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Effect of 781094, a new selective α-adrenoceptor antagonist, on the aggregatory responses of human blood platelets and on binding of [^{3}H]-dihydroergocryptine to these cells

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1 781094 (2-(2(1, 4-benzodioxanyl))-2-imidazoline hydrochloride) is a potent competitive inhibitor of the aggregatory responses of human platelets induced by adrenaline ($pA_2 = 7.3$) and UK-14304.

2 781094 is a more potent inhibitor of the pro-aggregatory response to clonidine than of that to methoxamine. The α_2/α_1-adrenoceptor selectivity ratio is 11.4.

3 781094 is a potent inhibitor of the binding of [^{3}H]-dihydroergocryptine to platelet lysates.

4 781094 has no effect on the aggregatory responses of human platelets induced by adenosine-5′-pyrophosphate (ADP), 5-hydroxytryptamine, thrombin, U-46619, or arginine-vasopressin provided that the platelet-rich plasma does not exhibit enhanced responsiveness. In some instances high concentrations of 781094 potentiate the aggregatory response to collagen.

5 In platelet-rich plasma which exhibits enhanced responsiveness, 781094 causes partial inhibition of the aggregatory responses to 5-hydroxytryptamine, ADP and vasopressin at concentrations similar to those required to inhibit the response to adrenaline.

6 781094 acts as a specific antagonist for the responses mediated by human platelet α-adrenoceptors and exhibits moderate selectivity for the α_2-adrenoceptors on this cell.

Introduction

Human blood platelets aggregate on exposure to a wide range of agonists including adrenaline, adenosine 5′-pyrophosphate (ADP), collagen, thrombin, vasopressin, 5-hydroxytryptamine and prostaglandin endoperoxides (cf. Gordon & Milner, 1976). In the case of adrenaline the aggregatory response is mediated primarily, if not exclusively, by an α_2-adrenoceptor (Grant & Scrutton, 1979; Hsu, Knapp & Halushka, 1979), although in at least some individuals physiological response studies suggest that α_1-adrenoceptors are also present on these cells (Grant & Scrutton, 1979; 1980). The human platelet therefore provides a suitable isolated cell system for analysis of the specificity and selectivity of drugs which are proposed to act at α-adrenoceptors. Such studies are described here for 781094 (2-(2(1, 4-benzodioxanyl))-2-imidazoline hydrochloride) a new drug which is postulated to act as a selective α_2-adrenoceptor antagonist (Chapleo, Doxey, Myers & Roach, 1981).

Methods

Blood was obtained by antecubital venepuncture from donors who had taken no drugs during the previous 14 days. For the aggregation studies coagulation was prevented by addition of 1/10th volume of acid citrate-dextrose (10 mM citrate) and platelet-rich plasma prepared by centrifugation for 20 min at 200 *g* and 20°C. Platelet-free plasma was prepared by centrifugation of an aliquot of the sedimented erythrocytes for 2 min at 12,000 *g*. Platelet-rich plasma was stored at 37°C in a tightly stoppered container and was used within 2.5 h after preparation. In all cases studies were performed using platelet-rich plasma from at least five different donors.

Platelet aggregation was monitored at 37°C using a Payton dual channel aggregometer interfaced with a Rikadenki Model 300 BD recorder and calibrated as described by Pearce, Wright, Egan & Scrutton (1978).

For radioligand binding studies 1/6th volume of acid-citrate dextrose prepared according to Aster &

Jandl (1964) was used as anticoagulant and washed platelets prepared as described by Knight & Scrutton (1980). Platelet lysates were prepared from the washed platelet preparations as described by Newman, Williams, Bishopric & Leflowitz (1978).

Binding studies were performed essentially as described by Newman *et al.* (1978). Platelet lysates (0.1 ml) were incubated for 25 min at 25°C with [^{3}H]-dihydroergocryptine plus appropriate additions in a total volume of 0.2 ml at the concentrations as indicated in the table legend. The platelet membranes were then rapidly separated from the suspension by filtration on Whatman GF/C glass fibre filters which had previously been soaked for at least 1 h in the suspension buffer. The filters were rapidly washed with 6 × 5 ml ice-cold 0.05 M Tris-Cl pH 7.35 containing 0.15 M NaCl and 0.02 M disodium edetate (EDTA) and then dried either under vacuum and/or in an oven at 50°C. The ^{3}H retained on the dry filters was estimated using a Beckman Model LS100C scintillation counter and Triton X-100/toluene (1:2, v/v) scintillation fluid containing 4 g 2, 5-diphenyloxazole per litre. Unless otherwise stated determinations were performed in triplicate. Values are expressed as means ± s.e.mean with the number of determinations in parentheses.

Collagen suspensions were prepared by homogenization of bovine achilles tendon collagen in 0.9% w/v NaCl solution (saline). The protein content of the resulting suspension was estimated by the biuret procedure (Layne, 1957).

Thrombin, arginine-vasopressin, adrenaline bitartrate, yohimbine and 5-hydroxytryptamine creatinine sulphate were obtained from Sigma Chemical Co.; adenosine-5′-pyrophosphate (ADP) from PL-Biochemicals Inc.; UK-14304 (5 - bromo - 6 - [2 - imidazolin - 2 ylamino] - quinoxaline) from Pfizer Ltd.; methoxamine hydrochloride from Wellcome Laboratories Ltd.; phentolamine mesylate from CIBA-Geigy Ltd.; U-46619 (11α, 9α-epoxymethanoprostaglandin H_2) from Upjohn Inc.; prazosin from Pfizer Ltd.; [^{3}H]-dihydroergocryptine (30 Ci/mmol) from the Radiochemical Centre; rauwolsin hydrochloride from Roth Chemie Gmbh; and 781094 from Reckitt & Colman Ltd.

Results

Effect of 781094 on the aggregatory response of human platelets to adrenaline and to UK-14304

As shown in Figure 1, 781094 causes potent and complete inhibition of the aggregatory response to both adrenaline (Figure 1a) and also the selective α_2-adrenoceptor agonist, UK-14304 (Ashton & Rawlins, 1978; Grant & Scrutton, 1980; Cambridge, 1981) (Figure 1b). In contrast to most platelet excitatory agonists, reduction by an antagonist of the extent of the initial phase of the response to adrenaline does not cause loss of the second phase of the response until the initial phase is virtually abolished (Figure 1). Since the extent of the initial phase is not readily defined, and since the total extent of response is not simply related to the effect of the antagonist, the degree of inhibition is most readily quantitated by measurement of the initial rate of aggregation as a function of antagonist concentration. The IC_{50} values obtained from these data, and calculated as described in Methods, are 0.24 ± 0.01 μM (5) (Figure 1a) and 0.26 ± 0.01 μM (5) (Figure 1b). No significant difference was observed in the extent of inhibition by 781094 when this drug was added from 30 s to 4 min before the agonists.

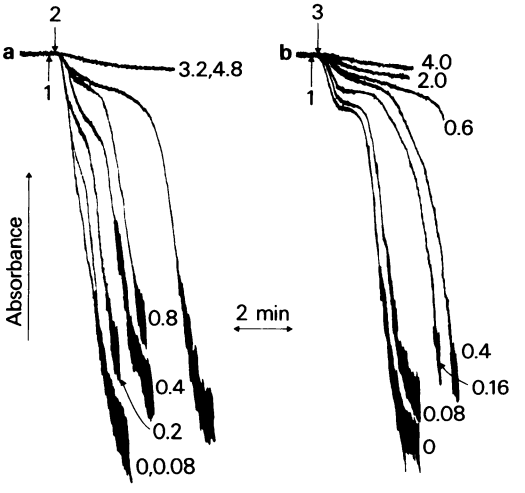

Figure 1 Effect of 781094 on the aggregatory responses of human platelets to adrenaline (a) and UK-14304 (b). Platelet-rich plasma was prepared using acid-citrate dextrose (10 mM citrate) and platelet aggregation monitored as described in Methods. Addition of 781094 alone at concentrations up to 200 μM failed to cause an aggregatory response. The additions were as follows: at (1) 781094 at the concentrations (μM) indicated by the figures to the right of the individual traces; at (2) 2 μM adrenaline; at (3) 4 μM UK-14304. The results shown are from a single experiment but are typical of those obtained in 5 similar experiments.

The effect of 781094 on the aggregatory response to adrenaline has been analysed further by measurement of dose-response curves for this agonist in the presence of a series of fixed concentrations of 781094. A parallel shift in the $\log_{10}$ dose-response relationship is observed on addition of increasing concentrations of 781094 (Figure 2a) and when plotted as described by Arunlakshana & Schild (1959) a linear relationship of approximately unit slope

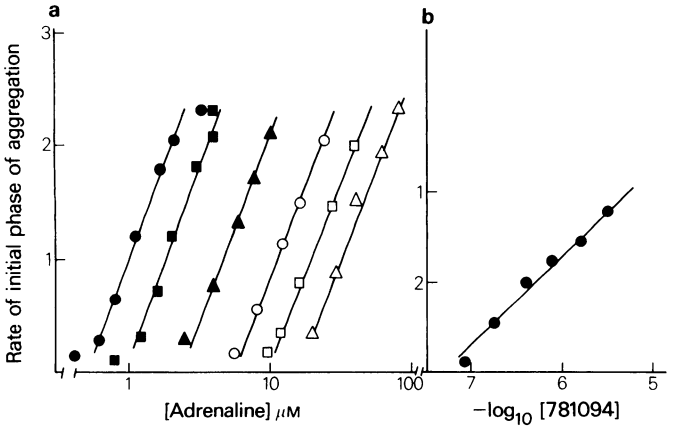

Figure 2 The relationship between the rate of the primary aggregatory response to human platelets to adrenaline and adrenaline concentration in the presence of various concentrations of 781094. Platelet-rich plasma was prepared and platelet aggregation monitored as described in Figure 1. (a) Shows the dose-response curves obtained and (b) the Schild plot derived from these data. In (a) the concentrations (μM) of 781094 employed were 0 (●), 0.08 (■), 0.2 (▲), 0.4 (○), 0.8 (□) and 1.6 (△). The results shown are from a single experiment but are typical of those obtained in 4–5 similar experiments. No significant change in sensitivity to adrenaline occurred during the course of the experiment. In (b) the slope of the line was calculated by linear regression analysis to be 1.0, $r = 0.992$. The slope had a 95% confidence range of 0.78 to 1.20.

($r = 0.992$) is obtained (Figure 2b). From this analysis the mean pA_2 is estimated as 7.3.

Effects of 781094 on the pro-aggregatory response of human platelets induced by clonidine and methoxamine

In order to obtain a direct measure of α_2/α_1 selectivity, we have also tested the effect of 781094 on the response of human platelets to selective α-adrenoceptor agonists such as clonidine (α_2), or methoxamine or phenylephrine (α_1). In contrast to adrenaline and UK-14304, these agonists do not induce an aggregatory response when added alone but do enhance the response of the platelets to agonists acting at a different receptor, e.g. ADP, when these latter agonists are added at a concentration which induces a sub-optimal response. Thus, as shown previously (Grant & Scrutton, 1980) and in Figure 3, addition of clonidine, or methoxamine prior to ADP increases the extent of the initial response to the sub-optimal dose of ADP and also, probably as a consequence of the enhanced primary response, may cause induction of a second wave of aggregation, as is shown in Figure 3. 781094 is a potent inhibitor of the pro-aggregatory response to clonidine and completely abolishes this response at a concentration of 4 μM (Figure 3a) without affecting the response to ADP. The IC_{50} value obtained from a series of such studies is 0.72 ± 0.06 (6) μM. Thus the concentration of 781094 required to cause 50% inhibition of the pro-aggregatory response to clonidine is three fold

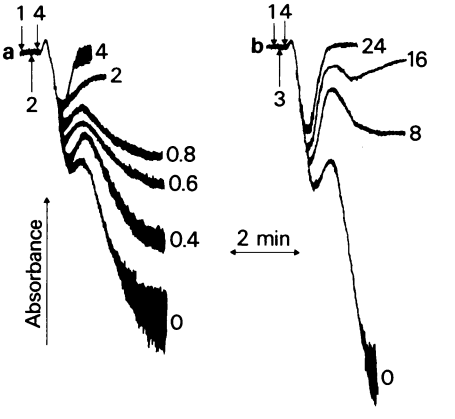

Figure 3 Effect of 781094 on the pro-aggregatory responses of human platelets to clonidine (a) and methoxamine (b). Platelet-rich plasma was prepared and platelet aggregation monitored as described for Figure 1. Addition of 781094 at concentrations up to 50 μM had no effect on the response to a sub-optimal dose of ADP. The additions were as follows: at (1) 781094 at the concentrations (μM) indicated by the figures to the right of the individual traces; at (2) 10 μM clonidine; at (3) 80 μM methoxamine; at (4) 1 μM (a) or 2 μM (b) ADP. In (a) the response to 1 μM ADP alone was identical with that observed in the presence of 4 μM 781094; in (b) the response to 2 μM ADP was identical to that observed in the presence of 24 μM 781094. The results shown are from a single experiment but are typical of those obtained in 4–6 similar experiments. Different platelet preparations were used for the studies shown in (a) and (b).

Table 1 Effect of 781094 on binding of [^{3}H]-dihydroergocryptine by platelet lypates: comparison with other α-adrenoceptor antagonists

Inhibitor	K_I (μM) *for inhibition of binding of [^{3}H]-dihydroergocryptine*
781094	0.007 ± 0.003 (4)
Yohimbine	0.008 ± 0.002 (3)
Indoramin	0.9 ± 0.2 (3)
Prazosin	1.8 ± 0.3 (3)

Binding studies were performed as described in Methods. The concentration of [^{3}H]-dihydroergocryptine employed was 6 nM. K_I values were calculated as described by Cheng & Prussoff (1973) using a K_L value of 3 nM (Newman *et al.*, 1978). The K_I values for inhibition by yohimbine and prazosin of the binding of [^{3}H]-dihydroergocryptine are similar to those obtained by Newman *et al.* (1978).

greater than that which gives 50% inhibition of the aggregatory response to adrenaline or UK-14304 (Figure 1).

Complete inhibition by 781094 of the pro-aggregatory responses to methoxamine (Figure 3b) and phenylephrine is also observed although the concentrations of the drug required to achieve this effect ($IC_{50} = 8.2 \pm 0.03$ μM) (4) (methoxamine) and $IC_{50} = 5.9 \pm 1.0$ μM (4) (phenylephrine) are approximately an order of magnitude greater than those which give blockade of the pro-aggregatory response to clonidine.

Effect of 781094 on the binding of [^{3}H]-dihydroergocryptine by platelet lysates

We have also examined the effect of 781094 on the binding of the non-selective α-adrenoceptor ligand, [^{3}H]-dihydroergocryptine, to human platelet lysates in comparison with drugs of established α-adrenoceptor sub-type selectivity. The data obtained in these studies are summarised as K_i values in Table 1.

Effect of 781094 on the response of human platelets to agonists other than adrenaline and UK-14304

The specificity of the action of 781094 as an antagonist at the platelet α-adrenoceptor has been assessed by testing the effect of this compound on the aggregatory responses induced by ADP, arginine-vasopressin, thrombin, 5-hydroxytryptamine, collagen and the stable prostaglandin endoperoxide/thromboxane A_2 analogue, U-46619. Addition of 781094 at concentrations up to 80 μM had no detectable effect on the aggregatory responses induced by saturating concentrations of ADP, vasopressin, thrombin and U-46619. The drug was also without effect when non-saturating concentrations of these agonists were used unless the platelet-rich plasma employed showed a biphasic response to 5-hydroxytryptamine (see below).

When aggregation was induced by collagen, concentrations of 781094 exceeding 10 μM caused a slight enhancement of the response of platelet-rich plasma obtained from some donors (Figure 4a). This effect appears to involve the early phase of the response to collagen since the effect was not observed in platelet-rich plasma which responded to collagen without a detectable initial increase in absorbance (Figure 4b).

In most samples 781094 when added at concentrations up to 100 μM also has no effect on the reversible aggregatory response which is typically observed on addition of 5-hydroxytryptamine (Figure 4d). However, effects of 781094 are observed for these less common platelet-rich plasma samples which exhibit a biphasic response to 5-hydroxytryptamine. In such cases as illustrated in Figure 4c, 781094 causes partial inhibition of the response to 5-hydroxytryptamine over a range of concentration ($IC_{50} = 0.3 \pm 0.04$ μM (3)) very similar to that over which this drug inhibits the entire aggregatory response to adrenaline or UK-14304 (Figure 1). This inhibition is primarily due to abolition of the second phase of the response to 5-hydroxytryptamine, but some reduction of the initial phase is also observed. However, addition of 781094 at concentrations up to 200 μM does not abolish the response to 5-hydroxytryptamine in these experiments. In platelet-rich plasma which exhibits a biphasic response to 5-hydroxytryptamine, addition of 781094 over a concentration range similar to that shown in Figure 4c also causes partial inhibition of the second phase of the response to non-saturating concentrations of other agonists e.g. ADP, vasopressin. Such inhibition has no significant effect on the initial phase of the response even at very high concentrations of 781094; and for ADP the effect on the second phase can be overcome by increasing the concentratons of this agonist. Thus in all cases where inhibition is observed the major effect of 781094 is to abolish the second phase of the aggregatory response.

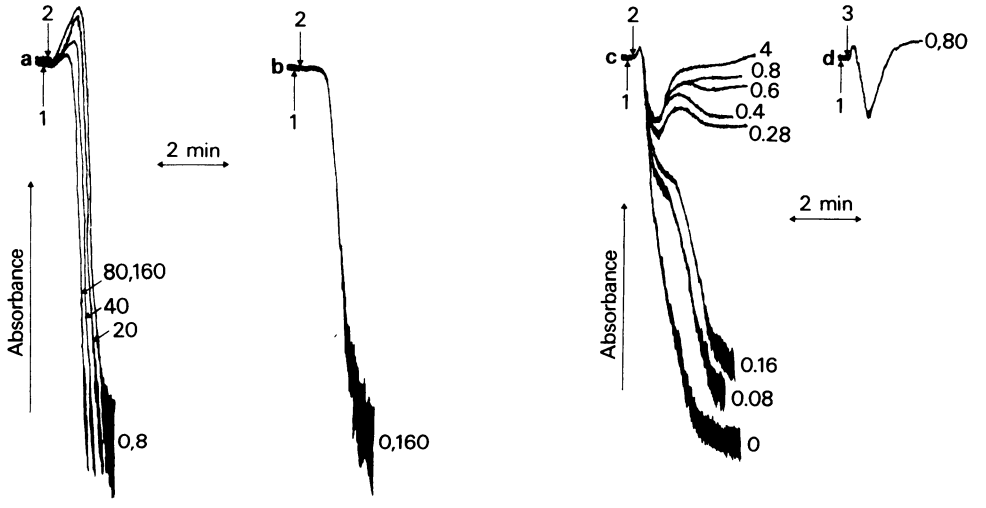

Figure 4 Effect of 781094 on the aggregatory response of human platelets to collagen (a,b) and 5-hydroxytryptamine (c,d). Platelet-rich plasma was prepared and platelet aggregation monitored as described in Figure 1. The additions were as follows: at (1) 781094 at the concentrations (μM) indicated by the figures to the right of the traces; at (2) collagen (4(b) or 10(a) μg/ml); at (3) 5-hydroxytryptamine (4(d) or 20 (c) μM). In (d) no difference in the extent of the aggregatory response was observed if the concentration of 5-hydroxytryptamine was increased to 20 μM. The results shown are from single experiments using different platelet preparations, but are typical of at least 3 similar experiments.

Discussion

The studies described here demonstrate that the new drug 781094 functions as a highly specific α-adrenoceptor antagonist with some selectivity for the α_2-adrenoceptor when tested in the human platelet system. It is equally potent as an inhibitor of the aggregatory responses induced either by a non-selective agonist (adrenaline) or by an agonist which acts selectively at α_2-adrenoceptors (UK-14304) (Figure 1), and exhibits properties consistent with its identification as a competitive agonist acting preferentially at the platelet α_2-adrenoceptor (Figure 2). Comparison with the IC_{50} values obtained previously for this system (Grant & Scrutton, 1980; Scrutton & Wallis, 1981) indicates that 781094 is twice as potent as yohimbine, and more than two orders of magnitude more potent than prazosin or indoramin as an inhibitor of the aggregatory responses induced by adrenaline or UK-14304. The effects of 781094 on the pro-aggregatory responses to clonidine and methoxamine demonstrate however that 781094 also causes inhibition of the α_1-adrenoceptor-mediated response, albeit at higher concentrations (Figure 3b). The α_2/α_1 selectivity factor calculated by comparison of the IC_{50} values for inhibition of clonidine and methoxamine stimulation of the response to ADP is 11.4. The α_2/α_1 selectivity factors for yohimbine and prazosin in these responses in the human platelet are greater than 16.7 and less than 0.04 respectively (Grant & Scrutton, 1980). Hence the physiological response studies indicate that 781094 is acting preferentially at the α_2-adrenoceptor although its preference for this subtype is less marked than that of yohimbine in the pro-aggregatory system. The selectivity of 781094 for α_2-adrenoceptors is also demonstrated by its effect on the binding of [^{3}H]-dihydroergocryptine to platelet lysates. In this system, 781094 behaves similarly to yohimbine and clearly differs from α_1-selective antagonists such as prazosin or indoramin (Table 1).

781094 is also highly specific as an antagonist at the platelet α-adrenoceptor. In platelet-rich plasma from most donors it has no effect on the responses to ADP, U-46619, thrombin, 5-hydroxytryptamine or vasopressin at concentrations up to two orders of magnitude greater than that required to abolish the response to a saturating concentration of adrenaline. In this respect 781094 is considerably more specific than yohimbine which causes marked inhibition of the primary aggregatory response to 5-hydroxytryptamine at concentrations not markedly higher than those required to abolish the response to a saturating concentration of adrenaline (Grant & Scrutton, 1979). However in some, although not all, platelet preparations high concentrations of 781094 enhance the aggregatory response to collagen (Figure 4a). The basis for this effect remains to be eluci-

dated but appears to result from enhancement of either adhesion or secretion resulting from adhesion.

The effects of 781094 on the responses to other agonists are more complex when examined in platelet-rich plasma in which a biphasic response to 5-hydroxytryptamine is observed (White, 1970) (Figure 4c). Enhanced responsiveness of platelets to all other excitatory agonists can be obtained in the presence of a concentration of adrenaline (or noradrenaline) which itself is just not adequate to induce an aggregatory response. This synergistic interaction is particularly marked for the noradrenaline/5-hydroxytryptamine agonist pair (Drummond, 1976; Scrutton & Wallis, 1981). The effect illustrated in Figure 4c could therefore arise from action of 781094 at the platelet α_2-adrenoceptor if the biphasic response to 5-hydroxytryptamine was generated by the presence of a low concentration of adrenaline (or noradrenaline) either in the plasma and/or secreted by the platelets on stimulation. This suggestion is supported by the observation that μmolar concentrations of both phentolamine and yohimbine also inhibit a biphasic aggregatory response to 5-hydroxytryptamine with properties similar to those described here for 781094 in Figure 4c. Furthermore, when a biphasic response to 5-hydroxytryptamine is induced by prior addition of a low concentration of noradrenaline, selective inhibition of the enhanced response can be observed on addition of phentolamine, yohimbine or 781094 over concentration-ranges similar to those required to affect the type of response shown in Figure 4c. Our observations therefore confirm the earlier suggestions of Besterman & Gillett (1973) and of Drummond (1976) that observation of a biphasic aggregatory response to 5-hydroxytryptamine is associated with enhanced responsiveness to other agonists and suggest that adrenaline or noradrenaline may be responsible for this effect. However addition of noradrenaline concentrations in the range 0.05–0.1 μM are required to induce a biphasic response to 5-hydroxytryptamine in platelet-rich plasma which does not exhibit this response. Such concentrations are far in excess of normal plasma noradrenaline levels which are in the range 1–2 nM (Da Prada & Zurcher, 1976), and even of those typically observed in patients with a phaeochromocytoma (10–16 nM) (Reid, Jones & Dollery, 1976). It therefore seems unlikely that circulating catecholamines are responsible for this effect since platelets showing a biphasic response to 5-hydroxytryptamine exhibit an ED_{50} for adrenaline and noradrenaline which is within the normal range.

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