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IRREVERSIBLE INHIBITION OF THE TXA_2/PGH_2 RECEPTOR OF HUMAN PLATELETS BY A PHOTOAFFINITY LIGAND

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Abstract—In order to tag the TXA_2/PGH_2 receptor of human platelets, we synthesized azido-BSP (= 4-[2-(4-azido-benzenesulfonylamino)-ethyl]phenoxyacetic acid), a photolabile derivative of the specific TXA_2/PGH_2 receptor antagonist sulotroban (= BM 13.177). If protected from UV light, azido-BSP competitively inhibited the shape change of human washed platelets stimulated by the TXA_2 mimetic U 46619. Schild analysis revealed a $pA_2 = 6.7$ (apparent $K_D = 0.2\ \mu mol/l$). Irreversible inhibition of the U 46619-induced platelet activation was achieved by irradiating for 5 min with UV light of 254 nm a platelet suspension containing azido-BSP. After subsequent washing, the platelets were stimulated with U 46619, ADP or PAF. Under these conditions azido-BSP inhibited the shape change, aggregation and [^{3}H]serotonin release induced by U 46619 but not the shape change induced by ADP or PAF. The concentrations of azido-BSP which blocked the U 46619-induced [^{3}H]serotonin release and the aggregation were 0.5 μmol/l and 1.0 μmol/l, respectively, whereas even 50.0 μmol/l of azido-BSP only partially inhibited the U 46619-stimulated shape change. Obviously, increasing numbers of thromboxane receptors have to be blocked in order to inhibit the [^{3}H]serotonin release, the aggregation and the shape change. Even at an azido-BSP concentration equal to 250 times the apparent dissociation constant, enough receptor sites remained active to allow U 46619 to induce the shape change. In sulotroban was added prior to irradiation, the blocking effect of azido-BSP decreased with increasing concentrations of sulotroban. These results show that azido-BSP is a specific and high affinity ligand of the TXA_2/PGH_2 receptor and that it covalently links to the receptor under irradiation. Azido-BSP is a new tool to identify and characterize the TXA_2/PGH_2 receptor.

The arachidonic acid metabolites prostaglandin H_2 (PGH_2) and thromboxane A_2 (TXA_2) are potent platelet agonists [1] which have been claimed to act via a common TXA_2/PGH_2 receptor [2, 3]. Competitive thromboxane receptor antagonists have been synthesized in the past. Most of them are chemically stable derivatives of the evanescent prostaglandin endoperoxides PGG_2 and PGH_2 [4] and TXA_2 [5–8]. Sulotroban is a non-prostanoic compound and the first non-toxic receptor antagonist which is now under clinical investigation [9]. Recently, Halushka and coworkers described the hydrodynamic properties of a TXA_2/PGH_2 binding site which they had solubilized from human platelet membranes [10]. Gel filtration experiments revealed a molecular weight of 180–220 kdal which was corrected to 140 kdal by calculation including the Stokes radius (5.25 nm) and the sedimentation coefficient (6.3 Svedberg units).

In order to isolate and purify the TXA_2/PGH_2 receptor, we synthesized a photolabile azido derivative of sulotroban. Covalent tagging of the thromboxane receptor by photaffinity labelling is a prerequisit for its differentiation from other platelet proteins. This procedure precludes substantial loss of the label during the purification steps. Azido-BSP turned out to be a competitive inhibitor of the platelet TXA_2/PGH_2 receptor as specific as sulotroban and enabled us to irreversibly block the receptor upon UV irradiation. Intact platelets instead of platelet membranes were treated with azido-BSP because only with the former one is able to control the efficiency of a receptor blockade by functional studies.

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MATERIALS AND METHODS

Biochemicals. The synthesis of azido-BSP (4-[2-(4 - azido - benzenesulfonylamino) - ethyl]phenoxyacetic acid) will be described elsewhere. Sulotroban (= BM 13.177) was a gift from Boehringer (Mannheim, F.R.G.); forskolin and PAF (L-alpha-lecithin, beta-acetyl, *O*-alkyl) were purchased from Calbiochem (Frankfurt, F.R.G.), apyrase from Sigma (Munich, F.R.G.), human serum albumin from Immuno (Heidelberg, F.R.G.), ADP from Boehringer (Mannheim, F.R.G.) and U 46619 from Upjohn (Kalamazoo, MI, USA). [^{3}H] serotonin was from Amersham Buchler (Braunschweig, F.R.G.). All other chemicals came from different commercial sources and were of the highest purity available. PAF and U 46619 (100 mM) were dissolved in dimethylsulfoxide of which 2 μl were added to 500 μl platelet suspension. The other agonists and antagonists were added as isotonic aqueous solutions in portions of 5μl per 500 μl platelet suspension.

Washed platelet suspension. Human blood was anticoagulated with ACD NIH-formula (0.8% (w/v) citric acid, 2.2% (w/v) sodium citrate, 2.45% (w/v) hydrous dextrose) and washed by differential centrifugation according to our previously described method [11–13]. The platelets were washed with a citrate medium pH 6.5 containing apyrase 50 μg/ml, forskolin 0.01 μmol/ml and human serum albumin 0.5 mg/ml. The final suspending medium of pH 7.4 contained TES (*N*-tris [hydroxymethyl]methyl-2-aminomethanesulfonic acid) 30 mM, KCl 5 mM, glucose 5 mM, $MgCl_2$ 67 μM, $CaCl_2$ 1 mM, albumin 330 μg/ml, apyrase 33 μg/ml and NaCl 162.5 mM.

Measurements of shape change, aggregation and [^{3}H] *serotonin release.* Platelet shape change and aggregation were followed turbidimetrically with two dual-channel aggregometers from LABOR GmbH (Hamburg, F.R.G.) at 37° [11, 14]. The stirring speed was 1000 rpm in aggregation and 400 rpm in shape change experiments. The latter were performed with added EDTA 2.5 mM to prevent aggregation. The percentage of shape change was calculated as follows: % = 100 × (amplitude of the increase in absorbance/amplitude of the increase in absorbance during full shape change with a high agonist concentration). In aggregation experiments, fibrinogen 300 μg/ml was added. In order to determine the amount of secretion, the platelets were preloaded with 28.86 kBq (= 0.78 μCi) [^{3}H] serotonin for 15 min. The release of [^{3}H]serotonin was calculated as the percentage of the total platelet-bound radioactivity released after 3 min of stimulation with U 46619. To obtain the platelet supernatant, the samples were rapidly cooled to about 0° and centrifuged at 0° for 30 sec (Eppendorf 3200 centrifuge).

Photoaffinity treatment. The treatment with the photoaffinity ligand azido-BSP was performed using suspensions of 5×10^8 platelets/ml in phosphate buffer pH 6.5 (7 mM phosphate, 5 mM KCl, 130 μM $MgCl_2$, 5 mM glucose, 2 mg/ml albumin, 200 μg/ml apyrase, 550 μM $CaCl_2$) in isotonic NaCl. After preincubating the azido-BSP-containing platelet suspensions in the dark for 1 min, the stirred suspensions were irradiated for 5 min with UV light of 254 nm at 25°. For this purpose a CAMAG mercury lamp (CAMAG 29010, CAMAG West Berlin) was installed 4 cm above the suspension's surface with an energy output of 8,375 mW/cm^2 at 254 nm wavelength. The controls were treated in parallel: one of them did not contain azido-BSP and to another azido-BSP was added only after the irradiation with UV light. Thereafter the platelet suspensions were centrifuged for 12 min at 220 *g* and the pellets were suspended in the washing medium pH 6.5 (10^{-8} M forskolin) to remove all the unbound azido-BSP (see under "washed platelet suspension"). After resting for 10 min at room temperature, the platelets were recentrifuged and the pellets suspended in the final suspending medium (see under "washed platelet suspension") and used for the platelet function tests.

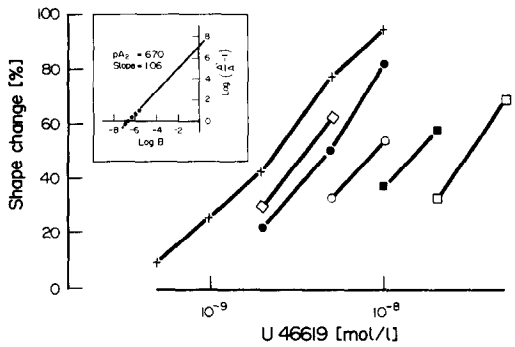


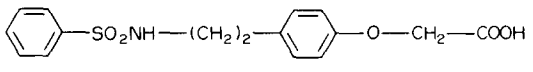
Fig. 2. Competitive inhibition by azido-BSP of the U 46619-induced shape change. Azido-BSP was added 1 min prior to U 46619 in the aggregometer, where it was protected from short-wave light. Platelet suspensions without azido BSP (×——×); with 0.1 μmol/l (◇——◇), 0.2 μmol/l (●——●), 0.5 μmol/l (○——○), 1.0 μmol/l (■——■) and 2.0 μmol/l (□——□) of azido-BSP. Insert: Schild plot. The values of U 46619 for the 50% shape change of different azido-BSP concentrations were taken into calculation of the ordinate scale. The slope and the pA_2 were determined by a computerized analysis program.

RESULTS

Azido-BSP is a photolabile derivative of sulotroban (Fig. 1). If protected from UV light, azido-BSP competitively inhibited the shape change of human platelets induced by the stable prostaglandin endoperoxide U 46619 [15] (Fig. 2). The Schild analysis [16] revealed a $pA_2 = 6.7 \pm 0.01$ (apparent $K_D = 0.2$ μmol/l) and a slope of 1.06 ± 0.02 (insert of Fig. 2).

In order to determine the appropriate wavelength for the photoactivation, UV spectra were recorded after treating an ethanolic solution of azido-BSP with light of short wave length for various times. Figure

Sulotroban



Azido-BSP

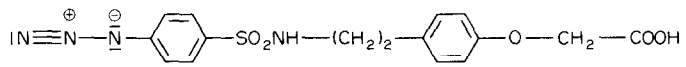


Fig. 1. Chemical structure of azido-BSP and sulotroban (= BM 13.1777).

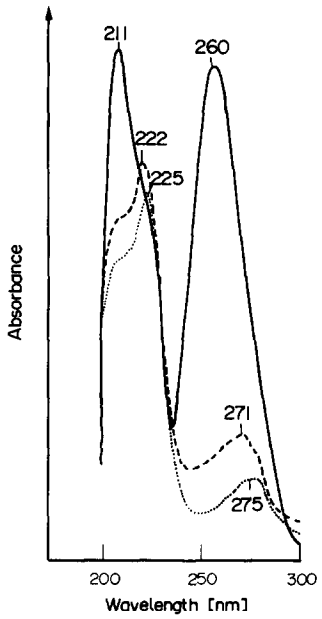


Fig. 3. Absorption spectra of an ethanolic solution of azido-BSP before (——) and after 1 min (- - - -) and 10 min (·····) irradiation with UV light of 254 nm wavelength.

3 shows absorption spectra at the beginning, after 1 min and 10 min of irradiation with UV light of 254 nm. The UV spectrum of azido-BSP exhibited two absorption maxima at 211 and 260 nm. Upon irradiation, the absorption at 260 nm was strongly decreased. Concomitantly a bathochrome shift of both maxima occurred. Irradiation with UV light of 366 nm was much less effective. Consequently, the photoaffinity coupling experiments were performed by irradiating platelet suspensions with UV light of 254 nm for 5 min.

In order to demonstrate the covalent linkage of azido-BSP to the TXA_2/PGH_2 receptor, we looked for an inhibition of the platelet response induced by the prostaglandin endoperoxide mimetic U 46619. Irradiation of the platelet suspensions in the presence of 0.5 μmol/l azido-BSP resulted in a complete blockade of the [^{3}H]serotonin release (Fig. 4a). At lower antagonist concentrations, the dose–response curves were less steep and did not achieve the maximum effect as compared to the control. Similar results were obtained for the U 46619-induced platelet aggregation but doubling of the azido-BSP concentration was necessary for a complete blockade of the aggregation (Fig. 4b). Here too, a decrease in the antagonist concentration led to dose–response curves typical for a partially irreversible inhibition [17]. The blockade of the shape change was only partial even if high concentrations of azido-BSP were present during the irradiation of the platelet suspension. Figure 4(c) shows concentration–response curves which reveal only a small shift towards higher agonist concentrations even at 50 μmol/l of azido-BSP, which is a hundred-fold the concentration sufficient for a complete blockade of the [^{3}H]serotonin release (compare to Fig. 4a). There was no difference between the dose–response curves of those control

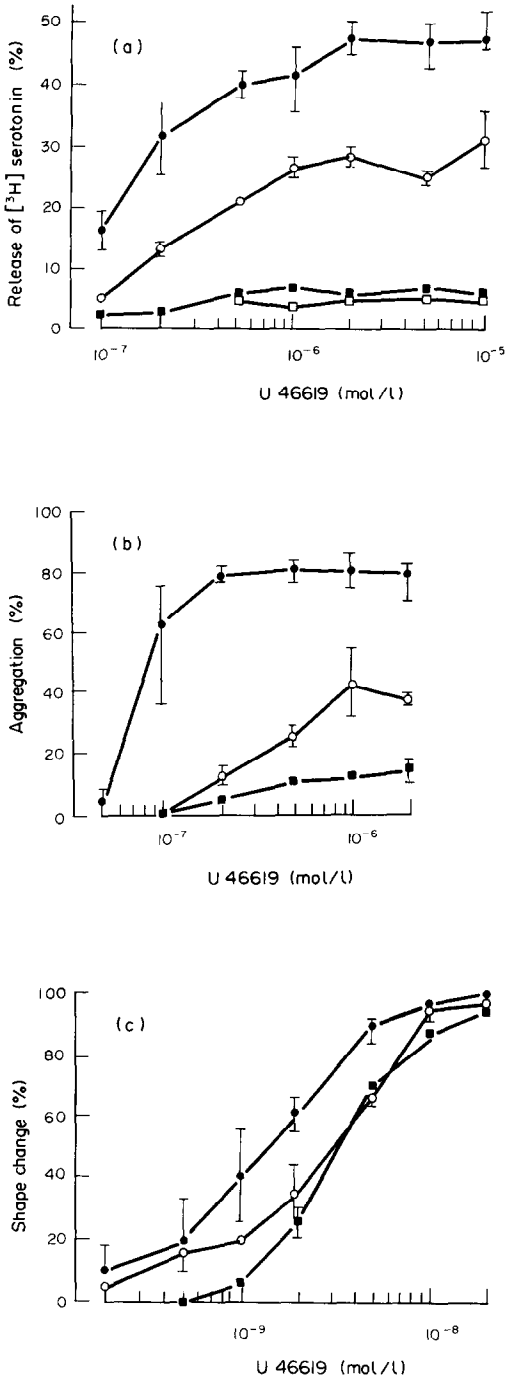


Fig. 4. Inhibition by azido-BSP of the U 46619-induced platelet activation.
(a) Inhibition of the [^{3}H]serotonin release. Platelet suspensions were irradiated (254 nm, 5 min, 25°) in the absence (●——●) and in the presence of 0.07 μmol/l (○——○), 0.5 μmol/l (■——■) and 1.0 μmol/l (□——□) of azido-BSP.
(b) Inhibition of the platelet aggregation. Platelet suspensions were irradiated with UV light in the absence (●——●) and in the presence of 0.5 μmol/l (○——○) and 1.0 μmol/l (■——■) of azido-BSP.
(c) Inhibition of the platelet shape change. Platelet suspensions were irradiated in the absence (●——●) and in the presence of 5.0 μmol/l (○——○) and 50.0 μmol/l (■——■) of azido-BSP.

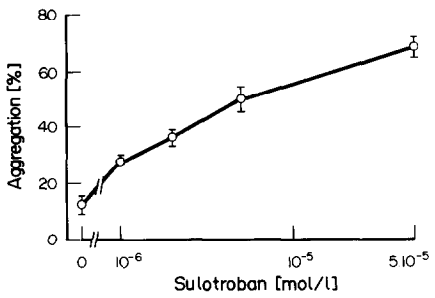


Fig. 5. Preventive effect of sulotroban on the photoaffinity inhibition by azido-BSP. Platelet suspensions were irradiated in the presence of 1.0 μmol/l of azido-BSP and various concentrations of sulotroban. After washing the suspensions, the aggregation was induced by 0.5 μmol/l U 46619.

suspensions containing azido-BSP but being protected from short-wave light, and those free of the photoaffinity ligand during irradiation. For this reason only one control curve was plotted for each kind of activation. The specificity of the blockade of the thromboxane receptor by azido-BSP was also demonstrated by its effect in the presence of the specific thromboxane antagonist sulotroban (BM 13.177). Depending on the concentration of sulotroban, a decrease in the blocking effect of azido-BSP resulted (Fig. 5). In contrast to the U 46619-induced platelet activation, neither the PAF- nor the ADP-induced shape change was influenced by the treatment with azido-BSP (see Table 1).

DISCUSSION

The UV spectrum of sulotroban exhibits three maxima at wavelengths of 235, 266 and 273 nm (spectrum not shown), whereas its photolabile azido derivative, azido-BSP, has only two maxima at 211 and 260 nm. The absorption at 260 nm was drastically decreased upon irradiation with short-wave light. Concomitantly a shift towards longer wavelengths (271 nm after 1 min and 275 nm after 5 min of irradiation) occurred. This is assumed to be due to the transformation of the azido moiety into several unknown reaction products via a nitrene intermediate. Thus, the observed shift of the absorbance maxima might reflect the heterogeneity of the resulting chromophores. If protected from short-wave light, the azido derivative acted like the competitive TXA_2/PGH_2 receptor antagonist sulotroban. The apparent affinity of azido-BSP ($pA_2 = 6.7$) was found to be about three times higher than that of sulotroban ($pA_2 = 6.17$) [18]. Thus, the introduction of the azido group into the molecule obviously improved the antagonistic activity.

Table 1. Absence of an effect of azido-BSP on the stimulation of the platelet shape change with PAF and ADP

Agonist	Irradiation	Shape change (%)
ADP 0.1 μmol/l	+	80 ± 4
ADP 0.1 μmol/l	−	78 ± 1
PAF 50 pmol/l	+	40 ± 7
PAF 50 pmol/l	−	48 + 5

Platelet suspensions were irradiated with or protected from UV light and subsequently washed as described under Methods. ($\bar{X}$ ± SEM).

The covalent coupling of an antagonist to its receptor leads to an irreversible inhibition of ligand binding and receptor activation. As binding studies are only indicatory for a binding site, we studied the inhibitory effect of azido-BSP on platelet activation by treating platelet suspensions with the photoaffinity ligand and UV light. U 46619 as a stable agonist of the TXA_2/PGH_2 receptor was used to stimulate the shape change, aggregation and secretion of human washed platelets. After UV irradiation, non-coupled antagonist had to be removed by repeated washings in order to prevent a competitive type inhibition by non-photolysed azido-BSP. The treatment with the photoaffinity ligand revealed concentration–response curves for the U 46619-induced release of [^{3}H]serotonin, which are typical for a partially irreversible or noncompetitive inhibition [17]. At each antagonist concentration, the concentration–response curves exhibited neither the slope nor the maximum effect of the control curve. The same type of inhibition was seen in the aggregation experiments, yet the antagonist concentration had to be doubled in order to attain a complete inhibition at high concentrations of U 46619. Even with very high concentrations of azido-BSP, the platelet shape change was inhibited only partially. It is concluded that the stimulation of the platelet shape change, aggregation and secretion requires an increasing number of receptor sites. Our results suggest that, even with the highest concentration of azido-BSP (50 μmol/l), a sufficiently high number of thromboxane receptor molecules remained for U 46619 to induce the complete shape change.

The specific blockade of the TXA_2/PGH_2 receptor by azido-BSP was confirmed by the effect of sulotroban, which inhibited the effect of azido-BSP and UV light depending on its concentration. Azido-BSP does not seem to attack other agonist receptors because no inhibition of the shape change induced by ADP or PAF was observed. Our photolabile ligand possesses not only high affinity for the thromboxane receptor but also can be easily derivatized via its free carboxylic group. Coupling with a radioactive moiety, for example, will allow combination of receptor specificity with detectability. By the functional experiments described in this paper, the feasibility of such derivatives can rapidly and easily be screened.

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REFERENCES

1. M. Hamberg, J. Svensson, T. Wakabayashi and B. Samuelsson, *Proc. natn. Acad. Sci. U.S.A.* **71**, 345 (1974).
2. D. E. McIntyre, *Haemostasis* **8**, 274 (1979).
3. R. A. Armstrong, R. L. Jones and N. H. Wilson, *Br. J. Pharmac.* **79**, 953 (1983).

4. G. C. Le Breton, D. L. Venton, S. E. Enke and P. V. Halushka, *Proc. natn. Acad. Sci. U.S.A.* **76**, 4097 (1979).
5. K. C. Nicolaou, R. L. Magolda, J. B. Smith, D. Aharony, E. F. Smith and A. M. Lefer, *Proc. natn. Acad. Sci. U.S.A.* **76**, 2566 (1979).
6. M. Katsura, T. Miyamoto, N. Hamanaka, K. Kondo, T. Terada, L. Oligoki, A. Kawasaki and M. Tsuboshima, *Prostaglandin Thromboxane Leukotriene Res.* **11**, 351 (1983).
7. R. L. Jones, N. M. Wilson, R. A. Armstrong, V. Peesapati and G. M. Smith, *Prostaglandin Thromboxane Leukotriene Res.* **11**, 345 (1983).
8. M. L. Ogletree, D. N. Harris, R. Greenberg, M. F. Haslanger and M. Nakane, *J. Pharmac. exp. Ther.* **234**, 435 (1985).
9. H. Patscheke, Ch. Staiger, G. Neugebauer, B. Kaufmann, K. Strein, R. Endele and K. Stegmeier, *Clin. Pharmac. Ther.* **39**, 145 (1986).
10. R. M. Burch, D. E. Mais, S. H. Pepkowitz and P. V. Halushka, *Biochem. biophys. Res. Commun.* **132**, 961 (1985).
11. H. Patscheke, *Haemostasis* **10**, 14 (1981).
12. H. Patscheke and P. Wörner, *Thrombosis Res.* **512**, 485 (1978).
13. H. Patscheke, *Haemostasis* **8**, 65 (1979).
14. H. Patscheke and K. Stegmeier, *Thrombosis Res.* **33**, 277 (1984).
15. R. A. Coleman, P. P. A. Humphrey, I. Kennedy, G. P. Levy and P. Lumley, *Br. J. Pharmac.* **73**, 773 (1981).
16. O. Arunlakshana and H. O. Schild, *Br. J. Pharmac.* **14**, 48 (1959).
17. R. F. Furchgott and P. Bursztyn, *Ann. N.Y. Acad. Sci.* **144**, 882 (1967).
18. H. Patscheke, K. Stegmeier, B. Müller-Beckmann, G. Sponer, C. Staiger and G. Neugebauer, *Biomed. biochim. Acta* **43**, 312 (1984).