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Comparison of the Potency of Five Potential β-Adrenoceptor Blocking Drugs and Eight Calcium Channel Blockers to Inhibit Platelet Aggregation and to Perturb Liposomal Membranes Prepared from Platelet Lipids

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Summary

Five potential β-adrenoceptor blocking (BAB) compounds, alkylesters of 4-[(2-hydroxy-3-alkylamino)propoxy] phenylcarbamic acid, and eight calcium channel blockers (CB), i.e. nifedipine, nimodipine, niludipine, nitrendipine, verapamil, gallopamil, mepamil and diltiazem, were compared as to their inhibitory effect on thrombin induced aggregation of washed rat platelets and their effect on dynamics/disorder of liposomal membranes prepared from platelet lipids, studied by EPR spectroscopy of a lipid spin probe. The anti-aggregatory potency of the BAB and CB drugs was effective within the concentration range of 0.01-1 mmol/l. The antiaggregatory potency of BAB increased in the order BL-143 < BL-243 < BL-343 < BL-443 < BL-543 and among the CB, nifedipine and diltiazem were the least potent, whereas nitrendipine and mepamil were the most potent drugs. The potency of the other CB tested was intermediate. The BAB drugs increased the dynamics/disorder of the liposomes in the same order as they inhibited platelet aggregation, whereas there was no relationship between antiaggregatory effect of CB and their influence on dynamics/disorder of the liposomes. Nifedipine, nimodipine, niludipine and nitrendipine had a minor perturbation effect on the liposomes, whereas verapamil, mepamil, gallopamil and diltiazem pronouncedly increased the dynamics/disorder of the hydrophobic part of the liposomes. The results indicate that the anti-aggregatory activity of BAB drugs may be mediated, at least partially, through their perturbation effect on the lipid part of biological membranes.

Key words

Calcium Blockers – β-Adrenoceptor blocking drugs – Platelet Aggregation – Liposome Perturbation – EPR spectroscopy

Introduction

Many drugs have the potency to inhibit platelet aggregation (for rev. see Seuter and Scriabine 1984). Different molecular mechanisms of the drug inhibition effect were suggested (Seuter and Scriabine 1984). A correlation was observed to exist between platelet membrane fluidity changes induced by some drugs and their anti-aggregatory potency. A positive correlation was established between the effects of the alcohols n-propyl, n-butyl, n-amyl, n-hexyl, ajoene (a compound isolated from garlic) and a polyunsaturated fatty acid on inhibition of platelet aggregation induced by ADP and perturbation of membrane lipids (Kitagawa *et al.* 1984, Rendu *et al.* 1989, Kitagawa *et al.* 1990a). An increase of fluidity of platelet membranes induced by alkyl alcohols, benzyl alcohol and phenolic compounds was found to be related to their inhibitory effects on platelet aggregation (Kitagawa *et al.* 1990b). Classical beta-adrenoceptor blocking (BAB) drugs were found to have an anti-aggregatory effect and they increased the fluidity of platelet membranes (Nosál *et al.* 1985).

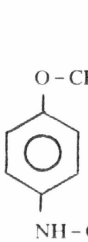
Some new alkylesters of 4-[(2-hydroxy-3-alkylamino) propoxy]phenylcarbamic acids were found to have β-adrenolytic, intrinsic sympathomimetic and local anesthetic activity (Bachratá *et al.* 1987, Račanská *et al.* 1990). In five of these substances known to have β-adrenolytic potency, we studied their anti-aggregatory effect.

Calcium channel blockers (CB) were reported to inhibit platelet aggregation (Ono *et al.* 1981, Schmunk and Lefer 1982, Kiyomoto *et al.* 1983), but the mechanism of the effect is not fully understand. The effect of CB on lipid dynamics/disorder was not studied so far.

In order to contribute to understanding of anti-aggregatory potency of BAB and CB drugs we compared the effect of eight CB and five BAB compounds on *in vitro* platelet aggregation and on dynamics/disorder of liposomes prepared from platelet total lipids.

Material and Methods

Chemicals. The new β-adrenoceptor blocking (BAB) drugs, alkylesters of 4-[(2-hydroxy-3-alkylamino)propoxy]phenyl carbamic acids (Scheme I.) were provided by Dr. Csöllei (Faculty of Pharmacy, Comenius University, Bratislava). Nifedipine (NIF) and Verapamil (VER) were from Sigma. Nimodipine (NIM), niludipine (NIL), nitrendipine (NIT), gallopamil (GAL), mepamil (MEP) and diltiazem (DIL) were provided by the Inst. for Drug Res., Modra, CSFR. Thrombin was from Imuna, Šarišské Michalany, CSFR. Spin probe, 1-palmitoyl-2-stearoyl phosphatidylcholine labelled by the doxyl group at the 16th carbon position (16-PC) was from Avanti Polar Lipids. Tyrode buffer contained (in mmol/l): NaCl 137, KCl 2.7, $NaHCO_3$ 12, NaH_2PO_4 0.4, $MgCl_2$ 1, glucose 5.6, 7.4 pH.



	X	Compound
O-CH_2-CH-CH_2-NH-$CH(CH_3)_2$	CH_3	BL-143
	C_2H_5	BL-243
	C_3H_7	BL-343
	C_4H_9	BL-443
	C_5H_{11}	BL-543

NH-COO-X

Scheme I. Chemical structure of the BAB compounds studied.

Platelet aggregation. Platelets were isolated from rat blood and their number was adjusted with calcium free Tyrode buffer to $2.5x10^5/\mu l$. The aggregation was induced by thrombin 0.46 UI/ml added 30 seconds after application of the drug (Nosál *et al.* 1983, Nosál *et al.* 1985) and measured according to Born (1962) in a dual channel Chrono-log aggregometer (Chrono-Log Corp., Havertown, PA, USA).

The drugs NIF, NIL, NIT and NIM were applied in DMSO. The final DMSO concentration in platelet suspension was 3 %. The anti-aggregatory potency of the drugs was expressed as the percentage change in light transmission compared to control H_2O 30 s, 60 s and 3 min after the beginning of aggregation.

Liposomes. Total lipids (TL) from platelets were isolated according to Folch *et al.* (1957) TL from platelets (1 mg) and the spin probe were mixed with the drug in chloroform:methanol = 2:1. The solvent was evaporated under nitrogen followed by evacuation. The dry samples were hydrated with 20 μl of the Tyrode solution. In order to attain equilibrium of the drugs in the liposomes the samples were subjected to vortex-freeze-thaw cycles several times. The drug/TL molar ratio in all samples was 1/2 and the spin probe/lipid ratio was 0.01.

EPR spectra were measured in glass capillaries (i.d. 1 mm) by Bruker 200 D Spectrometer. Typical instrument settings were: 10 mW microwave power, modulation amplitude of 0.1 mT.

To assess the relative efficiency of the drugs in perturbing the liposome membrane, the apparent order parameter S was calculated from the outer (Amax) and inner (A_{min}) splittings of the EPR spectra of the spin probe according to Marsh (1981). Decrease of the parameter S indicates higher disorder and/or dynamics of the hydrophobic part of the membrane. The membrane order and dynamics were not distinguished from the obtained EPR parameters. The EPR parameters were estimated to be within the relative error of ± 4 %.

Results

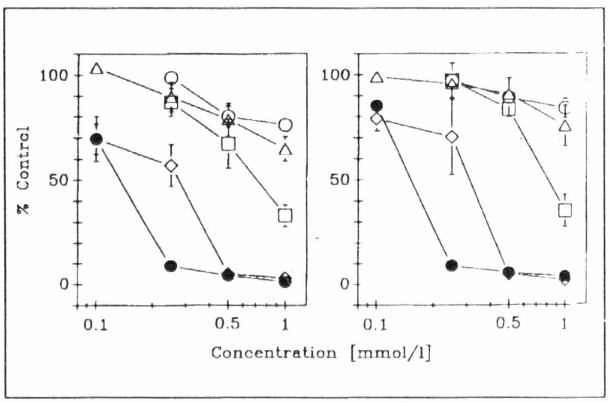


Fig. 1

Inhibitory effect of the BAB compounds: BL-143 (open circles), BL-243 (triangles), BL-343 (squares), BL-443 (diamonds) and BL-543 (filled circles) on platelet aggregation 60 s (left) and 3 min (right) after onset of aggregation. Means and standard deviations from 4-6 experiments are given.

BAB compounds

Platelet aggregation. Platelet aggregation curves after platelet treatment with the drugs were similar as shown in our previous studies (Nosál *et al.* 1983, Nosál *et al.* 1985). The BAB compounds were compared as to their effect on platelet aggregation induced by thrombin evaluated 60 s and 3 min after thrombin had been added (Fig. 1). The compounds had an anti-aggregatory potency at concentrations ≥0.1 mmol/l and the

order of their potency was: BL-143<BL-243<BL-343<BL-443<BL-543. The same order of the potency was obtained when the effect of compounds was evaluated 30 s, 60 s and 3 min after the onset of aggregation.

Liposome perturbation. The BAB and CB drugs influenced the EPR spectral parameters of the probe in the liposomes prepared from rat platelet lipids. The drugs increased disorder/dynamics of the hydrophobic part of the liposomal membranes. The effect of the BAB drugs on parameter S of the probe incorporated in liposomes prepared from TL isolated from platelets measured at 25 and 37 °C is shown in Fig. 2. The drugs decreased the parameter S and the order of their efficiency was the same as their anti-aggregatory potency.

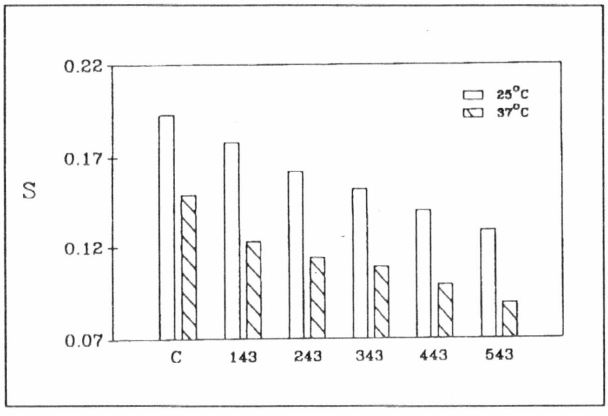


Fig. 2

Comparison of the effect of the BAB compounds on parameter S of 16-PC in platelet lipid liposomes (drug/TL molar ratio of 1/2) at 25 and 37 °C. C - control liposomes. For abbreviation see Material and Methods.

The comparison of the anti-aggregatory potency of the BAB drugs with their effect to increase dynamics/disorder of the hydrophobic membrane part of liposomes prepared from platelet lipids is shown in Fig. 3. There is a good relationship between the influence of the drugs on functional properties of platelets and on dynamics/disorder of membrane lipids.

CB drugs

Platelet aggregation. The CB were compared as to their effect on platelet aggregation induced by thrombin evaluated 60 s after thrombin had been added (Fig. 4). The drugs had an anti-aggregatory potency at concentrations >0.01 mmol/l. There was a difference between their anti-aggegatory potency. Of the dihydropyridines, NIF had the lowest and NIT the highest effect. VER, GAL, MEP and DIL also inhibited platelet aggregation in the order of potency MEP>VER>GAL>DIL.

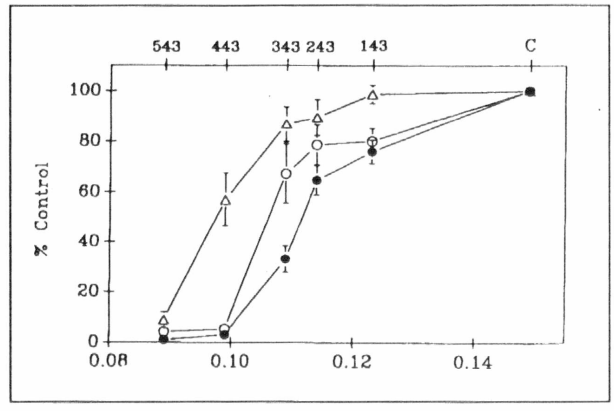


Fig. 3

Comparison of the effects of the BAB drugs on parameter S of spin probe in liposomes prepared from platelet lipids at 37 °C (data taken from Fig. 2) and their anti-aggregatory potency at concentrations of 0.25 (triangles), 0.5 (open circles) and 1 mmol/l (filled circles) (data taken from Fig. 1 left).

Liposome perturbation. The effect of CB on parameter S of the probe incorporated in liposomes prepared from TL isolated from platelets measured at 25 and 37 °C is shown in Fig. 5. The dihydropyridines, NIF, NIM, NIT and NIL had a minor effect on parameter S, while VER, GAL, MEP and DIL decreased the parameter S significantly. There was not correlation between anti-aggregatory effect of CB and their influence on dynamics/disorder of the liposomes.

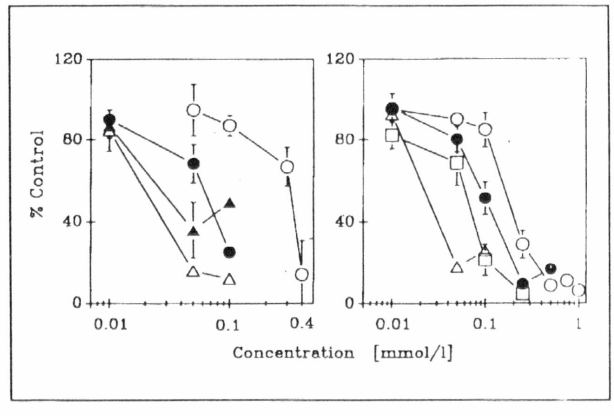


Fig. 4

Inhibitory effect of dihydropyridines (left) NIF (open circles), NIM (filled circles), NIL (filled triangles) and NIT (open triangles) and (right) of DIL (open circles), GAL (filled circles), MEP (open triangles) and VER (filled triangles) on platelet aggregation induced by thrombin. Means and standard deviations from 4-5 experiments are given.

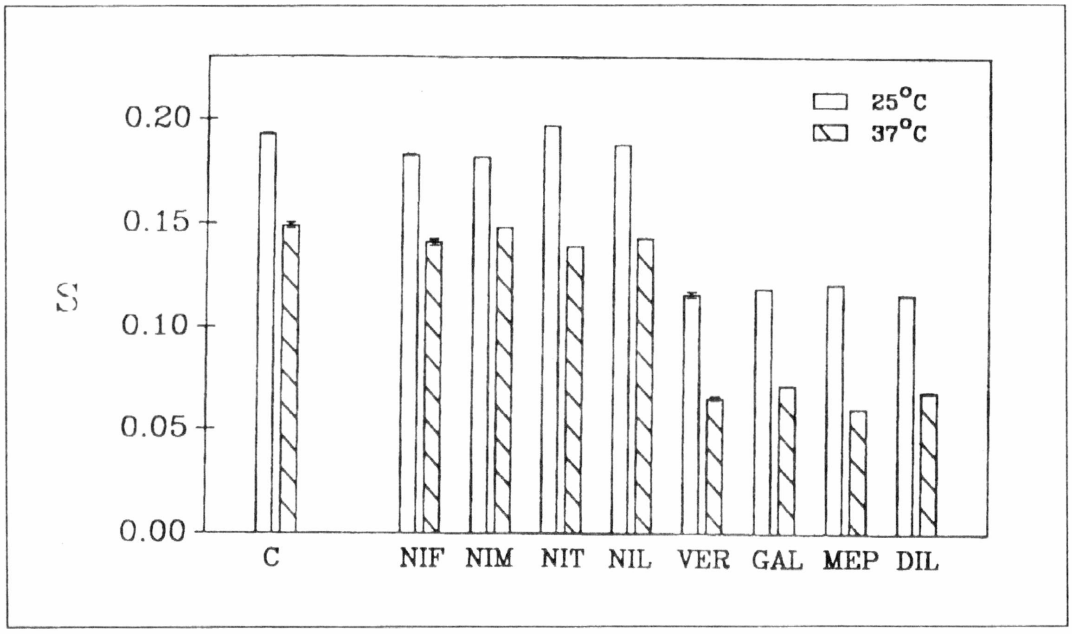


Fig. 5

Comparison of the effect of CB drugs on parameter S of 16-PC in platelet lipid liposomes (drug/TL molar ratio of 1/2) at 25 and 37 °C. C - control. For abbreviations see Material and Methods.

Discussion

In order to compare the potency of the BAB and CB drugs on the lipid part of platelet membranes, we isolated platelet total lipids and prepared liposomes. Since we were interested in the perturbation effect in the proximity of the drug incorporated in the liposomes, the drug/lipid molar ratio of 1/2 was chosen. Presuming that three lipid molecules are around the spin probe in the liposomes, then at the drug/lipid molar ratio of 1/2 the drug is in the proximity of the spin probe, and membrane perturbation around the drug can be detected.

BAB drugs

In our previous study, the perturbation properties of drugs were found to be most significant at the hydrocarbone membrane core of platelets and TL membranes (Nosál *et al.* 1985, Ondriaš *et al.* 1987 and 1989). Therefore we applied the spin probe labelled at the 16th carbon to detect the perturbation effect of the BAB drugs at the hydrocarbone membrane core.

A positive correlation was reported between the effects of alcohols, ajoene, polyunsaturated fatty acids and phenolic compounds on platelet aggregation and perturbation of platelet membranes (Kitagawa *et al.* 1984, Rendu *et al.* 1989, Kitagawa *et al.* 1990a, 1990b). The authors suggested that the drugs inhibited platelet aggregation due to perturbation of the lipid membrane.

In our study, BAB drugs inhibited platelet aggregation at relatively high concentrations (0.1-1 mmol/l). From this and from the good relationship of the anti-aggregatory and perturbation effect of the BAB drugs found in our study, it is also suggested that the anti-aggregatory activity of the studied BAB drugs may be mediated, at least partially, through their perturbation effect on the lipid part of platelet membranes. This is supported by findings that membrane proteins were influenced by properties of membrane lipids (Sanderman 1978) and that induction of platelet aggregation by thrombin affected platelet membrane dynamics/disorder (Rendu *et al.* 1985).

The partition coefficient (log P') of the BAB drugs studied in the octanol-buffer system by Bachratá *et al.* (1987) was: BL-143=0.053; BL-243=0.702; BL-343=0.924; BL-443=1.243 and BL-543=1.351. The anti-aggregatory and perturbation efficiency of the BAB drugs increased in the same order as their partition coefficient (BL-143<BL-243<BL-343<BL-443 <BL-543). This also support our assumption that the studied BAB drugs influenced platelet aggregation through their membrane perturbation. The perturbation effect of the studied drugs may have effect on lipid-protein interaction and/or formation of nonlamellar lipid structures.

The BAB compounds-receptor interaction is less probable, however if it is considered, the influence of drugs on receptors in platelet membranes may be supposed to be a complex reaction involving interaction with bulk membrane lipids. The nonspecific interaction of the drugs with lipid part of platelet membranes may

be important as a pathway to the receptor which may be located at the hydrophobic part of membrane proteins, comparably to the suggested interaction of calcium channel antagonists with receptors in sarcolemmal membranes (Herbette *et al.* 1989).

CB drugs

Calcium channel blockers inhibit platelet aggregation with a different potency (Ono *et al.* 1981, Schmunk and Lefer 1982, Kiyomoto *et al.* 1983). The order of the CB potency depended mainly on the agonist used to induce platelet aggregation. It was found that CB in platelets decreased production of tromboxane A2 (Mehta 1985), inhibited calcium influx (Jy and Haynes 1987) and decreased nucleotide release (Mehta 1985).

In our study we found that NIF, NIL, NIM and NIT had an insignificiant or minor perturbation effect, whereas the effect of VER, GAL, MEP and DIL on TL liposomes was pronounced.

Taking into account our results and the published data (Ono *et al.* 1981, Schmunk and Lefer 1982, Kiyomoto *et al.* 1983), it is evident that the order of potency of the CB to perturb the hydrophobic part of the lipid membrane does not correlate with their antiaggregatory effect. Therefore we suppose that membrane perturbation effect of CB drugs, namely dihydropyridines, may not play a pronounced role in their anti-aggregatory potency.

Some platelet-activating agonists are known to activate platelets by stimulating calcium entry (Jy and Haynes 1987). Thrombin was found to activate divalent cation selective channels in the platelet membrane (Zschauer *et al.* 1988). Blache *et al.* (1987) reported that nitrendipine inhibited thrombin-induced ^{45}Ca uptake by rat platelets. Taking into account these findings and our results that CB drugs inhibited platelet aggregation in calcium free solutions at relatively high concentrations (0.01-0.4 mmol/l), we suppose that the studied CB drugs may inhibit mobilization of Ca^{2+} from intracellular stores or interfere with platelet-activating agonists.

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