 3 h in a water bath. The pH during that period was controlled, and adjusted every 30 min with 1 N NaOH. Platelets were then centrifuged and resuspended in 10 ml of a Hepes-salt buffer containing glucose (5 mM), $CaCl_2$ (0.1 mM), $MgCl_2$ (1 mM), creatine phosphate (2 mM) and creatine phosphokinase (20 μ/ml). The platelet suspension was kept at 37°C for 60 min before beginning the experiment. Samples of platelet suspension were placed into aggregometer tubes and platelets were incubated with neomycin or saline whilst stirring for 1 min before exposure to the platelet agonists. After 1 min, or 1.5 min if collagen was used, the samples were transferred into chloroform/methanol/conc. HCl (100:200:2). The [^{3}H]inositol phosphates were extracted and separated on Dowex anion-exchange columns as described [19]. The fractions containing the inositol mono-, bis- and trisphosphates, respectively, were measured for radioactivity by liquid scintillation counting.

3. RESULTS

3.1. *Effects of neomycin on platelet-rich plasma*

Addition of neomycin to platelet-rich plasma effectively inhibits aggregation and secretion induced by thrombin or γ-thrombin (fig.1). ATP secretion is inhibited by lower concentrations of neomycin (ID_{50} ~0.2 mM) than platelet aggregation (ID_{50} ~0.7 mM). Concentrations of neomycin (1–1.5 mM) that inhibit ATP secretion by more than 90% lead to a reversible aggregation response by thrombin. A concentration of 2 mM neomycin blocks aggregation and secretion completely but not shape change. Platelet shape change can, however, be blocked by 5 mM neomycin. The effect of neomycin is almost instantaneous and it is observed when added 5 s before thrombin. Aspirin-treatment of platelet-rich plasma does not reduce platelet sensitivity to neomycin.

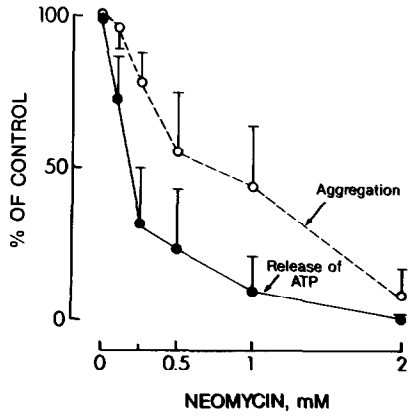


Fig.1. Inhibition of thrombin-induced responses of platelets in plasma by neomycin. Aspirinized or non-aspirinized platelet-rich plasma was incubated with neomycin for 1 min and stimulated with thrombin (0.25 U/ml) or γ-thrombin (90 nM) for 1 min. The results are means ± SD from 5 experiments and expressed as % of the thrombin-induced response in the absence of neomycin (44 ± 5% light transmission and 12 ± 2 nmol ATP secretion per 10^9 platelets).

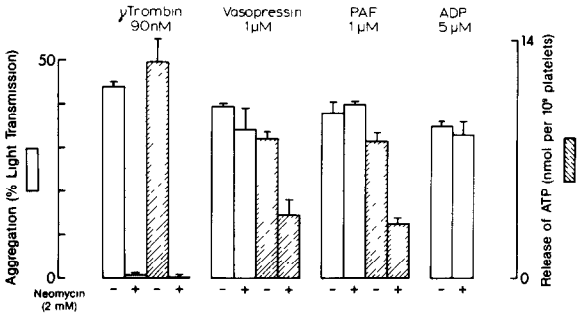
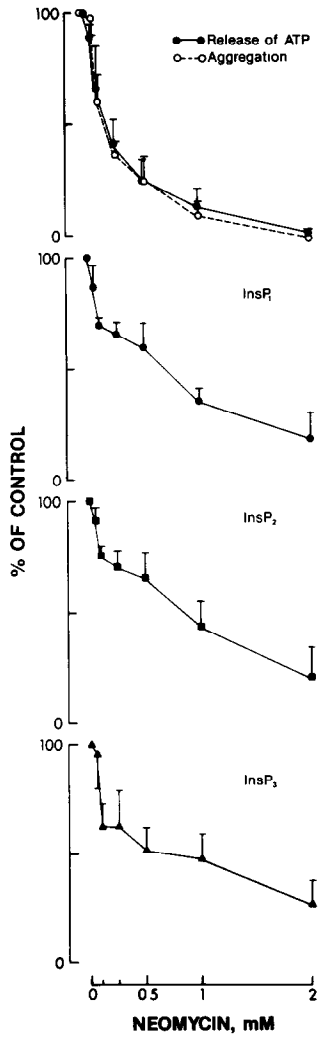


Fig.2. Effect of neomycin (2 mM) on aggregation and secretion of platelets in plasma stimulated by various agonists. Results (means ± SD) are from 4 experiments.



The responses of platelet-rich plasma to other agonists such as platelet-activating factor, ADP, vasopressin, epinephrine and collagen is not affected by neomycin (0.1–1 mM). The aggregation response to these platelet agonists is not reduced by high concentrations (2 mM) of neomycin, but a reduction of ATP secretion (up to 50%) is observed (fig.2).

3.2. *Effects of neomycin on washed platelets*

The effects of neomycin on the production of [^{3}H]inositol phosphates in washed platelets stimulated by thrombin are shown in fig.3. The platelets were aspirinized. The suspension buffer contained creatine phosphate/creatine phosphokinase to exclude effects of neomycin on platelet arachidonate metabolism and ADP release. Neomycin inhibits in a dose-dependent fashion the formation of $InsP_1$, $InsP_2$ and $InsP_3$ induced by thrombin. A concentration of 0.1 mM neomycin significantly inhibits inositol phosphate production, aggregation and ATP release caused by 0.25 units/ml thrombin (fig.3). Aggregation and ATP secretion in washed platelets are more effectively inhibited by neomycin (ID_{50} ~0.2 mM) than the production of the inositol phosphates (ID_{50} ~0.6–0.8 mM). Fig.3 also illustrates that there is no preferential inhibition of any of the inositol phosphates by neomycin.

Table 1 shows that the inositol phosphate production induced by collagen, vasopressin, PAF and prostaglandin endoperoxide analogs is not inhibited by 2 mM neomycin. Neomycin, however, reduces the secretion of ATP induced by those agonists by as much as 50% (table 1).

Fig.3. Dose-dependent inhibition of thrombin-induced platelet responses and inositol phosphate formation by neomycin. Platelet suspension prelabeled with [^{3}H]inositol were incubated with neomycin for 1 min and then with thrombin (0.25 U/ml) for 1 min. [^{3}H]Inositol phosphates were extracted and separated by Bio-Rad® columns as described in section 2. The ranges of inositol phosphate formation induced by thrombin in the absence of neomycin were 1000–3000 cpm for $InsP_1$, 700–2200 cpm for $InsP_2$ and 200–450 cpm for $InsP_3$. Results are shown as means ± SD from 4 experiments.

Table 1

Effect of neomycin (2 mM) on platelet inositol phosphate production and ATP secretion induced by various agonists

	^{3}H radioactivity (cpm)			ATP secretion (nmol per 10^9 platelets)
	$InsP_1$	$InsP_2$	$InsP_3$	
Control	2600	707	174	0
Neomycin	2643	655	165	0
Thrombin, 0.25 U/ml	3876	1460	427	134
+ neomycin	2901	1002	243	2
PAF, 1 μM	2960	801	207	8
+ neomycin	2992	793	200	3
Collagen, 25 μg/ml	3481	1292	305	43
+ neomycin	3507	1380	302	24
Vasopressin, 1 μM	2970	872	201	7
+ neomycin	3105	819	187	4
U44069, 1 μM	3057	896	183	18
+ neomycin	2974	882	176	4

For details see section 2. Mean of duplicate determinations of a typical experiment is shown

4. DISCUSSION

Neomycin (0.1–1 mM) selectively inhibits inositol phosphate production, aggregation and secretion induced by thrombin but not that caused by other platelet agonists. This agonist-selective platelet inhibition may indicate that neomycin acts prior to a general transduction mechanism. Activation of platelet proteases has been implicated in the mechanism of platelet activation by thrombin and trypsin [20]. Neomycin does not block trypsin-induced responses in platelets (unpublished), which indicates that neomycin probably does not affect platelet proteases. Neomycin most probably alters the interaction of thrombin with the platelet surface. This interaction is complex and involves at least two glycoproteins, glycoprotein Ib and V. Glycoprotein Ib might serve as the binding site for thrombin [21]; glycoprotein V is proteolytically cleaved by thrombin [22,23]. Studies using α- and γ-thrombin provide evidence for two types of thrombin receptors and coupling mechanisms [24]. Because neomycin blocks the action of γ-thrombin, the coupling mechanism involved with this receptor [24] might be affected by neomycin.

Platelet phospholipase C degrades all inositol phospholipids [15,25,26]. Since neomycin binds in vitro especially to phosphatidylinositol 4,5-bisphosphate [4,5], a selective inhibition of $InsP_3$ formation would have been expected. The fact that neomycin inhibited $InsP_1$, $InsP_2$ and $InsP_3$ formation in response to thrombin to the same degree supports the idea that neomycin inhibits the action of thrombin at a level prior to the activation of phospholipase C.

Thrombin-induced platelet responses are inhibited by neomycin more effectively than the production of inositol phosphates. For example at a concentration of 0.5 mM neomycin, platelet aggregation and ATP secretion are inhibited by 75%, but the formation of inositol phosphates is only inhibited by 40–50% (fig.3). This lack of an exact correlation between platelet responses and inositol phosphate formation is also observed with other platelet agonists (table 1). Neomycin (2 mM) reduced the secretion response to a number of agonists without inhibiting the inositol phosphate production (table 1). This could indicate that neomycin inhibits platelet secretion at steps which are distal to the production of inositol phosphates. One of those steps could be the inhibition of Ins 1,4,5-P_3 induced Ca^{2+} mobilization by neomycin [14].

In conclusion, our results show that neomycin has multiple effects on platelets that are unrelated to an interaction of that drug with inositol phospholipids.

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