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THE ROLE OF INTRACELLULAR CALCIUM IN A-23187 STIMULATED AND β-ADRENOCEPTOR BLOCKING DRUG TREATED BLOOD PLATELETS

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Abstract—A significant concentration-dependent difference was found between β-adrenoceptor blocking drugs in their ability to inhibit A23187-induced isolated platelet aggregation. In the absence of extracellular calcium ions the following rank order of potency to inhibit calcium ionophore stimulated platelet aggregation was shown: propranolol > bevantolol > alprenolol > metipranolol > oxprenolol > atenolol > pindolol > metoprolol ≈ sotalol ≈ practolol. The interruption of induced aggregation, as well as inhibition of aggregation, in the absence of extracellular calcium ions indicated interference of inhibitory β-adrenoceptor blocking drugs with intramembrane or intraplatelet calcium pools activated with A23187. This suggestion was supported by the reversal of the inhibitory effect of β-adrenoceptor blocking drugs in the presence of extracellular calcium ions. The effect was dose dependent and occurred within 30 sec after calcium administration. The results indicated that inhibitory β-adrenoceptor blocking drugs, possessing a cationic amphiphilic structure, suppressed calcium mobilization in A23187-stimulated platelets, most probably after entering platelets. This explains why lipophilic drugs are more effective than hydrophilic ones in calcium ionophore A23187-stimulated platelets.

Key words: calcium ionophore A23187; platelet aggregation; β-adrenoceptor blocking drugs

Platelet aggregation both *in vivo* and *in vitro* has been shown to be inhibited or reduced by cationic amphiphilic drugs [1, 2]. With one such series of drugs, β-adrenoceptor antagonists, both structure–activity relationships and dose- and time-dependent relationships have been reported for the inhibitory effects on stimulated platelet aggregation *in vitro* [3–5]. It seems likely that β-blockers inhibit stimulated platelet aggregation by interfering with phospholipase A_2 activation, thus reducing the availability of arachidonic acid for eicosanoid synthesis [6–8]. A good correlation was found between the inhibition of aggregation in β-blocker pre-treated and thrombin stimulated platelets on one side and the liberation of arachidonic acid, malondialdehyde formation and thromboxane production on the other [9–11]. Nevertheless, the inhibitory effect of metipranolol on platelet aggregation was demonstrated to be reversed by calcium ions, and propranolol was shown to alter the slow, as well as fast, exchangeable calcium pool in isolated platelets [12, 13]. These, together with other results demonstrating the displacement of calcium ions from membrane phospholipids with β-blocking drugs [14], led to this study on the role of platelet calcium in β-blocker inhibition of aggregation. To characterize the participation of intraplatelet calcium, platelets were stimulated with calcium ionophore A23187, which acted as an artificial calcium channel in the membrane and manipulated the concentration of extracellular Ca^{2+}.

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MATERIALS AND METHODS

Materials. Calcium ionophore A23187 (Calbiochem AG, Lucerne, Switzerland) was stored at −20° as 10 mmol/L stock solution in dimethyl sulphoxide (Serva, Heidelberg, Germany). The working solution (44 μmol/L) was obtained by diluting the stock solution in deionized water. Ca-free Tyrode's buffer contained 137 mmol/L NaCl, 2.7 mmol/L KCl, 11.9 mmol/L $NaHCO_3$, 0.4 mmol/L $NaH_2PO_4.2H_2O$, 1 mmol/L $MgCl_2.6H_2O$ and 5.6 mmol/L glucose, pH = 7.40.

Drugs: alprenolol (ALP) = 1-(*o*-allyloxyphenoxy)-3-(isopropylamino)-2-propanol (Hässle, Hälsingborg, Sweden); atenolol (ATE) = 2-*p*-[2-hydroxy - 3 - (isopropylamino)propoxy] - phenyl] - acetamide, practolol (PRA) = 4′-[2-hydroxy-3-(isopropylamino)propoxy]-acetanilide and propranolol (PRO) = 1 - (isopropylamino) - 3 - (1 - naphtyloxy)-2-propanol: ICI (Alderley Park, Cheshire, U.K.); bevantolol (BEV) = 1-[(3,4-dimethoxyphenethyl)amino] - 3 - (*m* - tolyloxy) - 2 - propanol (Parke Davis, Morris Plains, U.S.A.); metipranolol (MET) = (4 - hydroxy - 2,3,5 - trimethylphenoxy) - 3 - (isopropylamino) - 2 - propanol - 4 - acetate (Spofa Works, Praha, Bohemia); metoprolol (MTP) = 1 - (isopropylamino) - 3 - [*p* - (2 - methoxyethyl) - phenoxy] - 2 - propanol and oxprenolol (OXP) = 1 - [*o* - (allyloxy)phenoxy] - 3 - (isopropyl - amino)-2-propanol (Ciba Geigy, Basel, Switzerland); pindolol (PIN) = 1 - (4 - indolyloxy) - 3 - (isopropyl - amino) - 2 - propanol (Sandoz, Basel, Switzerland); sotalol (SOT) = 4′-[1-hydroxy-2-(isopropylamino) - ethyl]methane sulphonanilide (Bristol Myers, Ickenham, U.K.). All drugs were used as hydrochlorides, except metipranolol which was used as tartrate.

All other chemicals were of analytical grade from commercial sources.

Platelet preparation. Blood platelets were isolated as described previously [4, 5, 9]. Briefly, blood (9 mL) was collected through a polyethylene catheter from the common carotid artery of lightly anaesthetized male Wistar rats (350 g) into plastic thrombotest tubes containing 1 mL of 3.8% (0.129 mol/L) trisodium citrate. Platelet-rich plasma (PRP) was prepared by 15 min centrifugation (300 *g*) at room temperature (22°). Platelets were separated by subsequent centrifugation of PRP at 1000 *g* for 10 min, resuspended in Ca-free Tyrode's buffer (see Materials) with 5.4 mmol/L EDTA (pH = 6.9) and washed twice in Ca-free Tyrode's buffer by centrifugation (1000 *g*) for 6 min at room temperature. For aggregation studies platelets were resuspended after the final centrifugation in Tyrode's solution without EDTA.

Platelet counting. Platelets were counted in a Thrombocounter C (Coulter Electronics, Luton, U.K.) and adjusted to 2.5×10^8 cells/mL with Tyrode's solution.

Platelet aggregation. Platelet suspensions (450 μL) were incubated with test drugs (20 μL) for 30 sec at 37° before stimulation with calcium ionophore A23187 at a final concentration of 1.8 μmol/L. Aggregation was measured turbidimetrically according to Born [15] in a dual channel aggregometer (Chrono-log Corp., Harveton, U.S.A.). In indicated experiments calcium (20 μL) was added to samples at 30 sec aggregation. Each aggregation curve was recorded for 3 min. At 60 sec the absolute amplitude (in mm) of the aggregation curve was measured, or the percentage of light transmission was evaluated. Light transmittance through platelet-poor plasma represented 100% aggregation and that through platelet-rich plasma 0%.

Results are expressed as means ± SEM and the significance of differences tested by Student's *t*-test. A probability of $P < 0.05$ was considered to be statistically significant.

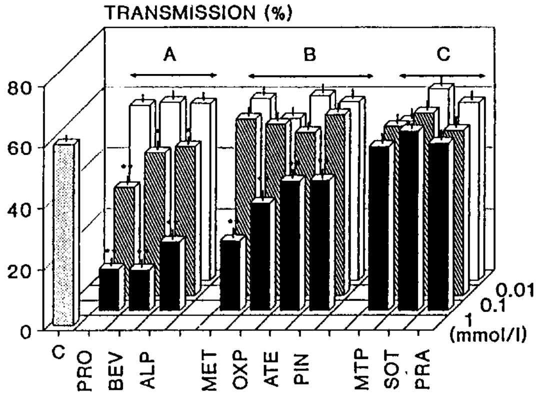

Fig. 1. Concentration-dependent effect of β-adrenoceptor blocking drugs on A23187-induced platelet aggregation in the absence of extracellular calcium. Isolated platelets were pre-treated with drugs for 30 sec at 37° before stimulation with A23187 at 1.8 μmol/L. (A) ALP, alprenolol; BEV, bevantolol; PRO, propranolol. (B) ATE, atenolol; MET, metipranolol; OXP, oxprenolol; PIN, pindolol. (C) MTP, metoprolol; PRA, practolol; SOT, sotalol; C, control. N, 6–8; $x \pm$ SEM; *$P \leq 0.05$, **$P \leq 0.01$.

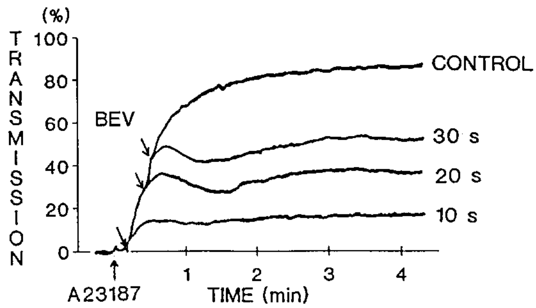

Fig. 2. Effect of bevantolol (1 mmol/L) on A-23187 induced platelet aggregation (representative aggregation curves) in the absence of extracellular calcium. Bevantolol was added to the cell suspension 10, 20 or 30 sec after A23187 (1.8 μmol/L).

RESULTS

Figure 1 shows the effect of β-adrenoceptor blocking drugs on A23187 stimulated isolated blood platelet aggregation in a Ca^{2+}-free medium. At 60 sec of aggregation light transmittance in control samples reached 59.25 ± 2.31%. The drugs in group A (propranolol, bevantolol and alprenolol), used at a concentration of 0.1 mmol/L, significantly decreased the transmittance to 35.25 ± 5.68, 46.62 ± 3.96 and 48.63 ± 3.26%, respectively, and at 1 mmol/L to 13.82 ± 1.46, 13.55 ± 5.04 and 22.62 ± 3.17%, respectively.

The compounds in group B (metipranolol, oxprenolol, atenolol and pindolol) were less effective. At a concentration of 1 mmol/L these drugs decreased significantly A23187-induced light transmittance to 22.99 ± 5.04, 35.16 ± 5.04, 42.31 ± 6.13 and 42.58 ± 2.32%, respectively, while at lower concentrations they were without significant effect. The drugs in group C (metoprolol, sotalol and practolol) showed no significant effects on light transmittance of platelets stimulated with A23187.

The effects of bevantolol added at a concentration of 1 mmol/L in a Ca^{2+}-free medium 10, 20 and 30 sec after A23187 stimulation of platelet aggregation are shown in Fig. 2. It was evident from the records of aggregation curves that immediately after bevantolol administration the development of aggregation was interrupted and the amplitude did not reach the control value.

Figure 3 shows the effect of pre-incubation time of platelets with alprenolol, metipranolol or propranolol for 30 sec, 5 and 15 min on A23187-induced aggregation in Ca^{2+}-free medium. Prolonging the exposure of platelets to β-blocking drugs before stimulation did not change their anti-

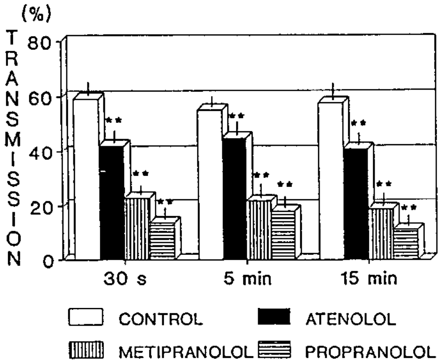

Fig. 3. Effect of pre-incubation (30 sec, 5 and 15 min, 37°) with atenolol (ATE), metipranolol (MET) and propranolol (PRO) at 1 mmol/L on A23187-induced platelet aggregation in the absence of extracellular calcium. C, control (A23187 = 1.8 μmol/L); N, 5–7; $x \pm$ SEM; **P ≤ 0.01.

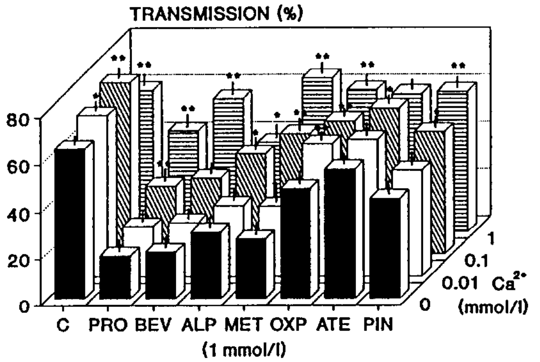

Fig. 4. Effect of extracellular calcium ions (0, 0.01, 0.1, 1.0 mmol/L) on aggregation of blood platelets pre-treated with β-blocking drugs (1 mmol/L, 30 sec, 37°) and subsequently stimulated with A23187 (1.8 μmol/L). PRO, propranolol; BEV, bevantolol; ALP, alprenolol; MET, metipranolol; OXP, oxprenolol; ATE, atenolol; PIN, pindolol; C, control. N, 5–7; $x \pm$ SEM; *P ≤ 0.05, **P ≤ 0.01.

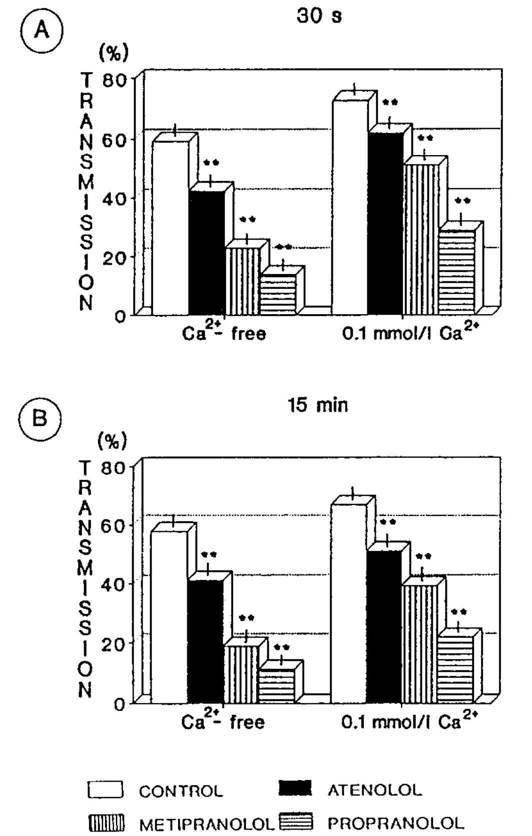

Fig. 5. Effect of pre-incubation time (30 sec, 15 min) with atenolol (ATE), metipranolol (MET) and propranolol (PRO) (1 mmol/L, 37°) in the presence of 0.1 mmol/L extracellular calcium on aggregation of isolated platelets stimulated with A23187. C, control; A23187, 1.8 μmol/L; N, 5–7; $x \pm$ SEM; **P ≤ 0.05.

aggregatory effect in platelets stimulated with A23187.

The effect of varying the concentration of Ca^{2+} in the incubation medium on the anti-aggregatory efficacy of β-blocking drugs is shown in Fig. 4. Extracellular calcium at concentrations of 0.1 and 1 mmol/L significantly reversed the inhibitory effect of all drugs tested. At a concentration of 0.01 mmol/L, extracellular calcium significantly reversed only the inhibitory effect of metipranolol and oxprenolol.

Figure 5 shows the effect of prolonging the pre-incubation time of platelets on the anti-aggregatory effects of atenolol, metipranolol and propranolol in the presence of 0.1 mmol/L extracellular Ca^{2+}. Prolonging the pre-incubation time from 30 sec to 15 min did not significantly change the modulatory effects of β-blocking drugs tested on stimulated platelet aggregation.

DISCUSSION

β-Adrenoceptor blocking drugs varied substantially in their capacity to inhibit A23187-induced platelet aggregation in calcium-free medium. In this work β-blockers were divided arbitrarily into three groups with respect to this property. The drugs in group A, were potent inhibitors of aggregation, whereas those in group B were 10–100 times less active. The drugs in group C were without activity. In studies using thrombin-, collagen- and ADP-stimulated aggregation, the activity of β-blocking drugs was suggested to be dependent on their lipid solubility and membrane perturbing activity [4, 5].

These results indicated that the inhibitory effect of β-blockers in the absence of extracellular calcium most probably prevented the mobilization of calcium from the suggested third calcium pool of A23187-stimulated platelets, located in the plasmic membrane or intracellularly [16]. For such inhibition to occur, drugs must just enter the platelets, where individual drugs may have different sites of action.

Depending on their liposolubility, β-blocking drugs, as well as other cationic amphiphilic drugs, displace calcium from membrane phospholipids and inhibit the hydrolysis of membrane phospholipids by calcium-dependent phospholipase A_2 [6, 7, 14, 17]. The inhibition of A23187-induced aggregation on the one hand and the alteration of intracellular calcium storage and calcium-dependent processes on the other, indicated interactions between the mobile intraplatelet calcium and the drugs investigated.

Calcium ionophore-A23187 activation of platelets bypassed plasma membrane receptors to mobilize intracellular calcium [18, 19]. This calcium was most probably utilized for phospholipase (PL)A_2 activation, since A23187 was demonstrated to increase arachidonic acid mobilization in platelets and other cell types [20–23]. As suggested, PLA_2 played a critical role in stimulus–response coupling in platelets and in releasing arachidonic acid from membrane phospholipids [9, 24, 25]. By interacting with anionic phospholipids, lipophilic β-blocking drugs formed complexes with a decreased susceptibility to enzymatic hydrolysis by phospholipases [2, 6, 7]. Inhibition of calcium dependent activation of PLA_2 by β-blocking drugs resulted in the inhibition of stimulated arachidonic acid liberation, malondialdehyde formation and thromboxane A_2 production in isolated platelets [9–11]. β-Blocking drugs have been demonstrated to enter the plasma membrane, to interact with membrane phospholipids of its inner leaflet, to increase membrane fluidity and to accumulate intracellularly [4, 26–28]. Moreover, lipophilic, non-selective β-blocking drugs displace calcium from intracellular membrane-binding sites and anionic membrane phospholipids [4, 14]. These findings also support the suggestion that lipophilic β-blocking drugs may interact with A23187 intracellularly.

Furthermore, inhibition of A23187-induced aggregation by β-blocking drugs was reversed, concentration dependently, by increasing the extracellular calcium concentration. This indicated firstly that the inhibitory effect of the drugs was not irreversible, and secondly that in the presence of inhibitory β-blocking drugs A23187 activated calcium-dependent second messengers in platelets.

Calcium ionophores failed to induce inositol phospholipid hydrolysis [19, 29–32]. Moreover, the degree of PLC activation was not enhanced in the presence of extracellular Ca^{2+}, which led to Ca^{2+} influx and to a large increase in cytosolic calcium concentration [33]. Therefore, β-blocking drugs presumably do not interfere with the PLC pathway in stimulated platelets. Nevertheless, in rat platelets a calcium-dependent phosphatidylcholine PLC was found, which could play a role in signal transduction by generating phosphatidylcholine-derived diacylglycerol [34].

Lipophilic β-blocking drugs, as demonstrated with bevantolol, interrupted the development of aggregation if added 10, 20 and 30 sec after A23187 administration. Such an immediate effect could occur only if inhibitory β-blocking drugs were acting intracellularly and interacting with rapid stimulatory pathways, such as thromboxane A_2 formation [11] or calmodulin-induced phosphorylation resulting in platelet shape changes [35]. Lipophilic β-blockers, particularly those in group A, may have interacted with A23187-induced phosphorylation of myosine light chain kinase and the 47 kDa cytosolic protein [32, 36]. Calmodulin was demonstrated to be inhibited with many cationic amphiphilic drugs and its inhibition with lipophilic β-blocking drugs cannot be excluded [37, 38]. Moreover, the loss of phosphatidylcholine in stimulated platelets with subsequent inhibition of arachidonic acid liberation due to trifluoperazine, alprenolol, metipranolol and propranolol indicated a possible involvement of calmodulin in PLA_2 activation [9, 39]. Protein kinase C was independent of calmodulin and was most probably indirectly activated by A23187. Its dependence on phosphatidylserine may indicate why many cationic amphiphilic drugs, including β-blockers, can bind to the hydrophobic domain of this enzyme by inhibiting its activity [1, 40–42].

These results indicate that β-blocking drugs inhibit platelet aggregation by interfering with calcium mobilization intracellularly. The degree of inhibition depended most probably on the physico-chemical properties of the drugs and on the direct interaction of these cationic amphiphilic drugs with second messengers. A direct effect of β-blocking drugs on phospholipase A_2 and calmodulin activation, as well as on other calcium-dependent pathways involved in platelet activation, is still to be proven.

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