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 μg ml⁻¹) both collagen (5 μg ml⁻¹) and adrenaline (16 μM) stimulated the aggregation of washed platelets. Collagen induced a transient rise (+4.97 ± 0.63 μM) in platelet Ca²⁺ concentration, [Ca²⁺]ᵢ, measured using the photoprotein aequorin, which coincided with the onset of aggregation. Adrenaline induced a smaller rise (+3.6 ± 0.96 μM) which, however, occurred after the onset of aggregation. Naftopidil, an α₁-adrenoreceptor antagonist produced a concentration-dependent inhibition of collagen-induced Ca²⁺ mobilization, maximum inhibition (22.9 ± 4.0%, P < 0.05) occurring with 40 μM naftopidil. The inhibition of Ca²⁺ mobilization was not reflected by a concentration-dependent inhibition of platelet aggregation, although 40 μM naftopidil produced statistically significant inhibition (23.3 ± 11.7%, P < 0.05). The adrenaline-induced rise in [Ca²⁺]ᵢ, was inhibited dose dependently by naftopidil (e.g. 40 μM naftopidil, 100 ± 0.0%, P < 0.05), as was aggregation (40 μM naftopidil, 100 ± 0.0%, P < 0.05). Doxazosin, another α₁-adrenoreceptor blocker, inhibited Ca²⁺ mobilization induced by collagen to similar extents as for naftopidil (30 μM doxazosin, 17.4 ± 2.5%, P < 0.05), but did not inhibit platelet aggregation. It also inhibited the adrenaline-induced rise in [Ca²⁺]ᵢ, in a concentration-dependent manner (30 μM doxazosin, 37.6 ± 13.7%, P < 0.05), significant inhibitions of platelet aggregation also being produced (30 μM, 49.6 ± 17.2%, P < 0.05). As expected, the calcium channel blocker nifedipine produced concentration-dependent inhibitions of both collagen-induced Ca²⁺ mobilization (e.g. 28 μM nifedipine, 47.8 ± 2.7%, P < 0.05) and aggregation (28 μM, 55.1 ± 9.2%, P < 0.05).

Conclusions These data indicate that the α₁-adrenoreceptor blockers, naftopidil and doxazosin, inhibit Ca²⁺ 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, α₂-adrenoreceptor antagonists, β-adrenoreceptor blockers and calcium antagonists all interfere with platelet aggregation [3–9]. α₁-Adrenoreceptor blockers, e.g. prazosin, urapidil and doxazosin also inhibit platelet aggregation [10–15]. In addition, we have reported that the experimental α₁-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 sub-threshold concentrations of collagen, serotonin or ADP [15]. Additionally, but less markedly, nifedipin inhibited platelet aggregation induced by adrenaline alone and to a lesser extent, serotonin. The results obtained with α₁-adrenoreceptor antagonists are paradoxical since, firstly, platelets are not thought to possess α₂-adrenoreceptors and secondly, platelet aggregation induced by adrenaline is mediated by α₁-adrenoreceptors [16]. It must therefore be concluded that α₁-adrenoreceptor

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acidified with acetic acid (3.2 m) is a prerequisite for platelet activation [17, 18]. Increases in 300 receptor antagonists on platelet function.

were prepared in DMSO and diluted with distilled water to tested at four concentrations. The lowest drug concentrations m associated with platelet activation. These include the at 2 min intervals with stirring. After the addition of the ff

Blood collection, platelet preparation and aequorin loading was conducted using PRP rather than washed platelets, i.e.

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 Photoprobes, 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.

Anticoagulant 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 1 m 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 (PGI2) 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 9%, 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 × 10⁶ platelets l⁻¹. CaCl₂ and MgCl₂ were then added to give a final concentration of 1 mM. Before commencement of experiments platelets were allowed to equilibrate for 20 min.

Methods
Measurement of platelet aggregation and calcium mobilization Platelet aggregation and aequorin luminescence were measured simultaneously using a lumi-aggregometer (PICA, Chrono-Log Corp, Haverton, PA, USA).

Samples of washed platelet suspension (800 μl) were mixed with fibrinogen solution (200 μl, final concentration, 300 μg ml⁻¹), previously prepared by suspension of 100 mg fibrinogen in 4 ml denoised 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 μg 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 proteins (95–97% to plasma proteins for naftopidil and 96–99% for doxazosin) and were consequently probably considerably lower than the actual free concentrations. The highest concentrations were also equivalent to the therapeu tic 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 μg ml⁻¹ collagen, respectively.

Intracellular calcium ion concentration ([Ca²⁺]ᵢ) was calculated from the fractional luminescence (L/Lₘₐₓ) which was obtained from the luminescence (L) of the aequorin-loaded platelet suspension and the maximal luminescence (Lₘₐₓ) recorded for appropriate aliquots of aequorin-loaded platelets lysed with Triton X-100 (final concentration 0.1%). Lₒ/ₘₐₓ, Lₘₐₓ was then converted to [Ca²⁺]ᵢ by reference to a calibration curve which relates fractional aequorin luminescence to [Ca²⁺]ᵢ in the presence of 1 mM Mg²⁺. Statistical analysis All values are expressed as means ± 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 ng ml⁻¹) and to a lesser extent adrenaline (16 μM) produced transient increases in [Ca²⁺]ᵢ (Figure 1). In the case of collagen, in control samples an increase of 4.97 ± 0.63 μM (n = 10) was observed, which coincided with the onset of platelet aggregation (Figure 1). With adrenaline an increase in [Ca²⁺]ᵢ of 3.6 ± 0.96 μM (n = 6) was obtained, 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 [Ca²⁺]ᵢ (Figure 2a), statistically significant inhibitions being recorded for 2 μM (12.6 ± 5.9%, P < 0.05, n = 6), 10 μM (13.8 ± 3.8%, P < 0.05, n = 7) and 40 μM (22.9 ± 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 μM (23.3 ± 11.7%, P < 0.05, n = 7).

The adrenaline-induced rise in [Ca²⁺]ᵢ was also inhibited in a dose-dependent manner by naftopidil (Figure 3a), albeit to a greater extent than that observed with collagen, with an estimated IC₅₀ (defined as the concentration of drug which produced 50% inhibition of the rise in [Ca²⁺]ᵢ) induced by adrenaline in the absence of drug) of 4.8 μM. Statistically significant inhibitions were observed with 10 μM (70.6 ± 9.9%, P < 0.05, n = 6) and 40 μM (100 ± 0%, P < 0.05, n = 6) naftopidil, but not with 2 μM (22.3 ± 7.3%, n = 6) or 0.4 μM naftopidil (2.8 ± 3.2%, n = 6). Contrasting with the results obtained with collagen, the inhibitions produced by naftopidil of the rise in [Ca²⁺]ᵢ induced by adrenaline were closely paralleled by inhibition of platelet aggregation (Figure 3a), statistically significant results being obtained with 10 μM (83.4 ± 2.9%, P < 0.05, n = 6) and 40 μM naftopidil (100 ± 0%, P < 0.05, n = 6) but not 2 μM (25.7 ± 11.4%, n = 6) or 0.4 μM (4.3 ± 7.3%, n = 6). The IC₅₀ calculated for naftopidil with respect to adrenaline-induced aggregation was 3.8 μM.

Doxazosin produced statistically significant concentration-dependent inhibitions of the collagen stimulated rise in [Ca²⁺]ᵢ, of the same order as naftopidil (Figure 2b): 1.5 μM (8.6 ± 3.5%, P < 0.05, n = 6), 7.5 μM (15 ± 3.8%, P < 0.05, n = 6) and 30 μM (37.4 ± 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 [Ca²⁺]ᵢ in a concentration-dependent manner (Figure 3b), although a statistically significant result was only obtained at a concentration of 30 μM (37.6 ± 13.7%, P < 0.05,

nifedipine were seen with 7.5 μM collagen. This was not paralleled by inhibitions of platelet aggregation, except at the highest concentration (40 μM) induced platelet aggregation, supports our proposal that plasma protein concentrations—aggregation, 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 α2-adrenoceptors 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+}], 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 permeabilisation procedure used may, to some extent, explain the responses to adrenaline observed in this study, given that stimulators of adenylate cyclase, e.g. PGI2 or PGE1, 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 naflopipid and doxazosin reduced slightly and to comparable extents the rise in platelet [Ca^{2+}], stimulated by collagen. This was not paralleled by inhibitions of platelet aggregation, except at the highest concentration (40 μM) of naflopipid 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+}] but is dependent on the generation of cyclooxygenase products [23, 24]. Thus, the rise in [Ca^{2+}], as measured by aequorin, induced by collagen is not the sole determinant of collagen-induced aggregation, but is dependent on the activation of cyclooxygenase products [23]. The rise in [Ca^{2+}], as measured by aequorin, induced by collagen is not the sole determinant of collagen-induced aggregation, but is dependent on the activation of cyclooxygenase products [23].

### Discussion

As previously reported [21] washes platelets loaded with aequorin responded to collagen with a substantial rise in [Ca^{2+}], 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 α2-adrenoceptors 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+}], 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 permeabilisation procedure used may, to some extent, explain the responses to adrenaline observed in this study, given that stimulators of adenylate cyclase, e.g. PGI2 or PGE1, 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.

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### References


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 \(\alpha_2\)-adrenoceptors 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 [Ca\(^{2+}\)]\(_i\), and platelet aggregation, may be mediated through the blockade of Ca\(^{2+}\) influx involving \(\alpha_2\)-adrenoceptor 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 thrombus-induced rise in platelet [Ca\(^{2+}\)]\(_i\) [31]. The concomitant inhibition by naftopidil of platelet aggregation and the rise in [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 \(\alpha_2\)-adrenoceptor blocking anti-hypertensive agents.

References


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