Biochimica et Biophysica Acta 821 (1985) 217–228
Elsevier

BBA 72772

The interaction of β-adrenoceptor blocking drugs with platelet aggregation, calcium displacement and fluidization of the membrane

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(Received February 22nd, 1985)
(Revised manuscript received June 19th, 1985)

Key words: Platelet aggregation; β-Adrenergic blocking drug; Calcium displacement; Membrane fluidity; ESR

β-Adrenoceptor blocking drugs interfere with adenosine diphosphate-stimulated platelet aggregation. Alprenolol, exaprolol, Kö 1124 and propranolol inhibited the aggregation, metipranolol decreased the extent and rate of aggregation significantly. Atenolol potentiated the aggregation measured by amplitude significantly. The interaction of β-adrenoceptor blocking drugs with aggregation correlated with the displacement of calcium ions from binding sites in isolated platelets and the fluidization of the whole platelets and isolated platelet membrane as measured with electron spin resonance of the spin probe. The most potent were highly liposoluble drugs alprenolol, exaprolol, metipranolol and propranolol which increased the calcium displacement and membrane fluidity, the least active was atenolol decreasing these phenomena. The inhibition by β-adrenoceptor blocking drugs of stimulated platelet aggregation is rather a result of unspecific than specific receptor interaction.

Introduction

Blood platelet aggregation plays an important role in many pathologic processes, ischemic heart disease being the most important one [1]. The benefitial effect of β-adrenoceptor blocking drugs (BAB drugs) in chronic ischemic heart disease has been generally accepted [2]. There is sufficient evidence that some β-adrenoceptor blocking drugs decrease stimulated platelet aggregation in vitro as well as in vivo [3–6]. We found that β-adrenoceptor blocking drugs interfere with ADP stimulated aggregation in three different ways [6]. Since the cyclic nucleotides play an important role in the regulation of platelet function [7], platelet aggregation is usually associated with changes in their level [8]. No relationship was found between the effect of β-adrenoceptor blocking drugs on ADP-stimulated platelet aggregation and the cAMP-cGMP levels in platelets [6]. The non-specific, β-receptor-independent interaction of β-adrenoceptor blocking drugs with platelet aggregation was proposed as a result of direct effect of β-adrenoceptor blocking drugs on negatively charged phospholipids in the membrane [9]. Studies with model phospholipid membrane clearly demonstrated that the amphiphilic β-adrenoceptor blocking drugs penetrate into the lipid bilayer [9–12]. It has been suggested that the potency of amphiphilic cationic drugs to inhibit platelet aggregation could correlate with their ability to increase membrane fluidity [13], and that the inhibition of aggregation may result from changes in calcium binding in the membrane [14].

The aim of our present study was to investigate the effect of different β-adrenoceptor blocking drugs on stimulated platelets in relation to membrane calcium displacement and platelet membrane fluidity.

Materials and Methods

Chemicals. Adenosine diphosphate and chlortetracycline (aureomycin): Serva (Heidelberg, F.R.G.). The chemical structure of the investigated β-adrenoceptor blocking drugs and their producers are shown in Table I. All drugs were used as hydrochlorides except metipranolol which was used as tartarate. Stearic acid spin label with dimethyloxazolidinyl group at the 5th and 16th carbon positions were from Syva (Palo Alto, U.S.A.). These will be refered to as I(12, 3) and I(1, 14), respectively. All other chemicals were of analytical grade from commercial sources.

Platelet preparation. Blood (9 ml) was collected through a polyethylene catheter from the common carotid artery of male Wistar rats (350 g) in light ether anesthesia into plastic tubes with 1 ml of 0.129 mol/l trisodium citrate. Platelet rich plasma was prepared by a 15 min centrifugation ($350 \times g$) at room temperature. Platelets were separated by subsequent centrifugation ($1000 \times g$) for 15 min at 22°C and platelet poor plasma was decanted. For ESR measurement the platelet pellet was resuspended in 9 ml of calcium-free Tyrode buffer pH 7.4, with 5 mmol/l EDTA. The platelets were washed twice in calcium-free Tyrode buffer by centrifugation ($1000 \times g$, 15 min, room temperature) and were resuspended in 1 ml/sample of calcium-free Tyrode buffer and β-adrenoceptor blocking drugs were added to obtain a 5 mmol/l concentration of the drug in the sample. After centrifugation (as above) the supernatant was decanted and the pellet resuspended in 130 μl of supernatant. In the samples taken for ESR studies, the number of platelets was $2.3 \cdot 10^7/\mu l$.

Platelet count. The number of platelets in platelet-rich plasma was determined in a Thrombocounter C (Coulter Electronic Ltd, Luton, U.K.). The platelet count in isolated and washed platelet samples was determined microscopically [15].

Platelet aggregation. The number of platelets in platelet rich plasma was adjusted with autologous platelet poor plasma to approximately $8 \cdot 10^5/\mu l$. Samples (450 μl) were preincubated for 3 min at 37°C and followed by incubation with β-adrenoceptor blocking drugs (20 μl) for 5 min. The aggregation was induced with ADP (8 μmol–20 μl) and measured according to Born [16], in a dual channel Chrono-log aggregometer (Chrono-log Corp., Haverton, PA, U.S.A.).

The extent (amplitude) and the rate of aggregation were evaluated from aggregation curves by means of a monoexponential equation

$$A = A_{max}((1 - \exp(-kt))) \quad (1)$$

where A and A_{max} are values of amplitude of aggregation curves in (cm) at given time and t_∞, t is time (s), and k is the rate constant of aggregation (s^{-1}). The data were fitted on Hewlett-Packard 9866 (USA-FRG) by use of nonlinear regression programme. The significance of calculated data was estimated according to Boxenbaum et al. [17] from the equation

$$t = \frac{A_{ij} - A_{ik}}{\left((SD_{ij})^2 + (SD_{ik})^2\right)^{1/2}} \quad (2)$$

where A_{ij} is the computer-estimated ith parameter in study j, A_{ik} is the computer-estimated ith parameter in study K, SD_{ij} is the asymptotic standard deviation of the ith computer-estimated parameter from study j, and SD_{ik} is the asymptotic standard deviation of the ith computer-estimated parameter from study k.

Platelet membrane preparation. Membranes from platelets were isolated according to Langer et al. [18]. Platelet rich plasma was isolated from 80 rats and platelets were washed after centrifugation (see above) two times in washing buffer (Tris-HCl 50 mmol/l, NaCl 150 mmol/l, EDTA 20 mmol/l (pH 7.4), $300 \times g$, 10 min, 4°C). Membranes were prepared by lysing and ultrasonication of platelets in low-ionic-strength buffer (Tris-HCl 5 mmol/l, EDTA 5 mmol/l (pH 7.4), Polytron speed 5, 15 s) and centrifuged at $30000 \times g$ for 10 min at 4°C. The pellet was resuspended in equal volume of after-lysing buffer (Tris-HCl 70 mmol/l, pH 7.4) and washed twice by centrifugation ($30000 \times g$, 10 min, 4°C). Isolated membranes were concentrated by centrifugation and stored at −80°C in incuba-

tion buffer (Tris-HCl 67 mmol/l, NaCl 160 mmol/l, KCl 6.7 mmol/l (pH 7.4)).

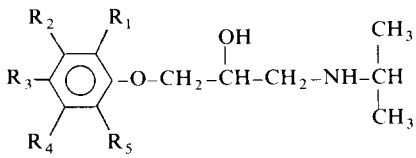
A sample was taken from each membrane preparation for electron microscopy. For ESR measurement samples of 0.9 mg protein per 40 μl sample (measured according to Lowry et al. [19]) were taken.

Electron microscopy. A sample of 50 μl from isolated membranes was dispersed in 1% agar (Difco) at a ratio 1 : 1 (v/v) and fixed for 2 h in glutaraldehyde (0.299 mol/l) and 1 h in OsO_4 (0.0393 mol/l) both in phosphate buffer. After embedding in Durcupan ACM (Fluka) ultrathin sections were contrasted with uranyl acetate and lead citrate (Serva), and observed in an electron microscope BS 500 (Tesla, Brno, C.S.S.R.).

Calcium displacement in isolated platelets. The method of Le Breton et al. [20] was adapted. Samples with platelet-rich plasma were incubated with chlortetracycline in a final concentration of

TABLE I

CHEMICAL STRUCTURE, ABBREVIATIONS AND PRODUCERS OF THE INVESTIGATED BETA-ADRENOCEPTOR BLOCKING DRUGS



Drug (Abbreviation)	Chemical structure	Producer
Alprenolol (ALP)	$R_1 = -CH_2-CH=CH_2$ $R_2 = R_3 = R_4 = R_5 = H$	Hässle, Hälsingborg, Sweden
Atenolol (ATE)	$R_1 = R_2 = R_4 = R_5 = H$ $R_3 = -CH_2-CO-NH_2$	ICI, Alderley Park, Cheshire, U.K.
Practolol (PRA)	$R_1 = R_2 = R_4 = R_5 = H$ $R_3 = -NH-CO-CH_3$	
Propranolol (PRO)	$R_1-R_2 = C_4H_4$ $R_3 = R_4 = R_5 = H$	
Doberol (DOB)	$R_1 = R_3 = R_4 = R_5 = H$ $R_2 = -CH_3$	Boehringer, Ingelheim, F.R.G.
Kö 1124 (Kö 1124)	$R_1 = R_3 = R_4 = R_5 = H$ $R_2 = -CH(CH_3)-CH_2-CH_3$	
Exaprolol (EXA)	$R_1 = C_6H_{12}$ $R_2 = R_3 = R_4 = R_5 = H$	Inst. for Drug Res., Modra, CSSR
Metipranolol (MET)	$R_1 = R_2 = R_4 = -CH_3$ $R_3 = -O-CO-CH_3$ $R_5 = H$	Spofa Works Praha, CSSR
Oxprenolol (OXP)	$R_1 = -O-CH_2-CH=CH_2$ $R_2 = R_3 = R_4 = R_5 = H$	Ciba-Geigy, Basel, Switzerland

50 μmol/l for 30 min at 25°C. After centrifugation (1000 × *g*, 10 min, 22°C) platelets were resuspended in calcium-free Tyrode buffer (NaCl 136.9 mmol/l, KCl 2.7 mmol/l, $NaHCO_3$ 11.9 mmol/l, $NaH_2PO_4 \cdot H_2O$ 0.36 mmol/l, $MgCl_2 \cdot 6\ H_2O$ 1 mmol/l, EDTA 5 mmol/l (pH 7.4)) to yield a final amount of approx. 10^6 platelets/μl of sample. Tubes (500 μl) were placed in a model 203 spectrofluorometer (Perkin Elmer Ltd., Beaconsfield, U.K.) and the initial fluorescence was read at 380 and 520 nm from the scale.

ESR measurements. In experiments with platelets, 10 μg of the spin label was layered onto the bottom of a plastic vial by evaporating an ethanol solution of the label. 50 μl of platelet suspension were added to the label and gently vortexed for 1 min. Thereafter the suspension was filled into a silanized glass capillary and equilibrated at temperatures for 4 min before measuring the ESR spectrum.

In experiments with isolated platelet membranes the spin probe in the amount of 7.5 μg was layered onto the bottom of the plastic vial by evaporating ethanol solution. The platelet membrane suspension with 0.9 mg of protein (40 μl)/sample and 10 μl of β-adrenoceptor blocking drug in Tyrode solution were added to the spin probe and vortexed for 1 min.

To increase the solubility of samples with exaprolol, metipranolol and practolol 6 mmol/l HCl was added. The final concentration of the β-adrenoceptor blocking drugs in the sample was 10 mmol/l.

All samples were kept frozen at −20°C for 1–3 days. Before measurement the samples were thawed, vortexed for 1 min and the suspension was filled into a glass capillary (i.d. 1 mm). The samples were equilibrated at temperatures indicated in Results for 4 min before measuring the ESR spectra.

The ESR spectra of spin probes in the platelets and isolated platelet membranes were recorded in an ESR-230 X-band spectrometer (G.D.R.) using 100 kHz modulation technique. Typical instrumental settings were: 5 mW microwave power and 0.08 mT modulation amplitude.

TABLE II

THE EFFECT OF β-ADRENOCEPTOR BLOCKING DRUGS ON THE AMPLITUDE (A_{max}) AND RATE (s^{-1}) OF AGGREGATION CURVES OF PLATELET-RICH PLASMA STIMULATED WITH ADP AS EVALUATED WITH A MONOEXPONENTIAL EQUATION

The significance of values was calculated according to Eqn. 2.

Expt. I	Drug Concn. (mol/l)	Atenolol Control 10^{-3}	Atenolol 10^{-4}	Practolol Doberol 10^{-3}	Oxprenolol 10^{-3}	Metipranolol 10^{-3}	Alprenolol Exaprolol Kö 1124 Propranolol 10^{-3}
A_{max} (cm)		5.2 ±0.06	5.5 ±0.06 **	5.1 ±0.09 **	4.7 ±0.1 **	2.8 ±0.16 **	0
k (s^{-1})		0.9 ±0.04	0.96±0.04	1.01±0.07	0.8 ±0.06	0.64±0.1 *	0
Expt. II A	Drug Concn. (mol/l)	Atenolol 10^{-5}	Atenolol 10^{-4}	Atenolol Control 10^{-3}			
A_{max} (cm)		5.9 ±0.06 **	5.5 ±0.06 **	5.2 ±0.06			
k (s^{-1})		0.96±0.03	0.96±0.04	0.91±0.04			
Expt. II B	Drug Concn. (mol/l)	Control	Propranolol 10^{-5}	Propranolol 10^{-4}	Propranolol 10^{-3}		
A_{max} (cm)		5.5 ±0.04	4.9 ±0.05 **	4.5 ±0.03 **	0		
k (s^{-1})		1.2 ±0.03	1.0 ±0.03 **	0.78±0.02 **	0		

* $P \leq 0.05$.
** $P \leq 0.01$.

Membrane fluidity. To estimate the relative efficiency of the β-adrenoceptor blocking drugs in perturbation of the platelet membrane, the order parameter S was calculated from the ESR spectra of spin label I(12, 3) incorporated into the membrane, the inner hyperfine splitting ($A'_{\perp}$) and polarity correction were estimated according to Gaffney [22]. In measurements where only one of the outer ($A'_{\parallel}$) or inner hyperfine splittings ($A'_{\perp}$) were used, relative order parameters $S_{\parallel}$ or $S_{\perp}$ were calculated according to Gordon and Sauerheber [37] from the $A'_{\parallel}$ or $A'_{\perp}$ values. The parameters S, $S_{\parallel}$ and $S_{\perp}$ represent values between 0 and 1. These extreme order parameters indicate that the spin probe samples fluidize and immobilize environments, respectively. The lower the S, $S_{\parallel}$ or $S_{\perp}$, the higher the disorder 'fluidity' of the hydrophobic part of the membrane. Parameter $A'_{\perp}$ is linearly proportional to the order parameter of spin probe in the membrane [21,22]. The higher the $A'_{\perp}$, the lower the order parameter and the higher the fluidity of the membrane. Because the motion of the spin probe I(1, 14) is not sufficiently anisotropic to allow resolution of the outer hyperfine splitting, in addition to parameter $A'_{\perp}$ the rotational correlation times τ_1 and τ_2 were evaluated from the ESR spectra [38,39] according to the formulae:

$$\tau_1 = 6.5 \cdot 10^{-10} \Delta H(0)\left[(h_0/h_{-1})^{1/2} - (h_0/h_{+1})^{1/2}\right]$$

$$\tau_2 = 6.5 \cdot 10^{-10} \Delta H(0)\left[(h_0/h_{-1})^{1/2} + (h_0/h_{+1})^{1/2} - 2\right]$$

where $H(0)$ is the peak-to-peak linewidth of the central field line and h_0, h_{-1} and h_{+1} are the peak-to-peak amplitudes of the first derivatives resonance of the central, high- and low-field peaks, respectively.

It is suggested from the similarity of these two values that the spin probe undergoes isotropic rotational diffusion. If these two values are substantially dissimilar, it is supposed that the spin probe undergoes anisotropic rotational diffusion [38]. To prove it we have used the value of $\tau_2 - \tau_1$, estimating the relative influence of the β-adrenoceptor blocking drugs on the degree of the probe I(1, 14) in the membrane.

The membrane polarity environment of the spin probes was evaluated as isotropic splitting constant A_o for the probe I(12, 3), $A_o = (A'_{\parallel} + 2A_{\perp})/3$, where $A_{\perp}$ was corrected according to Gaffney [22]. The constant A_o for probe I(1, 14) was evaluated directly from the spectrum as the distance between low and middle magnetic lines.

Results

Effect of β-adrenoceptor blocking drugs on ADP-stimulated platelet aggregation

Fig. 1 demonstrates representative aggregation curves from the structure-dependent relationship between β-adrenoceptor blocking drugs and ADP-stimulated aggregation. As evident from the figure, alprenolol, exaprolol, Kö 1124, metipranolol and propranolol visibly inhibited the aggregation induced with ADP. Doberol, oxprenolol and practolol have a less pronounced effect. Atenolol (10^{-4} mol/l) potentiated the stimulation by ADP.

The dose-dependent effect of atenolol and propranolol on ADP-stimulated aggregation is demonstrated in Fig. 2. Atenolol (A) seems to potentiate the aggregation. The concentration 10^{-3} mol/l was ineffective as compared with 10^{-5} mol/l. On the other hand propranolol (10^{-3} mol/l, B) blocked and significantly decreased (10^{-4} mol/l) the ADP-induced aggregation.

Table II summarizes the calculated parameters

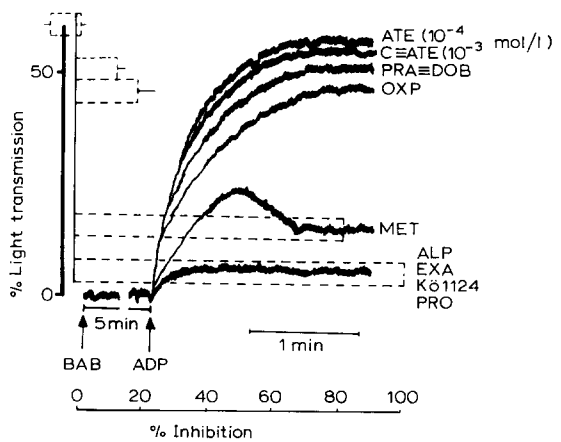


Fig. 1. The effect of β-adrenoceptor blocking (BAB) drugs on ADP (8 μmol/l) stimulated platelet aggregation. ALP, alprenolol; ATE, atenolol; DOB, doberol; EXA, exaprolol; MET, metipranolol; OXP, oxprenolol; PRA, practolol; PRO, propranolol; all in the concentration 10^{-3} mol/l, and ATE in a concentration of 10^{-4} mol/l. C, control (ADP only).

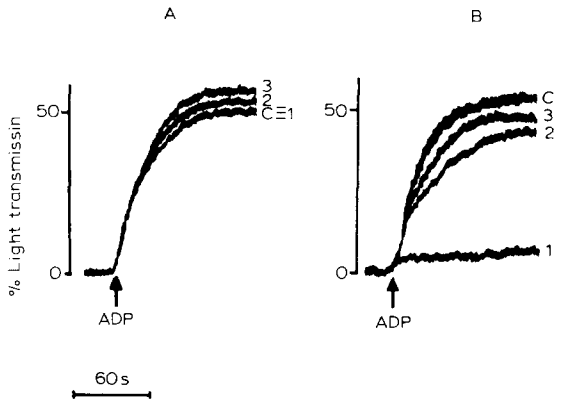


Fig. 2. The dose-dependent effect of atenolol (A) and propranolol (B) on platelet aggregation induced with ADP (8 μmol/l). Drug concentration used: 1, 10^{-3} mol/l; 2, 10^{-4} mol/l; 3, 10^{-5} mol/l; C, control (ADP only).

for amplitude and rate constant of aggregation curves. In experiment 1 the amplitude was significantly decreased for metipranolol (2.8 ± 0.16 cm), oxprenolol (4.7 ± 0.1 cm), practolol and doberol (5.1 ± 0.09 cm), all in the concentration of 10^{-3} mol/l as compared with the control. Alprenolol, exaprolol, Kö 1124 and propranolol blocked the aggregation induced with ADP, yet metipranolol significantly decreased the rate constant in comparison with control. As evident, in Expt. 2A atenolol increased the amplitude of aggregation curves significantly for concentrations 10^{-4} and 10^{-5} mol/l, respectively. This increase was dose-dependent. In Expt. 2B propranolol (10^{-3} mol/l) blocked both the amplitude and the rate of aggregation, and significantly inhibited (10^{-4} and 10^{-5}

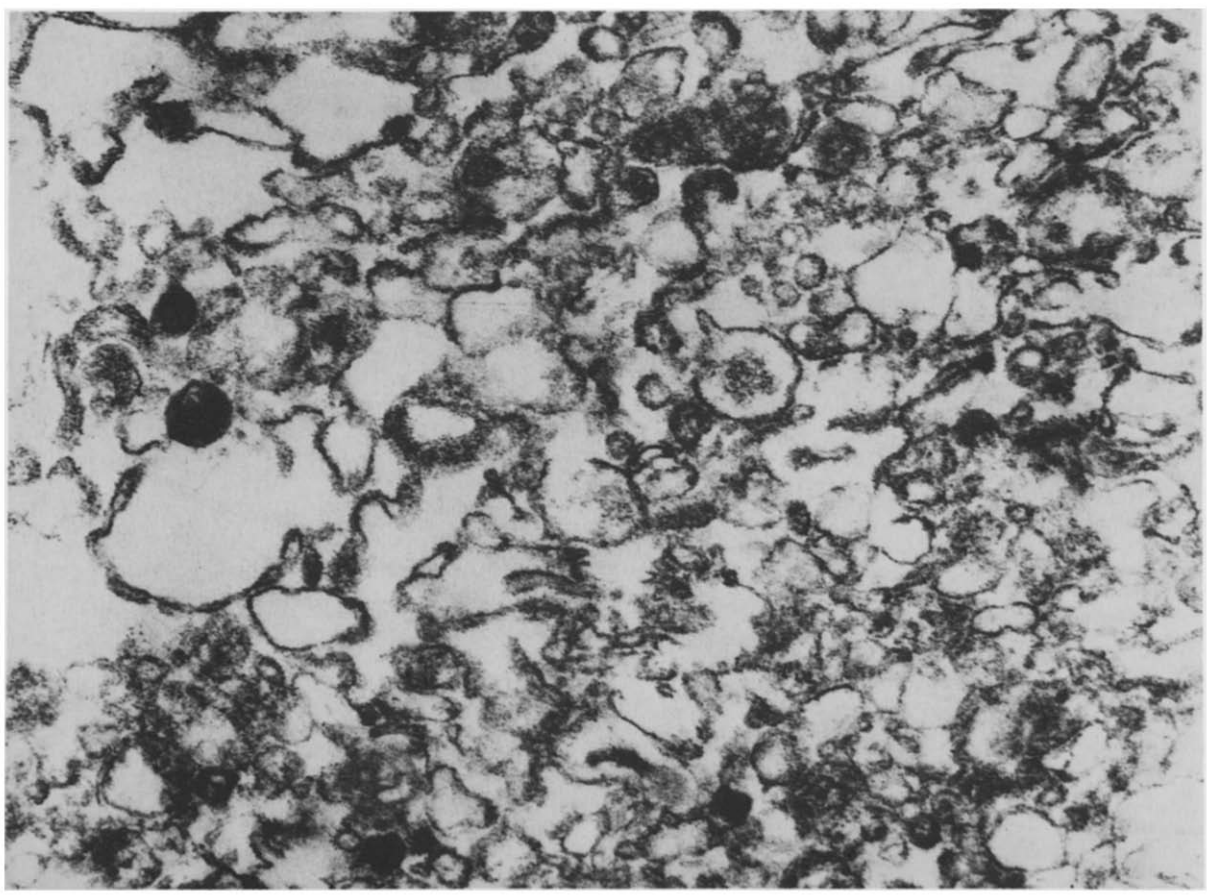


Fig. 4. The ultrastructure of isolated rat platelet membranes contrasted with lead citrate and uranyl acetate under the electron microscope. Plasma membranes are contaminated with rare dense bodies and mitochondrial membranes. Magnification 45000×.

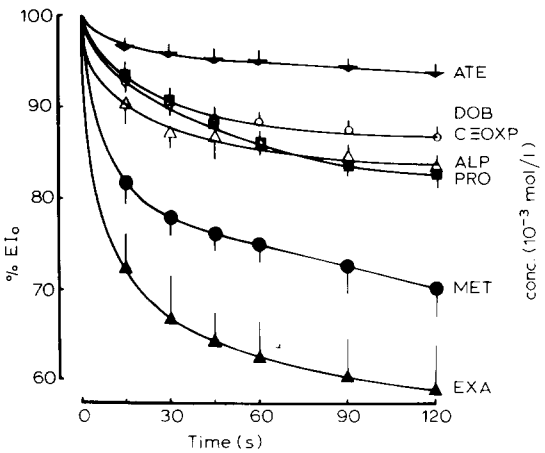


Fig. 3. The fluorescence emission intensity (EI) of the chlortetracycline-Ca^{2+} bound complex in platelets treated with β-adrenoceptor blocking drugs (10^{-3} mol/l) at 22°C. ALP, alprenolol; ATE, atenolol; DOB, doberol; EXA, exaprolol; MET, metipranolol; OXP, oxprenolol; PRO, propranolol. n = 6, $\bar{x} \pm$S.E. expressed as % EI_0.

mol/l) both values as compared with control.

Fig. 3 demonstrates the time-dependent change in fluorescence of the Ca^{2+}-chlortetracycline complex in platelets treated with β-adrenoceptor blocking drugs. The emission from the control cells declined by about 10% after 120 s of the initial value. Atenolol significantly inhibited the spontaneous decline in fluorescence intensity. Alprenolol and propranolol at the same concentration produced a comparable decrease in fluorescence, but doberol and oxprenolol had no effect. Metipranolol and exaprolol induced a very fast decline in the fluorescence of the Ca^{2+}-chlortetracycline complex, with the emission falling within the first 15 s to 82 and 72% of the starting value, respectively. This rapid decline was followed in the second phase by a small further decrease in fluorescence. The final fluorescence intensity was decreasing after 2 min in the rank order of potency: atenolol < control = oxprenolol = doberol < propranolol = alprenolol < metipranolol < exaprolol.

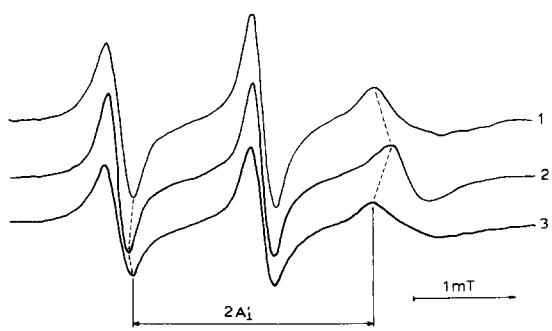


Fig. 5. Representative ESR spectra of spin probe I(1, 14) incorporated into isolated platelet membranes at 35°C 1, control; 2, exaprolol (10 mmol/l); 3, atenolol (10 mmol/l).

ESR measurements

The temperature dependence of the order parameters $S_{\parallel}$ and $S_{\perp}$ of the spin labels I(12, 3) and I(1, 14) incorporated into the platelets was investigated in the range between 20 to 37°C. The Arrhenius-type plots of order parameters are shown in Fig. 6. The dotted line represents the linear regression line obtained with the label I(12, 3) in human platelets by Sauerheber et al. [40]. It is noteworthy that the membrane lipid order parameter at the 5th carbon depth of stearic acid spin label is within experimental error, the same for both human and rat blood platelets. Furthermore, the absolute values of order parameter S in the 16th carbon depth of the platelet membrane are lower in comparison with S observed in the 5th carbon depth of membrane. This observation is consistent with the well known

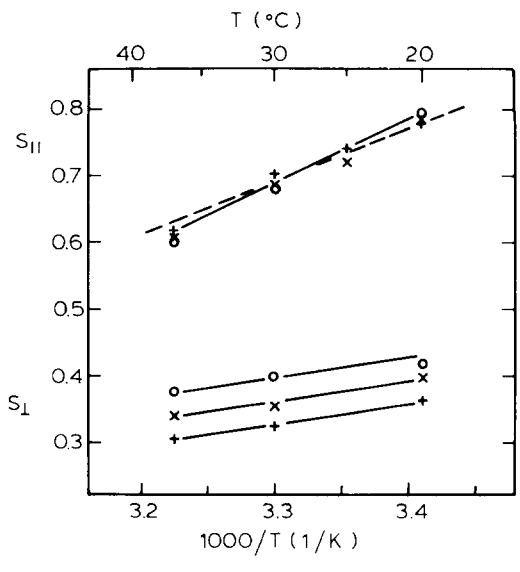


Fig. 6. Temperature dependence of the order parameters $S_{\parallel}$ (top) of spin probe I(12, 3) and $S_{\perp}$ (bottom) of spin probe I(1, 14) incorporated in the platelets. ×, control sample; +, propranolol (5 mmol/l); ○, atenolol (5 mmol/l).

'flexibility gradient' observed in model phospholipid and biological membranes [41]. It is also clear from Fig. 6 that the temperature gradients ($\Delta S/\Delta T$) are different in the different membrane depths. This figure shows also that β-adrenoceptor blocking drugs atenolol and propranolol at the 5 mmol/l concentration have no significant effect on the order parameter $S_{\parallel}$ in the 5th carbon depth of the platelet membrane, while atenolol increases, and propranolol decreases the order parameter $S_{\perp}$ at the 16th carbon depth of the membrane.

In experiments with isolated platelet membranes the ratio probe: membrane was sufficiently low to neglect probe-probe interaction in our study.

Fig. 7 shows the effect of β-adrenoceptor blocking drugs on the order parameter S of spin probe I(12, 3) incorporated into the isolated platelet membranes. The parameter S decreases with increasing the temperature in all samples. The values of S for control sample with 6 mmol/l HCl were lower than for control sample without HCl. Taking this into consideration as well as the fact that samples with practolol, metipranolol and exaprolol contained also 6 mmol/l HCl, the sequence of β-adrenoceptor blocking drug propensities to decrease parameter S in isolated plasma membranes was: exaprolol > Kö 1124 > metipranolol > propranolol ≈ doberol > practolol ≥ control ≈ atenolol. Within the experimental error β-adrenoceptor blocking drugs did not affect the isotropic splitting constant A_0. The A_0 detected by probe I(12, 3) was 1.52 ± 0.01 mT.

From the ESR spectra of spin probe I(1, 14) incorporated into isolated platelet membranes the parameter $A'_{\perp}$, τ_1 and τ_2 were evaluated.

Fig. 8 shows the temperature-dependent increase in the parameter $A'_{\perp}$ of the spin probe incorporated into isolated platelet membranes. β-Adrenoceptor blocking drugs were used in the concentration of 10 mmol/l with platelet membrane suspension in order to compare their relative efficiency modifying the mobility of the spin probe in the membrane. Because of high probe mobility in the membrane and outer hyperfine splitting, $A'_{\parallel}$

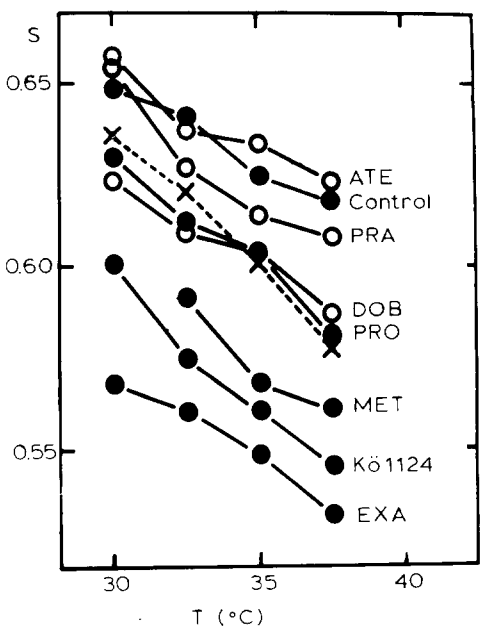


Fig. 7. The effect of β-adrenoceptor blocking drugs in 10 mmol/l concentration on the temperature dependence of the order parameter S of spin probe I(12, 3) incorporated in the isolated platelet membranes. ALP, alprenolol; ATE, atenolol; DOB, doberol; EXA, exaprolol; MET, metipranolol; PRA, practolol; PRO, propranolol. Broken line represents control sample containing 6 mmol/l HCl.

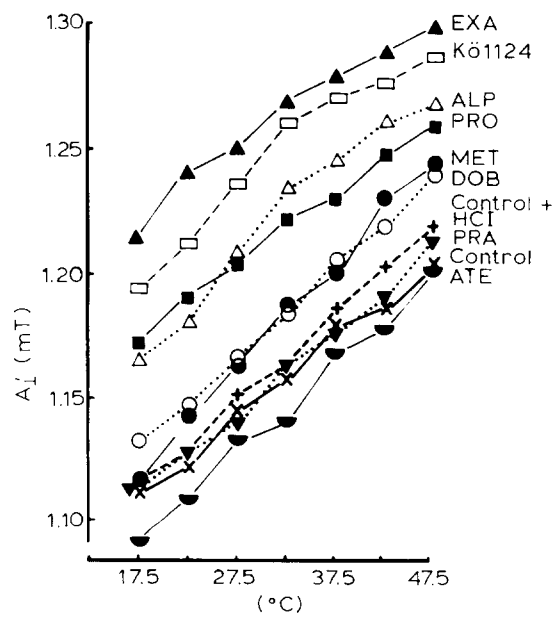


Fig. 8. Temperature dependence of the parameter $A'_{\perp}$ of the spin probe I(1, 14) incorporated into isolated platelet membranes treated with β-adrenoceptor blocking drugs at 10 mmol/l concentration. ALP, alprenolol; ATE, atenolol; DOB, doberol; EXA, exaprolol; MET, metipranolol; PRA, practolol; PRO, propranolol.

was not well distinguished on ESR spectra and therefore only the parameter $A'_{\perp}$ was evaluated (Fig. 5). No ESR signal was observed from the spin probe dissolved in the aqueous medium due to the low spin probe : membrane ratio and sedimentation of the membrane suspension during ESR experiment. It is evident from Fig. 8 that in the control sample the $A'_{\perp}$ increases linearly with temperature from 1.11 to 1.20 mT. β-Adrenoceptor blocking drugs change the temperature dependence for parameter $A'_{\perp}$ parallel to the control curve almost linearly. The relative order of β-adrenoceptor blocking drug propensities to increase the parameter $A'_{\perp}$ was: exaprolol ≥ Kö 1124 > alprenolol ≥ propranolol > metipranolol ≈ doberol > control ≥ practolol > atenolol. The effect of propranolol, Kö 1124 and exaprolol on parameter $A'_{\perp}$ was equivalent to heating the control sample by about 20, 25 and 30 Cdeg, respectively. The β-adrenoceptor blocking drugs slightly increased (less than 1%) the isotropic splitting constant A_0 of probe I(1, 14) in isolated platelet membranes. The A_0 values for the control sample at 17.5°C and 47.5°C were 1.40 mT and 1.41 mT, respectively. The A_0 values for the sample with 10 mmol/l of exaprolol at 17.5°C and 47.5°C were 1.41 mT and 1.42 mT, respectively. The A_0 values for other β-adrenoceptor blocking drugs were within the A_0 values for exaprolol and the control sample.

Fig. 9 demonstrates the effect of β-adrenoceptor blocking drugs on rotational correlation time τ_2 of spin probe I(1, 14) in isolated platelet membranes. The correlation time decreases with increasing temperature of the sample. The lipophilic β-adrenoceptor blocking drugs exaprolol, Kö 1124, alprenolol, propranolol, doberol and metipranolol decreased the correlation time. The τ_2 values for

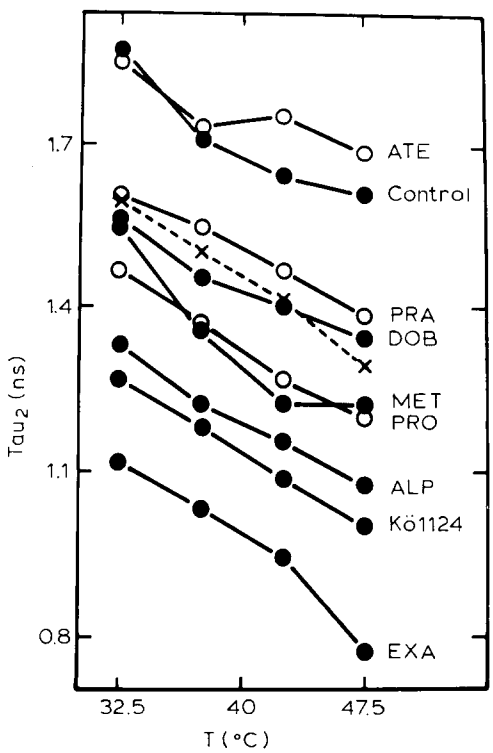


Fig. 9. The effect of β-adrenoceptor blocking drugs in 10 mmol/l concentration on the temperature dependence of the rotational correlation time τ_2 of spin probe I(1, 14) incorporated into isolated platelet membranes. ALP, alprenolol; ATE, atenolol; DOB, doberol; EXA, exaprolol; MET, metipranolol; PRA, practolol; PRO, propranolol.

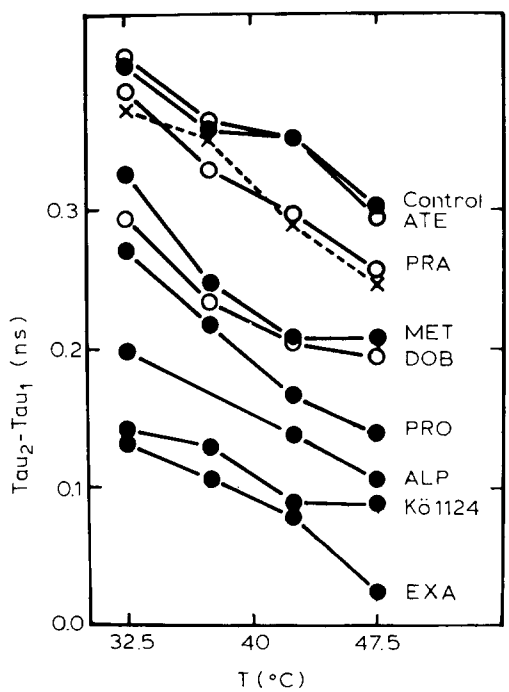


Fig. 10. Temperature dependence of parameter $\tau_2 - \tau_1$ of spin probe I(1, 14) incorporated into isolated platelet membranes treated with β-adrenoceptor blocking drugs at 10 mmol/l concentration. ALP, alprenolol; ATE, atenolol; DOB, doberol; EXA, exaprolol; MET, metipranolol; PRA, practolol; PRO, propranolol.

the control sample with HCl are lower than the values for the control sample without HCl. Taking this into account, the β-adrenoceptor blocking drug propensities to decrease the correlation time τ_2 were in the rank order of potency: exaprolol > Kö 1124 > alprenolol > propranolol ≈ metipranolol > doberol > practolol > control ≈ atenolol.

Fig. 10 shows the effect of β-adrenoceptor blocking drugs on anisotropic motion of spin probe I(1, 14) in isolated platelet membranes. The values of $\tau_2 - \tau_1$ decreased with increasing the temperature, indicating that the motion of the probe was more isotropic with increasing temperatures. The β-adrenoceptor blocking drugs decreased the parameter $\tau_2 - \tau_1$ with the propensities which were similar to those decreasing the correlation time τ_2 and increasing the parameter $A'_\perp$ in the sample.

Discussion

The aggregation of blood platelets is a complex event and no single pathway for activation of mediators can be postulated. We have found that β-adrenoceptor blocking drugs interfere with ADP-stimulated aggregation. The inhibition or potentiation of aggregation indicated that the rate of aggregation as well as the amplitude (extent of aggregation) are structure dependent and concentration limited. The mathematical analysis of aggregation curves with monoexponential nonlinear equation revealed that the potentiation of aggregation with atenolol was significant only in the value of amplitude. The inhibition of aggregation induced with alprenolol, exaprolol, Kö 1124, propranolol as well as metipranolol is significant in both measured parameters: the rate and extent of aggregation. Moreover, this effect is evident from the decline of the aggregation curves soon after ADP administration.

Platelet aggregation stimulated with adenosine diphosphate was shown to be initiated by the binding of ADP to specific membrane receptors [23]. The latent fibrinogen-glycoprotein receptors and newly formed calcium ion binding sites were revealed as initial step of stimulation [24–26].

Calcium displacement from binding sites in blood platelets as measured with Ca^{2+}-chlortetracycline complex fluorescence technique corresponds to the results obtained in aggregation studies. Atenolol, the drug with lower liposolubility diminished the measured emission intensity in comparison with spontaneous calcium displacement. On the other hand, the highly liposoluble drugs metipranolol and exaprolol rapidly declined the fluorescence intensity of the Ca^{2+}-chlortetracycline complex. This might indicate an immediate calcium displacement from binding sites in platelet membranes.

The fluorescence of the Ca^{2+}-chlortetracycline complex is caused by its association with membrane probably due to the interaction with polar headgroups of membrane lipids [27]. The change in the fluorescence intensity in our experimental conditions could thus indicate the change in the membrane affinity for the Ca^{2+}-chlortetracycline complex [27,28]. The Ca^{2+}-chlortetracycline fluorescence technique fails to establish the binding site in platelets from which calcium is being displaced.

Incorporation of β-adrenoceptor blocking drugs into platelet membrane was shown to result in perturbation of its structure. It was demonstrated that propranolol (as well as several other amphiphilic drugs) inhibited the formation of prostaglandins, endoperoxides and thromboxane A_2 from phospholipid arachidonate, probably as a result of interaction with calcium dependent phospholipase A_2 [14,19]. The dose-dependent decrease in malondialdehyde production in platelets treated with β-adrenoceptor blocking drugs might also be a result of interaction of these drugs with membrane phospholipids [30].

β-Adrenoceptor blocking drugs and membrane fluidity

The results of our ESR experiments demonstrated that β-adrenoceptor blocking drugs influence the spin probes in the platelets or in the isolated platelet membranes. The stearic acid spin probes principally reside in the plasma surface membrane of intact platelets [40].

Propranolol and atenolol influenced the fluidity of the membrane of intact platelets at the 16th carbon membrane depth, whereas they had no effect at the 5th carbon depth. We suppose that the effect of propranolol may be explained by its spatial incorporation in the membrane. It has been found that the *n*-naphthalene moiety of proprano-

lol partitions into the hydrophobic part of lipid bilayer and the charged amine side chain is positioned in the aqueous headgroup region [12,31]. It is supposed that such incorporation of the drug into the membrane may induce a 'free volume' at the hydrocarbon lipid core resulting in higher molecular freedom of the lipid chains at the 16th carbon depth [32]. Therefore, the disordering (fluidizing) effect of such incorporated drugs is 2- to 10-times higher at the hydrocarbone core of the membrane than at the 5th carbon depth, as was found also for local anesthetics [32,33,42]. It may be supposed that the membrane concentration of propranolol is low enough to induce a significant effect at the 5th carbon depth, but is detected at the 16th carbon depth. The mechanism by which atenolol decreases the membrane fluidity in platelets or in isolated membranes remains unexplained and the interaction of this drug with the polar membrane part might be of importance.

Propranolol and other lipophilic β-adrenoceptor blocking drugs increased the fluidity of the isolated platelet membranes detected by the probe I(12, 3), whereas the hydrophilic drugs atenolol and practolol slightly increased it. The effect of drugs on membrane perturbation was found to be dependent on the lipid : buffer ratio in the sample [32]. Because the lipid : buffer ratio in platelets and isolated platelet membranes was unknown, it was difficult to compare the effect of drugs on these membranes.

HCl increased the fluidity of the isolated platelet membranes, which might be connected with the dependence of vertical position of probes I(12, 3) and I(1, 14) in the membrane versus pH as it was found for the phospholipid membranes [43]. The isotropic splitting constants A_o of the spin probes in isolated platelet membranes were comparable to those found for spin-labelled phospholipids incorporated into egg phosphatidylcholine aqueous dispersion, measured at 5th, 10th and 12th carbon depth [44]. Slightly higher A_o values in isolated platelet membranes indicate that these membranes possess a more polar hydrophobic membrane region than egg phosphatidylcholine bilayers.

The lipophilic β-adrenoceptor blocking drugs decreased the parameter $A'_\perp$, τ_2 and $\tau_2 - \tau_1$, indicating that the β-adrenoceptor blocking drugs disordered membranes, increased the mobility and increased the measure of isotropic motion in the hydrophobic core of the membranes. Atenolol and practolol had slightly opposite or nonsignificant effects on these parameters. The β-adrenoceptor blocking drug propensities on these parameters were comparable and were roughly in the order of potency: exaprolol ≥ Kö 1124 > alprenolol ≥ propranolol > doberol ≥ metipranolol > control ≥ practolol ≥ atenolol. This order corresponded with their potency to inhibit platelet aggregation or to displace calcium from binding sites in platelets. The liposoluble drugs exaprolol, Kö 1124, alprenolol and propranolol were the most active, whereas the hydrophilic drugs atenolol and practolol were least potent. Because the perturbation effect of some drugs was found to depend on lipid : buffer ratio in the sample [32], the purpose of this ESR study was to find the order of the β-adrenoceptor blocking drug propensities in influencing platelet membranes. The physico-chemical state of lipid bilayers is thought to be largely responsible for the maintanance of many membrane enzyme activities [35].

Propranolol has influenced the thermally-induced structural transition in the intact human erythrocyte membrane and reduced the temperature of gel to liquid-crystalline phase transition in phospholipid bilayers [10,34]. Moreover, hydrophilic practolol has had no effect on the lipid phase transition [10]. Propranolol and timolol most probably perturb functional properties of the calcium pump protein in the membrane indirectly, by partitioning into the bulk lipid matrix of the membrane [12]. Changes in membrane fluidity as a result of altered cholesterol content or incorporated alcohols correlated with the change in platelet aggregability [27,36].

Our results confirm previous findings concerning nonspecific interaction of β-adrenoceptor blocking drugs with platelet aggregation. It is suggested that β-adrenoceptor blocking drug activities affecting platelet aggregation and calcium displacement may be mediated, at least in part, through their perturbation effect on platelet membranes.

Acknowledgments

The authors wish to thank Ing. Karel Květoň, CSc., Department of Physics, Czech Technical

University, Prague for his original HP-9830 computer program of nonlinear regression analysis, Ing. Mária Ďurišová, CSc., Institute of Experimental Pharmacology CPS, for kind help in evaluating the data and Dr. Magda Kouřilová, Department of Foreign Languages, Comenius University Bratislava for her help with the translation of the manuscript.

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