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SHAPE CHANGES INDUCED BY BIOLOGICALLY ACTIVE PEPTIDES AND NERVE GROWTH FACTOR IN BLOOD PLATELETS OF RABBITS

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1 Nerve growth factor (NGF), substance P (SP) and thymopoietin all caused shape change reactions of rapid onset in rabbit platelets. NGF had the highest maximal effect, and SP the lowest EC_{50} (concentration causing half maximal shape change). The action of SP was reversible within 5 min, whereas that of NGF lasted for at least 1 h. A series of other peptides were inactive.

2 After preincubation of platelets with SP, a second application of SP no longer caused a shape change reaction, whereas the effect of NGF was not influenced.

3 An oxidized NGF-derivative without biological activity did not cause a shape change reaction, neither did epidermal growth factor.

4 Prostaglandin E_1 (PGE_1) and pretreatment of the platelets with 3% butanol, which counteract the shape changes caused by 5-hydroxytryptamine (5-HT) and adenosine 3′, 5′-diphosphate, also antagonized those induced by NGF and SP. Neither heparin nor methysergide, an antagonist of 5-HT-receptors, influenced the shape change induced by NGF or SP. The action of NGF was also antagonized by a specific antibody to NGF.

5 Thymopoietin, like the basic polypeptide polyornithine (mol. wt. 40,000), was not antagonized by PGE_1 and butanol. Heparin, which counteracted the effect of polyornithine, did not influence that of thymopoietin.

6 In conclusion, different modes of action are involved in the shape change of blood platelets induced by polypeptides and proteins. SP and NGF may act by stimulating specific membrane receptors.

Introduction

Various compounds induce a shape change reaction of blood platelets, characterized by a transition from the normal, discoid shape into a spheroid form. This change can be quantified by measuring the light absorption of the platelet suspension (Born, Dearnley, Foulks & Sharp, 1978). One group of shape change-inducing agents, represented by substances like 5-hydroxytryptamine (5-HT) and adenosine 3′, 5′-diphosphate (ADP), probably acts through specific receptors at the plasma membrane, as has been shown by shape change and binding studies with specific agonists and antagonists of 5-HT and ADP receptors (Born, 1970; Drummond, 1976; Graf & Pletscher, 1979; Adler & Handin, 1979). Another group includes basic proteins and polypeptides, such as myelin basic protein, polyornithine and polylysine, which are all positively charged (called basic peptides in the following). They seem to exert their effect by unspecific, physicochemical (e.g. electrostatic) interaction with the negatively charged membrane surface. This is suggested by the finding that heparin, a polyanion, counteracts the shape change caused by the basic peptides, but not that elicited by 5-HT. Specific antagonists of the 5-HT-induced shape change do not diminish the shape change caused by basic peptides (Laubscher, Pletscher, Honegger & Richards, 1979).

The receptor that is responsible for the 5-HT-induced shape change reaction in platelets generally reacts to the same agonists and antagonists as the neuronal 5-HT-receptors in some areas of the central nervous system (CNS) (Graf & Pletscher, 1979). In addition, those basic peptides which induced a shape change also caused depolarization of CNS-neurones (Laubscher *et al.*, 1979). It was therefore of interest to investigate whether other biologically active peptides and proteins also caused a shape change reaction in platelets. The present work shows that this is in fact the case and that peptides and proteins with various modes of action can be distinguished.

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Methods

Isolation of platelets

Male or female rabbits, 2.5–3.5 kg, were bled after injections of Hypnorm (Philips Duphar, Amsterdam) (0.5 ml/kg i.p.) or under light ether anaesthesia through a polyethylene cannula inserted in a carotid artery. The whole blood was collected in plastic tubes and mixed with 1/10 volume 3.8% trisodium citrate · $2H_2O$ (TSC). Platelet-rich plasma (PRP) was prepared by centrifugation of the whole blood at 600 g for 10 min.

PRP (20 ml) was put on a gradient consisting of 4 ml 20% dextran T_{10} (bottom layer) and 7 ml 10% dextran (upper layer). The dextran solutions were prepared from a 30% stock solution of dextran T_{10} in water containing 2.25% bovine albumin, 0.15% glucose and 0.3% TSC (all w/v), pH 7.4. For the dilutions, Tris buffer (0.14 M NaCl, 0.0076 M KCl, 0.0056 M glucose and 0.0076 M tris (hydroxymethylamino)methane, pH 7.4) plus 3.8% TSC (9:1, v/v) was used. After centrifugation at 4500 *g* for 10 min the platelets, which had banded between the two layers, were removed by puncturing the tube from the bottom. The platelets were then counted in a Coulter counter and diluted with 8–12 volumes Tris plus TSC, so that the final platelet concentration was $1-2 \times 10^5/\mu l$. No fibrinogen was added, since in preliminary experiments supplementation of the final platelet suspension with bovine fibrinogen (1 or 5 g/l) did not enhance the ADP-response. For more details see Graf, Laubscher, Richards & Pletscher (1979).

For the experiments with butanol pretreatment, 4.5 ml 4% butanol (in Tris, final butanol concentration 3% v/v) or 4.5 ml Tris alone were added to 1.5 ml samples of the platelet suspension removed from the gradient (see above). These suspensions were incubated for 15 min in ice-water. The platelets were then re-isolated on a dextran gradient, as indicated before, and diluted with Tris plus TSC in order to give a final platelet concentration of $2 \times 10^5/\mu l$.

Light absorption

Platelets suspended in Tris-TSC (500 µl samples) were preincubated for 30 min at 37°C. The platelets were stirred at 1000 rev/min with a magnetic stirrer and the various substances (in volumes of 2.5–7.5 µl H_2O) were added. The increase in light absorption as an indication of the shape change reaction was measured with an Elvi 840 aggregometer and a Rikadenki B 361 recorder. Potential antagonists were added 1 min before the agonists. For details see Graf *et al.*, 1979.

Ultramorphology

Suspensions of platelets in Tris ($1-2 \times 10^5$ platelets/µl) were stirred and incubated for 3 min either with nerve growth factor (NGF) 10^{-6} M or with the solvent (H_2O) alone. While stirring they were fixed with an equal volume of 6% glutaraldehyde in 0.15 M phosphate buffered saline (PBS), pH 7.3, and kept overnight at 4°C. After centrifugation (1800 *g*, 12 min) each pellet was washed in PBS and postfixed in 2% osmium tetroxide in the same buffer for 1 h. These procedures were carried out at 4°C. After dehydration in graded alcohol and embedding in Epon, ultrathin sections were prepared and stained with uranyl acetate and lead citrate.

Calculations

The maximal values of the light absorption (maximal effect) reached with the agonists are given as a percentage of the value obtained with 5-HT 10^{-6} M (concentration at which 5-HT has its maximal effect). The values for the EC_{50} (molar concentration of substance causing half the maximal effect) and IC_{50} (concentration of antagonist causing 50% inhibition of the shape change induced by the agonist) were determined graphically. Statistical analyses were performed with Student's *t* test using the absolute maximal values (i.e. the deflection of the recorder in mV).

Materials

Standard NGF was prepared as the 2.5 S subunit from the male mouse submandibular gland according to the procedure of Bocchini & Angeletti (1969). The biological activity was 250 biological units/µg of protein, as determined by the bioassay method of Fenton (1970), using dorsal root ganglia of 10 day-old chicken embryos. The purity of standard 2.5 S NGF was assessed by 15% polyacrylamide sodium dodecyl gel electrophoresis. For control experiments part of this preparation was freed of renin by preparative isoelectric focusing in a pH-gradient (pH 7–10) in 7.5% acrylamide gels (Server & Shooter, 1976). Antibodies against 2.5 S NGF were raised in goats and purified by affinity chromatography according to Stöckel, Gagnon, Guroff & Thoenen (1976). The biological activity of the antibodies was tested by the inhibition of the NGF-mediated fibre outgrowth in the dorsal root ganglion (Fenton, 1970), in which 30 ng of purified NGF-antibodies inhibited the effect of 1 ng of 2.5 S NGF. Biologically inactive NGF was obtained by oxidation of the tryptophan residues with N-bromosuccinimide as described by Frazier, Angeletti, Sherman & Bradshaw (1973). Epidermal growth factor was prepared according to Savage & Cohen (1972).

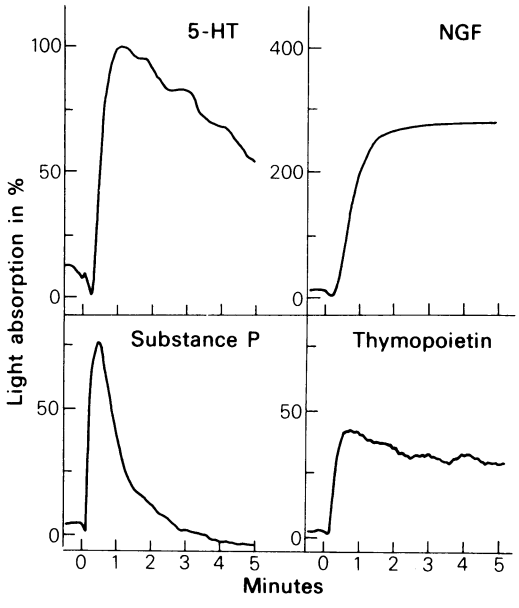

Figure 1 Shape change induced by 5-hydroxytryptamine (5-HT, 10^{-6} M), nerve growth factor (NGF, 10^{-6} M), substance P (5×10^{-8} M) and thymopoietin (2×10^{-5} M) in platelets of rabbits. Individual experiments.

Arginine-vasotocin, secretin, neurotensin and heparin (150 iu/mg protein) were kindly supplied by F. Hoffmann-La Roche Inc. Basel, Switzerland. Tuftsin was purchased from Bachem, Bubendorf, Switzerland, and polyornithine (mol. wt. 40,000) Sigma, U.S.A. The other polypeptides, i.e. substance P(SP), Met- and Leu-enkephalin, D-Ala^2-Metenkephalin, somatostatin, synthetic human calcitonin (cibacalcin), thymopoietin II-(29-41) (thymopoietin) (Schlesinger, Goldstein, Scheid & Boyse, 1975), wasp kinin (Pisano, 1968), chemotactic peptide (For-Met-Leu-Phe-OH), were generous gifts from CIBA-GEIGY Inc. Basel, Switzerland. The remaining substances were of analytical grade, obtained from commercial sources.

Table 1 Shape change induced by 5-hydroxytryptamine (5-HT), adenosine 3', 5'-diphosphate (ADP), nerve growth factor (NGF), substance P (SP), thymopoietin and polyornithine in rabbit platelets incubated in Tris-TSC

Agonist	*EC_{50} (M)*	*Max. effect (%)*
5-HT	$(2.1 \pm 0.3) \times 10^{-7}$	100
ADP	$(1.3 \pm 0.2) \times 10^{-7}$	120 ± 4.5
NGF	$(5.7 \pm 0.9) \times 10^{-7}$	214 ± 33
SP	$(9.5 \pm 2.5) \times 10^{-9}$	87 ± 31
Thymopoietin	$(8.9 \pm 2.3) \times 10^{-6}$	64 ± 34
Polyornithine	$(1.8 \pm 0.3) \times 10^{-8}$	194 ± 18

EC_{50} is the concentration of agonist causing half the maximal effect. The maximal shape change is given as a percentage of that induced by 5-HT 10^{-6} M. Each value is an average with s.e.mean of 3–6 experiments.

Results

Shape change

In rabbit platelets that had been isolated on a dextran gradient and resuspended in buffer solution, NGF, SP and thymopoietin caused a shape change reaction which had a rapid onset (Figure 1). SP showed the lowest EC_{50} value, which was of the order of that of polyornithine. The EC_{50} of thymopoietin was highest, i.e. more than two orders of magnitude higher than that of SP (Table 1). NGF, like polyornithine, induced a maximal shape change significantly ($P < 0.01$) greater than that of 5-HT. The average maximal effects of SP and thymopoietin were smaller, although these differences were not significant ($P > 0.05$) due to the relatively large variability in polypeptide values. The actions of NGF and thymopoietin persisted for 1 h (maximal time of measurement), whereas that of SP was reversed within 5 min.

Other peptides

The other polypeptides tested, including neuropeptides, i.e. Met- and Leu-enkephalin, D-Ala^2-Metenkephalin, somatostatin, neurotensin, tuftsin, secretin, calcitonin, wasp kinin, chemotactic peptide and epidermal growth factor, did not induce a shape change reaction in rabbit platelets in concentrations of 10^{-5} M and higher. A derivative of NGF (10^{-5} M), in which the three tryptophan residues were oxidized with N-bromosuccinimide, was also inactive.

Preincubation with substance P

SP (10^{-7} and 10^{-6} M) no longer caused a shape change reaction when added to the platelet suspension 10 min after a first addition of 10^{-7} M SP (at a time when the light absorption had returned to normal). In contrast, the shape change reaction induced by 5×10^{-7} and 10^{-6} M NGF was not influenced by preincubation of the platelets for 10 min with 10^{-7} M SP.

Prevention of shape change

The shape change reaction caused by NGF and SP in

Table 2 Inhibition by prostaglandin E_1 (PGE_1), methysergide, heparin and washing with 3% butanol of the shape change of rabbit platelets induced by 5-hydroxytryptamine (5-HT) 10^{-6} M, adenosine 3', 5'-diphosphate (ADP) 10^{-6} M, nerve growth factor (NGF) 10^{-6} M, substance P 5×10^{-8} M, thymopoietin 2×10^{-5} M and polyornithine 5×10^{-7} M

	IC_{50} values of			
Agonist	*PGE_1 (nM)*	*Methysergide (nM)*	*Heparin (iu)*	*Shape change after butanol*
5-HT	5.4 ± 0.2	6.2 ± 0.1	> 10	—
ADP	34.0 ± 5.0	> 100	> 10	—
NGF	8.9 ± 2.1	> 100	> 10	—
Substance P	1.1 ± 0.4	> 100	> 10	—
Thymopoietin	> 1000	> 100	> 10	+
Polyornithine	> 1000	> 100	2.3 ± 0.8	+

IC_{50} is the concentration of the inhibitors causing 50% inhibition of the shape change induced by the agonists. Each value is an average with s.e.mean of 3–4 experiments.
+ = shape change reaction present
− = no shape change reaction.

platelets suspended in buffer was counteracted by low concentrations of prostaglandin E_1 (PGE_1) as well as by previous washing of the platelets with 3% (v/v) butanol. These measures also antagonized the actions of 5-HT and ADP but not those of thymopoietin and polyornithine which were sometimes even enhanced by butanol (Table 2). Preincubation of the platelets in ice-water without butanol (controls to the butanol experiments) caused no decrease in their responses to NGF, SP, 5-HT or ADP. Anti-NGF-antibodies (50 fold excess on a weight basis) abolished the shape change reaction due to NGF 10^{-6} M, but had no effect on that due to polyornithine 10^{-7} M even in a 100 fold excess. In addition, methysergide, in concentrations that completely blocked the 5-HT-receptor (10^{-7} M) (Graf & Pletscher, 1979), did not antagonize the shape changes caused by NGF, SP or thymopoietin. Finally, heparin (10 iu/ml), which has been shown to abolish the action of polyornithine (Laubscher *et al.*, 1979), did not counteract the effects of NGF, SP or thymopoietin (Table 2). Methylamine (10^{-2} M), which prevents endocytosis of proteins (Maxfield, Willingham, Davies & Pastan, 1979), had no influence on the action of NGF.

Electronmicroscopy

On electronmicroscopy the majority of the control platelets exhibited a discoid form with a smooth cellular surface. After incubation with NGF 10^{-6} M for 3 min, most of the platelets had assumed a spheroid shape with extrusion of blebs and short pseudopods. However, the granular contents of the cells were not changed by NGF, the 5-HT-storage organelles (dense bodies) and the α-granules having the same appearance as those in normal platelets (Figure 2).

Discussion

Our results show that the mechanism of action of NGF and SP in causing the shape change reaction is different from that of basic peptides, such as polyornithine, which are thought to have an unspecific (electrostatic) interaction with the platelet membrane (Laubscher *et al.*, 1979). Firstly, PGE_1 and butanol treatment of the platelets abolished the shape change induced by NGF and SP, but not that induced by polyornithine. It has been suggested that PGE_1 counteracts aggregation (e.g. that mediated through receptor stimulation) by elevating cyclic adenosine 3', 5'-monophosphate (cyclic AMP) (Haslam, Davidson, Fox & Lynham, 1978), whereas butanol removes superficial proteins, which might be essential for an intact receptor function in membranes (Noll, Matranga, Cascino & Vitorelli, 1979). Secondly, heparin, a polyanion which antagonizes the shape change induced by polyornithine, did not have any effect on the shape change induced by NGF and SP. Finally, an anti-NGF-antibody which was purified by affinity chromatography (sepharose-coupled NGF) abolished the action of NGF without affecting that of polyornithine.

On the other hand, the actions of PGE_1, butanol and heparin on the shape-changing effects of NGF and SP were closely comparable with their actions on the shape-changing effects of 5-HT and ADP, which are thought to be mediated by specific receptors. Therefore, NGF and SP might also act by stimulation of specific membrane receptors. In the case of NGF this hypothesis is supported by the finding that a biologically inactive NGF-derivative, whose three tryptophan residues were oxidized (abolishing its stimulatory effect on neurite outgrowth in sensory neurones and its inducing action on tyrosine hydroxylase in sympathetic neurones), no longer

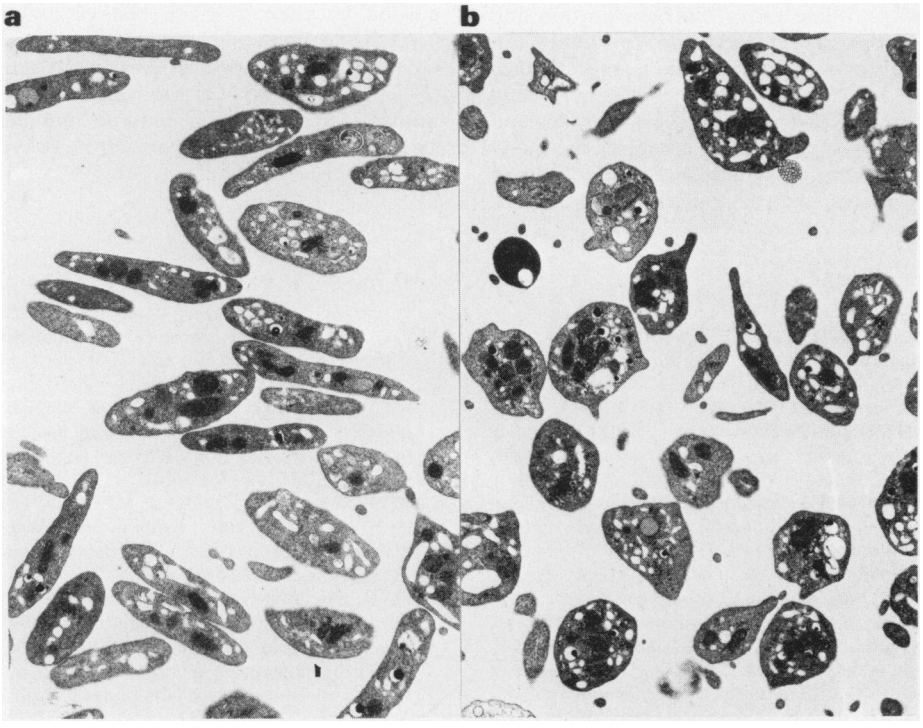

Figure 2 Electronmicrographs of rabbit platelets incubated for 3 min in buffer stirred at 1000 rev/min with a magnetic stirrer. Magnification × 7600. The same platelet dilutions were used as in the experiments in which shape change was measured ($1-2 \times 10^5$ platelets/μl). The incubation was followed by fixation as indicated in the methods section. (a) Without addition of nerve growth factor (NGF); (b) addition of NGF 10^{-6} M (final concentration). Notice the discoid shape in (a), and the spheroid form with extrusion of blebs and short pseudopods in (b).

caused a shape change reaction. In addition, epidermal growth factor, another biologically active polypeptide isolated from male mouse submandibulary glands, was inactive as a shape change inducer.

The shape change reactions induced by NGF and SP were not due to 5-HT released from the platelets. In fact, the 5-HT-receptor antagonist methysergide, which abolishes the shape change caused by 5-HT (Graf & Pletscher, 1979), did not diminish the action of NGF and SP. Also, certain possible contaminants of salivary gland NGF, i.e. renin (Cozzari, Angeletti, Lazar, Orth & Gross, 1973), kallikrein and tuftsin, were not responsible for the shape change reaction. Thus, a renin-free 2.5 S NGF (see 'Materials') had the same action as standard NGF. Furthermore, trasylol, an inhibitor of kallikrein, did not influence the NGF-response and tuftsin was inactive as a shape change inducer.

Whether NGF and SP act on two separate receptors remains to be clarified by use of labelled ligands. The presence of two distinct receptors could explain why preincubation of platelets with SP abolished the shape change-inducing effect of a second addition of SP, but not of NGF. The rapid onset of the NGF- and SP-induced shape change reaction as well as the ineffectiveness of methylamine regarding the NGF-induced shape change would be in agreement with a superficial localization of the receptors, i.e. at the plasma membrane.

The present results show that the mechanism of action of thymopoietin (mol. wt. 1611) is different from that of NGF and SP, since PGE_1 and butanol did not counteract the thymopoietin-induced shape change. However, an unspecific effect similar to that of polyornithine can also be excluded, because only basic proteins of high molecular weight (e.g. polyornithine 40,000 but not 4000) (Laubscher *et al.*, 1979) caused a shape change reaction and because the action of thymopoietin was not antagonized by heparin.

Therefore, with regard to their shape change-inducing action in rabbit platelets, four types of polypeptides and proteins can be distinguished: (1) inactive ones like enkephalins, somatostatin, neurotensin, tuftsin, etc.; (2) those possibly acting via membrane receptors, e.g. NGF and SP; (3) those showing an unspecific (probably electrostatic) interaction with the plasma membrane, e.g. basic pep-

tides like polyornithine and myelin basic protein, and (4) those having a yet unknown (probably not receptor-mediated mode of action, e.g. thymopoietin.

The presence of receptors for neuropeptides and hormone-like proteins on platelets raises the question of the physiological significance of these receptors. It is of interest that the concentration of NGF needed to elicit a shape change reaction (EC_{50} 5.7×10^{-7} M) was much higher than that required to induce fibre outgrowth in dissociated neuronal cells (EC_{50} 2×10^{-11} M). On the other hand, it was of a similar order to that needed to enhance tyrosine hydroxylase activity in organ cultures of sympathetic ganglia (Otten & Thoenen, 1977).

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