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β-ADRENOCEPTOR ANTAGONISTS AND HUMAN PLATELETS: RELATIONSHIP OF EFFECTS TO LIPID SOLUBILITY

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Abstract—Six β-adrenoceptor antagonists (propranolol (+ and − isomers); ICI-118,551; oxprenolol; timolol; metoprolol; and practolol (+ and − isomers), chosen to represent a spectrum of physicochemical and pharmacological properties, inhibited the response of human platelets to all aggregating agents tested. For any given aggregating agent the extent of inhibition correlated with the lipid solubility of the β-adrenoceptor antagonist and showed no relation to other properties of these compounds.

Inhibition of aggregation by the β-adrenoceptor antagonists was manifested as a parallel shift to the right in the dose–response curve. Analysis of these data according to Arunlakshana and Schild (*Br. J. Pharmac.* **14**, 48–58 (1969)) showed a dependence of the apparent pA_2 on the agonist employed and gave a slope approximating unity when ADP, 9,11-epoxymethanoprostaglandin H_2 (U-46619), adrenaline, 1-*O*-alkyl-2-acetyl-sn-glyceryl-3-phosphorylcholine (PAF) or arachidonate were used as agonists. Slopes significantly greater than unity, and approaching a value of 2, were obtained when this analysis was applied to data obtained using collagen in the presence or absence of aspirin, 12-*O*-tetradecanoylphorbol-13-acetate (TPA), or a divalent cation ionophore (A-23187) as agonists.

Inhibition by (±) propranolol of secretion induced by collagen was manifested as a parallel shift to the right in the dose–response curve for collagen. The Schild plot of these data has a slope of unity.

(±)-Propranolol inhibited thromboxane B_2 production induced by collagen but over a similar concentration range had little effect on conversion of arachidonate to thromboxane B_2.

(±)Propranolol had no significant effect on the level of cyclic-3′,5′-AMP (cAMP) in unstimulated platelets or on the increase in the level caused by addition of forskolin, but caused partial inhibition of the increase in platelet cAMP induced by prostaglandin E_1. It also completely abolished inhibition by ADP of the increase in [cyclic-3′,5′-AMP] induced by prostaglandin E_1.

These data are interpreted on the basis of a model in which interaction of propranolol with phosphatidylserine and phosphatidylinositol causes inhibition of phospholipases C and A_2, inhibition of protein kinase C and alteration of membrane receptor properties as a consequence of distortion of their microenvironment.

Pharmacological and radioligand binding studies have shown the presence on human platelets of a significant population of β_2-adrenoceptors [1, 2]. Occupancy of these receptors by a selective β-adrenoceptor agonist, e.g. isoprenaline, causes inhibition of the aggregatory response of platelets to all excitatory stimuli as a consequence of the increase in the level of cAMP§ [1, 3]. A similar effect can be shown *in vitro* for occupancy of the β_2-adrenoceptors by adrenaline provided that the studies are performed in the presence of a α-adrenoceptor antagonist [4]. In both cases the inhibitory effect can be blocked by addition of a β-adrenoceptor antagonist, e.g. (-) propranolol or ICI-118,551 [1, 2, 4], and further *in vitro* studies showed that, as predicted, the aggregatory response to adrenaline could be enhanced by addition of such an antagonist [2, 4]. However, this latter response has been duplicated in the *ex vivo* situation in only one study [5] and in the overwhelming majority of such analyses β-adrenoceptor antagonists have been found to inhibit the responses to a range of excitatory stimuli including adrenaline [6–8]. These inhibitory effects of β-adrenoceptor antagonists on platelet function observed both *ex vivo* and also *in vivo* can be duplicated in *in vitro* studies provided that concentrations in the range 0.01–1.0 mM are employed [6, 9]. Such concentrations, however, are well in excess of the range required to achieve blockade of the human platelet β-adrenoceptor. For example, such blockade is achieved using (-)-propranolol with an IC_{50} of 0.1 μM [1]. Furthermore, in contrast to the situation for blockade of the β-adrenoceptor [1], direct inhibition of platelet function by β-adrenoceptor antagonists shows no discrimination between the effects of the (+) and (−) isomers of the antagonists where these have been tested [6, 10].

Many adrenoceptor antagonists, e.g. propranolol, have local anaesthetic and 'membrane-stabilizing' activity [11–13]. The extent of activity for these latter properties is generally correlated with the lipid solubility of the β-adrenoceptor antagonist and appears to be related to interaction of these drugs with membrane phospholipids [14, 15]. We have therefore explored in greater detail the properties of inhibition of a range of platelet responses by selected β-adreno-

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§ Abbreviations: TPA 12-*O*-tetradecanoylphorbol-13-acetate; PAF, 1-*O*-alkyl-2-acetyl-sn-glyceryl-3-phosphorylcholine; cAMP, cyclic-3′,5′-AMP.

ceptor antagonists and have attempted to relate the effects we have observed to different properties possessed by some examples of this class of drugs with particular reference to propranolol.

MATERIALS AND METHODS

Preparation of platelet-rich plasma. Blood was obtained by antecubital venepuncture from donors who had denied taking medication during the previous 14 days. For the aggregation studies coagulation was prevented by addition of 1/10th volume of acid citrate dextrose [16] to give the final blood citrate concentration as 10 mM and platelet-rich plasma prepared by centrifugation for 20 min at 200 *g* and 20°. The residual red and white cell contamination was further reduced by centrifugation of the platelet-rich plasma for 5 min at 280 *g* and 20°. 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° in a tightly stoppered container and used within 2.5 hr of preparation.

Aggregometry and lumiaggregometry. The aggregatory response of platelets in platelet-rich plasma was monitored using a Payton dual channel aggregometer interfaced with a Rikadenki Model 300 BD dual channel recorder calibrated as described by Pearce *et al.* [16]. Quantitation was based on measurement of the extent of the response except for adrenaline where the initial rate of the response was measured. (+) or (−) Propranolol (10–100 μM) proved equally effective in causing inhibition of aggregation induced by A23187, collagen, U-46619 or ADP irrespective of the time (0–30 min) for which the platelet-rich plasma was pre-incubated with the β-adrenoceptor antagonist prior to addition of the aggregating agent. As a consequence of these results the β-antagonist under study was pre-incubated with the platelet-rich plasma for 1 min at 37° prior to the addition of the aggregating agent in all the studies described.

Solutions of ADP (PL-Biochemicals) and (−)-adrenaline (Sigma) were prepared in 0.9% (w/v) NaCl. Stock solutions of U-46619, PAF, TPA (PL-Biochemicals) and A-23187 (Calbiochem) were prepared in methanol and dilutions then made using 0.9% (w/v) NaCl. Arachidonic acid (Sigma) was dissolved in 0.2 M Na_2CO_3 under N_2 to give a 0.1 M stock solution which was further diluted as required using 0.9% NaCl. Collagen (Hormon Chemie) was obtained as a stock solution (1 mg/ml) in isotonic glucose pH 2.7 and was diluted in this medium. The β-adrenoceptor antagonists were dissolved either in 0.9% (w/v) NaCl or in methanol, and dilutions were made using 0.9% (w/v) NaCl. In all cases the final methanol concentration in the platelet-rich plasma after all additions did not exceed 1% (v/v). Control experiments showed that the aggregatory and inhibitory responses were not affected by this concentration of methanol.

Aggregation and secretion were monitored simultaneously using a Payton 1020 Lumiaggregation module as described by Ambler and Wallis [17]. The extent of ATP secretion was estimated using a luciferin–luciferase enzyme system as an ATP monitoring reagent (LKB Wallac). The order of additions (made at 30 sec) to the platelet-rich plasma (360 μl) were: test drug/luciferin–luciferase reagent (40 μl/aggregating agent (2–20 μl). Secretion was standardized with the addition of a standard ATP solution to each cuvette giving a final concentration of 2.5 μM after the peak response had been reached.

Thromboxane B_2 radioimmunoassay. This assay was performed essentially as described by Butler *et al.* [18]. Samples for thromboxane B_2 analysis were taken from the platelet aggregometer 1 min after maximal aggregation had occurred. The sample (0.4 ml) was tipped into a tube containing 10 mg/ml indomethacin in methanol (20 μl) and immediately centrifuged for 2 min at 12,000 *g* to remove the platelets. The supernatant was then rapidly frozen in solid CO_2. In this way thromboxane B_2 was determined in the same sample as that used for aggregation studies.

The incubation mixture consisted of: test plasma (0.1 ml), Tricine buffered saline (0.1 ml), [^{3}H]-thromboxane B_2 (10,000 dpm) (0.05 ml) and anti-TXB_2 antiserum (0.05 ml). Thromboxane B_2 standards (0.01–100 ng/ml) were made up in control rat plasma. Control rat plasma was prepared from rats which had been predosed with aspirin (30 mg/kg p.o.) 2 hr prior to bleeding into 0.1 volumes of 2% EDTA pH 7.2 containing 2.0 mg/ml indomethacin. Tricine buffered saline consisted of 0.1 M Tricine, 0.154 M NaCl, 0.015 M NaN_3 and 0.1% (w/v) gelatine adjusted to pH 8.0. Incubation was carried out for 2 hr at 37°. The free and protein bound [^{3}H]-thromboxane B_2 were separated by the addition of saturated $(NH_4)_2SO_4$ (0.3 ml) and centrifugation at 12,000 *g* for 2 min. The pellet was washed by suspending it in 60% saturated $(NH_4)_2SO_4$ (0.6 ml) and recentrifuged, redissolved in deionized water (0.6 ml) and prepared for scintillation counting by addition of Lumagel (Lumac, Basel) (4.0 ml).

The binding of [^{3}H]-thromboxane B_2 in the absence of added unlabelled thromboxane B_2 was approx. 50% of the maximum binding of the label. The lowest detectable concentration of thromboxane B_2 in plasma samples was 0.1 ng/ml.

Platelet cyclic AMP assay. Platelet cAMP levels were measured as % ^{14}C present in cAMP using the procedures described by Haslam *et al.* [19]. Platelet-rich plasma was incubated with 0.5 μCi 8-(^{14}C)-adenine per ml plasma (175 μM adenine) for 90 min at 37°. Aliquots (0.25 ml) of this [^{14}C]-labelled platelet-rich plasma were then incubated with either prostaglandin E_1 (1 μM), forskolin (5 μM) or saline in the presence of increasing concentrations (0–400 μM) of (±) propranolol. All experiments were performed in the presence of 25 μM papaverine. After 1 min 0.025 ml of 6 M $HClO_4$ was added and [^{14}C]-cAMP isolated by chromatography on columns of Dowex-50W-H^+ and of alumina as described by Salomon *et al.* [20].

Lipid soiubilities of β-antagonists. The lipid solubilities of oxprenolol, timolol and metoprolol were taken from Wood and Robinson [21] and of practolol from Hellenbrecht *et al.* [22] and are expressed as the partition coefficient between n-octanol and H_2O at 37°. The lipid solubility of ICI-118,551 expressed on the same basis is 8.0 [23].

Quantitation of results. Where data permitted the parallel shifts in the dose–response curve in the presence of increasing concentrations of β-adrenoceptor antagonists were subjected to analysis as described by Arunlakshana and Shild [24]. Resultant 'apparent pA_2' values were regarded as a measure of the ability of the β-adrenoceptor antagonist to inhibit the action of a given aggregating agent. Thromboxane B_2 data were not subjected to such analysis as full dose–response curves were not obtained.

Results are expressed where applicable as the mean ± SEM. Statistical analysis was carried out by Student's *t*-test and linear regression analysis was calculated according to Tallarida and Jacobs [25].

Materials. (+), (−) and (±) propranolol, (+),(−) practolol and ICI 118,551 (erythro-DL-1(7-methylindan-4-yloxy)-3-isopropylamino-butan-2-ol) were obtained from I.C.I. Ltd., metoprolol and oxprenolol from Ciba-Geigy; timolol from Merck; acetylsalicylic acid (aspirin) and papaverine from Sigma Chemical Co; U-46619, thromboxane B_2 and prostaglandin E_1 from Upjohn Co. Inc; A-23187 and forskolin from Calbiochem; and rabbit anti-serum to thromboxane B_2 from Seragen Inc. 1-*O*-Alkyl-2-acetyl-sn-glyceryl-3-phosphorylcholine (PAF) was a kind gift from Dr R. Goeschke (Ciba-Geigy).

RESULTS

Effect of a range of β-adrenoceptor antagonists on a given aggregatory response

The effects of 6 β-adrenoceptor antagonists (propranolol, ICI 118,551, oxprenolol, timolol, metoprolol and practolol) were assessed for their effects on the aggregatory responses induced by A-23187, collagen, U-46619, ADP, adrenaline, PAF and TPA. Both the (+) and (−) isomers of propranolol and practolol were tested. Figure 1 shows dose–response curves for inhibition by all these β-adrenoceptor antagonists of the aggregatory response to 1.75 μM U-46619 (Fig. 1). The potency order for the inhibition of aggregation was (+) propranolol = (−) propranolol > ICI 118,551 > oxprenolol > timolol > (+) practolol = (−) practolol. An identical potency order was observed when similar studies were performed using the other aggregating agents indicated above. In all cases each β-adrenoceptor antagonist caused complete inhibition regardless of the agent which was used to induce the aggregatory response. Furthermore the extent of inhibition by either propranolol or practolol was the same whether the (+) or (−) isomer was used as illustrated in Fig. 1. Thus the inhibitory effect fails to show any stereospecificity in accord with previous reports [6, 10]. All subsequent studies were therefore performed using (−) or (±)-propranolol.

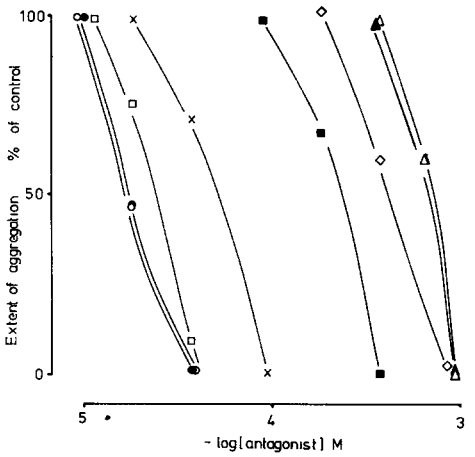

Fig. 1. Dose–response curves for inhibition of an aggregatory response induced by U-46619 by β-adrenoceptor antagonists. Platelet-rich plasma was preincubated with the β-adrenoceptor antagonists for 1 min at 37° prior to the addition of 1.75 μM U-46619 and aggregation measured using a Payton aggregometer as described in Methods. The β-adrenoceptor antagonists used were (+) propranolol (●), (−) propranolol (○), ICI 118,551 (□), oxprenolol (X), timolol (■), metoprolol (◇) and (+) (△) and (−) (▲) practolol. The results are from a single experiment but are typical of three similar experiments.

Consideration of the various physicochemical and pharmacological properties possessed by the β-adrenoceptor antagonists which have been examined suggested that the effects illustrated in Fig. 1 could not be explained by their sub-type selectivity or degree of agonist activity. However, the potency order correlates well with the lipid solubility of these compounds as expressed by their partition coefficient between n-octanol and H_2O (data not shown).

Effect of variation of the concentration of aggregating agent on the extent of inhibition by β-adrenoceptor antagonists

Further insight into the properties of inhibition by β-adrenoceptor antagonists was obtained when the extent of the inhibition was measured as a function of the concentration of the aggregating agent used to induce the response. For all aggregating agents used the addition of the β-adrenoceptor antagonist caused a parallel shift in the dose–response curve to the right. Thus there is an apparently competitive relationship between the effects of the aggregating agents and of the β-adrenoceptor antagonists.

When data of this type were subjected to analysis as described by Arunlakshana and Schild [24], linear relationships were obtained which have slopes approximating to unity if the studies were performed using aggregating agents which cause the aggregatory response as a direct consequence of occupancy of a plasma membrane receptor, e.g. adrenaline, ADP, U-46619 and PAF. Apparent pA_2 values and slopes derived from such Schild plots are summarized in Table 1A. Table 1A also includes data of this type obtained using arachidonate as aggregating agent, since the aggregatory response in this latter system is attributable to conversion of arachidonate to thromboxane A_2, and possibly also to prostaglandin endoperoxides formed as intermediates, which are then believed to act at a plasma membrane thromboxane receptor. The very close agreement between the properties of inhibition by propranolol observed for arachidonate and for the thromboxane A_2/prostaglandin endoperoxide mimetic, U-46619 (Table 1A), are in accordance with such a mechanism. It is also apparent from Table 1A that the ability of a given β-adrenoceptor antagonist to inhibit the aggregatory response, as measured by the appar-

Table 1. Apparent pA_2 values and Schild plot slopes for the effect of (±) propranolol on aggregation

Aggregating agent	Apparent pA_2	Slope
(A)		
Adrenaline	5.1	0.97 ($r = 0.99$)
Collagen (secretion)	4.9	1.08 ($r = 0.98$)
ADP	4.6	1.04 ($r = 0.99$)
U-46619	4.4	1.12 ($r = 0.98$)
Arachidonic acid	4.4	1.17 ($r = 0.98$)
PAF	4.0	1.23 ($r = 0.98$)
(B)		
Collagen (aggregation)	5.0	1.60 ($r = 0.94$)
Collagen (+ aspirin) (aggregation)	4.4	1.96 ($r = 0.97$)
TPA	4.4	1.98 ($r = 0.99$)
A-23187	3.6	1.75 ($r = 0.99$)

Apparent pA_2 values and slopes were obtained by analysis of data similar to those shown in Fig. 4 as described by Arunlakshana and Schild [24]. In all cases except for collagen (secretion) the primary data were derived from studies performed as described in Methods using a Payton aggregometer. The data for collagen (secretion) were obtained using a Payton Lumiaggregometer. Linear regression analyses of the Schild plots yielded the correlation coefficients (r). The basis for division of the Table into (A) and (B) is considered in the Discussion.

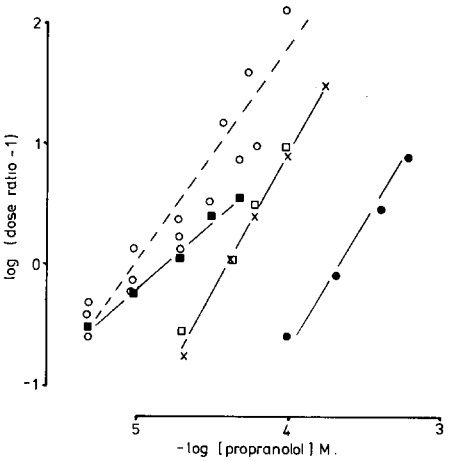

Fig. 3. Schild plot for inhibition by (±) propranolol of aggregation induced by TPA (×), A-23187 (●) and collagen in the presence (□) and absence (○) of aspirin (100 μM) and of ATP secretion induced by collagen (■). The data shown is from three experiments similar to those illustrated in Fig. 4 for collagen. The studies were performed as described in Methods. The data shown for aggregation induced by TPA, A-23187 and collagen in the presence and absence of aspirin were obtained using a Payton aggregometer and those for secretion induced by collagen using a Payton lumiaggregometer in the presence of the luciferin/luciferase reagent.

ent pA_2 value, is a function of the aggregating agent employed with the order of potency being adrenaline > ADP > U-46619 = arachidonate > PAF.

Conversely addition of increasing concentrations of an aggregating agent causes a parallel shift to the right in the dose–response curves for inhibition by β-adrenoceptor antagonists. Analysis of these data as described by Arunlakshana and Schild [24] again yields linear plots with a slop approximating 1 as shown in Fig. 2A for the effects of U-46619 on the dose–response curves for inhibition by (±) propranolol, oxprenolol and metoprolol. The apparent pA_2 values for U-46619 derived from these data for the 3 β-adrenoceptor antagonists studied in this way show a strong correlation ($P < 0.01$) with the *n*-octanol:H_2O partition coefficient (Fig. 2B). This relationship supports the concept that the effect is a function of the lipid solubility of these drugs, as suggested by the correlation of the potency order with this parameter which has already been noted.

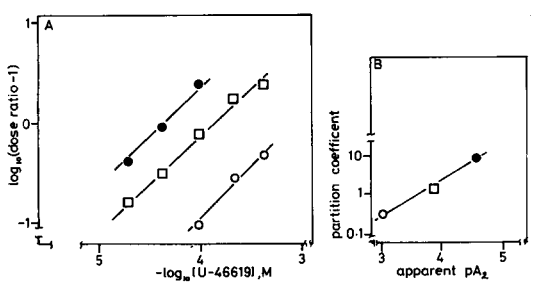

Fig. 2. (A) Schild plot analysis of the shift in the dose–response curve for propranolol (●), oxprenolol (□) and metoprolol (○) induced by U-46619. (B) Correlation of apparent pA_2 values for U-46619 obtained from studies using propranolol (●), oxprenolol (□) and metoprolol (○) as antagonist with the n-octanol/H_2O partition coefficients for these three drugs. The plots shown were obtained from data in which the dose–response curve for inhibition of aggregation by these antagonists was determined in the presence of a series of fixed concentrations of U-46619. Platelet aggregation was measured and quantitated as described in Methods using a Payton Aggregometer. The data shown are from a single experiment but are representative of the pattern seen in two other such experiments.

Studies have also been performed using aggregating agents for which the aggregatory response is either unrelated to occupancy of a plasma membrane receptor, e.g. A-23187, TPA, or is an indirect consequence of such occupancy, e.g. collagen. The data qualitatively resemble those obtained for agents acting directly via a plasma membrane receptor in that a parallel shift to the right of the dose–response curve is observed. Analysis of the data by the procedure described by Arunlakshana and Schild [24] gives linear relationships but with slopes which are significantly greater than 1 and which in most instances approximate a value of 2. This is illustrated in Fig. 3 for the effect of (±) propranolol on the dose–response curves for aggregation induced by A-23187, by TPA and by collagen in the presence or absence of aspirin. For studies performed in the presence of aspirin the platelet-rich plasma was incubated for at least 10 min at 37° with 0.1 mM aspirin before addition of collagen. Control studies showed that under these conditions the response to arachidonate was abolished. Comparison of the responses to collagen observed in the presence and absence of aspirin permits assessment of the extent

to which the effects observed can be attributed to inhibition of the response to thromboxane A_2 produced as a consequence of stimulation by collagen. The apparent pA_2 values and slopes derived from the Schild plot analysis are summarized in Table 1B. The only exception to a linear Schild plot for this group of aggregating agents may occur for the effect of (±) propranolol on aggregation induced by collagen in the absence of aspirin where the data points are fitted equally well by a linear plot with a slope of 1.6 (r = 0.94) or a biphasic plot with limiting slopes of 1 and 2 (r = 0.85/0.95). Distinction between these two possiblities could not be obtained since the small extent of the shift in the collagen dose–response curve at (±) propranolol concentrations below 5 μM could not be measured with sufficient accuracy while at (±) propranolol concentrations in excess of 100 μM the shift in the dose–response curve was so large that the necessary determinations could not be made with the collagen preparations available to us.

Effect of (±) propranolol on amine storage granule secretion induced by collagen

Secretion of the contents of the amine and protein storage granules is an early event in the response to collagen. By the use of the lumiaggregometer it is possible to monitor both aggregation and ATP secretion simultaneously. Figure 4 shows data obtained when collagen dose–response curves were obtained for both aggregation (A) and ATP secretion (B) in the presence of a series of fixed concentrations of (±) propranolol. Parallel shifts to the right are observed for both responses but particularly at higher (±) propranolol concentrations the shift is clearly greater for aggregation than for ATP secretion. This is reflected in Fig. 4 and Table 1 which show that the slope of the Schild plot for inhibition of ATP secretion induced by collagen is significantly lower than that observed for inhibition of aggregation.

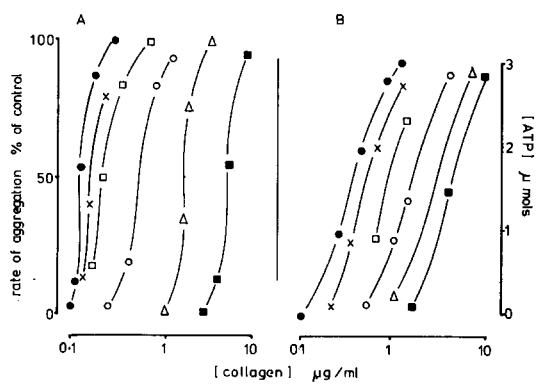

Fig. 4. Relationship between the extent of aggregation (A) and the extent of ATP secretion (B) induced by collagen in the presence of increasing concentrations of (±) propranolol. Platelet-rich plasma was reincubated for 1 min at 37° with 0 μM (●), 10 μM (×), 20 μM (□), 40 μM (○), 60 μM (▲) and 100 μM (■) (±) propranolol before addition of collagen at the concentrations as indicated. ATP secretion and aggregation were measured simultaneously using a Payton lumiaggregometer as described in Methods. The data shown is typical of three similar experiments.

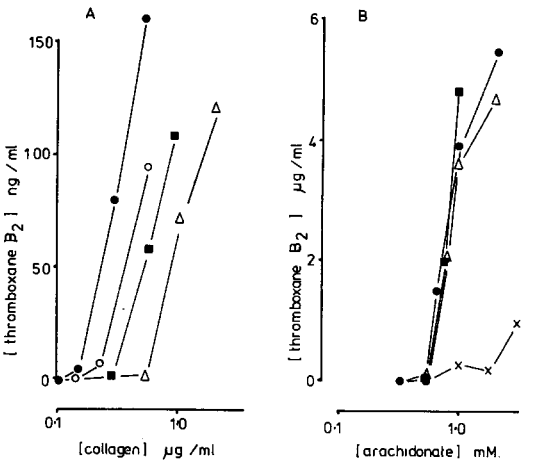

Fig. 5. Relationship between the extent of thromboxane B_2 production induced by collagen (A) and arachidonate (B) and the concentration of these agents in the presence of a series of increasing concentrations of (±) propranolol. The assay of thromboxane B_2 was performed as described in Methods. Thromboxane B_2 production was terminated 1 min after maximal aggregation had occurred. The results shown are from a single experiment but are representative of two other such experiments. The (±) propranolol concentrations used were 0 μM (●), 20 μM (○), 40 μM (■), 100 μM (△) and 400 μM (×).

Effect of (±) propranolol on thromboxane B_2 production induced by collagen and arachidonate

Another early event in the response of platelets to collagen in the absence of aspirin is the synthesis of thromboxane A_2 which can be measured as the appearance of the stable degradation product thromboxane B_2 [18]. Comparison of the effects of (±) propranolol on thromboxane B_2 synthesis induced by collagen and by arachidonate further permits localization of the site of inhibition by this drug. The results of such studies are shown in Fig. 5 as collagen (A) or arachidonate (B) dose–response curves at a series of fixed concentrations of (±) propranolol. Although complete dose–response curves were not obtained, it is clear that (±) propranolol causes inhibition of thromboxane B_2 synthesis induced by collagen over the same concentration range as it causes inhibition of secretion and of aggregation induced by this agonist (Figs 4, 5). In contrast, over this range no effect is observed on thromboxane B_2 synthesis induced by addition of arachidonate, although at higher (±) propranolol concentrations, some inhibition is observed (Fig. 5B). Furthermore, the extent of the shift in the collagen dose–response curve for thromboxane B_2 synthesis (Fig. 5A) is more comparable to that characterizing inhibition of ATP secretion (Fig. 8B) than to that observed for inhibition of aggregation (Fig. 8A).

Effect of (±) propranolol on platelet cAMP production in the absence of agonist, in the presence of forskolin and in the presence of prostaglandin E_1 with or without ADP

Further insight into the mechanism of the inhibition by (±) propranolol can be obtained by ex-

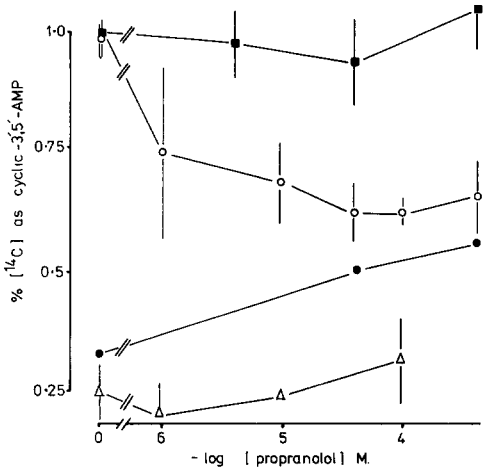

Fig. 6. Effect of (±) propranolol on the level of platelet cyclic 3',5'-AMP in the absence of additions (△), or in the presence of forskolin (■), prostaglandin E_1 (○) or of prostaglandin E_1 and ADP (●). The level of cyclic-3'5'-AMP in intact platelets was measured after 1 min incubation as described in Methods. The concentrations of forskolin, prostaglandin E_1 and ADP used were 5, 1 and 20 μM respectively. The level of cyclic-3',5'-AMP expressed at % ^{14}C incorporated was 0.15% in the absence of additions, 0.7% in the presence of forskolin and 0.6% in the presence of prostaglandin E_1. These latter values have been normalized as a value of 1.0 and all other values expressed relative to this normalized value. The points shown are mean ± SEM for three determinations.

amining the effect of this drug on the level of cAMP in the resting platelet and after stimulation of adenylate cyclase by forskolin [26] or by prostaglandin E_1 [27]. Furthermore, the effect of (±) propranolol on inhibition by ADP of the response of cAMP to prostaglandin E_1 was also examined. As shown in Fig. 6, (±) propranolol when added at concentrations in the range 1–400 μM has no effect on resting levels of cAMP in the unstimulated platelets or the elevated level of this metabolite produced by stimulation of adenylate cyclase by forskolin. However, partial (ca. 50%) inhibition of the response of platelet to 1 μM prostaglandin E_1 is observed at a saturating dose of propranolol, although such inhibition occurs over a range of concentrations (Fig. 6) similar to that over which (±) propranolol inhibits the aggregatory response to excitatory agonists such as ADP and U-46619. In addition, Fig. 6 demonstrates that addition of (±) propranolol over this concentration range completely prevents the inhibition by 20 μM ADP of the increase in cAMP induced by 1 μM prostaglandin E_1.

DISCUSSION

The data presented here provide a more complete account than has been available previously of the effect of β-adrenoceptor antagonists, and in particular of propranolol, on various responses of human platelets. In accordance with the recent findings of Weksler *et al.* [6] and of Nathan *et al.* [9] but in contrast to earlier reports [28] propranolol and other β-adrenoceptor antagonists can cause complete inhibition of the aggregatory responses to all agents tested (Fig. 1) and of the secretory response and the synthesis of thromboxane B_2 induced by collagen (Figs 4, 5) and of the inhibitory effect of ADP on enhanced cAMP formation induced by prostaglandin E_1 (Fig. 6). Propranolol also causes partial inhibition of enhanced cAMP synthesis induced by prostaglandin E_1 but has no effect on enhanced cAMP synthesis induced by forskolin (Fig. 6) or on thromboxane B_2 synthesis from arachidonate over a similar range of concentrations (Fig. 5).

These widespread effects cannot be due to inhibition of either cyclooxygenase or, as suggested previously [29], of thromboxane synthetase, and seem more likely to relate to the known interaction of propranolol and other β-adrenoceptor antagonists with phospholipids [14, 15]. This latter explanation is consistent with the correlation of the extent of inhibition with the lipid solubility of the β-adrenoceptor antagonist used (Fig. 2B) and also with the time course and the lack of stereoselectivity (Fig. 1) which characterizes this inhibitory effect. The properties observed for inhibition of the aggregatory and secretory responses as summarized in Table 1 further suggest that several different mechanisms may be operative even though such mechanisms may all originate from a single mode of action for the β-adrenoceptor antagonists.

All aggregating agents for which the aggregatory response is not due to occupancy of a plasma membrane receptor, e.g. A-23187, TPA [30, 31], or for which this response is a secondary consequence of secretion induced by such occupancy, e.g. collagen, give Schild plots for inhibition by propranolol with slopes approximating a value of 2 (Table 1B), suggesting a complex mechanism of inhibition. This inhibitory effect could be explained if binding of propranolol to phosphatidylserine and phosphatidylinositol on the inner leaflet of the plasma membrane [15] impaired the availability of the former phospholipid for activation of protein kinase C [32] and prevented hydrolysis of the latter phospholipid either by phosphatidylinositol-specific phospholipase C and/or by phospholipase A_2 [33]. Proposal of the former effect is necessary to explain inhibition by propranolol of the aggregatory response to TPA which mimics the effect of 1,2-diacylglycerol in enhancing the activity of protein kinase C at low [Ca^{2+}] [30, 34]. The latter postulate is consistent with the data of Vanderhoek and Feinstein [35] and is required to explain selective inhibition by propranolol of thromboxane B_2 synthesis induced by collagen without a comparable effect if exogenous arachidonate is provided (Fig. 5). The quantitative similarity in the properties of inhibition by propranolol of the aggregatory responses induced by TPA and by collagen in the presence of aspirin (Table 1B) suggests further that activation of protein kinase C by 1,2-diacylglycerol is a crucial event in the response to collagen observed in the presence of cyclooxygenase blockade. Provision of 1,2-diacylglycerol depends in turn on the action of phospholipase C on a phosphoinositide which may also be restricted by binding of propranolol to these phospholipids.

These postulates may not, however, provide an adequate explanation for inhibition by propranolol of the aggregatory response to A-23187 which is believed to result from mobilization of Ca^{2+} into the cytosol by this ionophore [36, 37], although enhanced breakdown of phosphatidylinositol and phosphatidylcholine also occurs under these conditions [31]. Among the agents tested the aggregatory response to A-23187 is the least sensitive to inhibition by propranolol (Table 1) and it is known that binding to phospholipids other than phosphatidylserine and phosphatidylinositol occurs when higher concentrations of propranolol are used [15]. Such a more general effect might cause membrane disorganization and so directly impair the ionophoretic effect in accordance with the observation that propranolol inhibits $^{45}Ca^{2+}$ influx into platelets induced by A-23187 [6]. However, if Ca^{2+} mobilization is induced by a product of phosphatidylinositol breakdown as suggested by Michell *et al.* [38], then inhibition of the response to A-23187 could also result from binding of propranolol to a phosphoinositide as suggested above.

In contrast, inhibition by propranolol and by other β-adrenoceptor antagonists of the responses which are a direct consequence of occupancy of a plasma membrane receptor is characterized by Schild plots having a slope approximating 1. This group includes the secretory response to collagen and the aggregatory responses to adrenaline, ADP, U-46619 and PAF, as well as to arachidonate which causes this response as a consequence of formation of thromboxane A_2 and possibly also prostaglandin endoperoxides [39] (Table 1A). As would be predicted on this basis, inhibition of the aggregatory response to arachidonate occurs with properties (apparent pA_2, slope) which are not significantly different from those observed for U-46619 (Table 1A). The properties observed are those expected if propranolol were acting as a competitive antagonist at the receptors responsible for the responses to all these agonists. However, the failure to observe stereoselective inhibition by (+) and (−) propranolol or practolol (Fig. 1) is not compatible with such a mode of action which is in any case unlikely on the grounds of structural similarity. It is therefore more probable that the interaction of propranolol and other β-adrenoceptor antagonists with plasma membrane phospholipids causes a perturbation in receptor properties such that their affinity for the excitatory agonist is decreased. This explanation is in accordance with the finding that propranolol inhibits receptor-mediated effects on adenylate cyclase by prostaglandin E_1 and ADP but has no effect on the enzyme itself or on activation by forskolin which bypasses the receptor [26] (Fig. 6). The significant differences observed between the apparent pA_2 values for inhibition of aggregation induced by the various agonists (Table 1A) as well as the finding of partial inhibition of the response to prostaglandin E_1 (Fig. 6) suggests further that receptors may exhibit different sensitivities to this perturbation of their phospholipid environment.

In addition to considerations of the mechanism of inhibition as discussed above, the results summarized in Table 1A have important implications for studies in which lipophilic drugs are used as inhibitors of cellular responses. When a Schild plot having a slope of approximately 1 is obtained for an inhibitory effect, this finding is usually taken as being indicative of competitive interaction with the agonist at its receptor or with an element, e.g. a Ca^{2+} channel, closely associated with the receptor. For example, such properties have been observed, and interpretations suggested, for inhibition by diltiazem and nicardipine of platelet aggregation induced by PAF, U-46619 and ADP [40, 41] and for inhibition by verapamil of such aggregation induced by adrenaline, ADP and A-23187 [42]. The data summarized in Table 1A suggest, however, that such conclusions are premature unless studies have been performed which exclude interaction with membrane phospholipids as an explanation for the inhibitory effect. It is of interest in this respect that the lipid solubility of verapamil is similar to that of propranolol and is considerably greater than that of nicardipine [43].

Finally it is of interest to assess the possible clinical implications of our data since β-adrenoceptor antagonists are now widely used in regimens designed to protect patients at risk from myocardial infarction [13]. A platelet component in myocardial protection by β-adrenoceptor antagonists appears to be excluded by the wide difference between the plasma levels of these drugs achieved in clinical practice (<0.1 μM) and the concentrations (10–1000 μM) required to observe *in vitro* inhibition of platelet responses (Table 1). However, several factors complicate such a simple comparison. First, studies using [^{3}H]-propranolol and [^{3}H]-dihydroalprenolol show that platelets accumulate these lipophilic drugs to a marked extent [6]. Hence the plasma drug level is not likely to be a good indicator of the concentration of drug present in the platelet and, second, patients suffering from hypertension and related disease often possess platelets which are unusually responsive to excitatory agonists and which may therefore respond atypically to β-adrenoceptor antagonists.

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