Biochem. J. (1991) 273, 241–243 (Printed in Great Britain)

Neomycin does not interfere with the inositol phospholipid metabolism, but blocks binding of α-thrombin to intact human platelets

Ole-Bjørn TYSNES,* Eli JOHANESSEN and Vidar M. STEEN
Department of Biochemistry, University of Bergen, Årstadveien 19, N-5009 Bergen, Norway

Neomycin was demonstrated to inhibit the binding of thrombin to intact human platelets. The effects of neomycin on both thrombin binding and thrombin-induced changes in inositol phospholipid metabolism could be reproduced by the thrombin antagonist hirudin. We propose that neomycin inhibits thrombin-induced platelet activation by interference with the cellular receptor.

INTRODUCTION

Receptor-mediated increase in inositol phospholipid metabolism represents one of the major trans-membrane signal-transducing mechanisms (reviewed in [1]). Upon cellular activation, phosphatidylinositol 4,5-bisphosphate (PIP_2) is hydrolysed to inositol 1,4,5-trisphosphate and diacylglycerol, which both act as intracellular messenger molecules [2,3]. The polycationic antibiotic neomycin binds strongly and relatively specifically to PIP_2 [4–6]. On the basis of this property, neomycin has frequently been used to perturb the turnover of the inositol phospholipids in cells [7–10]. However, experiments on permeabilized cells have revealed that very different concentrations of neomycin are required to affect the inositol phospholipid turnover in the various cell types. In platelets only 10 μM was required [11], whereas in mast cells [12] and sea-urchin eggs [13] 1 mM was necessary to inhibit the agonist-induced breakdown of PIP_2. Moreover, the data obtained by the use of neomycin have been difficult to interpret, since polyanionic molecules to which it binds also include inositol 1,4,5-trisphosphate and ATP [14], and at high concentrations the drug has even been shown to induce Ca^{2+} release and stimulate GTPases [15]. The specificity and the mechanism of the inhibitory effects of neomycin have therefore frequently been debated (reviewed in [16]).

We have recently suggested that neomycin may interfere with thrombin-induced platelet activation at a level before the inositol phospholipid metabolism [17,18]. Here we give evidence that neomycin affects inositol phospholipid metabolism in thrombin-stimulated intact human platelets by blocking the binding of thrombin to its receptor.

MATERIALS AND METHODS

Materials

Stock solutions of bovine thrombin were obtained from Hoffman–La Roche. [^{32}P]P_i (code PBS-11; carrier free) and ^{125}I (code IMS 30) were obtained from Amersham. α-Thrombin was purified as described by Brosstad [19] and labelled with ^{125}I by the Enzymobead lactoperoxidase reagent (Bio-Rad; no. 170-6001).

Platelet isolation, labelling and incubation

Platelet-rich plasma was obtained by differential centrifugation of freshly drawn human venous blood anticoagulated with ACD [20].

In some experiments (Fig. 1) the cells were labelled with [^{32}P]P_i (0.1 mCi/ml) for 1 h at 37 °C before gel-filtration through Sepharose 2B into a nominally Ca^{2+}- and phosphate-free Tyrode's buffer [20]. The final platelet number was standardized at 3.5×10^8/ml and the cells were incubated at 37 °C. Neomycin was added 90 s before the cells were exposed to thrombin, and after another 90 s 0.5 ml of the platelet suspension was withdrawn and mixed with 2 ml of chloroform/methanol/conc. HCl (20:40:1, by vol.; 0 °C).

In another method (Fig. 2), unlabelled gel-filtered platelets were exposed to neomycin 90 s before addition of ^{125}I-labelled purified thrombin. After 60 s, the cells were mixed with 1 M-formaldehyde/50 mM-EDTA (0 °C) and immediately thereafter centrifuged through silicone oil as described by Holmsen *et al.* [21]. The radioactivity in the pellet was subsequently determined in a γ-radiation spectrometer.

Phospholipid extraction and chromatography

The lipids were extracted from chloroform/methanol/HCl by addition of 0.5 ml of water and 0.5 ml of chloroform [20]. Phospholipids were separated by t.l.c. in chloroform/methanol/20% (v/v) methylamine (30:18:5, by vol.) [20] and detected by overnight radioautography. The spots were subsequently scraped off the plates for determination of radioactivity by liquid-scintillation counting.

RESULTS AND DISCUSSION

We have previously suggested that the inhibitory effects of neomycin on thrombin-induced platelet activation may be unrelated to the ability of the drug to bind to phospholipids involved in the signal-transduction process [17,18]. The inositol phospholipids are generally believed to be located mainly to the inner leaflet of the plasma membrane [22], and it is still unclear whether the highly polar neomycin molecule can penetrate the intact cellular plasma membrane to interact with the phospholipids on the cytosolic side.

In a recent paper we have demonstrated that changes in the steady-state levels of the inositol phospholipids [phosphatidylinositol 4-phosphate (PIP) and PIP_2] are metabolically tightly

Abbreviations used: PA, phosphatidic acid; PIP, phosphatidylinositol 4-phosphate; PIP_2 phosphatidylinositol 4,5-bisphosphate.

* To whom correspondence should be addressed.

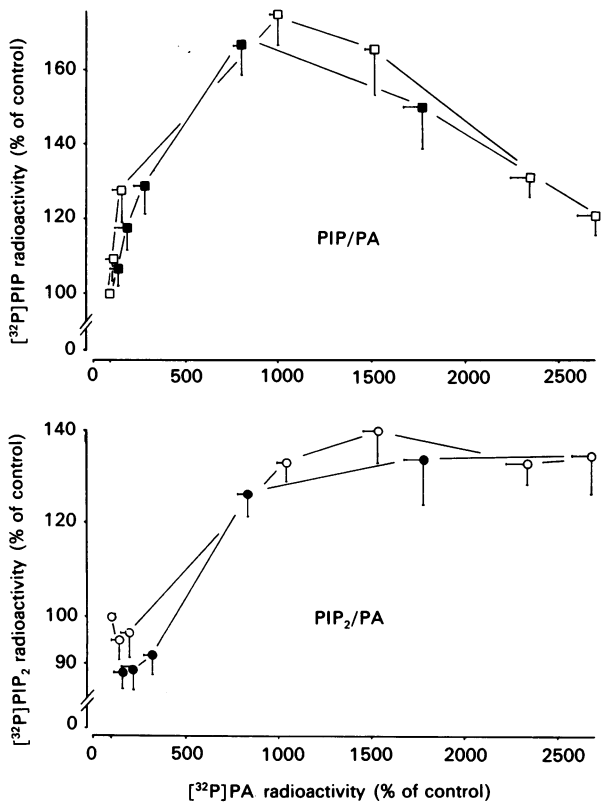

Fig. 1. Relationship between [^{32}P]PIP_2, [^{32}P]PIP and [^{32}P]PA in thrombin-stimulated platelets in the absence and presence of neomycin

[^{32}P]P_i-prelabelled platelets were stimulated with increasing concentrations of thrombin (0.01, 0.03, 0.05, 0.1, 0.3 and 0.5 unit/ml; □, ○) or with a constant concentration of thrombin (0.5 unit/ml) together with increasing concentrations of neomycin (1, 2, 4, 6, 8 mM; ■, ●). The data represent the [^{32}P]PIP_2/[^{32}P]PA and [^{32}P]PIP/[^{32}P]PA relationships for each dose of thrombin and are expressed as means ± S.D. of four independent determinations.

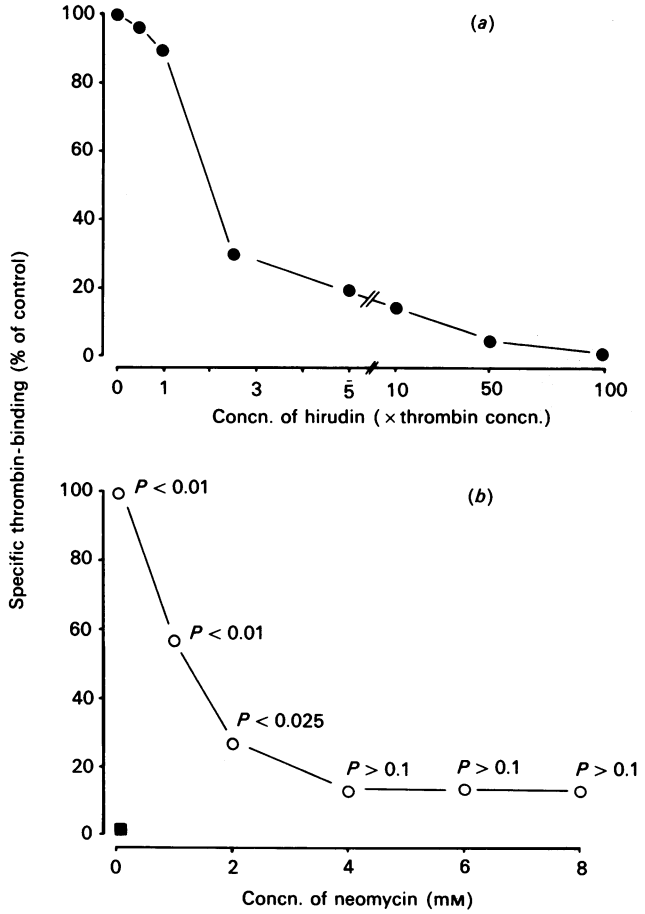

Fig. 2. Effects of hirudin and neomycin on the binding of thrombin to human platelets

Unlabelled gel-filtered platelets were exposed to 0.1 unit of ^{125}I-labelled thrombin/ml. At 90 s before addition of the agonist, either hirudin (*a*) or neomycin (*b*) was added. The data on hirudin represent means of duplicate experiments. The data on neomycin as well as maximal inhibition with hirudin (*b*; ■) represent means of 11 independent determinations. Statistical difference from maximal inhibition with hirudin was tested with Student's paired *t* test.

coupled to phospholipase C activation [measured as the accumulation of phosphatidic acid (PA)] [23]. This metabolic relationship between the inositol phospholipids and PA was not affected by compounds which directly or indirectly affect the thrombin-induced phospholipase C activation (e.g. the thrombin antagonist hirudin; [23]). In contrast, drugs which interfere with specific steps in the polyphosphoinositide cycle (e.g. chlorpromazine or prostaglandin E_1) alter the PIP_2/PA and PIP/PA relationships upon thrombin stimulation [18]. In Fig. 1 we demonstrate that, like increasing concentrations of hirudin, the addition of increasing concentrations of neomycin did not affect the [^{32}P]PIP/[^{32}P]PA or [^{32}P]PIP_2/[^{32}P]PA relationships as compared with the effects obtained with thrombin alone. It is therefore likely that neomycin interferes with the platelet-activating process at a level after or before the inositol phospholipid metabolism.

Previously we have shown that neomycin failed to inhibit the platelet activation when the cells were stimulated by ionomycin or phorbol ester, thereby short-circuiting the inositol lipid signalling system [17]. To investigate whether the thrombin–receptor interaction was affected by the drug, unlabelled platelets were stimulated by ^{125}I-labelled α-thrombin. Fig. 2 shows the inhibitory effects of hirudin (panel *a*) and neomycin (panel *b*) on thrombin binding. The maximal inhibition obtained by hirudin is referred to as the zero point, and was used as a control for maximal inhibition in the experiments with increasing concentrations of neomycin. When neomycin was used at concentrations of 4 mM or more, there was no statistically significant difference in thrombin binding in cells exposed to neomycin or hirudin (Fig. 2). To ensure that the inhibitory action of the drug was unrelated to an interaction between thrombin and neomycin, we tested whether neomycin affected the ability of thrombin to transfer fibrinogen to fibrin, thereby clotting platelet-poor plasma. In contrast with hirudin, no such effect was found with 8 mM-neomycin (results not shown).

Our data clearly demonstrate that neomycin decreases the thrombin binding in intact human platelets. The concentration of neomycin needed to inhibit binding was similar to that required for inhibition of platelet functions [10,17]. Both the decrease in binding and the thrombin-induced changes in inositol phospholipid metabolism can be reproduced by addition of the thrombin antagonist hirudin. The inhibitory action of neomycin on receptor-mediated cellular activation is therefore not related to the ability of the drug to bind to the phospholipase C-hydrolysable inositol phospholipids, as claimed by most authors [7–15]. Neomycin has previously been shown to inhibit not only thrombin-induced platelet activation but also collagen- and platelet-activating-factor-induced aggregation and secretion [10]. These agonists are all believed to activate the platelet through the inositol phospholipid system, but there is little knowledge about the structure of the cellular receptors. In BHK cells, neomycin

inhibited the internalization of Herpes Simplex Virus 1 [24]. This process has been proposed to be dependent on the inositol phospholipid metabolism [25]. However, those authors have shown that neomycin probably inhibits virus internalization by interfering with the cellular receptor function [26,27]. Inositol phospholipids have been demonstrated as structural elements of some cellular receptors [28]. Although never demonstrated, the thrombin receptor may contain these lipids as well as other negatively charged molecules which may interact with neomycin at millimolar concentrations. We therefore propose that neomycin inhibits the signal-transduction process by interfering with the cellular receptor function.

REFERENCES

1. Berridge, M. (1989) J. Am. Med. Assoc. **262**, 1834–1841
2. Nishizuka, Y. (1984) Nature (London) **308**, 693–698
3. Berridge, M. & Irvine, R. F. (1984) Nature (London) **312**, 315–321
4. Schacht, J. (1976) J. Neurochem. **27**, 1119–1124
5. Schacht, J. (1989) J. Lipid Res. **19**, 1063–1067
6. Wang, B. M., Weiner, N. D., Takada, A. & Schacht, J. (1984) Biochem. Pharmacol. **33**, 3257–3262
7. Schibeci, A. & Schacht, J. (1977) Biochem. Pharmacol. **26**, 1769–1774
8. Downes, C. P. & Michell, R. H. (1981) Biochem. J. **198**, 133–140
9. Carney, D. H., Scott, D. L., Gordon, E. A. & LaBelle, E. F. (1985) Cell **42**, 479–488
10. Tysnes, O.-B., Verhoeven, A. J. M. & Holmsen, H. (1987) Biochem. Biophys. Res. Commun. **144**, 454–462
11. Rock, C. O. & Jackowski, S. (1987) J. Biol. Chem. **262**, 5492–5498
12. Cockcroft, S., Howell, T. W. & Gomperts, B. D. (1987) J. Cell Biol. **105**, 2745–2750
13. Whitaker, M. & Aitchison, M. (1985) FEBS Lett. **182**, 119–124
14. Prentki, M., Deeney, J., Matchinsky, F. M. & Joseph, S. K. (1986) FEBS Lett. **197**, 285–288
15. Herrmann, E. & Jakobs, K. H. (1988) FEBS Lett. **229**, 49–53
16. Gabev, E., Kasianowicz, J., Abbott, T. & McLaughlin, S. (1989) Biochim. Biophys. Acta **979**, 105–112
17. Tysnes, O.-B., Steen, V. M. & Holmsen, H. (1988) Eur. J. Biochem. **177**, 219–223
18. Tysnes, O.-B., Steen, V. M. & Holmsen, H. (1990) FEBS Lett. **264**, 33–36
19. Brosstad, F. (1977) Thromb. Res. **11**, 119–130
20. Tysnes, O.-B., Aarbakke, G. M., Verhoeven, A. J. M. & Holmsen, H. (1985) Thromb. Res. **40**, 329–338
21. Holmsen, H., Dangelmaier, C. A. & Rongved, S. (1984) Biochem. J. **222**, 157–167
22. Berridge, M. J. (1984) Biochem. J. **220**, 345–360
23. Steen, V. M., Tysnes, O.-B. & Holmsen, H. (1989) Biochem. J. **263**, 621–624
24. Langeland, N., Haarr, L. & Holmsen, H. (1986) Biochem. Biophys. Res. Commun. **141**, 198–203
25. Langeland, N., Haarr, L. & Holmsen, H. (1986) Biochem. J. **237**, 707–712
26. Langeland, N., Holmsen, H., Lillehaug, J. R. & Haarr, L. (1987) J. Virol. **61**, 3388–3393
27. Langeland, N., Moore, L. J., Holmsen, H. & Haarr, L. (1988) J. Gen. Virol. **69**, 1137–1145
28. Low, M. G. (1987) Biochem. J. **244**, 1–13

Received 21 September 1990/23 October 1990; accepted 2 November 1990