Br. J. Pharmac. (1984), **81**, 091–102

Mammalian platelet adrenoceptors

R. Kerry, M.C. Scrutton & R.B. Wallis*

Department of Biochemistry, King's College, Strand, London, WC2R 2LS and CIBA-GEIGY Pharmaceuticals Division*, Wimblehurst Road, Horsham, West Sussex, RH12 4AB

1 Mammalian platelets vary widely in their responses to catecholamines in part because these agonists can act via excitatory α- and inhibitory β-adrenoceptors. In the absence of antagonists, adrenaline enhances the response of rabbit platelets to an excitatory agonist, e.g. adenosine-5′-O-(1-thiodiphosphate) (ADP-α-s) acting at another receptor, but has no effect on the response of rat or guinea pig platelets to such an agonist. In the presence of a β-adrenoceptor antagonist, adrenaline enhances the response of rat, but not guinea-pig platelets to ADP-α-S and the extent of the enhanced effect on rabbit platelets is increased. In the presence of an α-adrenoceptor antagonist, adrenaline inhibits the response of rabbit and rat platelets to ADP-α-S but has no such effect on the response of guinea-pig platelets.

2 Studies using selective agonists and antagonists demonstrate that the excitatory response of rat platelets to adrenaline is mediated by an α_2-adrenoceptor, and the inhibitory response of rabbit platelets to adrenaline by a β_2-adrenoceptor as is the case in other species which have been examined.

3 The mean α_2-adrenoceptor density on human, rabbit, rat and guinea-pig platelets as assessed using [^{3}H]-yohimbine as radioligand is obtained as 258, 270, 42 and < 10 receptors per platelet.

4 The mean β_2-adrenoceptor density on human, rabbit, rat and guinea-pig platelets as assessed using (−)-[^{125}I]-iodocyanopindolol is obtained as 66, 14, 41 and < 5 receptors per platelet.

5 The nature of the effect of adrenaline on the response of mammalian platelets to other excitatory agonists, e.g. ADP-α-S, appears therefore to be largely determined by the absolute number of α_2-adrenoceptors and by the relative content of α_2- and β_2-adrenoceptors on these cells for the species which have been examined in this analysis.

Introduction

With the exception of those obtained from man and some other primates (O'Brien, 1963; Loeb & Mackey, 1973), platelets from mammalian species do not aggregate on exposure to adrenaline in the absence of another excitatory agonist (Drummond, 1976; Dodds, 1978). Typically such platelets either fail to show such an aggregatory response to adrenaline even in the presence of a second excitatory agonist, e.g. rat platelets (Yu & Latour, 1977), or exhibit a proaggregatory response in which addition of adrenaline enhances the aggregatory response to a sub-optimal concentration of another excitatory agonist such as ADP, e.g. rabbit or cat platelets (Drummond, 1976; Yu & Latour, 1977).

Both radioligand binding and/or physiological response studies suggest that the aggregatory response of human platelets, and the pro-aggregatory response of rabbit platelets, induced by adrenaline is mediated by an α_2-adrenoceptor (Hsu, Knapp & Hulushra, 1979; Hoffman, Delean, Wood, Schocken & Lefkowitz, 1979; Grant & Scrutton, 1979; 1980). Evidence has also been presented for the presence of α-adrenoceptors on rat platelets (Yu & Latour, 1977) although this observation has not been confirmed by others (Motulsky & Insel, 1982).

The presence of β-adrenoceptors which mediate inhibition of platelet function by adrenaline has been shown for human, rabbit and rat platelets (Mills & Smith, 1971; Yu & Latour, 1977). More recently, this receptor has been identified as a β_2-adrenoceptor for human and rat platelets by an analysis of the inhibitory response using selective β-adrenoceptor agonists and antagonists (Kerry & Scrutton, 1983a).

The studies of Yu & Latour (1977) suggested that the difference in the nature of the response of rabbit and rat platelets to adrenaline resulted from a differ-

ence in the efficacy of this agonist at the α- and β-adrenoceptors. We have now confirmed and extended these observations and in addition have provided insight into the molecular basis for the different modes of response to adrenaline observed for rabbit, rat and guinea-pig platelets. A preliminary report of some of these findings has been presented (Kerry & Scrutton, 1983b).

Methods

Preparation of platelets

Platelet-rich plasma was prepared from rabbits, rats and guinea-pigs as described previously (Hallam, Scrutton & Wallis, 1981; Kerry & Scrutton, 1983a). For studies of the aggregatory response, blood was taken into 0.1 volume 3.8% (w/v) trisodium citrate as anticoagulant and these aggregation studies were performed on platelet-rich plasma at 37°C using a Payton Instruments dual channel aggregation module as described by Kerry & Scrutton (1983a). In these studies the α- or β-adrenoceptor antagonist was added 15 s before the α- or β-adrenoceptor agonist (or to adrenaline), followed 15 s later by adenosine - 5′ - O - (1 - thiodiphosphate) (ADP-α-S) to induce the aggregatory response. Specific details are given in the figure legends. Quantitation was based on measurement of the extent of the aggregatory response. No difference was observed in the extent of the effect of the antagonists if the time interval before addition of the adrenoceptor agonist was varied in the range 15–120 s.

For radioligand binding studies, platelet-rich plasma from the species as above, and from human volunteers, was prepared using 0.17 volume acid citrate dextrose (Aster & Jandl, 1964) as anticoagulant. The platelet-rich plasma was prepared by centrifugation at 200 *g* and 20°C for 20 min, and was then centrifuged for a further 5 min at 200 *g* (20°C) to reduce contamination by erythrocytes and white cells and the platelets then collected by centrifugation for 15 min at 380 *g* (4°C). The supernatant fraction was discarded and the platelets were washed by suspension in an equal volume of 0.05 M Tris-Cl pH 7.5 containing 0.1 M NaCl and 0.005 M disodium edetate (EDTA) (the Tris-Cl buffer) followed by centrifugation for 15 min at 380 *g*. The platelet pellet was resuspended in the Tris-Cl buffer to give cell concentrations in the range $2-9 \times 10^8$ platelets ml^{-1}. Platelet counts were estimated using a Model D Coulter Counter equipped with a 70 μm window.

The extent of contamination by erythrocytes and white cells in the final platelet suspension did not exceed 0.02% as assessed by light microscopic analysis.

Radioligand binding studies

Except where otherwise indicated, aliquots (0.1 ml) of this washed platelet preparation were incubated with [^{3}H]-yohimbine for 30 min at 25°C, or with (−)-[^{125}I]-iodocyanopindolol for 60 min at 37°C. The final volume including agonists or antagonists as indicated was 0.2 ml. Incubations were terminated by rapid filtration on Whatman GF/C glass fibre filter discs using a Millipore filtration manifold, followed by immediate washing with 3 × 5 ml of the Tris-Cl buffer at 4°C. When [^{3}H]-yohimbine was used as radioligand the filters were dried at 50°C and retained radioactivity estimated using an LKB-Wallac Model 1216 Rackbeta liquid scintillation counter with Lumagel (LKB-Wallac) as scintillation fluid.

When (−)-[^{125}I]-iodocyanopindolol was used as radioligand, the ^{125}I retained on the filters was estimated directly using a Packard Instruments Autogamma 500C gamma counter.

Binding of [^{3}H]-yohimbine which was related to occupation of α-adrenoceptors was defined as the difference between the extent of ^{3}H retained on the filters after incubation in the absence and presence of 10 μM phentolamine. At saturating concentrations of [^{3}H]-yohimbine the contribution of α-adrenoceptor-specific binding expressed as a per cent of total binding was 44 ± 10%, 54 ± 8% and 25 ± 4% for human, rabbit and rat platelets respectively. Binding of (−)-[^{125}I]-iodocyanopindolol which was related to occupation of β-adrenoceptors was defined as the difference between the extent of ^{125}I retained on the filters after incubation in the absence and in the presence of 1 μM (−)-propranolol. At saturating concentrations of (−)-[^{125}I]-iodocyanopindolol the contribution of β-adrenoceptor-specific binding expressed as a per cent of total binding was 80 ± 7%, 40 ± 8% and 85 ± 5% for human, rabbit and rat platelets respectively.

All data are expressed as means ± s.e.mean and represent duplicate or triplicate determinations from two or three experiments. Where appropriate the data were subjected to linear regression analysis and 95% confidence limits were calculated as described by Tallarida & Jacobs (1979).

For both radioligands a linear relationship was observed between the extent of binding and platelet number over the range used in these studies.

Materials

Hybrid rabbits (3/4 New Zealand white: 1/4 lop, 2–3 kg) were bred at Ciba-Geigy Pharmaceuticals Division; COB-Wistar (300–500 g) rats were obtained from Charles River (UK) Ltd. and Dunkin-Hartley guinea-pigs from Porcellus Ltd. 9-[^{3}H]-

yohimbine (75 Ci mmol^{-1}) and 3-(−)-[^{125}I]-iodocyanopindolol (2257 Ci mmol^{-1}) were obtained from Amersham International p.l.c. ADP-α-S (adenosine-5′-O-(1-thiodiphosphate) was a kind gift from Dr N.J. Cusack. Atenolol, (−)- and (+)-propranolol, practolol and ICI-118,551 (erythro-DL-1(7-methylindan-4-yloxy)-3-isopropylaminobutan-2-ol) were obtained from Imperial Chemical Industries; UK-14304 (5-bromo-6-[2-imidazolin-2-ylamino]-quinoxaline) and prazosin from Pfizer Inc.; methoxamine and butoxamine from Wellcome Research Laboratories; indoramin from Wyeth Laboratories; phentolamine mesylate from Ciba-Geigy Pharmaceuticals; RX 781094 (2-(2(1,4-benzodioxanyl))-2-imidazoline hydrochloride) from Reckitt and Colman Ltd; and (−)-adrenaline and (−)- and (+)-isoprenaline from Sigma Chemical Co.

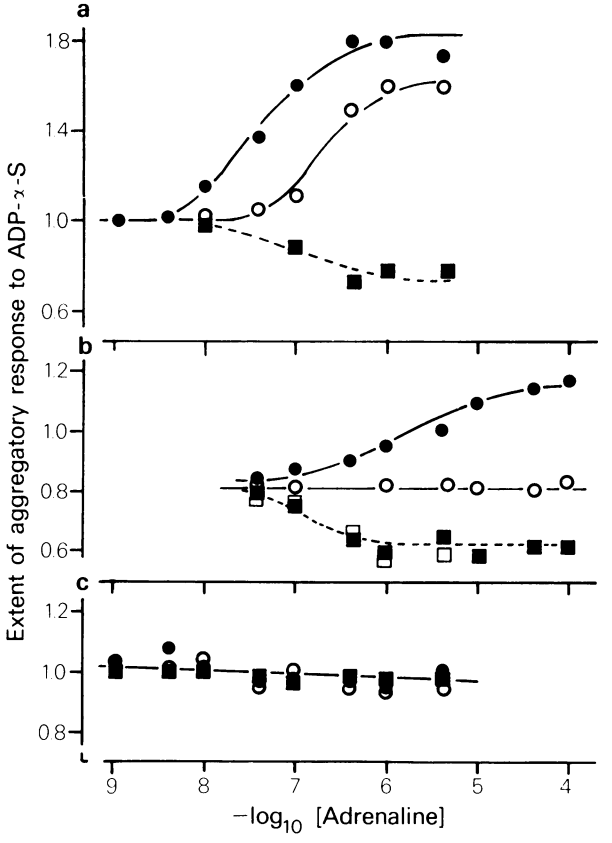

Figure 1 Effect of adrenaline on the aggregatory response of rabbit (a), rat (b) or guinea-pig (c) platelets to ADP-α-S. Platelet-rich plasma was prepared and platelet aggregation monitored and quantitated as described in Methods. The results shown are from a single experiment but are typical of those from three similar experiments. The adrenoceptor antagonists when present were added 15 s before adrenaline (or isoprenaline (b)) followed 15 s later by 2 μM ADP-α-S. The points represented as (○) show results obtained with adrenaline and ADP-α-S; those represented as (■), results obtained with phentolamine (10 μM), adrenaline and ADP-α-S; those represented as (●), results obtained with (−)-propranolol (10 μM), adrenaline and ADP-α-S; and those represented as (□), results obtained with (−)-isoprenaline and ADP-α-S.

Results

The effect of adrenoceptor antagonists on the aggregatory responses of rabbit, rat and guinea-pig platelets

The results of such studies are shown in Figure 1. In rabbit platelets addition of a β-adrenoceptor antagonist (10 μM (−)-propranolol) enhances the pre-existing pro-aggregatory response to adrenaline (Figure 1a) whereas in rat platelets such an addition reveals this pro-aggregatory response (Figure 1b). In this system however, guinea-pig platelets fail to show a pro-aggregatory response to adrenaline even in the presence of β-adrenoceptor blockade (Figure 1c) and no such response can be induced by addition of selective α-adrenoceptor agonists, e.g. UK 14304 or methoxamine (data not shown).

Conversely addition of an α-adrenoceptor antagonist (10 μM phentolamine) reveals an inhibitory response to adrenaline in the case of both rabbit and rat platelets (Figure 1a, b), but guinea-pig platelets fail to show such a response under these conditions (Figure 1c). Furthermore the aggregatory response of guinea-pig platelets to ADP-α-S cannot be inhibited by addition of a selective β-adrenoceptor agonist, e.g. (−)-isoprenaline.

While the EC_{50} for adrenaline acting as an inhibitory (β-adrenoceptor) agonist is in the same range for both rabbit ($EC_{50} = 0.1 \pm 0.03$ μM) and rat ($EC_{50} = 0.46 \pm 0.14$ μM) platelets (Table 1, Figure 1), this is not the case for its action as an excitatory agonist. Thus the EC_{50} for the pro-aggregatory response to adrenaline is 3.30 ± 0.27 μM for rat platelets but 0.06 ± 0.009 μM for rabbit platelets (Table 2, Figure 1).

Definition of the sub-type of the rat platelet α-adrenoceptor and the rabbit platelet β-adrenoceptor by analysis of the aggregatory response

Studies defining the sub-type of the rat platelet α-adrenoceptor and the rabbit platelet β-adrenoceptor have not been reported previously. Addition of UK-14304 causes a pro-aggregatory response of rat platelets in the absence of β-adrenoceptor blockade. This response was observed over a concentration

Table 1 Properties of the inhibitory response of rabbit platelets induced by β-adrenoceptor agonists and the effect of β-adrenoceptor antagonists on this response

	Agonist (Antagonist)	*EC_{50}* (μM)		*Relative maximal extent of inhibition*	*IC_{50}* (μM)	
A	(−)-Adrenaline	0.11 ± 0.03	(3)	1.0		
	(−)-Isoprenaline	0.13 ± 0.02	(5)	1.9 ± 0.1		
	(+)-Isoprenaline	60.0	(2)	1.7		
B	(−)-Propranolol				0.3 ± 0.2	(3)
	(+)-Propranolol				50.0	(1)
	Butoxamine				0.7	(2)
	ICI-118,551				3.0	(2)
	Atenolol				> 100	(2)

Platelet-rich plasma was prepared and platelet aggregation monitored and quantitated as described in Methods. The studies in **A** and **B** were performed in the presence of 10 μM phentolamine. In **A** the β-adrenoceptor agonist was added 15 s before 4 μM ADP-α-S which was used to induce the response: in **B** the β-adrenoceptor antagonist was added 15 s before 10 μM isoprenaline and followed 15 s later by 4 μM ADP-α-S. The values given are means ± s.e.mean (where indicated) with the number of experiments in parentheses.

range and to a maximal extent similar to that which characterizes the response to adrenaline in the presence of propranolol. In contrast, addition of methoxamine has no such effect. Furthermore the pro-aggregatory response of rat platelets to adrenaline which is revealed on addition of a β-adrenoceptor

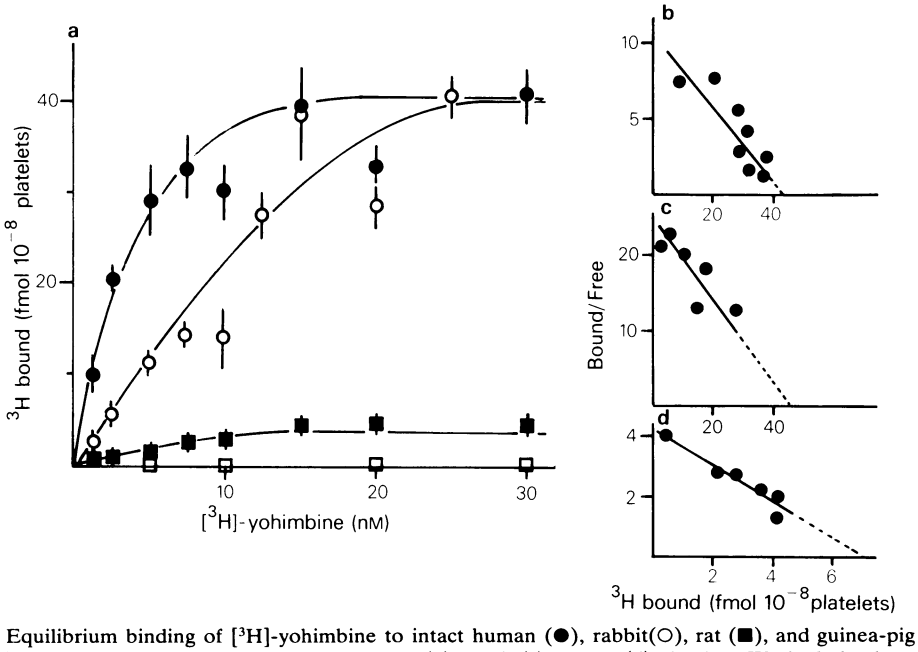

Figure 2 Equilibrium binding of [^{3}H]-yohimbine to intact human (●), rabbit(○), rat (■), and guinea-pig (□) platelets (a); and Scatchard plots of the data for human (b) rabbit (c) and rat (d) platelets. Washed platelets were prepared and [^{3}H]-yohimbine binding studies performed in the presence and absence of 10 μM phentolamine as described in Methods. In (a) the results which are from two (human, rabbit, guinea-pig) or 3 (rat) experiments with determinations performed in triplicate are plotted as means with the bars showing the s.e.mean. For the determinations using rat and guinea-pig platelets the studies were performed using more concentrated cell suspensions with cell counts in the range 8–9 × 10^8 platelets ml^{-1}. In (b-d) the mean values are plotted and the line of best fit to the data determined by linear regression analysis. The correlation coefficients (r) obtained by such analysis were 0.816 (b), 0.820 (c) and 0.924 (d).

Table 2 Properties of the proaggregatory response of rat platelets induced by α-adrenoceptor agonists and the effect of α-adrenoceptor antagonists on this response

Agonist (Antagonist)	*EC_{50}* (μM)	*Relative maximal extent of enhancement*	*IC_{50}* (μM)
A (−)-Adrenaline (in the presence of 10 μM (−)-propranolol)	3.3 ± 0.27 (3)	1.0	
UK-14304	1.2 ± 0.5 (4)	1.0	
Methoxamine	> 10 (2)	—	
B RX-781094			0.2 (2)
Indoramin			> 10 (2)
Prazosin			> 10 (2)

Platelet-rich plasma was prepared and platelet aggregation monitored and quantitated as described in Methods. The studies in **B**, and in **A** where specified, were performed in the presence of 10 μM propranolol. In **A** the α-adrenoceptor agonist was added 15 s before 4 μM ADP-α-S which was used to induce the response. No response was observed to UK-14304 in the absence of ADP-α-S. In **B** the α-adrenoceptor antagonists were added 15 s before 10 μM adrenaline and followed 15 s later by 3 μM ADP-α-S. The values given are means ± s.e.mean (where indicated) with the number of experiments in parentheses.

antagonist is completely inhibited by RX-781094 but is insensitive to inhibition by prazosin or indoramin (Table 2).

The rabbit platelet β-adrenoceptor displays the expected stereospecificity since the (−)-isomers of isoprenaline and propranolol are two orders of magnitude more effective as agonist and antagonist respectively than the (+)-isomers based on comparison of EC_{50} values (Table 1). Furthermore β_2-adrenoceptor antagonists (butoxamine, ICI-118, 551) cause effective blockade of the inhibitory response to isoprenaline, whereas β_1-adrenoceptor antagonists, e.g. atenolol are ineffective (Table 1).

Radioligand binding studies using [^{3}H]-yohimbine

Saturable binding of [^{3}H]-yohimbine, which is specific for the α-adrenoceptor as defined by the

Table 3 Parameters describing the binding of [^{3}H]-yohimbine and (−)-[^{125}I]-iodocyanopindolol to rabbit, rat and human platelets

Parameter	*Source of Platelets*					
	Human		*Rat*		*Rabbit*	
A *[^{3}H]-yohimbine*						
K_d (binding isotherm) (nM)	3	(1.5–4.5)	15	(14–16)	19	(17–21)
K_d (kinetic analysis) (nM)		—	7	(4–10)	23	(10–36)
B_{max} (receptors/platelet)	258	(245–271)	42	(40–44)	270	(256–284)
B (−)-[^{125}I]-iodocyanopindolol						
K_d (binding isotherm) (nM)	0.17	(0.16–0.18)	0.10	(0.09–0.11)	0.037	(0.035–0.039)
K_d (kinetic analysis) (nM)	0.06	(0.045–0.075)	0.066	(0.062–0.070)	0.005	(0.002–0.008)
B_{max} (receptors/platelet)	66	(62–70)	41	(36–46)	14	(13–15)

The studies were performed and the data analysed as described for Figures 2, 4, and 5. The results shown are from at least two experiments using triplicate determinations and are given as the means derived from linear regression analyses of the data together with the 95% confidence limits in parentheses.

extent of inhibition of binding by 10 μM phentolamine, is observed for intact rabbit and rat platelets as well as for human platelets (Figure 2a). The latter were included in this study as a control to permit comparison with other analyses employing this radioligand. The extent of such specific binding expressed as a per cent of total binding is given in 'Methods'. No such specific binding of [^{3}H]-yohimbine to intact guinea-pig platelets could be demonstrated (Figure 2a). Although the dissociation constants for receptor-specific binding of this radioligand to human, rabbit and rat platelets are all in the same (nM) range, the maximal extent of specific binding is clearly much greater for human and rabbit platelets than for rat platelets (Figure 2a). These conclusions are substantiated by Scatchard plot analysis of the data (Figure 2, b-d) to give K_d and B_{max} (receptor density) values which are summarized in Table 3. The density of α-adrenoceptor on human and rabbit platelets as assessed using [^{3}H]-yohimbine is similar and is approximately 6 fold greater than the density of these receptors on rat platelets. Studies using selective α-adrenoceptor antagonists confirm that the [^{3}H]-yohimbine binding sites on rabbit platelets have properties characteristic of α_2-adrenoceptors. Thus as shown in Figure 3 for rabbit platelets, RX-781094 is considerably more potent as an inhibitor of [^{3}H]-yohimbine binding than is prazosin. Table 4 summarizes the K_I values obtained in such studies on both rat and rabbit platelets. For both species the order of potency for inhibition of [^{3}H]-yohimbine binding is RX-780194 > phentolamine > prazosin as expected for an α_2-adrenoceptor, although it is notable that the discrimination is more marked for rat than for rabbit platelets.

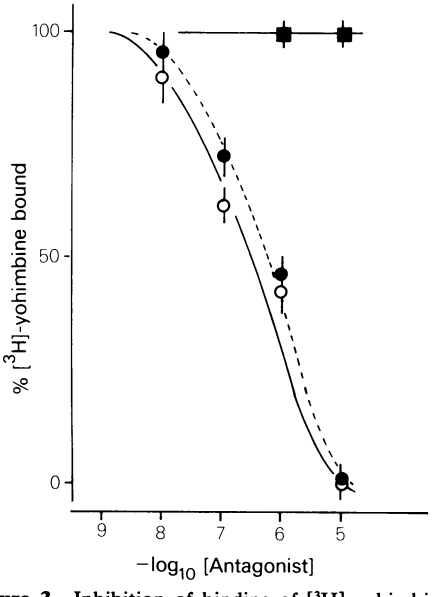

Figure 3 Inhibition of binding of [^{3}H]-yohimbine to intact rabbit platelets by α-adrenoceptor antagonists. Washed rabbit platelets were prepared and radioligand binding studies performed at 22°C in the presence of 15 μM [^{3}H]-yohimbine and the concentrations of the α-adrenoceptor antagonists as indicated. Data are shown for inhibition by RX-781094 (○), phentolamine (●) and prazosin (■). The results are from at least two experiments using triplicate determinations and are shown as means with the bars indicating the s.e.mean.

Table 4 Properties of inhibition of binding of [^{3}H]-yohimbine to rat and rabbit platelets by α-adrenoceptor antagonists

Antagonist	*Mean* K_I (μM) *Rabbit platelets*		*Rat platelets*	
RX-781094	0.28	(2)	0.032	(2)
Phentolamine	0.42	(2)	0.013	(2)
Prazosin	4.50	(2)	2.00	(2)

Washed platelets were prepared and radioligand binding studies performed as described in Methods. The concentration of [^{3}H]-yohimbine used was 15 μM and the incubation time was 30 min at 22°C. The K_I values were obtained by analysis of data similar to that for rabbit platelets as shown in Figure 3. The values are the means of two experiments in which the IC_{50} values differed by no more than 5 fold.

We have also examined the time course for association of [^{3}H]-yohimbine with rat and rabbit platelets, and for dissociation of this radioligand following addition of 10 μM phentolamine. In each case binding follows a monophasic relationship and equilibrium is reached within 10 min (Figure 4a). Analysis of these time courses as described by Williams & Lefkovitz (1978) gives the results for rat platelets shown in Figure 4b. From these plots k_{ob} is obtained as 1.7×10^{-3} s^{-1} ($1.5 - 1.9 \times 10^{-3}$ s^{-1}) and k_{-1} as 5.5×10^{-4} s^{-1} ($5 - 6 \times 10^{-4}$ s^{-1}). From these rate constants we obtained the values for K_d (kinetic analysis) which are given in Table 3. These values are in reasonable agreement with the values for K_d derived by analysis of the saturation curves.

Radioligand binding studies using (−)-[^{125}I]-iodocyanopindolol

Saturable binding of (−)-[^{125}I]-iodocyanopindolol, which was specific for the β-adrenoceptor as defined by the extent of inhibition of binding by 1 μM (−)-propranolol, was observed for intact rabbit, rat and human platelets. The extent of such specific binding expressed as a per cent of total binding is given in

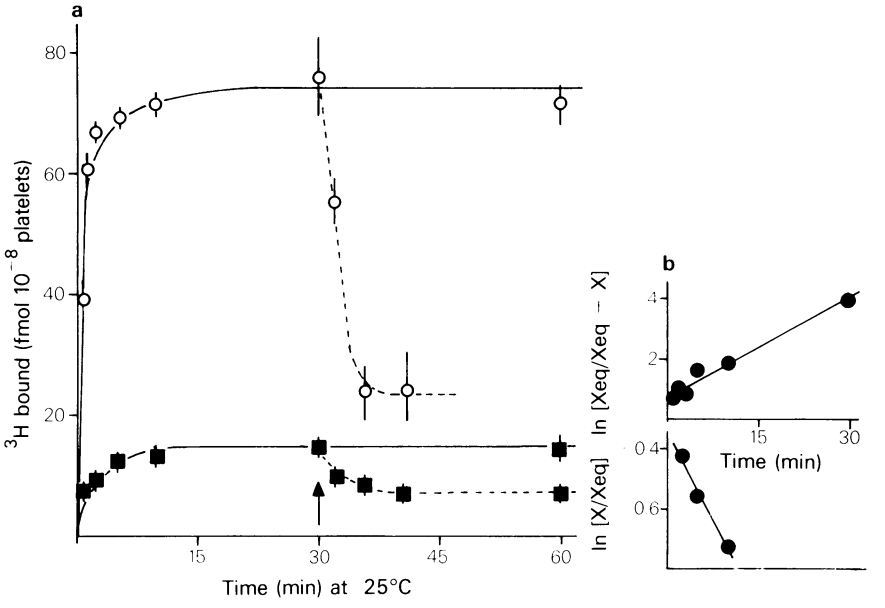

Figure 4 Kinetics of [^{3}H]-yohimbine binding to intact rabbit (○) and rat (■) platelets. Washed platelets were prepared as described in Methods, [^{3}H]-yohimbine added to a final concentration of 15 nM, and the suspension incubated at 25°C. Aliquots (0.2 ml) were withdrawn at the times indicated and the extent of binding assessed as described in Methods. At the time indicated by the arrow, 10 μM phentolamine was added to induce dissociation of bound [^{3}H]-yohimbine. A parallel incubation was performed in which 10 μM phentolamine was present throughout. The results in (a) are mean values from at least two experiments using triplicate determinations with the bars showing the s.e.mean and are given as total ^{3}H bound. In panel (b) the data for rat platelets are presented as described by Williams & Lefkowitz (1978) to permit calculation of k_{ob} and k_{-1} and in the case of the association of [^{3}H]-yohimbine (upper section) refer to specific binding. The lines shown are drawn on the basis of linear regression analysis of the data and have correlation coefficients (r) of 0.986 (upper line) and 0.738 (lower line).

Methods. No such specific binding of (−)-[^{125}I]-iodocyanopindolol to intact guinea-pig platelets could be demonstrated over a range of (−)-iodocyanopindolol which shows such binding in the other species (Figure 5a). Analyses of the data from Figure 5a according to Scatchard (1949) gives the results illustrated in panels b-d of Figure 5 and permits calculation of the K_d and B_{max} (receptor density) values given in Table 3. Human and rat platelets show similar affinities and receptor densities for (−)-[^{125}I]-iodocyanopindolol, whereas for rabbit platelets the affinity is higher and the receptor density is lower.

Studies on the inhibition of the binding of iodocyanopindolol by both agonists and antagonists confirm that the binding has the properties expected for a receptor with characteristics typical of a β_2-adrenoceptor. Thus as shown in Figure 6 for human platelets, (−)-propranolol is markedly more potent as an inhibitor than (+)-propranolol and in addition (−)-isoprenaline also causes inhibition, albeit at higher concentrations. Furthermore from the K_I values obtained by analysis of the inhibition curves (see legend to Figure 6) the order of potency of inhibition is obtained as ICI-118,551 > (−)-propranolol > (−)-practolol. Results similar to those shown in Figure 6 have also been obtained for rabbit and rat platelets (data not shown).

To provide further evidence that the sites identified by (−)-[^{125}I]-iodocyanopindolol have the properties expected for a receptor, we have studied the effect of variation of incubation temperature on the rate of association of (−)-[^{125}I]-cyanopindolol with human platelets, as well as determining the rate of dissociation of this radioligand induced by addition of 1 μM (−)-propranolol at a single temperature. The results demonstrate a marked increase in the rate of association as the temperature increased (Figure 7) with k_{ob} increasing from 8×10^{-5} ($7 - 9 \times 10^{-5}$) s^{-1} at 4°C to 1.7×10^{-3} ($1.6 - 1.8 \times 10^{-3}$) s^{-1} at 37°C. Even so, equilibrium is reached only after 30 min even at 37°C.

From the values of k_{ob} and k_{-1} (which was obtained from the rate of dissociation at 37°C) we calculated a value for K_d which as shown in Table 3 is in reasonable agreement with that obtained from analysis of the

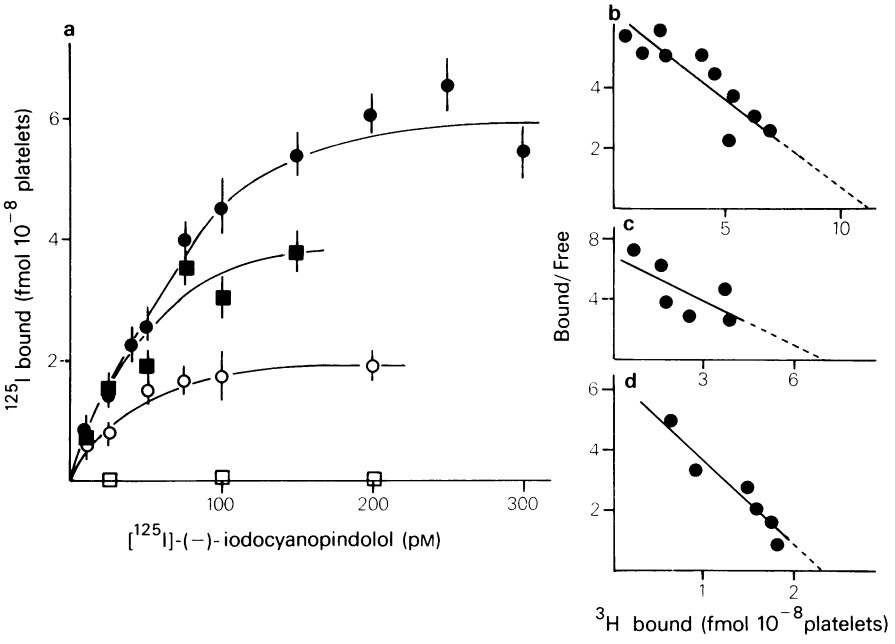

Figure 5 Equilibrium binding of (−)-[^{125}I]-iodocyanopindolol to intact human (●), rabbit (○), rat (■), and guinea-pig (□) platelets (a); and Scatchard plots of the data for human (b), rabbit (c) and rat (d) platelets. Washed platelets were prepared and (−)-[^{125}I]-iodocyanopindolol binding studies performed as described in Methods. In (a) the results, which are from two (human, rat, guinea-pig) or 3 (rabbit) experiments with determinations performed in triplicate, are plotted as means with the bars showing the s.e.mean. For the determinations using rabbit and guinea-pig platelets the studies were performed using more concentrated cell suspensions with cell counts in the range $8-9 \times 10^8$ platelets ml^{-1}. In (b-d) the mean values are plotted and the line of best fit determined by linear regression analysis. The correlation coefficients (r) obtained by such analysis were 0.666 (b), 0.926 (c) and 0.617 (d).

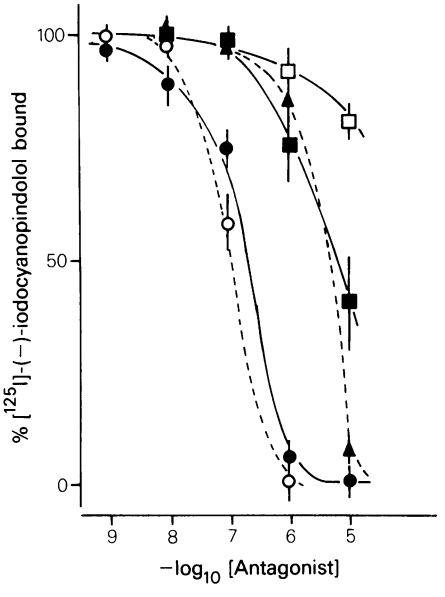

Figure 6 Inhibition of binding of (−)-[^{125}I]-iodocyanopindolol to intact human platelets by β-adrenoceptor agonists and antagonists. Washed human platelets were prepared and radioligand binding studies performed at 37°C in the presence of 0.1 nM (−)-[^{125}I]-iodocyanopindolol and the β-adrenoceptor agonists and antagonists at the concentrations indicated. Data are shown for inhibition by (−)-propranolol (●), (+)-propranolol (▲), ICI-118,551 (○), (−)-practolol (□) and (−)-isoprenaline (■). The results shown are from at least two experiments using triplicate determinations and are shown as means with the bars indicating the s.e.mean. The mean K_I values calculated from these data are ICI-118,551 (0.07 μM), (−)-propranolol (0.13 μM), (+)-propranolol (2.5 μM), (−)-practolol (>6 μM) and (−)-isoprenaline (3.1 μM).

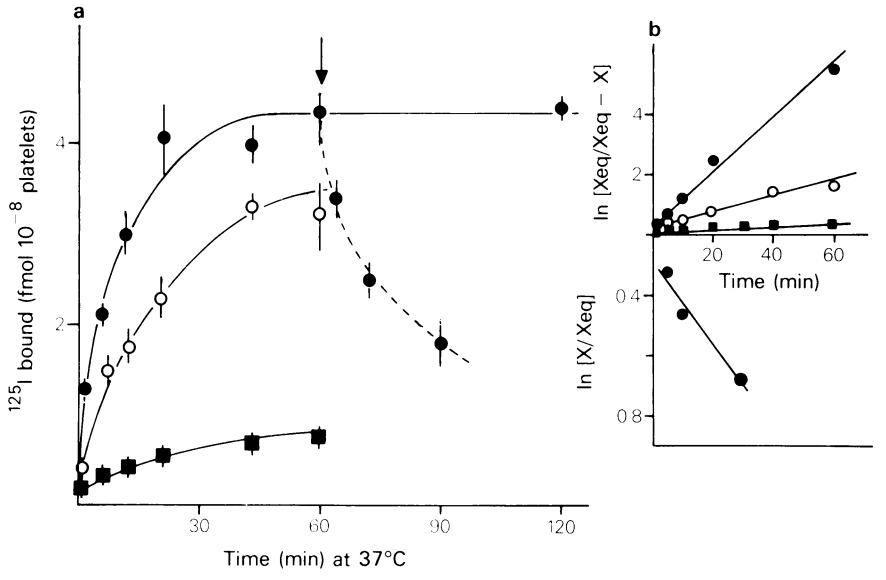

Figure 7 Kinetics of binding of (−)-[^{125}I]-iodocyanopindolol to intact human platelets measured at different temperatures. Washed human platelets were prepared as described in Methods, (−)-[^{125}I]-iodocyanopindolol added to a final concentration of 0.1 nM and the suspension incubated at 4°C (■), 22°C (○), or 37°C (●). Aliquots (0.2 ml) were withdrawn at the times indicated and the extent of binding assessed as described in Methods. In the sample incubated at 37°C 1 μM (−)-propranolol was added at the time indicated by the arrow to induce dissociation of bound (−)-[^{125}I]-iodocyanopindolol. Parallel incubations were performed at all temperatures in which 1 μM(−)-propranolol was present throughout. The results in (a) are mean values from at least two experiments using triplicate determinations with the bars showing the s.e.mean and are given as total ^{125}I bound. In panel (b) the data are presented as described by Williams & Lefkowitz (1978) to permit calculation of k_{ob} and k_{-1} (for 37°C data only). In the case of the rates of association (k_{ob}) panel (b) is based on specific binding. The lines shown are drawn on the basis of linear regression analyses of the data and have correlation coefficients (r) of 0.976 (●), 0.903 (○) and 0.938 (■) (upper section); and 0.959 (●) (lower section).

binding isotherm. Similar studies have been performed at 37°C for rat and rabbit platelets and analysed to give K_d values as shown in Table 3. For rat platelets there is good agreement between the K_d values obtained from kinetic and binding isotherm analysis, but a wider divergence between these values is observed for rabbit platelets.

Discussion

The aggregation and radioligand binding studies described here complement and extend previous studies in which we and others have defined the adrenoceptor sub-types present on human (Grant & Scrutton, 1979; Hsu, Knapp & Halushka, 1979; Hoffman *et al.*, 1979; Kerry & Scrutton, 1983a), rabbit (Grant & Scrutton, 1980) and rat (Kerry & Scrutton, 1983a) platelets. It seems clear that when adrenoceptors are present on mammalian platelets the β-adrenoceptor is likely to be of the β_2-sub-type and the α-adrenoceptor is likely to be predominantly, if not exclusively, of the α_2-sub-type (Grant & Scrutton, 1979). However, within this overall classification there appears to be wide differences in the properties of the receptors in different species. This is most strikingly seen for the rabbit platelet β-adrenoceptor for which ICI-118,551 is much less effective as an antagonist than are (−)-propranolol or butoxamine (Table 1) in contrast to the relationships between the IC_{50}s for these antagonists observed for the human and rat platelet β-adrenoceptors. In addition, for the rabbit α-adrenoceptor the difference between K_I values for phentolamine (or RX-781094) and prazosin as inhibitors of the binding of [^{3}H]-yohimbine are much less marked than is the case for either the rat (Table 4) or human (Daiguji, Meltzer & U'Prichard, 1981; Motulsky, Shattil & Insel, 1981) α-adrenoceptors. The K_I values reported here for phentolamine and prazosin in the rat platelet system (Table 4) are quantitatively very similar to those obtained for human platelets by Motulsky, Shattil & Insel (1981).

Estimates of α-adrenoceptor density using [^{3}H]-

yohimbine have not previously been published for rat platelets, although both Motulsky & Insel (1982) and Glusa & Markwardt (1983) suggested that no α_2-adrenoceptors are present on these cells, based on their failure to observe specific binding of this radioligand, and also in the case of Glusa & Markwardt (1983) based on failure to detect enhancement by adrenaline of a sub-optimal response to ADP. The discrepancy in respect to the radioligand binding data probably arises since the receptor density is close to the limit of detection using ^{3}H-radioligands and the studies have to be conducted in the face of a high level of non-specific binding (see Methods). It should however be noted that the results obtained for binding of [^{3}H]-yohimbine to rat and guinea-pig platelets are clearly different (Figure 2) and that for rat platelets the 95% confidence limits for the estimate of B_{max} (Table 3) indicate minimal divergence between the contributing data. Hence, given that a pro-aggregatory response to adrenaline which is mediated by α-adrenoceptors can clearly be demonstrated for rat platelets both by us (Figure 1) and by Yu & Latour, (1977) it seems reasonable to conclude that the B_{max} values obtained by use of [^{3}H]-yohimbine as radioligand (Tables 3 and 4) provide an acceptable estimate of the α_2-adrenoceptor density even though this determination pushes the available technology to its usable limit. It would therefore be desirable to confirm the estimate of α_2-adrenoceptor density on rat platelets by use of a radioligand having a higher specific radioactivity.

For human platelets the value for α_2-adrenoceptor density reported here is similar to that obtained by other workers (Motulsky *et al.*, 1981; Glusa & Markwardt, 1983) in studies which used intact platelets and [^{3}H]-yohimbine as radioligand, but is approximately 3 fold greater than the density estimated using this radioligand and platelet lysates (Daiguji *et al.*, 1981). However, the K_d for [^{3}H]-yohimbine is similar in all these studies. For rabbit platelets our data give a higher α_2-adrenoceptor density and a less favourable K_d than those reported by Glusa & Markwardt (1983).

Platelet β-adrenoceptor densities have not previously been estimated using (−)-[^{125}I]-cyanopindolol as radioligand. However, for human platelets Steer & Atlas (1982) have reported that [^{125}I]-iodohydroxybenzylpindolol, a related β-adrenoceptor radioligand, binds to 24 ± 4 sites per platelet with a mean K_d of 58 μM, thus giving a β-adrenoceptor density that is approximately one third of that estimated here (Table 3). The data of Steer & Atlas (1982) appear less likely to be reliable than those given here since specific binding of [^{125}I]-iodohydroxybenzylpindolol constituted only 20–30 per cent of total binding in contrast to the value of 80 ± 7 per cent seen in our studies using (−)-[^{125}I]-iodocyanopindolol. Similar studies have not previously been reported for rat or rabbit platelets using (−)-[^{125}I]-iodocyanopindolol or any other β-adrenoceptor radioligand, but in these studies give adequate estimates of β_2-adrenoceptor densities in each case. The value for rabbit platelets is again close to the limit of detection but is supported by the minimal divergence between contributing data as expressed in the 95% confidence limits for B_{max} (Table 3), and by the failure to detect any such receptors on guinea-pig platelets (Figure 5). It is also notable that the K_d values obtained for binding of (−)-[^{125}I]-iodocyanopindolol to human and rabbit platelets are close to an order of magnitude higher than those obtained in studies of binding of this radioligand to membrane fractions from other tissues (cf. Engel, 1981). It is possible that this discrepancy reflects the use of intact cells in this study with the associated problems of cellular uptake of radioligand. However, the observation that (−)-[^{125}I]-iodocyanopindolol can be displaced from human platelets by subsequent addition of (−)-propranolol (Figure 7) and that stereospecific inhibition of the binding of this radioligand can be observed (Figure 6) argue against this possibility. The use of (−)-[^{125}I]-iodocyanopindolol therefore allows estimates to be made of β-adrenoceptor density on a cell for which previous attempts (Kerry & Scrutton, 1983c) using the classic β-adrenoceptor radioligand, [^{3}H]-dihydroalprenolol (Williams & Lefkowitz, 1978) had failed to reveal any detectable receptor-mediated binding. The high specific radioactivity of (−)-[^{125}I]-iodocyanopindolol and its high affinity for the β-adrenoceptor (Table 3) permit the use of ligand concentrations at which little interference from lipid solubility occurs in contrast to the situation with [^{3}H]-dihydroalprenolol in this system (Kerry & Scrutton, 1983c). It is however notable that the rate of association of (−)-[^{125}I]-iodocyanopindolol (Figure 7) is much slower than might be expected on the basis of the rapidity with which β-adrenoceptor antagonists, e.g. propranolol, pindolol, block the inhibitory response to isoprenaline (see Methods). Such a discrepancy in time courses for binding of radioligand and for initiation of the physiological effect is frequently observed in studies of this type (cf. Williams & Lefkowitz, 1978) but has not been satisfactorily explained.

The differences in α- and β-adrenoceptor density seen for the platelets from the four species examined in this study provide at least a partial explanation for the differences which are observed in the responses of these platelets to adrenaline. Two factors appear to be of importance: the absolute density of α_2-adrenoceptors and the ratio of the α_2:β_2-adrenoceptor densities which varies from 19 in rabbit platelets to 3 in human platelets and approximately 1

in rat platelets (Table 3). Platelets which show a pro-aggregatory response to adrenaline in the absence of a β-adrenoceptor antagonist, e.g. those from humans and rabbits, exhibit high absolute α_2-adrenoceptor densities and an α_2:β_2-adrenoceptor density ratio in excess of 1. In contrast, platelets that fail to respond to adrenaline may either contain finite but approximately equal numbers of α_2- and β_2-adrenoceptors (α_2:β_2-adrenoceptor density ratio of approximately 1), e.g. rat, or may be devoid of adrenoceptors, e.g. guinea-pig. These latter two situations can be distinguished in aggregation studies since as shown in Figure 1, a pro-aggregatory or an inhibitory response to adrenaline can be elicited for rat but not for guinea-pig platelets by addition of a β-adrenoceptor and an α-adrenoceptor antagonist respectively. The radioligand binding data (Figure 2 and 4) demonstrate clearly that the failure of guinea-pig platelets to respond to adrenaline even under these conditions is due to the absence of detectable numbers of both α- and β-adrenoceptors on these cells. Our studies therefore confirm and extend the postulate of Yu & Latour (1977) that the nature of the response of platelets to adrenaline is determined by the relative efficiency with which this catecholamine acts as an α- and a β-adrenoceptor agonist for these cells, and demonstrate the molecular basis for this postulate. In addition, the results obtained for the aggregatory responses are more reliable than those described previously by Yu & Latour (1977) since they were obtained over a wide range of adrenaline concentrations and in the presence of a second excitatory agonist (ADP-α-S) which does not inhibit adenylate cyclase (Cusack & Hourani, 1981) and which therefore is unlikely to interfere with the adrenergic responses. However the concept proposed here offers no explanation for the difference in behaviour of human and rabbit platelets on exposure to adrenaline in the absence of a second excitatory agonist, since no manipulations performed in these studies have led to demonstration of an aggregatory response of rabbit platelets to adrenaline in the absence of a second excitatory agonist. Hence the presence of such a direct aggregatory response to adrenaline is more likely to be related to the ability of this agonist to activate its stimulus-response coupling mechanism than to the α_2:β_2-adrenoceptor balance.

Our studies also provide no insight into the reasons why platelets from these four mammalian species should differ so widely in the nature of their responses, or lack of response, to adrenaline, although for rat platelets the situation could be envisaged as a mechanism by which a cell which possesses α_2-adrenoceptors might respond selectively to noradrenaline in the presence of adrenaline. However, the findings have practical importance since rabbits, rats and guinea-pigs are all used in small animal models of thrombosis. If such models are employed to investigate the effects of drugs acting at adrenoceptors, the species differences described here will need to be borne in mind especially if the results obtained are to be relevant to human thrombotic disease.

R.K. is supported by CIBA-GEIGY Ltd. We are grateful to Dr J.R. Smith and Mr J. Ambler for provision of rabbit, rat and guinea-pig blood.

References

ASTER, J.H. & JANDL, J. (1964). Platelet sequestration in man. *J. clin. Invest.*, **43,** 843–855.

CUSACK, N. J. & HOURANI, S.M.O. (1981). Effect of **R**p and **S**p diastereoisomers of adenosine 5′-O-(1-thiodiphosphate) on human platelets. *Br. J. Pharmac.*, **73,** 409–412.

DAIGUJI, M., MELTZER, H.J. & U'PRICHARD, D.C. (1981). Human platelet α_2-adrenergic receptors: labelling with ^{3}H-yohimbine, a selective antagonist ligand. *Life Sci.*, **28,** 2705–2717.

DODDS, W.J. (1978). Platelet function in animals. In *Platelets: a Multidisciplinary Approach*, ed Gaetano, G. & Garattini, S. pp. 45–59. New York: Raven Press Inc.

DRUMMOND, A.H. (1976). Platelet-biogenic amine interactions. In *Platelets in Biology and Pathology*, ed. Gordon, J. L. pp. 203–239. Amsterdam: Elsevier/North Holland Biomedical Press.

ENGEL, G. (1981). Subclasses of beta-adrenoceptors – a quantitative estimation of $beta_1$ and $beta_2$-adrenoceptors in guinea-pig and human lung. *Postgrad. Med. J.*, **57,** (Suppl. 1) 77–83.

GLUSA, E & MARKWARDT, F. (1983). Characterisation of α_2-adrenoceptors on blood platelets from various species using ^{3}H-yohimbine. *Haemostasis*, **13,** 96–101.

GRANT, J.A. & SCRUTTON, M.C. (1979). Novel α_2-adrenoceptors primarily responsible for inducing platelet aggregation. *Nature*, **227,** 659–661.

GRANT, J.A. & SCRUTTON, M.C. (1980). Interaction of selective α-adrenoceptor agonists and antagonists with human and rabbit blood platelets. *Br. J. Pharmac.*, **71,** 121–134.

HALLAM, T.J., SCRUTTON, M.C. & WALLIS, R.B. (1981). Responses of rabbit platelets to adrenaline induced by other agonists. *Thrombos. Res.*, **20,** 437–446.

HOFFMAN, B.B., DELEAN, A., WOOD, C.L., SCHOCKEN, D.D. & LEFKOWITZ, R.J. (1979). Alpha-adrenergic subtypes: a quantitative assessment by ligand binding. *Life Sci.*, **24,** 1739–1746.

HSU, C.Y., KNAPP, D.R. & HALUSHKA, P.V. (1979). Effects of alpha-adrenergic agents on human platelet aggregation. *J. Pharmac. exp. Ther.*, **208,** 366–370.

KERRY, R. & SCRUTTON, M.C. (1983a). Platelet β-adrenoceptors. *Br. J. Pharmac..*, **79,** 681–691.

KERRY, R. & SCRUTTON, M.C. (1983b). Species differences

in the response of rat, rabbit and guinea-pig blood platelets to adrenaline. *Br. J. Pharmac.*, **78,** 157P.

KERRY, R. & SCRUTTON, M.C. (1983c). Binding of [^{3}H]-dihydroalprenolol and [^{3}H]-acetobutolol to human blood platelets is not related to occupancy of β-adrenoceptors. *Thrombos. Res.*, **29,** 583–594.

LOEB, W.F. & MACKEY, B. (1973). A comparative study of platelet aggregation in primates. *J. med. Primatol.*, **2,** 195–205.

MILLS, D.C.B. & SMITH, J.B. (1971). The influence on platelet aggregation of drugs that affect the accumulation of adenosine-3′,5′-cyclic monophosphate in platelets. *Biochem.*, **121,** 185–196.

MOTULSKY, H. & INSEL, P.A. (1982). Adrenergic receptors in man: direct identification, physiologic regulation and clinical alterations. *New Eng. J. Med.*, **307,** 18–29.

MOTULSKY, H.J., SHATTIL, S. & INSEL, P.A. (1981). Characterisation of α_2-adrenergic receptors on human platelets using [^{3}H]-yohimbine. *Biochem. biophys. Res. Comm.*, **97,** 1562–1570.

O'BRIEN, J.R. (1963). Some effects of adrenaline and anti-adrenaline compounds on platelets *in vitro* and *in vivo*. *Nature*, **200,** 763–764.

SCATCHARD, G. (1949). The attractions of proteins for small molecules and ions. *Ann. N.Y. Acad. Sci.*, **51,** 660–672.

STEER, M. & ATLAS, D.C. (1982). Demonstration of human platelet β-adrenergic receptors using ^{125}I-labelled cyanopindolol and ^{125}I-labelled hydroxybenzyl pindolol. *Biochem. biophys. Acta*, **686,** 240–244.

TALLARIDA, R.J. & JACOBS, L.S. (1979). *The Dose Response Relation in Pharmacology*. pp. 100–102. Heidelberg: Springer-Verlag.

WILLIAMS, L.T. & LEFKOWITZ, R.J. (1979). *Receptor Binding Studies in Adrenergic Pharmacology*. pp. 53–109. New York: Raven Press Inc,

YU, S.K. & LATOUR, J.G. (1977). Potentiation by α- and inhibition by β-adrenergic stimulations of rat platelet aggregation. *Thrombos. Haemostas.*, **37,** 413–422.

(*Received May 30, 1983.*
Revised August 8, 1983.)