***In vitro* adrenaline and collagen-induced mobilization of platelet calcium and its inhibition by naftopidil, doxazosin and nifedipine**

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Aims The aim of the study was to obtain further information regarding the modes of action of doxazosin, naftopidil and nifedipine on platelet function.

Methods We conducted an *in vitro* study of drug influences on adrenaline and collagen-induced mobilization of platelet calcium.

Results In the presence of fibrinogen ($300 \mu\text{g ml}^{-1}$) both collagen ($5 \mu\text{g ml}^{-1}$) and adrenaline ($16 \mu\text{M}$) stimulated the aggregation of washed platelets. Collagen induced a transient rise ($+4.97 \pm 0.63 \mu\text{M}$) in platelet Ca^{2+} concentration, $[\text{Ca}^{2+}]_i$, as measured using the photoprotein aequorin, which coincided with the onset of aggregation. Adrenaline induced a smaller rise ($+3.6 \pm 0.96 \mu\text{M}$) which, however, occurred after the onset of aggregation. Naftopidil, an α_1 -adrenoreceptor antagonist produced a concentration-dependent inhibition of collagen-induced Ca^{2+} mobilization, maximum inhibition ($22.9 \pm 4\%$, $P < 0.05$) occurring with $40 \mu\text{M}$ naftopidil. The inhibition of Ca^{2+} mobilization was not reflected by a concentration-dependent inhibition of platelet aggregation, although $40 \mu\text{M}$ naftopidil produced statistically significant inhibition ($23.3 \pm 11.7\%$, $P < 0.05$). The adrenaline-induced rise in $[\text{Ca}^{2+}]_i$ was inhibited dose dependently by naftopidil (e.g. $40 \mu\text{M}$ naftopidil, $100 \pm 0\%$, $P < 0.05$), as was aggregation ($40 \mu\text{M}$ naftopidil, $100 \pm 0\%$, $P < 0.05$). Doxazosin, another α_1 -adrenoreceptor blocker, inhibited Ca^{2+} mobilization induced by collagen to similar extents as for naftopidil ($30 \mu\text{M}$ doxazosin, $17.4 \pm 2.5\%$, $P < 0.05$), but did not inhibit platelet aggregation. It also inhibited the adrenaline-induced rise in $[\text{Ca}^{2+}]_i$ in a concentration-dependent manner ($30 \mu\text{M}$ doxazosin, $37.6 \pm 13.7\%$, $P < 0.05$), significant inhibitions of platelet aggregation also being produced ($30 \mu\text{M}$, $49.6 \pm 17.2\%$, $P < 0.05$). As expected, the calcium channel blocker nifedipine produced concentration-dependent inhibitions of both collagen-induced Ca^{2+} mobilization (e.g. $28 \mu\text{M}$ nifedipine, $47.8 \pm 2.7\%$, $P < 0.05$) and aggregation ($28 \mu\text{M}$, $55.1 \pm 9.2\%$, $P < 0.05$).

Conclusions These data indicate that the α_1 -adrenoreceptor blockers, naftopidil and doxazosin, inhibit Ca^{2+} mobilization, this mechanism being possibly the means whereby these drugs inhibit platelet aggregation.

Keywords: platelets, calcium mobilization, naftopidil, doxazosin, nifedipine

Introduction

Platelet activity is increased in hypertension and may contribute to the increased cardiovascular complications, i.e. thrombosis and atherosclerosis, commonly observed in this condition [1, 2]. It has been reported that various anti-hypertensive drugs reduce platelet sensitivity and therefore by extrapolation, presumably thrombosis [2]. For example, α_2 -adrenoreceptor antagonists, β -adrenoreceptor blockers and calcium antagonists all interfere with platelet aggregation [3–9]. α_1 -Adrenoreceptor blockers, e.g. prazosin, urapidil and doxazosin also inhibit platelet aggregation [10–15]. In addition, we have reported that the experimental α_1 -

adrenoreceptor antagonist, naftopidil, inhibits platelet function [15]. Using a more sensitive experimental system for assessing platelet aggregation we demonstrated that naftopidil and, to a lesser extent, doxazosin, at pharmacological concentrations, inhibited aggregation induced by sub-threshold concentrations of adrenaline (the threshold concentration being defined as the minimum concentration of agonist sufficient to induce secondary irreversible aggregation) in combination with subthreshold concentrations of collagen, serotonin or ADP [15]. Additionally, but less markedly, naftopidil inhibited platelet aggregation induced by adrenaline alone and to a lesser extent, serotonin. The results obtained with α_1 -adrenoreceptor antagonists are paradoxical since firstly, platelets are not thought to possess α_1 -adrenoreceptors and secondly, platelet aggregation induced by adrenaline is mediated by α_2 -adrenoreceptors [16]. It must therefore be concluded that α_1 -adrenoreceptor

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blockers produce their inhibitory effects on platelet function through other mechanisms.

One possible mechanism is that of intracellular calcium mobilization. The mobilization of intra-platelet calcium is a prerequisite for platelet activation [17, 18]. Increases in platelet free Ca^{2+} cause the activation of various mechanisms associated with platelet activation. These include the activation of phospholipase A_2 , an enzyme which regulates arachidonic acid and prostaglandin synthesis, the activation of Ca^{2+} dependent proteases (calpains) and mechanisms mediating conformational changes of cytoskeletal proteins [17, 18].

In light of the central role played by Ca^{2+} mobilization in platelet activation, the decision was therefore made to examine the effects of naftopidil and doxazosin, in comparison with the well known Ca^{2+} channel blocker, nifedipine, on this process. Thus, the aim was to obtain further information regarding the modes of action of α_1 -adreno-receptor antagonists on platelet function.

Methods

Chemicals

Adrenaline, fibrinogen, nifedipine and dimethylsulphoxide (DMSO) were purchased from the Sigma Chemical Co Ltd, Poole, UK. Collagen fibrils were from Nycomed Arzneimittel, Munich, Germany. Aequorin was obtained from Friday Harbor Photoproteins, Friday Harbor, WA, USA. Naftopidil was supplied by Asta Pharma, Frankfurt, Germany and its solutions prepared in dimethyl formamide acidified with acetic acid (3.2:1 v/v) followed by dilution with distilled water, as previously described [15]. Doxazosin mesylate came from Pfizer Ltd, Sandwich UK and its solutions prepared in distilled water. Nifedipine solutions were prepared in DMSO and diluted with distilled water to the desired concentrations.

Subjects

Healthy non-smoking subjects aged 28–54 years, none of whom had taken any aspirin-like drugs during the previous 2 weeks.

Blood collection, platelet preparation and aequorin loading

Antecubital venous blood was collected into acid citrate-dextrose anticoagulant (0.8% w/v citric acid; 2.2% w/v trisodium citrate; 2.4% w/v glucose), the anticoagulant: blood ratio being 1:9. Platelet-rich plasma (PRP) was prepared by centrifugation of the anticoagulated blood and acidified to pH 6.0 with 1M citric acid to inhibit binding of fibrinogen to its receptor [19]. The platelets were sedimented by centrifugation of the PRP (800 g, 20 min) and then resuspended in a wash solution consisting of HEPES pH 7.3 (10 mM), NaCl (140 mM), KCl (2.7 mM), EGTA (5 mM), glucose (5 mM) and bovine serum albumin (1 mg ml^{-1}). The platelets were then re-sedimented by centrifugation and resuspended in 50 μl of the HEPES buffered solution to produce a thick suspension. The platelets were then loaded with aequorin using a DMSO permeabilisation procedure

[20] as modified by Cooper *et al.* [21] in which prostacyclin (PGI_2) is omitted. Aequorin loading was conducted at room temperature (22°C) as follows. The volume of the platelet suspension was determined (typically 100 μl) and aequorin (300 μg in 15 μl EGTA solution) added. DMSO was then added in three equal volumes (final concentration 6%, v/v) at 2 min intervals with stirring. After the addition of the final aliquot of DMSO, the cells were incubated for a further 4 min whereupon they were transferred to a 1.5 ml microcentrifuge tube, diluted with 1 ml of the HEPES-buffered saline in which the EGTA was omitted. Platelets were sedimented by centrifugation (12,000 g, 15 s) and the washing procedure repeated twice. Finally the platelets were resuspended in the same solution and diluted to a platelet count of 300×10^9 platelets l^{-1} . CaCl_2 and MgCl_2 were then added to give a final concentration of 1 mM. Before commencement of experiments platelets were allowed to equilibrate for 20 min.

Measurement of platelet aggregation and calcium mobilization

Platelet aggregation and aequorin luminescence were measured simultaneously using a lumi-aggregometer (PICA, Chrono-Log Corp, Havertown, PA, USA).

Samples of washed platelet suspension (800 μl) were mixed with fibrinogen solution (200 μl , final concentration, 300 $\mu\text{g} \text{ ml}^{-1}$), previously prepared by suspension of 100 mg of fibrinogen in 4 ml deionised water followed by dialysis *vs* HEPES-buffered saline (minus EGTA) and determination of the protein concentration of the solution, and incubated for 1 min at 37°C. Drug vehicle (10 μl) or drug solution was then added and the incubation continued for 1 min whereupon collagen (5 $\mu\text{g} \text{ ml}^{-1}$) or adrenaline (16 μM) was added to stimulate platelet aggregation. As previously described [15] naftopidil and doxazosin were tested at four concentrations. The lowest drug concentrations were equivalent to reported therapeutic plasma free concentrations but without taking account of their binding to protein (95–97% to plasma proteins for naftopidil and 98–99% for doxazosin) and were consequently probably considerably lower than the actual free concentrations. The highest concentrations were also equivalent to the therapeutic plasma free concentrations but after having taken account of protein binding. Admittedly, our previous study of the effects of naftopidil and doxazosin on platelet function was conducted using PRP rather than washed platelets, i.e. platelet preparations from which plasma proteins have been removed. Proteins which may bind drugs (i.e. fibrinogen and especially albumin) were, however, present in washed platelet suspensions. Thus, it is our contention, as previously stated [15], that the results obtained with the higher drug concentrations are in fact probably more reflective of the situation occurring *in vivo*, if one considers the concentrations of drug remaining in the medium following protein binding. Thus, naftopidil was tested at concentrations of 0.4, 2, 10 and 40 μM (equivalent to 0.157, 0.79, 3.93 and 15.7 mg l^{-1}) and doxazosin at 0.3, 1.5, 7.5 and 30 μM (0.164, 0.82, 4.1 and 16.4 mg l^{-1}). Nifedipine was tested at concentrations of 0.28, 1.4, 7 and 28 μM (0.097, 0.49, 2.42 and 9.7 mg l^{-1}), 0.2–0.28 μM being equivalent to the reported therapeutic free drug plasma concentration [22].

Aggregation was expressed as a percentage of light transmission with 0% and 100% transmission being that recorded for the unstimulated platelet suspension and the suspension stimulated with $10 \mu\text{g ml}^{-1}$ collagen, respectively.

Intracellular calcium ion concentration ($[\text{Ca}^{2+}]_i$) was calculated from the fractional luminescence (L/L_{max}) which was obtained from the luminescence (L) of the aequorin-loaded platelet suspension and the maximal luminescence (L_{max}) recorded for appropriate aliquots of aequorin-loaded platelets lysed with Triton X-100 (final concentration 0.1%). $\text{Log}_{10} L/L_{\text{max}}$ was then converted to $[\text{Ca}^{2+}]_i$ by reference to a calibration curve which relates fractional aequorin luminescence to $[\text{Ca}^{2+}]$ in the presence of 1 mM Mg^{2+} .

Statistical analysis

All values are expressed as means $\pm$ s.e.mean. Data were analysed by Student's *t*-test for paired data or by ANOVA and Dunnett's test for multiple comparison.

Results

Collagen ($5 \mu\text{g ml}^{-1}$) and to a lesser extent adrenaline ($16 \mu\text{M}$) produced transient increases in $[\text{Ca}^{2+}]_i$ (Figure 1). In the case of collagen, in control samples an increase of $4.97 \pm 0.63 \mu\text{M}$ ($n=10$) was observed, which coincided with the onset of platelet aggregation (Figure 1). With adrenaline an increase in $[\text{Ca}^{2+}]_i$ of $3.6 \pm 0.96 \mu\text{M}$ ($n=6$) was recorded, however, unlike with collagen, this increase occurred after the onset of aggregation (Figure 1).

Naftopidil produced a concentration-dependent inhibition of the collagen-induced rise in $[\text{Ca}^{2+}]_i$ (Figure 2a), statistically significant inhibitions being recorded for $2 \mu\text{M}$ ($12.6 \pm 5.9\%$, $P < 0.05$, $n=6$), $10 \mu\text{M}$ ($13.8 \pm 3.8\%$, $P < 0.05$, $n=7$) and $40 \mu\text{M}$ ($22.9 \pm 4\%$, $P < 0.05$, $n=7$). With collagen-induced platelet aggregation however, a statistically significant inhibition was observed with naftopidil only at a concentration of $40 \mu\text{M}$ ($23.3 \pm 11.7\%$, $P < 0.05$, $n=7$).

The adrenaline-induced rise in $[\text{Ca}^{2+}]_i$ was also inhibited

Figure 1 Aggregation and $\Delta [\text{Ca}^{2+}]_i$ responses induced by collagen ($5 \mu\text{g ml}^{-1}$) and adrenaline ($16 \mu\text{M}$) in the presence of fibrinogen ($300 \mu\text{g ml}^{-1}$). $\blacktriangle$ indicate points of addition of agonists.

Figure 2 Effects of naftopidil and doxazosin on collagen ($5 \mu\text{g ml}^{-1}$) induced platelet $[\text{Ca}^{2+}]_i$ mobilization ($\square$) and aggregation ($\blacksquare$) in washed platelet suspensions. Data are means $\pm$ s.e.mean ($n=6-8$), $*P < 0.05$.

in a dose-dependent manner by naftopidil (Figure 3a), albeit to a greater extent than that observed with collagen, with an estimated IC_{50} (defined as the concentration of drug which produced 50% inhibition of the rise in $[\text{Ca}^{2+}]_i$ induced by adrenaline in the absence of drug) of $4.8 \mu\text{M}$. Statistically significant inhibitions were observed with $10 \mu\text{M}$ ($70.6 \pm 9.5\%$, $P < 0.05$, $n=6$) and $40 \mu\text{M}$ ($100 \pm 0\%$, $P < 0.05$, $n=6$) naftopidil, but not with $2 \mu\text{M}$ ($22.3 \pm 7.3\%$, $n=6$) or $0.4 \mu\text{M}$ ($4.3 \pm 7.3\%$, $n=6$). Contrasting with the results obtained with collagen, the inhibitions produced by naftopidil of the rise in $[\text{Ca}^{2+}]_i$ induced by adrenaline were closely paralleled by inhibition of platelet aggregation (Figure 3a), statistically significant results being obtained with $10 \mu\text{M}$ ($83.4 \pm 2.9\%$, $P < 0.05$, $n=6$) and $40 \mu\text{M}$ naftopidil ($100 \pm 0\%$, $P < 0.05$, $n=6$) but not $2 \mu\text{M}$ ($25.7 \pm 11.4\%$, $n=6$) or $0.4 \mu\text{M}$ ($4.3 \pm 7.3\%$, $n=6$). The IC_{50} calculated for naftopidil with respect to adrenaline-induced aggregation was $3.8 \mu\text{M}$.

Doxazosin produced statistically significant concentration-dependent inhibitions of the collagen stimulated rise in $[\text{Ca}^{2+}]_i$ of the same order as naftopidil (Figure 2b): $1.5 \mu\text{M}$ ($8.6 \pm 3.5\%$, $P < 0.05$, $n=6$), $7.5 \mu\text{M}$ ($15 \pm 3.8\%$, $P < 0.05$, $n=6$) and $30 \mu\text{M}$ ($17.4 \pm 2.5\%$, $P < 0.05$, $n=8$). Unlike with naftopidil, however, these inhibitions were not mirrored by statistically significant inhibitions of platelet aggregation (Figure 2b). Doxazosin also inhibited the adrenaline-induced rise in $[\text{Ca}^{2+}]_i$ in a concentration-dependent manner (Figure 3b), although a statistically significant result was only obtained at a concentration of $30 \mu\text{M}$ ($37.6 \pm 13.7\%$, $P < 0.05$,

Figure 3 Effects of naftopidil and doxazosin on adrenaline (16 μM) induced platelet $[Ca^{2+}]_i$ mobilisation ($\square$) and aggregation ($\blacksquare$) in washed platelet suspensions. Data are means $\pm$ s.e.mean ($n=6$), * $P<0.05$.

$n=6$). Statistically significant inhibitions of platelet aggregation were seen with 7.5 μM ($35.8 \pm 12.6\%$, $P<0.05$, $n=6$) and 30 μM ($49.6 \pm 17.2\%$, $P<0.05$, $n=6$) doxazosin (Figure 3b).

As expected, nifedipine produced concentration-dependent inhibitions of collagen stimulated Ca^{2+} mobilization (1.4 μM , $14.5 \pm 4.3\%$, $P<0.05$, $n=7$; 7 μM , $19.3 \pm 3.5\%$, $P<0.05$, $n=7$; 28 μM , $47.8 \pm 2.7\%$, $P<0.05$, $n=9$) which were paralleled by statistically significant inhibitions of platelet aggregation (1.4 μM , $24 \pm 9.2\%$, $P<0.05$, $n=7$; 7 μM , $40.1 \pm 11.4\%$, $P<0.05$, $n=7$; 28 μM , $55.1 \pm 9.2\%$, $P<0.05$, $n=9$) (Figure 4).

Figure 4 Effect of nifedipine on collagen (5 $\mu g\ ml^{-1}$) induced platelet $[Ca^{2+}]_i$ mobilisation ($\square$) and aggregation ($\blacksquare$) in washed platelet suspensions. Data are means $\pm$ s.e.mean ($n=8-9$), * $P<0.05$.

Discussion

As previously reported [21] washed platelets loaded with aequorin responded to collagen with a substantial rise in $[Ca^{2+}]_i$, which coincides with aggregation. Perhaps surprisingly, adrenaline was also found to induce near maximal platelet aggregation and $[Ca^{2+}]$ mobilization, although the latter process occurred after the onset of aggregation. Previous studies involving washed platelet preparations and gel filtered platelets have yielded conflicting results when adrenaline has been used as the primary agonist [23, 24]. Lanza *et al.* [23] have proposed that the aggregation of washed platelets induced by adrenaline reflects the pre-activation of platelets during processing, as adrenaline by itself is not thought to induce modifications of washed platelet morphology, metabolism or function, but rather interacts with platelet α_2 -adrenoreceptors to potentiate the effects of other platelet agonists. The platelet membrane permeabilisation procedure employed for loading platelets with aequorin may also contribute to the mobilization of $[Ca^{2+}]_i$ induced by adrenaline [23]. Thus, apart from the fact that fibrinogen was included in all platelet incubations, platelet pre-activation as a consequence of the cellular processing procedure used may, to some extent, explain the responses to adrenaline observed in this study, given that stimulators of adenylate cyclase, e.g. PGI_2 or PGE_1 , which are frequently included in platelet washing solutions to reduce mechanical activation of platelets, such as by centrifugation or pellet resuspension, were omitted. Reproducible responses to adrenaline and clear-cut responses to the drugs tested were however obtained, indicating that variable and non-specific effects produced by platelet processing were minimal.

Both naftopidil and doxazosin reduced slightly and to comparable extents the rise in platelet $[Ca^{2+}]_i$ stimulated by collagen. This was not paralleled by inhibitions of platelet aggregation, except at the highest concentration (40 μM) of naftopidil tested, essentially providing confirmation of our previous findings obtained with platelet-rich plasma [15]. It has been suggested that collagen-induced platelet activation, which is mediated by the activation of phospholipase C, is relatively insensitive to increases in $[Ca^{2+}]_i$ but is dependent on the generation of cyclooxygenase products [25]. Thus, the rise in $[Ca^{2+}]_i$, as measured by aequorin, induced by collagen is not the sole determinant of collagen-induced responses [26] and, therefore, the inhibition of this rise may not interfere with these responses. The inhibitions produced by naftopidil and doxazosin of the collagen-induced increases in $[Ca^{2+}]_i$ may therefore involve the blockade of Ca^{2+} influx, as extracellular Ca^{2+} is known to contribute to the aequorin-registered rise in $[Ca^{2+}]_i$ caused by collagen [26].

Naftopidil produced marked concentration-dependent inhibitory effects on adrenaline-induced platelet $[Ca^{2+}]_i$ mobilization and aggregation, far in excess of those observed on collagen stimulation, whilst doxazosin was less potent in its effects. Platelet aggregation was also inhibited by both drugs, albeit to a greater extent by naftopidil. The fact that both drugs had this effect, whilst a previous study [15] which entailed studying their effects in platelet-rich plasma failed to demonstrate strong effects on adrenaline-induced aggregation, supports our proposal that plasma protein

binding of drugs, resulting in reductions in available free drug concentrations, can lead to the masking of the inhibitory effects of potential anti-thrombotic agents.

Adrenaline is the only stimulator of human platelets known to be fully dependent on the presence of extracellular Ca^{2+} for platelet activation [27]. Generally, the activation of platelet α_2 -adrenoreceptors by adrenaline triggers calcium influx via receptor-operated calcium channels [28]. Thus the inhibitory effects of naftopidil and doxazosin on the adrenaline stimulated rise in platelet $[\text{Ca}^{2+}]_i$ and platelet aggregation, may be mediated through the blockade of Ca^{2+} influx involving α_2 -adrenoreceptor activation, or receptor-operated calcium channels and/or voltage operational calcium channels. Indeed, naftopidil has been reported to inhibit Ca^{2+} entry into vascular and cardiac muscle, possibly via L-type calcium channels [29, 30], and the thrombin-induced rise in platelet $[\text{Ca}^{2+}]_i$ [31]. The concomitant inhibition by naftopidil of platelet aggregation and the rise in $[\text{Ca}^{2+}]_i$ observed in this study is consistent with the concept of adrenaline-induced aggregation being associated with Ca^{2+} influx.

The inhibitory effects of nifedipine on platelet function have been well documented [32–38] and was used in this study as a positive control. Thus, we found that nifedipine inhibited both collagen stimulated platelet Ca^{2+} mobilization and aggregation in a concentration-dependent manner. These findings are consistent with the blockade by nifedipine of a dihydropyridine-sensitive transmembrane calcium channel with the consequent inhibition of intracellular Ca^{2+} -dependent signal transduction mechanisms involved in platelet aggregation, e.g. thromboxane A_2 synthesis [39].

At this stage in our investigations we cannot predict with confidence if the data obtained in this study is of clinical relevance. It is however important to note that previous *in vitro* platelet studies which have demonstrated inhibitory effects for drugs have often been followed up by *in vivo* studies yielding similar findings. Indeed, Hernandez *et al.* [13] followed up an *in vitro* study on the inhibitory actions of doxazosin on platelet aggregation with an *ex vivo* study in hypertensive patients which demonstrated similar inhibitory effects for the drug [14]. In conclusion, what we can say about the present study is that it provides further evidence for the platelet anti-aggregatory actions of α_1 -adrenoreceptor blocking anti-hypertensive agents.

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