

Stimulation and inhibition of human platelet membrane high-affinity GTPase by neomycin

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The effect of the inositol phospholipid-binding antibiotic neomycin was studied on high-affinity GTPase in human platelet membranes. At low concentrations (up to 1 mM), neomycin by itself stimulated a high-affinity GTPase. This GTPase stimulation was additive with that caused by the hormonal factors, prostaglandin E_1 and epinephrine, but not with thrombin. At concentrations higher than 1 mM, neomycin reduced control GTPase activity and eliminated the stimulation caused by thrombin. The data suggest that neomycin by a presently unknown mechanism can regulate activity states of signal transducing GTP-binding proteins.

Neomycin; GTP-binding protein; GTPase; Phospholipase C; (Human platelet)

1. INTRODUCTION

The aminoglycoside antibiotic neomycin binds specifically to polyphosphoinositides in comparison to other phospholipids [1,2]. Therefore, this antibiotic has been used in many studies to inhibit polyphosphoinositide-degrading phospholipase C and subsequent cellular processes [3–7]. Because of the polarity of the compound, neomycin is usually added to permeabilized cells or subcellular fractions in order to gain access of the antibiotic to the cytoplasmic site of the plasma membrane.

In the last few years, ample functional evidence has been presented that receptor-mediated activation of phospholipase C by hormones and neurotransmitters involves the intermediate action of guanine nucleotide-binding proteins (G-proteins) [8–10], located at the inner surface of the plasma membrane. The nature of the phospholipase C-coupling G-protein(s) has not been identified so far. In order to gain more insight

into the inhibitory action of neomycin on receptor-stimulated phospholipase C we studied whether, in addition to its well known binding to the substrates of phospholipase C, polyphosphoinositides, neomycin may also affect the activator of the phospholipase C, namely the G-proteins. The function of these proteins can be measured in crude membrane preparations as high-affinity GTPase activity [10,11]. In the course of these studies, we observed that in human platelet membranes neomycin by itself can markedly modify the activity of high-affinity GTPases.

2. MATERIALS AND METHODS

2.1. Materials

Neomycin sulfate, thrombin (human), epinephrine, prostaglandin E_1 (PGE $_1$) and indomethacin were obtained from Sigma, Deisenhofen, FRG. GTP, ATP, creatine phosphate and creatine kinase were purchased from Boehringer, Mannheim. [γ - 32 P]GTP was prepared as described in [12].

2.2. Preparation of human platelet membranes

Crude membranes of human platelets were prepared as in [13] with 5 mM EDTA used throughout the membrane preparation procedure. Small aliquots frozen at -70°C were thawed immediately before the GTPase assay and recentrifuged for 10 min at $30000 \times g$ in 10 mM triethanolamine-HCl, pH 7.4,

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containing 5 mM EDTA, and the membranes were resuspended in 10 mM triethanolamine-HCl, pH 7.4.

2.3. GTPase assay

High-affinity GTPase activity was determined with a reaction mixture containing, if not otherwise indicated, 0.1–0.3 μ M [γ - 32 P]GTP (0.1 μ Ci/tube), 0.1 mM ATP, 0.1 mM EGTA, 2 mM $MgCl_2$, 1 mM dithiothreitol, 5 mM creatine phosphate, 0.4 mg/ml creatine kinase, 2 mg/ml bovine serum albumin and the additions indicated in 50 mM triethanolamine-HCl, pH 7.4, in a total volume of 100 μ l. Reactions were started by adding platelet membranes (5–10 μ g protein/tube) to the pre-equilibrated reaction mixture and conducted for 10 min at 25°C. Stopping of the reactions and isolation of $^{32}P_i$ formed were performed with the charcoal method as in [13]. High-affinity GTPase activity was calculated from the difference between total $^{32}P_i$ formed and $^{32}P_i$ formed in the presence of 50 μ M GTP, which amounted to 5–10% of total $^{32}P_i$ formation.

3. RESULTS

When human platelet membrane GTPase activity was studied with 0.1 μ M GTP as substrate, neomycin added at concentrations from 0.1 to 10 mM caused a biphasic response (fig.1). At concentrations up to about 1 mM, neomycin increased GTP hydrolysis with a maximal increase of 60–80% observed at 0.8 mM neomycin. Half-maximal stimulation was observed at 0.1–0.2 mM neomycin. At above 1 mM, neomycin reduced GTPase activity, being similar to or slightly less than control activity at 10 mM neomycin. To determine whether the GTPase stimulation observed at the low concentrations of neomycin was due to a high-affinity GTPase, substrate saturation experiments were performed. As shown in fig.2, neomycin added at 0.8 mM increased GTPase activity for any low GTP concentration studied. The apparent K_m values of the GTPase observed in the absence and presence of neomycin were very similar, 0.19 ± 0.05 and 0.23 ± 0.03 μ M (SE, $n = 3$), respectively, while the V_{max} of the high-affinity GTPase was increased by neomycin by about 75%. Since it has recently been described that neomycin's stimulatory effects on phospholipase C activity in permeabilized platelets are blocked by addition of EGTA or indomethacin [14], we performed similar experiments to those shown in fig.1 in the absence of these agents and in the presence of either 1 mM EGTA or 10 μ M indomethacin. As illustrated in fig.3, neither the stimulatory nor the inhibitory phase of neomycin's actions on human

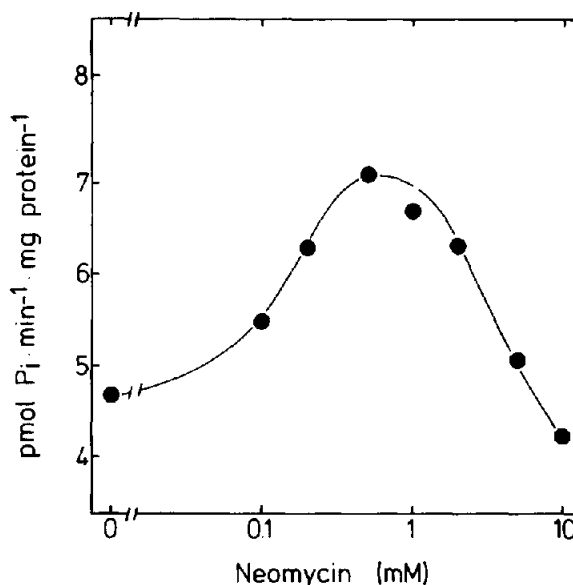


Fig.1. Influence of neomycin on platelet membrane GTPase activity. GTPase activity of human platelet membranes was studied with 0.1 μ M GTP as substrate at the indicated concentrations of neomycin.

platelet membrane GTPase was essentially altered by the presence of these agents.

In human platelet membranes, a wide variety of hormone receptors after activation by their specific agonists interact with guanine nucleotide-binding proteins. This interaction can be monitored as an increase in the respective G-protein GTPase activity [13,15–18]. Therefore, we were interested in whether the stimulation of the hormone receptor-dependent GTPases is modulated by neomycin being present at either stimulatory or inhibitory concentrations. As shown in fig.4, neomycin (0.8 mM) increased GTPase activity to an extent similar to that of PGE_1 (10 μ M) and epinephrine (30 μ M), which activate the stimulatory (G_s) and inhibitory (G_i) G-protein of the adenylate cyclase system, respectively. When combined with neomycin at the low stimulatory concentration (0.8 mM), at least partial additivity between the effects of the hormonal agents and that of neomycin was observed. The most effective GTPase activator in platelet membranes is thrombin, which activates both G_i and an as yet unidentified G-protein coupling to phospholipase C [16–18]. In the presence of thrombin (0.1 U/ml), addition of neomycin (0.8 mM) had only a minimal effect.

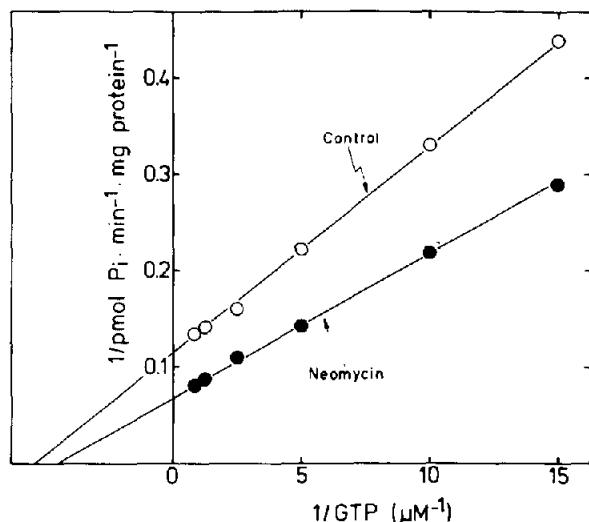


Fig. 2. Activation of a high-affinity GTPase by neomycin. GTPase activity was studied at various concentrations of GTP in the absence (○) and presence (●) of 0.8 mM neomycin. Double-reciprocal plots are shown.

The effects of neomycin added at a complete concentration-response curve on basal and thrombin-stimulated GTPase are shown in fig. 5. Up to 1 mM, neomycin had only a very small ef-

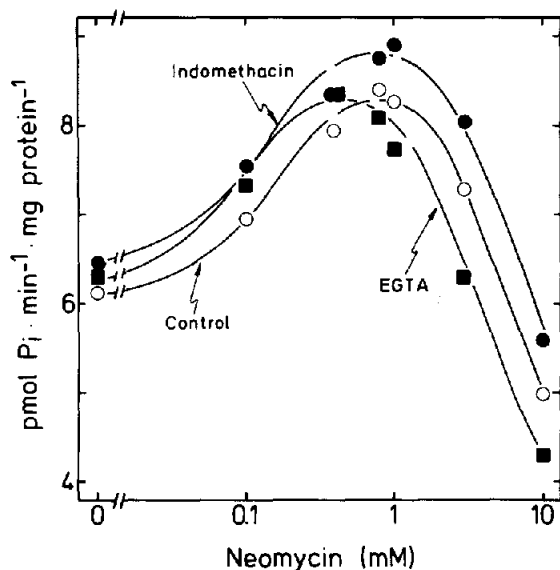


Fig. 3. Influence of EGTA and indomethacin on regulation of GTPase by neomycin. GTPase activity was studied with 0.2 μM GTP as substrate in the absence (○) and presence of either 1 mM EGTA (■) or 10 μM indomethacin (●) at the indicated concentrations of neomycin.

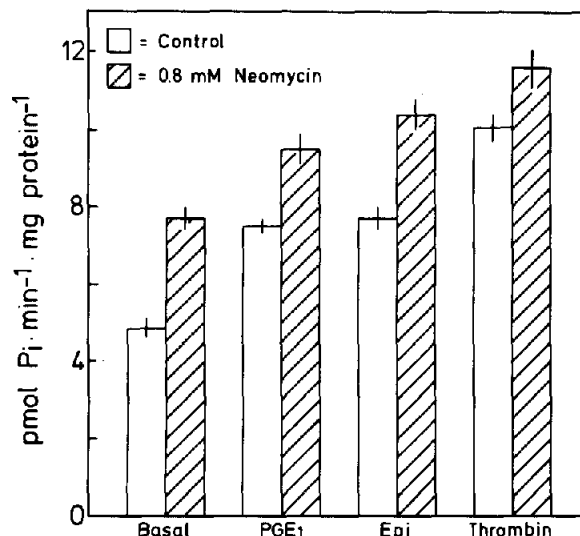


Fig. 4. Influence of neomycin on hormone-stimulated GTPase activity. GTPase activity was studied with 0.1 μM GTP as substrate in the absence (open bars) and presence (hatched bars) of 0.8 mM neomycin without (basal) and with 10 μM PGE₁, 30 μM epinephrine (Epi) or 0.1 U/ml thrombin as indicated.

fect on thrombin-stimulated GTPase activity, while basal activity was increased by about 60%. At higher concentrations, neomycin not only reduced control GTPase activity, but also even

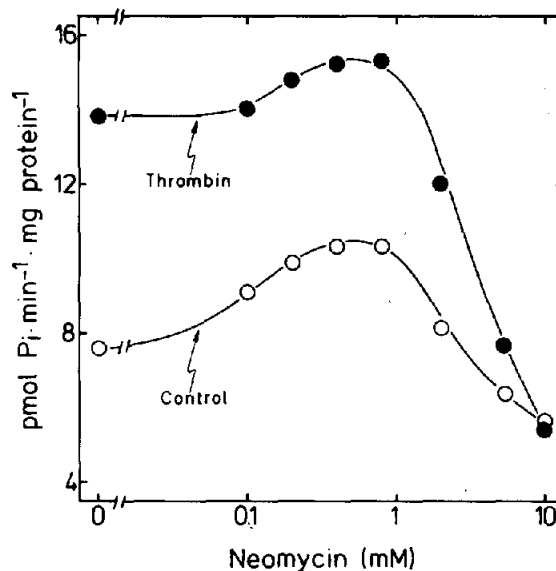


Fig. 5. Influence of neomycin on thrombin-stimulated GTPase activity. GTPase activity was studied with 0.3 μM GTP as substrate in the absence (○) and presence (●) of 0.1 U/ml thrombin at the indicated concentrations of neomycin.

more specifically reduced GTPase stimulation by thrombin, which was completely absent at 10 mM neomycin.

4. DISCUSSION

The present data demonstrate that the aminoglycoside antibiotic neomycin at rather low concentrations can be a very effective stimulator of a high-affinity GTPase in platelet membranes. The extent of GTPase stimulation caused by neomycin was similar to that induced by the hormonal factors PGE₁ and epinephrine, activating the stimulatory and inhibitory G-protein of the adenylate cyclase system, respectively. When combined with one of these agents, both at maximally effective concentrations, at least partial additivity in GTPase activation was observed. In contrast, in combination with thrombin, which activates G_i and the phospholipase C-coupling G-protein, at a just maximally effective concentration [16,17], no additivity was observed. At high concentrations (>1 mM), neomycin reduced basal GTPase and even more specifically attenuated the GTPase stimulation caused by thrombin.

These biphasic responses observed with neomycin on high-affinity GTPase are in excellent agreement with recently published data on neomycin's action on permeabilized human platelets [14,19]. At low concentrations, with a maximal response observed between 0.4 and 1 mM, neomycin was shown to induce inositol phosphate and phosphatidic acid formation as well as arachidonic release, platelet aggregation and serotonin release. At higher concentrations, neomycin inhibited platelet aggregation and serotonin release. At 10 mM, the stimulatory effects of neomycin were lost [14]. This bell-shaped pattern of concentration response curve was also found in studying neomycin's actions on high-affinity GTPase in platelet membranes.

The intriguing question is how neomycin exerts its effects on high-affinity GTPase. From the functional studies, in which an activation of G-protein-regulated effector systems by neomycin has been observed [14,19], it appears to be unlikely that neomycin simply activates the GTP-hydrolyzing activity of a G-protein. Such increased hydrolysis would induce inhibition rather than stimulation of effector enzymes such as the phospholipase C. It

may be speculated that neomycin binds directly to a G-protein and, thereby, by itself mimics the interaction of an agonist-activated receptor with a G-protein. From the combined data, it seems most likely that the G-protein activated by neomycin is of the same nature as that activated by thrombin. It is also feasible that the hydrophilic compound neomycin somehow induces the coupling of an agonist-free receptor with the G-protein and, thereby, also mimics the action of an agonist-activated receptor. At high concentrations, neomycin may additionally inhibit the binding of GTP to the G-protein and, thereby, decrease control activity and agonist (thrombin)-stimulated GTP hydrolysis. Last but not least, although not very likely from the functional studies, it cannot be excluded that neomycin by binding to polyphosphoinositides somehow regulates the activity state of membrane proteins such as G-proteins and phospholipase C. Recently, evidence has been presented that inositol-containing phospholipids can constitute an anchoring domain for certain plasma membrane proteins [20]. The final answer to this question may be obtained in studying neomycin's effects on the purified membrane proteins.

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