Neomycin cannot be used as a selective inhibitor of inositol phospholipid hydrolysis in intact or semi-permeabilized human platelets

Aminoglycosides activate semi-permeabilized platelets

Thomas POLASCIK,* Philip P. GODFREY† and Stephen P. WATSON*

*Department of Pharmacology, University of Oxford, South Parks Road, Oxford OX1 3QT, and †Department of Clinical Pharmacology, Radcliffe Infirmary, Woodstock Road, Oxford OX2 6HE, U.K.

High concentrations of neomycin (2–10 mM) inhibited aggregation, but not shape change, of intact platelets by collagen, ADP and the Ca^{2+} ionophore, A23187, the last two studies being carried out in the presence of the cyclo-oxygenase inhibitor indomethacin. In contrast, over the same range of concentrations neomycin inhibited both aggregation and shape change induced by thrombin. Under these conditions activation of platelets by collagen and by thrombin, but not by A23187 or by ADP, is believed to be dependent on the hydrolysis of membrane inositol phospholipids. These data therefore suggest that the inhibitory action of neomycin on intact platelets is not related to its previously reported inhibitory effect on phosphoinositide metabolism. The selective inhibition of thrombin-induced shape change indicates a second site of action of neomycin on intact platelets. On platelets rendered semi-permeable with saponin, neomycin and a second aminoglycoside antibiotic, streptomycin (each 0.06–2 mM), stimulated secretion and aggregation responses. These effects were inhibited by indomethacin and by EGTA. Activation of semi-permeabilized platelets by neomycin is associated with the formation of inositol phosphates and phosphatidic acid, indicating activation by phospholipase C. This effect is also inhibited by indomethacin, implying that it is secondary to the formation of prostaglandins and endoperoxides. These results are discussed in the context of the use of neomycin as a selective inhibitor of polyphosphoinositide metabolism.

INTRODUCTION

Receptor-stimulated hydrolysis of membrane inositol phospholipids has now become recognized as a major transmembrane signalling pathway within the body (for review see [1] and [2]). The hydrolysis of phosphatidylinositol 4,5-bisphosphate generates inositol trisphosphate and 1,2-diacylglycerol, both of which appear to function as second messengers. Inositol trisphosphate stimulates the release of Ca^{2+} from non-mitochondrial stores within the cell, and 1,2-diacylglycerol activates a Ca^{2+} phosphatidylserine-dependent protein kinase, named protein kinase C [1,2]. In the human platelet a variety of agonists stimulate the hydrolysis of phosphatidylinositol bisphosphate, including thrombin [3], collagen [4] and thromboxanes [4], and thus induce the formation of inositol trisphosphate and 1,2-diacylglycerol. These two second messengers are thought to act in synergy to produce secretion and aggregation responses in the platelet [5]. In contrast, other stimuli are believed to activate platelets independently of inositol phospholipid metabolism. ADP at concentrations which produce a similar degree of platelet activation to thrombin does not stimulate inositol phospholipid hydrolysis [6], whereas the Ca^{2+} ionophore A23187, in the presence of cyclo-oxygenase inhibitors, activates platelets by the mobilization of Ca^{2+} [7].

The aminoglycoside antibiotic neomycin binds strongly and selectively to polyphosphoinositides relative to other phospholipids [8–10], and has therefore been used as a potential and relatively specific inhibitor of

inositol phospholipid metabolism. For example, the introduction of neomycin (0.01–0.1 mM) into semi-permeabilized mast cells inhibits secretion induced by guanosine 5'-[α -thio]triphosphate, a process thought to be brought about by the hydrolysis of inositol phospholipids [11]; similarly, the addition of neomycin (0.4–10 mM) to the cytoplasmic side of the plasma membrane in sea-urchin eggs inhibits Ca^{2+} -mediated hydrolysis of inositol phospholipids and subsequent exocytosis [12]. It was necessary in those studies to permeabilize the surface membrane in order to allow the polar neomycin access to the inner leaflet of the plasma membrane, where the polyphosphoinositides are located. There is also a single report, however, showing that at higher concentrations neomycin (2 mM) is inhibitory on intact cells, i.e. neomycin inhibits thrombin-stimulated inositol phospholipid metabolism and cellular proliferation in hamster fibroblasts [13].

Neomycin binds to polyphosphoinositides at concentrations of 0.01–0.1 mM upwards [9,10], and has been shown to inhibit phospholipase C, phospholipases A and phosphatidylinositol 4-monophosphate kinase at these concentrations, using subcellular fragments of brain [14,15], kidney [16,17], ear [18] and erythrocyte [19] membranes. At higher concentrations, neomycin also binds to other phospholipids, and has been shown to inhibit phosphatidylinositol kinase [19], the binding of Ca^{2+} to surface membranes, and Ca^{2+} entry [14]. Other aminoglycosides, including gentamycin and streptomycin, also bind to polyphosphoinositides, but they are less potent than neomycin in this action [17,18]. The

present study investigates whether neomycin selectively inhibits inositol phospholipid hydrolysis in human platelets, and can therefore be used to define further the role of this pathway in platelet activation.

METHODS

Blood (50 ml), anti-coagulated with citrate (15%, except for experiments carried out with platelet-rich plasma, when 3% was used), was taken on the day of the experiment from volunteers who had denied taking aspirin in the previous 2 weeks [3]. Platelet-rich plasma was prepared by centrifuging at 200 *g* for 20 min, and was labelled with 10 μ Ci of [3 H]5-hydroxytryptamine for 1 h at room temperature. Prostacyclin (100 ng) was added, and the platelet-rich plasma was centrifuged at 1000 *g* for 10 min; prostacyclin increases cyclic AMP, and thus prevents aggregation during centrifugation [20]. The platelet-poor plasma was discarded, and 5 ml of 'intracellular' or 'extracellular' (see below) buffer was added to the platelet pellet and also discarded; this ensures the near-complete removal of residual amounts of plasma, which are known to interfere with the action of saponin [21]. The platelets were then resuspended at a concentration of 2×10^8 – 3×10^8 /ml in either 'intracellular' buffer [composition (mM): KCl 140, NaH_2PO_4 0.42, MgCl_2 1, glucose 1, NaHCO_3 11.9, Hepes 5; adjusted to pH 7.35] [20] or 'extracellular' buffer [composition (mM): NaCl 134, NaHCO_3 12, KCl 2.9, Na_2HPO_4 0.34, MgCl_2 1, glucose 5, Hepes 5, adjusted to pH 7.4]. These buffers were designed to mimic the intracellular [22] or extracellular ionic environments found *in vivo*. The platelets were then left at room temperature for 30 min to allow the action of prostacyclin to wear off. In some experiments platelets were prelabelled with [32 P]P_i (1 mCi/3 ml) or [3 H]inositol (66 μ Ci/ml) for 1 h and 3 h respectively in extracellular buffer, and washed in the presence of prostacyclin (1 μ g) before experimentation.

Experiments were carried out in a Born lumiaggregometer at 37 °C with stirring at 600 rev./min; the platelets (0.5 ml final volume) were prewarmed to 37 °C for 2 min before experimentation. The following time cycles were used: neomycin, streptomycin, indomethacin and EGTA were given at the start of the experiment, and saponin, thrombin, collagen, ADP or A23187 was added 60 s later, and the mixture was left for a further 60 s. The reaction was then stopped by transferring to an equal volume of 6% (v/v) glutaraldehyde for measurements of [3 H]5-hydroxytryptamine release [23], by transferring to 1.88 ml of chloroform/methanol (1:2, v/v) for analyses of phosphatidic acid [24], or by transfer to chloroform/methanol/HCl (50:100:1, by vol.) for analyses of inositol phosphates. Phosphatidic acid was separated from other phospholipids by t.l.c. and measured by scintillation counting [24]. Total inositol phosphates were separated on Dowex anion-exchange columns [3,4]. Inositol and glycerophosphoinositol were eluted together with 16 ml of 60 mM-ammonium formate/5 mM-sodium tetraborate, and inositol mono-, bis- and tris-phosphates were also eluted together with 16 ml of 800 mM-ammonium formate/0.1 M-formic acid. Light-transmission was recorded throughout the experiment and was displayed on a chart recorder in order to monitor aggregation. The concentration of saponin used in these studies was estimated as described previously by

Fig. 1. Concentration-response curve for neomycin inhibition of 5-hydroxytryptamine secretion by thrombin from washed platelets

Washed platelets, prelabelled with [3 H]5-hydroxytryptamine, were incubated with various concentrations of neomycin for 1 min before challenge with a just sub-maximal concentration of thrombin (0.06 unit/ml) for a further 1 min. [3 H]5-Hydroxytryptamine secretion was measured as described in the Methods section. Results are shown as means $\pm$ S.E.M. for four separate experiments; ***P* < 0.01 relative to thrombin alone.

measuring the leakage of ATP by using luciferin-luciferase [21]. Results are shown as means $\pm$ S.E.M. from at least three separate experiments. Statistical comparisons were made by using a Wilcoxon Rank Test and by Student's *t* test.

Neomycin sulphate, streptomycin sulphate, indomethacin, thrombin, ADP and A23187 were purchased from Sigma, Poole, Dorset, U.K. Collagen was from Horm-Chemie, Munich, Germany. [3 H]5-Hydroxytryptamine, [32 P]P_i and [3 H]inositol were from Amersham. All other reagents were of analytical grade.

RESULTS

Intact platelets

On its own, neomycin has no observable action on washed platelets. In contrast, relatively high concentrations of neomycin (2–10 mM) inhibited secretion (Fig. 1), shape change and aggregation (Fig. 2a) induced by a concentration of thrombin which was just sub-maximal for aggregation. On the other hand, neomycin inhibited aggregation, but not shape change, induced by the Ca^{2+} ionophore A23187, in the presence of indomethacin. Similarly, on platelets suspended in platelet-rich plasma, neomycin (2–10 mM) inhibited aggregation, but not shape change, induced by collagen or by ADP, the latter experiments also being carried out in the presence of indomethacin (10 μ M) (Fig. 2b).

Semi-permeabilized platelets

Experiments on platelets rendered semi-permeable with saponin were carried out in a buffer designed to mimic more closely the intracellular ionic environment of the platelet [22]. The concentration-response curves for secretion and aggregation responses to thrombin obtained by using this 'intracellular' buffer (for composition see the Methods section) were similar to those obtained with the 'extracellular' buffer (results not shown).

Fig. 2. Increasing concentrations of neomycin inhibit platelet aggregation by thrombin, collagen, A23187 and ADP

Platelets were incubated with various concentrations of neomycin for 1 min before challenge with a just sub-maximal concentration of agonist for a further 1 min. Indomethacin ($10 \mu\text{M}$) was present in the experiments involving A23187 and ADP. An increase in light transmission represents the change in shape of the platelets from a disc to a sphere with pseudopodia; a decrease reflects platelet aggregation. Δ shows when the agonist was administered, and the decrease in A at this time point is a dilution artifact. The results are shown as typical traces from one experiment, which is representative of three others. (a) Washed platelets were challenged with either (i) thrombin (0.06 unit/ml) or (ii) A23187 (300 nM). (b) Platelets suspended in plasma were challenged with either (i) collagen ($2 \mu\text{g/ml}$) or (ii) ADP ($2 \mu\text{M}$).

With platelets rendered permeable with saponin, relatively low concentrations of neomycin (0.06 – 2 mM) stimulated secretion and aggregation (Figs. 3 and 4). The concentration–response curves for these effects are bell-shaped, with inhibition seen at concentrations above 2 mM (Figs. 3 and 4). This presumably reflects an inhibitory action similar to that described above for intact cells. The activation of semi-permeabilized platelets by neomycin sulphate is inhibited completely by indomethacin (Table 1) and by 1 mM -EGTA (results not shown), but is not mimicked by K_2SO_4 (results not shown). With platelets prelabelled with $[^{32}\text{P}]\text{P}_i$ or $[^3\text{H}]\text{inositol}$, neomycin (0.4 mM) stimulated formation of $[^{32}\text{P}]\text{phosphatidic acid}$ and inositol $[^3\text{H}]\text{phosphates}$ after permeabilization with saponin (Table 1); the formation of phosphatidic acid was inhibited completely by indomethacin (Table 1) (not studied for inositol phosphates).

A second aminoglycoside antibiotic, streptomycin, also stimulated aggregation and secretion responses in saponin-permeabilized platelets at relatively low concentrations (0.06 – 2 mM), and again inhibition was observed at higher concentrations (Figs. 3 and 4). Activation of semi-permeabilized platelets by streptomycin is inhibited completely by indomethacin ($10 \mu\text{M}$) and by EGTA (1 mM) (results not shown).

Concentrations of neomycin that were below threshold for secretion and aggregation responses (i.e. 0.006 – 0.02 mM) had no effect on the activation of saponin-permeabilized platelets by thrombin (results not shown).

DISCUSSION

The present study has shown that the aminoglycoside antibiotic neomycin inhibits aggregation of intact platelets by thrombin, collagen, ADP and A23187. Since, in the presence of indomethacin, A23187 [7] and ADP [6] do not activate platelets through inositol phospholipid hydrolysis, the inhibitory action of neomycin is unlikely to be related to an action on these lipids. This result is

Fig. 3. Aminoglycosides induce aggregation of saponin-permeabilized human platelets

Washed platelets were incubated with neomycin or streptomycin for 1 min before addition of saponin (arrowhead) and left for a further 1 min. For description of traces see Fig. 2. The results are shown as typical traces from one experiment, which is representative of five others.

Table 1. Neomycin stimulates [³H]5-hydroxytryptamine secretion and [³²P]phosphatidic acid and [³H]inositol phosphate formation in semi-permeabilized platelets

Platelets were prelabelled with [³²P]P_i, [³H]inositol or [³H]5-hydroxytryptamine (5-HT) and challenged with saponin (17 µg/ml) alone or in the presence of neomycin (0.4 mM) for 60 s. Release of [³H]5-HT and formation of [³²P]phosphatidic acid (PA) or total [³H]inositol phosphates (IPs) were measured as described in the Methods section. Results are shown as means ± s.e.m. for three or four experiments performed in triplicate or quadruplicate: **P* < 0.05 relative to saponin alone. Results for [³H]5-HT secretion are given as percentage of total in tissue, and those for [³²P]PA and [³H]IPs as d.p.m.

Parameter	Basal	Saponin	Saponin + neomycin	Saponin + neomycin + indomethacin
[³ H]5-HT release	0	23.0 ± 4.4	60.3 ± 10.0*	21.6 ± 3.9
[³² P]PA formation	696 ± 131	694 ± 72	1481 ± 191*	756 ± 96
[³ H]IPs formation	4804 ± 465	4873 ± 314	6493 ± 500*	—

Fig. 4. Aminoglycosides stimulate 5-hydroxytryptamine secretion from saponin-permeabilized human platelets

Washed platelets, prelabelled with [³H]5-hydroxytryptamine, were incubated with neomycin or streptomycin for 1 min before addition of saponin and left for a further 1 min. 5-Hydroxytryptamine secretion was estimated as described in the Methods section. Note that saponin causes a significant release of 5-hydroxytryptamine, as reported previously by ourselves [21] and others [22]. Results are means ± s.e.m. from four experiments: **P* < 0.05, ***P* < 0.01 relative to saponin alone.

perhaps not surprising, since neomycin does not readily cross cell membranes, and the inositol phospholipids are predominantly located on the inner leaflet of the membrane. The concentration of neomycin required to inhibit aggregation [2–10 mM] is high enough that binding to other anionic phospholipids, e.g. phosphatidylserine and phosphatidic acid, is likely to occur [25], and this may account for its inhibitory action on aggregation. The selective inhibition of aggregation rather than of shape change indicates that neomycin may inhibit the cross-linking of platelets by fibrinogen or the binding of fibrinogen to its receptor. The selective inhibition of thrombin-induced shape change suggests a second site of action for neomycin in intact platelets; this site is unlikely to be related to the inositol phospholipids,

since neomycin did not prevent shape change induced by collagen.

With semi-permeabilized platelets, relatively low concentrations of neomycin, and also streptomycin, stimulate secretion and aggregation, together with the formation of phosphatidic acid and inositol phosphates (not studied for streptomycin). All these effects are fully inhibited by indomethacin and EGTA. The rapid formation of phosphatidic acid [26] and inositol phosphates indicates activation of phospholipase C, and the sensitivity to indomethacin suggests that this occurs secondary to cyclo-oxygenase activation. The mechanism whereby neomycin activates phospholipase C is not known, but it is interesting that the present observations resemble those reported previously for activation of saponin-permeabilized platelets by inositol trisphosphate [21,22,27]. In this case it was hypothesized that the release of Ca²⁺ by inositol trisphosphate from intracellular stores led to the activation of phospholipase A₂. In this context, it may be worth considering that the lethal action of aminoglycosides on bacteria is associated with the leakage of small ions, an effect linked to the disruption of membrane structure [28]. Clearly, a direct study of the action of neomycin on Ca²⁺ mobilization from intracellular organelles is required.

The ability of neomycin to activate semi-permeabilized platelets at concentrations known to bind to polyphosphoinositides [8–16] is not easy to explain. There is no reason to suspect that neomycin will not bind to inositol phospholipids in platelet membranes, and yet activation by neomycin involves the hydrolysis of inositol phospholipids. Possible explanations which may account for this apparent paradox include compartmentalization within the semi-permeabilized platelets, or kinetic differences.

Since this paper was submitted, Siess & Lapetina [29] have reported that neomycin inhibits activation of intact platelets by thrombin, an observation that has been confirmed in the present study. They also reported that neomycin had no effect on platelet activation by other agonists, but in the present study we observed a significant inhibition of aggregation induced by collagen, ADP and A23187.

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