On the Relationship between Aggregation and Cyclic Nucleotides in Platelets Treated with Betaadrenoceptor Blocking Drugs

R. Nosáľ, V. Jančinová and M. Petríková

Institute of Experimental Pharmacology, Centre of Physiological Sciences, Slovak Academy of Sciences, Dúbravská cesta 26, 842 16 Bratislava, Czechoslovakia

Abstract. The inhibition of aggregation of rat platelets treated with betaadrenoceptor blocking drugs alprenolol, exaprolol, metipranolol, practolol and propranolol, and stimulated with adenosinediphosphate was shown to be dose-dependent. Atenolol in all concentrations potentiated the aggregation. The stimulated aggregation was potentiated by low and decreased by high concentrations of doberol. Cyclic adenosinemonophosphate was decreased in platelets treated with all the betaadrenoceptor blocking drugs used. On the other hand, a dose-dependent increase in the cyclic guanosinemonophosphate content was evident in betaadrenoceptor blocker-treated platelets. The interaction of betaadrenoceptor blocking drugs with the aggregation of blood platelets seems not to be mediated through cyclic nucleotides.

Key words: Blood platelets — Aggregation — BAB drugs — Cyclic nucleotides

Introduction

Platelet aggregation is a complex event and no sigle pathway for release and/or activity of mediators can be postulated. In studing the mechanisms regulating platelet functions and aggregation the opposite action of Ca^{2+} ions and Cyclic AMP as well as an increase in platelet cyclic GMP have been observed by several authors. The role of cyclic nucleotides in the process of aggregation has not been fully understood, and contradictory results have been published on this subject (Haslam et al. 1978a).

Many drugs are known to interfere with platelet aggregation in different manner. Acetylosalicylic acid affects prostaglandin synthesis in platelets (Vargaftig et al. 1980), lidocain and nifedipine are thought to interfere with the calcium transport in platelets (Vanhoutte and Van Neuten 1980, Vanhoutte 1981). The observations that betaadrenoceptor blocking drugs (BAB drugs) diminish the aggregability of platelets in patients with ischemic heart disease (Weksler et al.

1977) as well as that both in human and animal platelets in vitro (Rubegni et al. 1975, Nosáľ and Menyhardtová 1975, Nathan et al. 1977) make the investigation of the effect of these drugs on platelets in relation to aggregation and formation of cyclic nucleotide attractive.

Material and Methods

Blood (9 ml) was collected through a plastic catheter into Na_3-citrate (0.129 mol.l^{-1}, 1 ml) from the common carotid artery of male Wister rats (350 g) under light ether anesthesia. After centrifugation (250 × g, 20 min, 22 °C) the platelet rich plasma (PRP) was removed and the blood was centrifuged again (1000 × g, 20 min, 22 °C) to obtain platelet poor plasma (PPP). Platelet counts in PRP were determined microscopically (Brecher and Cronkite 1950) and they were adjusted with autologous PPP to 8×10^5/ml for aggregation studies and to 5×10^8/ml for cyclic nucleotide determination. Platelet aggregation was measured according to Born (1962) in a dual channel aggrometer (Chrono-log aggrometer USA) at 37 ° using 450 µl samples. The scale of the recorder was adjusted with PRP and PPP and the aggregation was induced with ADP (8×10^{-6} mol.l^{-1}).

For cAMP and cGMP determinations PRP was incubated with BAB drugs at 37 °C for 15 min. Teophylline (10^{-3} mol.l^{-1}) was added to each sample to inhibit the activity of cyclic nucleotidephosphodiesterase. The incubation of BAB drugs with PRP was terminated at 0 °C with $HClO_4$ (1 mol.l^{-1}) and the samples were further treated according to Cailla et al. (1973) and Johnson et al. (1974), including centrifugation of tubes (2000 × g at 4 °C) and neutralization of samples with 6 mol.l^{-1} K_2CO_3. After the centrifugation cAMP and cGMP were determined in samples using cAMP and cGMP assay kits (Amersham).

The cAMP-assay kit TRK 432 Amersham with a charcoal separation step gives a detection limit in ^{3}H-labelled cAMP for binding to a protein with a high specificity and affinity to cAMP. The assay for cGMP is based on the competition between an unlabelled and a fixed quantity of tritium-labelled cGMP for binding to an antiserum with a high specificity and affinity to cGMP.

The cAMP-assay kit TRK 432 Amersham with a charcoal sepatation step gives a detection limit in the range from 0.2 to 16 pmol-sample cAMP. The cGMP-ria kit TRK 500 Amersham shows a high specific activity in the range up to 8 pmol/sample and a detection limit of 0.04 pmol.

Statistics: all samples were processed in duplicate. Results were expressed as means from four to six experiments ± S.E.M. The results were evaluated by the Student's *t*-test.

Material: ADP (adenosinediphosphate): Serva, alprenolol (ALP-Hässle): o-(alkyl-phenoxy)-3-isopropylamino-2-propanol. HCl, atenolol (ATE ICI): 1-(p-carbamoylmethylphenoxy)-3-isopropyl--amino-2-propanol. HCl, doberol (DOB-Boehringer): 1-(3′-methylphenoxy)-2-hydroxy-3-isopropyl--aminopropanol). HCl, exaprolol (EXA-Institute for Drug Research Modra CSSR): 1-(2. cyclohexyphenoxy)-3-isopropylamino-2-propanol). HCl, metipranolol (MET-Spofa): 1-(2,3,5-trimethyl-4-acetoxyphenoxy)-3-isopropylamino-3-propanol) tartarate, oxprenolol(OXP-Ciba): 1-(o--alyloxy-phenoxy)-3-isopropylamino-2-propanol).HCl, practolol(PRA-ICI): 4′-2-(hydroxy-3-isopropylaminopropane-acetanilide).HCl, propranolol(PRO-ICI): 1-isopropylamino-3-(naphtoxy-2-propanol) HCl. All other chemicals were of analytical grade from commercial sources.

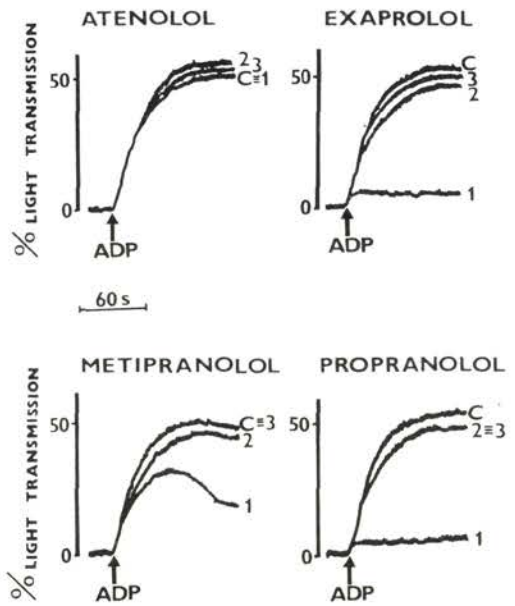

Fig. 1. The effect of atenolol, exaprolol, metipranolol and propranolol in concentrations of 10^{-3} (1), 10^{-4}(2) and 10^{-5} (3) mol.l^{-1} (preincubation 5 min at 37 °C) on rat platelet aggregation induced with ADP (8×10^{-6} mol.l^{-1}). C-control.

Results

For aggragation studies PRP was equilibrated in aggregometer at 37° for 1 min. The amplitude of the aggregation curves (baseline) was not changed when BAB drugs were added to PRP.

Fig. 1 illustrates the dose-dependent modulation of the aggregation curves of rat PRP pretreated (5 min at 37 °C) with atenolol, exaprolol, metipranolol, or propranolol. The control curves represent aggregation induced with ADP alone. ATE in concentrations of 10^{-5} and 10^{-4} mol.l^{-1} potentiated the ADP-induced aggregation of PRP, while 10^{-3} mol.l^{-1} had no effect. Similar effects were observed after 0.5; 1; 5; and 15 min of preincubation. EXA in concentrations of 10^{-5} and 10^{-4} mol.l^{-1} slightly inhibited the aggregation; the addition of 10^{-3} mol.l^{-1} resulted in a complete inhibition of aggregation. MET decreased the effect of ADP in concentrations of 10^{-3} and 10^{-4} mol.l^{-1} resulting in diminution of the amplitude of the aggregation curves. The latter drug was ineffective in a concentration of 10^{-5} mol.l^{-1}. ADP-induced aggregation of platelets was blocked by PRO in a concentra-

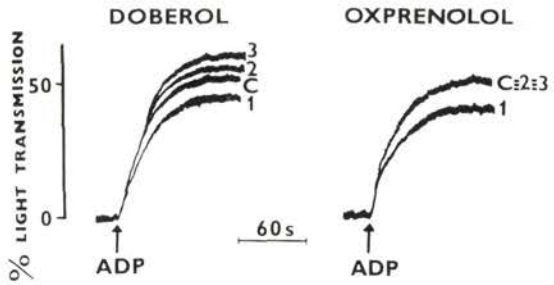

Fig. 2. The effect of doberol and oxprenolol in concentrations of 10^{-3}(1), 10^{-4}(2) and 10^{-5}(3) mol.l^{-1} (preincubation 5 min at 37 °C) on rat platelet aggregation induced with ADP (8×10^{-6} mol.l^{-1}). C — controls.

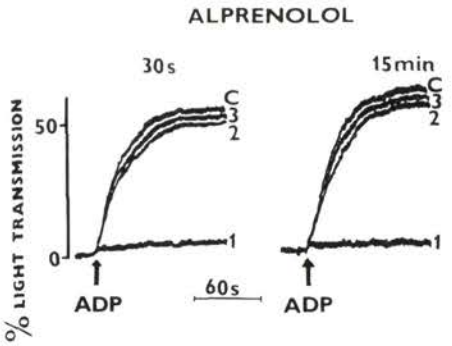

Fig. 3. The effect of preincubation with alprenolol for 0.5 and 15 min on rat platelet aggregation stimulated with ADP (8×10^{-6}mol.l^{-1}) at 37 °C. Alprenolol concentration: 1 — 10^{-3} mol.l^{-1}; 2 — 10^{-4}mol.l^{-1}; 3 — 10^{-5} mol.l^{-1}1. C — control.

tion of 10^{-3} mol.l^{-1} and it proportionally decreased after the addition of 10^{-5} and 10^{-4} mol.l^{-1}, respectively.

The effect of practolol was similar to that of metipranolol (see Fig. 1). When added in concentrations of 10^{-4}; 10^{-5}, and 10^{-3} mol.l^{-1}, practolol caused a decrease in the aplitudes of the aggregation curves to 45 % and 40 % respectively. The control value was 51 % of light transmission.

Fig. 2 shows the effect of DOB and OXP preincubation for 5 min on ADP-induced aggregation in PRP. DOB in concentrations of 10^{-4} and 10^{-5} mol.l^{-1} potentiated ADP-induced aggregation whereas a concentration of 10^{-3} mol.l^{-1} resulted in a clear decrease in the amplitude of the aggregation curve. OXP was ineffective in concentrations of 10^{-4} and 10^{-5} mol.l^{-1}; in a concentration of 10^{-3} mol.l^{-1} it decreased the amplitude of the aggregation curve from 52 to 41 % of light transmission.

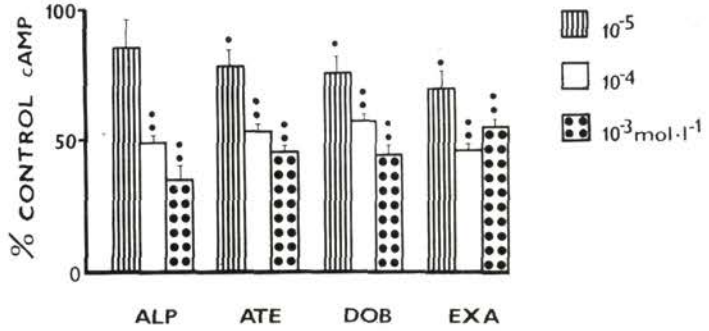

Fig. 4. Dose-dependent effect of alprenolol (ALP), atenolol (ATE), doberol (DOB) and exaprolol (EXA) on cAMP level in rat platelets treated 15 min at 37 °C. Each value is the mean from 6 experiments ± S.E.M. ●p ≦ 0.05, ●●, p ≦ 0.01,

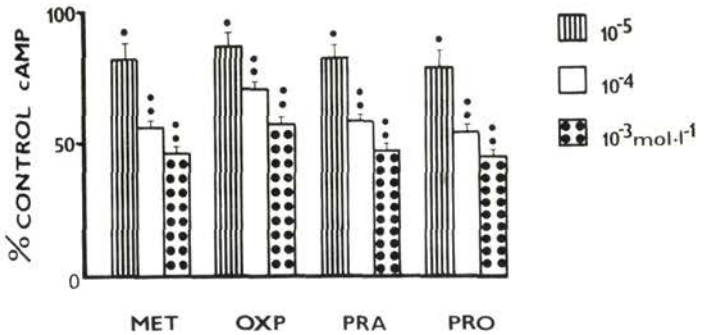

Fig. 5. Dose-dependent effect of metipranolol (MET), exprenolol (OXP), practolol (PRA) and propranolol (PRO) on cAMP level in rat platelets treated 15 min at 37 °C. Each value is the mean from 6 experiments ± S.E.M. ●p ≦ 0.05, ●●p ≦ 0.01

The effect of ALP (10^{-5} to 10^{-3} mol.l^{-1}) on ADP-induced aggregation is shown in Fig. 3. It is evident that the dose-dependent inhibition of aggregation was time-limited and the response of platelets to ADP after 0.5 and 15 min preincubation with ALP was identical. Similar effects were observed for all the BAB drugs used: identical responses were recorded after preincubation for 0.5; 1; 5; and 15 min. This indicated that the effect of BAB drugs on platelets, resulting either in potentiation or inhibition of aggregation, is very fast (30 s) and remains unchaged after 15 min of exposure.

The cAMP content in untreated platelets after 15 min of incubation at 37 °C was 18.3 ± 1.34 pmol/10^9 platelets. Fig. 4 and 5 show the level of cAMP in platelets treated with BAB drugs at 37 °C for 15 min, expressed as relative values. With the

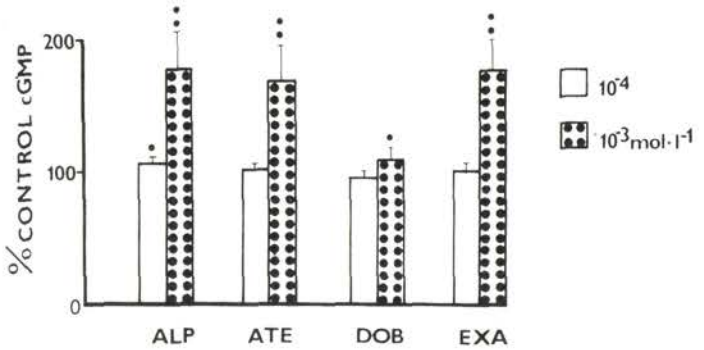

Fig. 6. The effect of alprenolol (ALP), atenolol (ATE), doberol (DOB) and exaprolol (EXA) in concentrations of 10^{-4} and 10^{-3} mol.l^{-1} on cGMP level in rat platelets treated 15 min at 37 °C. Each value is the mean from 4 experiments ± S.E.M. ●p ≦ 0.05, ●●p ≦ 0.01.

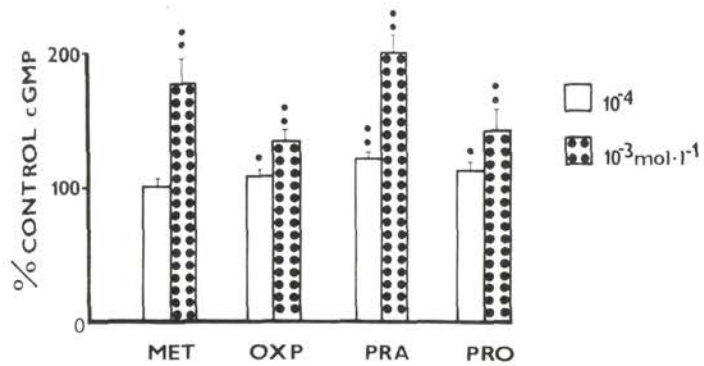

Fig. 7. The effect of metipranolol (MET), oxprenolol (OXP), practolol (PRA) and propranolol (PRO) in concentrations of 10^{-4} and 10^{-3} mol.l^{-1}on cGMP level in rat platelets treated 15 min at 37 °C. Each value is the mean from 4 experiments ± S.E.M. ●p ≦ 0.05, ●●p ≦ 0.01.

exception of ALP, 10^{-5} mol.l^{-1} of all other BAB drugs used caused a decrease in the cAMP level in platelets. Of all the BAB drugs tested the most effective was EXA which decreased, in the above concentration, the cAMP content to 70 % of the control value. The addition of 10^{-4} mol.l^{-1} of this drug resulted in a further significant decrease in cAMP. The most effective was ALP (46 % of the control value), the least effective was OXP (72 % of the control value). A further raising of the concentration of BAB drugs to 10^{-3} mol.l^{-1} resulted in a decline of the cAMP content ranging between 35 % (ALP) and 59 % (OXP) of the control value.

The effect of BAB drugs on the cGMP content in untreated rat platelets (controls) after 15 min of incubation at 37 °C was 1.68 ± 0.38 pmol/10^9 platelets.

In a concentration of 10^{-5} mol.l^{-1} ALP, ATE, DOB, EXA, MET, OXP and PRO did not influence the cGMP level in platelets. PRA was the only drug to increase significantly the cGMP content (to 112 %).

In a concentration of 10^{-3} mol.l^{-1} all the BAB drugs used significantly increased the cGMP content. The most effective was PRA (204 % of the control value), the least active of all the BAB drugs was DOB (111 % of the control value). A significant increase in cGMP was observed in platelets treated with 10^{-4} mol.l^{-1} ALP, OXP, PRA or PRO. The highest content of cGMP in platelets treated with BAB drugs (10^{-3} mol.l^{-1}) was as follows (in pmol/10^9 platelets): PRA (3.39), ALP (3.16), EXA (3.01), MET (2.96), ATE (2.89), PRO (2,44), OXP (2.30) and DOB (1.86).

Discussion

The thrombocyte shows several advantages as a model for the analysis of biochemical changes associated with pharmacological effects. Platelets may be obtained in a pure suspension (free from other cells) and they respond to a number of various agonists. They show two basic types of reaction: shape changes with aggregation and release of their granule constituents; the latter is analogous to the secretion by exocytosis in other cells.

Aggregation of platelets induced with ADP may be influenced by BAB drugs. As it is evident from the present results three types of interaciton may occur. First, drugs like ATE potentiate aggregation. Inhibitory drugs inhibit aggregation in a dose-dependent manner; ALP, EXA, MET, OXP, PRA and PRO all show the second type of interaction. Finally, DOB in lower concentrations (10^{-4} and 10^{-5} mol.l^{-1}) potentiates and in a higher concentration (10^{-3} mol.l^{-1}) inhibits aggregation; thus it represents the third type.

The mechanism by which ADP induces aggregation is not known. Some ADP receptors and ADP membrane binding proteins have been identified (Bennet et al. 1978; Adler and Handin 1979). In addition, ADP decreases the elevated cAMP content in platelets probably by inhibiting adenylate cyslase (Barber 1976) and the principal role of calcium in ADP-induced platelet aggregation has been suggested (Nosáľ and Menyhardtová 1975; Shattil and Benett 1981).

The interaction of BAB drugs with ADP-induced platelet aggregation may be of different origin. All these drugs alter the cyclic nucleotide level in platelets. The decrease in cAMP was dose-dependent: the higher the dose of BAB drugs, the lower the level of cAMP. On the other hand, in equimolar concentrations these drugs increased the level of cGMP. It can be suggested that such changes result from an altered synthesis rather than from degradation of cyclic nucleotides, since the concentration of theophylline in the samples was sufficiently high to block phosphodiesterase.

Comparing the effect of BAB drugs on rat platelets no relationship between the interference with aggregation and the level of cyclic nucleotides can be suggested. This is also supported by the observation that the selective $beta_1$ antagonists atenolol and practolol have opposite activities on aggregation and parallel effects on cyclic nucleotides. The increase in cGMP observed in platelets treated with BAB drugs does not explain the increase in cGMP during platelet aggregation (Haslam et al. 1978 b).

Platelets form a bidirectional system in that, in addition to stimulatory antagonists, they also respond in a negative way by reversal of inhibition of aggregation and inhibition of the release reaction in the presence of certain inhibitory agonists. Substantial evidence exist that beta adrenergic agonists act by increasing the platelet cyclic AMP concentration (Haslam 1975). On the contrary, some evidence suggest, that the effect of BAB drugs on platelets might be a result of a non-specific rather than a specific interaction that involves the interference with the beta adrenergic receptor (Grobecker et al. 1973). Such an effect was also demonstrated in red cells, nerves, the skeletal and cardiac muscle (Lucchesi and Iwami 1968; Langslet 1970; Godin et al. 1976).

The opposite effect of BAB drugs on aggregation and cyclic nucleotide formation in platelets observed in our experiments may be explained in the light of recent findings. The importance of calcium in regulating a variety of platelet physiological processes is well known. Calcium modulates the aggregation as well as the activity of the adenylate cyslase (Rodan and Feinstein 1976) and decreases the fluidity of membrane phospholipids (Lüsher and Massini 1975). BAB drugs may interfere with stimulated aggregation by affecting the availability of calcium ions in platelets. Propranolol displaces calcium ions from their membrane sites and inhibits lipid-facilitated transport of calcium inside the cell (Seeman 1966; Nayler 1966). Moreover propranolol interacts with prostaglandin synthesis thus inhibiting formation of thromboxane A_2 which is considered as intracellular calcium ionophore in platelets. This interaction is located at the level of phospholipase A_2 activation in the platelet membrane (Dachary-Prigent et al. 1979).

The interaction of BAB drugs with the synthesis of cyclic nucleotides seems to be of unspecific origin. Owing to their physico-chemical properties BAB drugs interact with membrane phospholipids resulting in their changed fluidity (Godin et al. 1976). Moreover membrane phospholipids directly modify the activity of different enzymes which are an integral part of the protein structure of membranes. Thus the drug-stimulated increase or decrease in membrane phospholipide fluidity results in the activation or inhibition of several membrane enzymes (Mc Elhaney 1982) including adenylate cyclase (Klein et al. 1978; Dipple and Honslay 1978) and Mg^{2+}, Na^+, K^+ ATPases.

Based on our results it can be suggested that the interaction of BAB drugs with platelets results in a change of the membrane fluidity. The perturbation in

phospholipids is followed by such a membrane calcium availability that limits the triggering of aggregation. The dose-dependent increase and/or decrease in cyclic nucleotides opposite for cAMP and cGMP may result from the effect of BAB drugs on both synthetizing enzymes which may be modulated by the change in the fluidity of platelet membranes. This hypothesis should be proved by measuring the activity of adenylate and guanylate cyclases in platelets provided that the degradation by phosphodiesterase in experiments with theophylline will be prevented.

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