Pergamon

Biochemical Pharmacology, Vol. 48, No. 7, pp. 1319–1326, 1994.

0006-2952/94 $7.00 + 0.00

0006-2952(94)00321-1

EFFECTS OF EXTRACELLULAR DIVALENT CATIONS ON RESPONSES OF HUMAN BLOOD PLATELETS TO ADENOSINE 5′-DIPHOSPHATE

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(*Received* 24 *March* 1994; *accepted* 4 *July* 1994)

Abstract—The effects of extracellular divalent cations on the responses of human platelets to adenosine 5′-diphosphate (ADP) and on its inhibition by the competitive antagonist adenosine 5′-triphosphate (ATP) were investigated. Two responses were studied, shape change and the inhibition of prostaglandin E_1 (PGE_1)-stimulated adenylate cyclase, and experiments were carried out in the presence of divalent cations (Ca^{2+} and Mg^{2+}, 1 mM) or in their absence. For each response there was a small leftward shift of the concentration–response curve to ADP in the absence of divalent cations compared to that in their presence, and this leftward shift disappeared when the results were plotted in terms of ADP^{3-} rather than total ADP concentration. The shape change results were, however, complicated by a reduction in the maximal response to ADP in the absence of divalent cations. For each response there was also a marked increase in the pA_2 value of ATP in the absence of divalent cations compared to that in their presence, and this difference disappeared if the results were calculated in terms of ATP^{4-} instead of total ATP. These results suggest that the human platelet ADP receptor, in common with other receptors for adenine nucleotides, recognises predominantly the uncomplexed forms of ADP and ATP as ligands.

Key words: ADP; ATP; shape change; adenylate cyclase; P_2-purinoceptors; human platelets

Adenine nucleotides such as ADP‡ and ATP have pharmacological effects on many tissues, and these effects are mediated by cell surface receptors known as P_2-purinoceptors. At least five classes of P_2-purinoceptor (P_{2X}, P_{2Y}, P_{2U}, P_{2Z} and P_{2T}) have been proposed, based largely on the structure–activity relationships of agonists [1, 2]. Of these, the P_{2T}-purinoceptor is unique in that ATP is an antagonist at this receptor whereas it is an agonist at the other subtypes. It also has a very limited distribution, being found only on blood platelets and their precursors, the megakaryocytes. The endogenous agonist is ADP, which causes platelets to change shape, aggregate and release the contents of intracellular storage granules, including ADP and ATP. ADP plays an important role in platelet function, being one of the feedback mediators amplifying platelet activation, and may also play a role in triggering aggregation, as it is released in high concentrations when cells are damaged. The mechanism whereby ADP induces these changes in platelets is not fully understood, but in common with other aggregating agents an increase in cytosolic calcium is thought to be of major importance [3, 4]. In confirmation of this, the structure–activity relationships for agonists and antagonists in causing increases in cytosolic calcium levels correlate very well with the structure–activity relationships for inducing aggregation [5–7]. The way in which ADP causes increases in cytosolic calcium is not fully understood. There is evidence for a receptor-operated cation channel as well as for mobilization of calcium from internal stores, although ADP has not been consistently shown to activate the phospholipase C pathway [8, 4]. Another biochemical event associated with ADP-induced platelet activation is inhibition of adenylate cyclase, which is independent of aggregation, and there has been a certain amount of controversy over whether these two effects are mediated by one type of ADP receptor on human platelets or two types (see Refs 9–11 for review).

In physiological buffers, and indeed in biological fluids such as plasma, ATP exists as a mixture of several species including the divalent cation complexes, $Mg{\cdot}ATP^{2-}$ and $Ca{\cdot}ATP^{2-}$, and the tetrabasic form, ATP^{4-}. It is now widely accepted that the P_{2Z} receptor responds to this tetrabasic form of ATP rather than any of the other species of ATP found in solution [12]. Evidence is also accumulating that the preferred agonist at other ATP receptors is this tetrabasic form of ATP rather than, for example, the magnesium complex which is well known to act as a substrate of many enzymes. For example, it has been suggested that ATP^{4-} is the agonist species at the P_{2X} receptor on the guinea-pig vas deferens [13] and that ATP^{4-} and UTP^{4-} are the agonists at the P_{2Y} and P_{2U} receptors present on aortic endothelial

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‡ Abbreviations: ADP, adenosine 5′-diphosphate; ATP, adenosine 5′-triphosphate; PGE_1, prostaglandin E_1; cyclic AMP, adenosine 3′:5′-cyclic monophosphate; PRP, platelet-rich plasma; IBMX, isobutylmethylxanthine

cells [14]. ATP^{4-} has also been proposed to be the agonist at P_{2U} receptors on NG108–15 cells [15] and at the (unusual) P_2-purinoceptor on rat megakaryocytes [16]. It would seem therefore that a characteristic of ATP receptors is that they require the uncomplexed form of ATP and do not recognize the divalent cation complexes, whilst the opposite is true of ATP-utilizing enzymes.

In the absence of extracellular divalent cations platelets are unable to aggregate in response to stimulatory agonists. Aggregation requires the binding of fibrinogen to fibrinogen receptors exposed in the platelet glycoprotein IIb/IIIa complex, with the fibrinogen forming the cross-links between the platelets. Formation of this glycoprotein IIb/IIIa complex requires the presence of extracellular divalent cations [17]; thus in the absence of divalent cations this complex cannot form and the platelets do not aggregate. However, in the absence of divalent cations platelets are still able to undergo a shape change response. This would suggest that extracellular divalent cations are not required for the binding of ADP to the ADP receptor or for the production of this response. Also, two analogues of ADP, 2-azido-ADP and 2-methylthio-ADP have both been shown to bind to platelets in the absence of divalent cations [18, 19], further suggesting that extracellular divalent cations are not required for the binding of ADP to its receptor. However, the divalent cation requirement of the response of platelets to ADP has never been studied in detail and it is not known whether the ADP receptor responds to all ADP species or simply to the uncomplexed form of ADP, ADP^{3-}. Similarly, it is not known which form (or forms) of ATP (divalent cation complexes or ATP^{4-}) acts as the antagonist at this receptor.

We therefore studied the effects of varying the extracellular concentration of magnesium and calcium on the ability of ADP to activate platelets. Two responses of platelets to ADP were studied, shape change and inhibition of adenylate cyclase, both of which occur in the absence of extracellular calcium. ADP-induced platelet shape change, like aggregation, is, however, thought to be triggered by the increase in cytosolic calcium concentration, and this increase is, in part, caused by influx of calcium into the platelet from the extracellular medium [4]. Removal of extracellular calcium may therefore attenuate this response. The effect of extracellular divalent cation concentration was, therefore, also determined on the ability of ADP to inhibit PGE_1-stimulated adenylate cyclase activity, as this response does not appear to be due to the increase in cytosolic calcium concentration, but is an independent effect [8–10]. As changes in the potency of an agonist may reflect changes in both the affinity of the agonist for the receptor and its efficacy in inducing the response, the effects of varying the extracellular divalent cation concentration were also determined on the ability of ATP to antagonize these responses.

MATERIALS AND METHODS

Measurement of platelet shape change. Blood was drawn from healthy human volunteers who had not taken drugs for 10 days, into one-sixth of a volume of acid-citrate-dextrose anticoagulant (85 mM trisodium citrate, 71 mM citric acid, 111 mM glucose) and centrifuged at 260 *g* for 20 min to obtain PRP. Platelets were obtained by centrifuging the PRP at 680 *g* for 20 min in the presence of prostacyclin (1 μM). The supernatant plasma was discarded and the platelets were suspended in 10 mL of nominally divalent cation-free HEPES–saline (145 mM NaCl, 5 mM KCl, 10 mM glucose, 10 mM HEPES, 2 mg/mL BSA adjusted to pH 7.4 with NaOH 1 M). The platelets were again collected by centrifugation at 680 *g* for 20 min in the presence of prostacyclin (1 μM) and resuspended at 1×10^8/mL in nominally divalent cation-free HEPES–saline. The second centrifugation step was employed to help remove residual plasma proteins which may allow the platelets to aggregate during shape change measurement. Aliquots (0.3 mL) of platelet suspension were incubated in siliconized glass aggregometer tubes at 37° for 3 min in the presence of 1 mM $MgCl_2$, 1 mM $CaCl_2$, both 1 mM $MgCl_2$ and 1 mM $CaCl_2$, 1 mM EGTA or an equivalent volume of water and transferred to a Chrono-Log Lumi-Aggrometer. Shape change was quantified as the maximal decrease (in arbitrary units) in light transmission through the stirred sample at 37° on addition of the agonist, relative to a 50% dilution of the platelet suspension (i.e. 5×10^7 platelets/mL). Diluted platelet suspension was used as the reference rather than HEPES–saline to magnify the responses. Under the above conditions, i.e. in the absence of fibrinogen (and other plasma proteins), aggregation in response to ADP is negligible as measured in the aggregometer. ADP was added in 6 μL of water and ATP, where used, was added simultaneously with the ADP.

Log concentration–response curves were obtained in triplicate in blood obtained from at least three donors. EC_{50} values were obtained by regression analysis of the linear portion of the triplicate concentration–response curves, and dose ratios for ATP were determined from the EC_{50} values in the presence and absence of the antagonist. The apparent K_d values for ATP were obtained from the dose ratios using the formula K_d = concentration of ATP/(dose ratio − 1), and pA_2 values were calculated as $-\log_{10} k_d$. Statistical significance was determined by analysis of variance or, when only two sets of conditions were compared, Student's *t*-test. P values greater than 0.05 were taken as indicating no significant difference between values being compared.

Measurement of inhibition of PGE_1-stimulated adenylate cyclase. PRP was incubated with 4 μM [^{14}C]adenine for 45 min at 37°. The platelets were then collected by centrifugation at 680 *g* for 20 min in the presence of prostacyclin (1 μM) and resuspended at 1.1×10^8/mL in HEPES–saline. Platelet cyclic AMP levels were determined by a modification of the method of Haslam and Rosson [20]. Platelets were suspended at 1.1×10^8/mL in nominally divalent cation-free HEPES–saline which was supplemented with either $MgCl_2$ (1 mM) and $CaCl_2$ (1 mM), or EGTA (1 mM), and preincubated for 3 min at 37° before addition of solutions (50 μL) of ADP and/or ATP which contained PGE_1 (10 μM),

to stimulate adenylate cyclase, and IBMX (1 mM), to inhibit phosphodiesterase. In some experiments apyrase (0.5 U/mL) was also included in the incubation mixture to degrade any ADP released by the platelets and remaining in the suspension. After 30 sec the reaction was terminated by addition of 3 M perchloric acid (100 μL) containing [^{3}H]cyclic AMP (~20,000 dpm) to estimate recovery. After at least 30 min on ice the samples were centrifuged in a microfuge and a 450 μL aliquot of the supernatant was applied to columns of AG50W-X8 [H^+] (1.3 mL) to remove the adenine and adenosine. The adenine nucleotides were eluted with 1 mM KH_2PO_4 (pH 7.5) and the cyclic AMP-containing eluate was twice treated with nascent barium sulphate (by addition of 0.3 mL of 0.25 M zinc sulphate and 0.3 mL of 0.25 M barium hydroxide) and centrifuged to remove adenine nucleotides other than cyclic AMP. An aliquot (5–6 mL) of the supernatant was lyophilized, the ^{3}H and ^{14}C were estimated by scintillation counting, and ^{14}C dpm were corrected for recovery of [^{3}H]cyclic AMP. Log concentration–response curves were obtained in triplicate in blood from at least three donors and presented as [^{14}C]cyclic AMP dpm in the sample. EC_{50} values were obtained by regression analysis of the linear portion of these concentration–response curves. Dose ratios for ATP were determined from the EC_{50} values in the presence and absence of ATP, and apparent K_d values were calculated using the formula K_d = concentration of ATP/(dose ratio − 1). Statistical significance was determined using Student's *t*-test, and P values greater than 0.05 were taken as indicating no significant difference between values being compared.

Determination of nucleotide release from platelets. Platelets were resuspended at 1×10^8/mL in nominally divalent cation-free HEPES–saline, and aliquots (1.8 mL) were incubated at 37° in the presence of either 1 mM $MgCl_2$ and 1 mM $CaCl_2$, or 1 mM EGTA. After 3.5 min, aliquots (0.5 mL) were removed and layered onto a mixture of silicone oils (4 parts Dow Corning 704 EU diffusion pump fluid and 1 part Dow Corning 200/0.65 cs silicone fluid) (200 μL). The platelets were separated from the suspending medium by centrifugation in a swing-out microfuge for 1 min and aliquots (300–400 μL) of the supernatant were removed and stored at −20° before HPLC analysis. The concentration of ADP released from the platelets was determined by ion-paired reverse-phase HPLC on a Techsphere 5ODS column, and quantified as the height of the peak on the chart recorder trace, compared to standard samples of ADP. Two buffers were used, A (100 mM KH_2PO_4, pH 7.0, containing 8 mM tetrabutylammonium hydrogen sulphate) and B (a mixture of buffer A and methanol, 70:30, v/v), and the column was eluted at a flow rate of 1.3 mL/min with the following gradient: 2.5 min at 100% A, 7.5 min linear gradient up to 100% buffer B, 10 min at 100% B.

Calculation of free ADP^{3-} and ATP^{4-} concentrations. In solutions containing only a single divalent cation species (i.e. Mg^{2+} or Ca^{2+} alone) the free ADP^{3-} or ATP^{4-} concentration was calculated using the method of Dahlquist and Diamant [21] by solving the following quadratic equation:

$$K_M[AXP]_F^2 + [AXP]_F(K_M[M]_T - K_M[AXP]_T + 1) - [AXP]_T = 0,$$

where M represents either Ca^{2+} or Mg^{2+}; AXP represents either ADP or ATP; K_M is the stability constant of the M·AXP complex: $\log K_M$ = 2.80 for Ca·ADP, 3.30 for Mg·ADP, 3.94 for Ca·ATP and 4.28 for Mg·ATP [22, 23]; $[AXP]_F$ is the concentration of free ADP^{3-} or ATP^{4-}; $[AXP]_T$ is the total concentration of AXP; and $[M]_T$ is the total concentration of M.

The stability constants used for these calculations are those quoted in Refs 22 and 23 at 40°. From the data presented in these papers, which give stability constants at 0.4, 12, 25 and 40°, these values are unlikely to change by more than 0.03 log units at 37° even in the most extreme case (MgATP), and this change is almost certainly within the error which the authors quote on their determinations (±0.02 log units). In solutions containing both 1 mM calcium and 1 mM magnesium the $[AXP]_F$ was approximated as the mean of the $[AXP]_F$ calculated in the presence of 2 mM calcium and in the presence of 2 mM magnesium. No adjustment was made to the free divalent cation concentration when determining $[AXP]_F$ in solutions containing both ADP and ATP, as the change due to the presence of the other nucleotide would not exceed ~10% even at the highest concentrations used (i.e. in solutions containing 100 μM ATP and 100 μM ADP). In solutions with no added divalent cations it was assumed that $[AXP]_F = [AXP]_T$. No adjustment was made for the presence of protonated forms, as even for the species with the highest pK_a value (ATP^{4-}) the proportion of protonated forms is unlikely to exceed ~10% [22, 23].

Materials. ADP, ATP, BSA, IBMX, PGE_1, prostacyclin, apyrase and tetrabutylammonium hydrogen sulphate were obtained from Sigma (Poole, U.K.). AG50W-X8 [H^+] was from Bio Rad (Hemel Hempstead, U.K.). [2,8-^{3}H]cyclic AMP (41.5 Ci/mmol) in 50% aqueous ethanol and [U-^{14}C]adenine (270 mCi/mmol) in 2% ethanol were from Amersham International (Amersham, U.K.). HPLC solvents were from Fisons (Loughborough, U.K.); all other chemicals were AnalaR grade from BDH (Poole, U.K.). PGE_1 was dissolved at 1 mM in 50% aqueous ethanol and prostacyclin was dissolved at 100 μg/mL in 10 mM NaOH. Both compounds were stored in solution at −20°. IBMX was dissolved in nominally divalent cation-free HEPES–saline and nucleotides were dissolved in water. IBMX was made up freshly each day whilst nucleotides were stored frozen.

RESULTS

ADP induced platelet shape change in a concentration-dependent manner under all conditions tested, but there was a slight decrease in the maximal response to ADP in the absence of added divalent cations (Fig. 1). The pD_2 values ($-\log EC_{50}$) obtained for ADP were not significantly different ($P > 0.05$) under the various conditions tested (the pD_2 values under these various conditions are shown

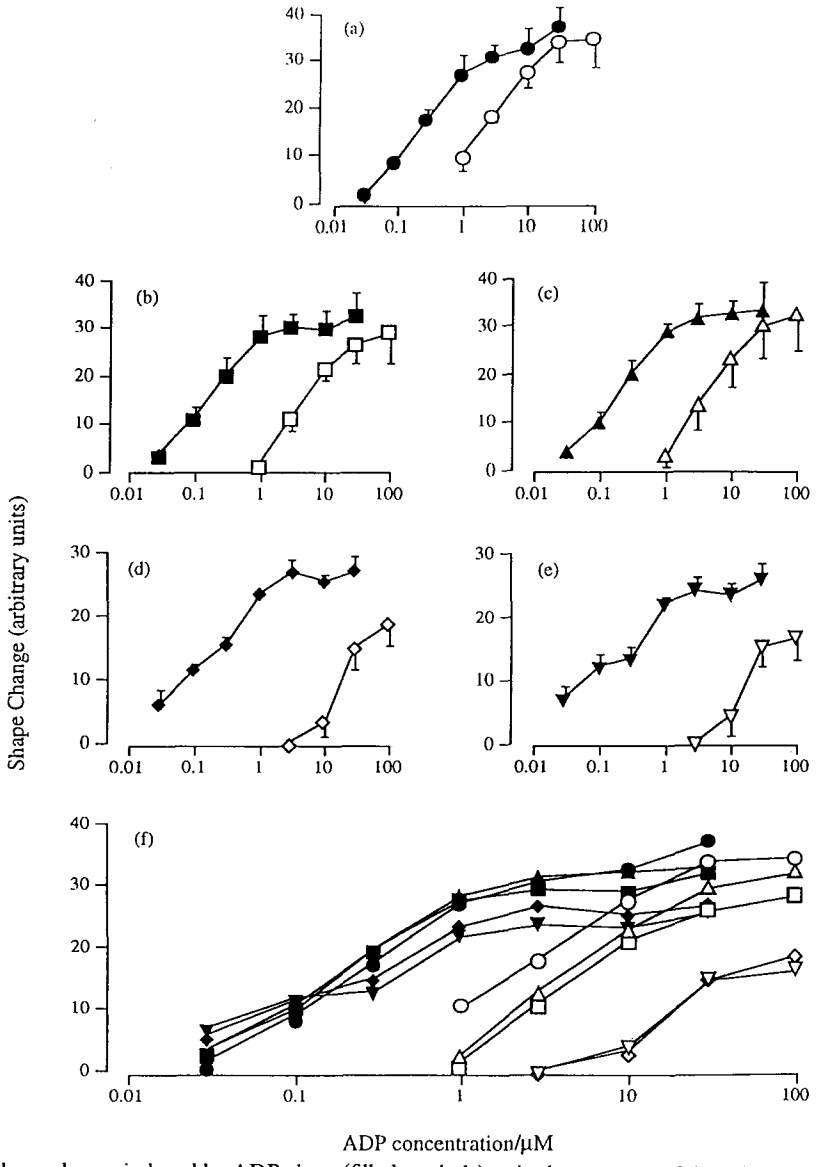

Fig. 1. Shape change induced by ADP alone (filled symbols) or in the presence of ATP (100 μM) (open symbols), in the presence of (a) 1 mM $MgCl_2$ and 1 mM $CaCl_2$ (●, ○), (b) 1 mM $CaCl_2$ (■, □), (c) 1 mM $MgCl_2$ (▲, △), (d) no added divalent cations (◆, ◇), or (e) 1 mM EGTA (▼, ▽). Each point is the mean of three triplicate determinations using blood from three different donors, and vertical bars show SEM. (f) A comparison of the data presented in (a)–(e), with the same symbols and with the error bars omitted for clarity.

Table 1. pD_2 values for induction of shape change by ADP and by ADP^{3-}, and pA_2 values for the inhibition of this response by ATP or by ATP^{4-}

Conditions	pD_2 for ADP	pA_2 for ATP	pD_2 for ADP^{3-}	pA_2 for ATP^{4-}
1 mM $MgCl_2$ + 1 mM $CaCl_2$	6.42 ± 0.07	4.88 ± 0.11	6.97 ± 0.06	6.31 ± 0.06
1 mM $CaCl_2$	6.67 ± 0.12	5.42 ± 0.16	6.90 ± 0.11	6.38 ± 0.16
1 mM $MgCl_2$	6.59 ± 0.14	5.36 ± 0.16	6.99 ± 0.16	6.72 ± 0.16
No added divalent cations	(6.70 ± 0.10)	(6.31 ± 0.14)	6.70 ± 0.10	6.31 ± 0.14
1 mM EGTA	(6.71 ± 0.10)	(6.37 ± 0.16)	6.71 ± 0.10	6.37 ± 0.16

pD_2 values for ADP^{3-} and pA_2 values for ATP^{4-} were obtained using calculated concentrations of these ionic species. In the absence of divalent cations or the presence of EGTA the nucleotides were assumed to be entirely in the uncomplexed form. Values are the means ± SEM of three determinations.

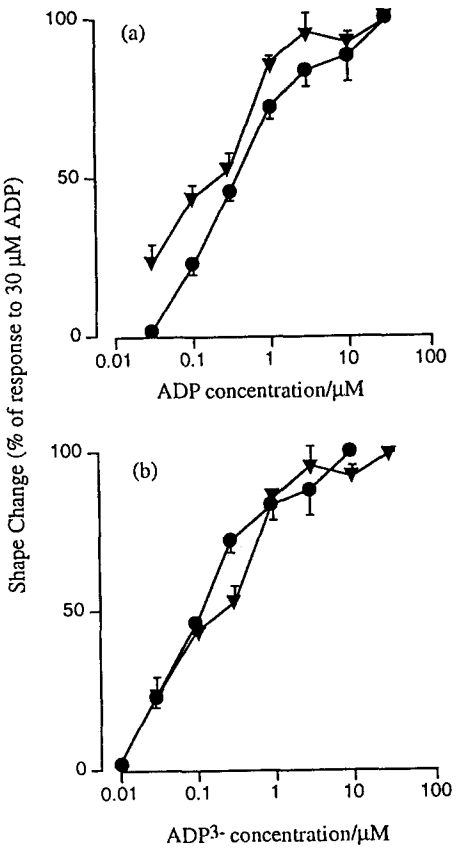

Fig. 2. Shape change induced by ADP in the presence of 1 mM $CaCl_2$ and 1 mM $MgCl_2$ (●) or in their absence (▼), calculated as a percentage of the response to 30 μM ADP. (a) Data presented in terms of the total ADP concentration; (b) data presented in terms of ADP^{3-}. Each point is the mean of at least three triplicate determinations using blood from three different donors. Vertical bars show SEM.

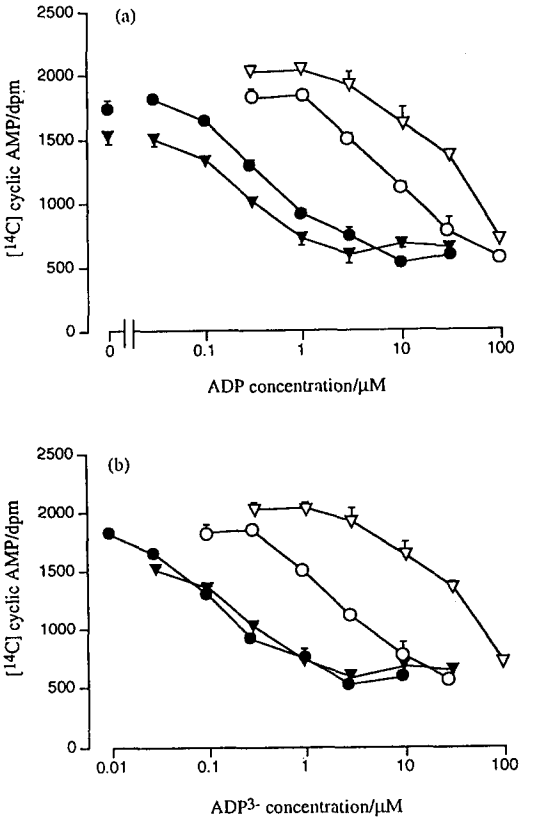

Fig. 3. Inhibition of PGE_1-stimulated cyclic AMP accumulation by ADP alone (filled symbols) or in the presence of ATP (100 μM) (open symbols). Experiments were performed in the presence of either 1 mM $CaCl_2$ and 1 mM $MgCl_2$ (●, ○), or 1 mM EGTA (▼, ▽). Unconnected symbols in (a) show the levels of cyclic AMP in platelets incubated in the presence of PGE_1, in the presence of divalent cations (●) or of EGTA (▼). (a) Data presented in terms of total ADP concentration, and (b) data presented in terms of ADP^{3-}. The data are representative of three similar experiments using blood from three different donors; each point is the mean of at least three determinations and the vertical bars show SEM.

in Table 1). This shape change was inhibited by ATP in all cases (Fig. 1), although this inhibition by ATP was significantly greater in the presence of EGTA and in the absence of added divalent cations than in the presence of divalent cations ($P < 0.05$) (the pA_2 values for ATP under these conditions are also shown in Table 1). This increase in pA_2 value corresponds to an 18.2-fold increase in the affinity (apparent K_d) of ATP for the ADP receptor in the presence of EGTA compared to the presence of both calcium and magnesium.

If the data from Fig. 1 are recalculated in terms of the concentration of ADP^{3-} present rather than in terms of the total ADP concentration, ADP again shows no significant difference in potency in inducing the response in the presence or absence of divalent cations (see Table 1). However, when the pA_2 values for inhibition of these responses by ATP under the different conditions are calculated using the concentration of free ATP^{4-} present rather than the total ATP concentration these values are no longer significantly different (Table 1).

When the data from Fig. 1 are calculated as percentages of the response to 30 μM ADP (Fig. 2a) to make allowances for the differing maximal responses, the log concentration–response curve for ADP in the absence of divalent cations is shifted to the left of the log concentration–response curve in the presence of divalent cations, and a small but significant difference between the pD_2 values for ADP is seen. The pD_2 values of ADP were 6.43 ± 0.06 ($EC_{50} = 0.37$ μM) in the presence of divalent cations and 6.76 ± 0.10 ($EC_{50} = 0.17$ μM) in their absence ($P < 0.05$). When these data are calculated in terms of ADP^{3-} rather than total ADP concentration (Fig. 2b), the curves become almost superimposable and there is now no significant difference between the pD_2 values, which are 6.96 ± 0.07 and 6.76 ± 0.10, respectively, in the presence and absence of divalent cations.

ADP also inhibited PGE_1-induced accumulation of cyclic AMP by platelets both in the presence of

divalent cations and in the presence of 1 mM EGTA; however, in this case there was a significant leftward shift in the log concentration–response curve in the absence of divalent cations (Fig. 3a). The pD_2 values were 6.48 ± 0.10 in the presence of divalent cations (EC_{50} = 0.33 μM) and 7.21 ± 0.24 in the presence of EGTA (EC_{50} = 0.07 μM) ($P < 0.05$). Again, ATP inhibited the response to ADP and this inhibition was significantly greater in the absence of divalent cations than in their presence, the pA_2 values being 6.59 ± 0.26 and 5.24 ± 0.05, respectively ($P < 0.01$). This corresponds to an approximately 22-fold increase in the apparent affinity of ATP for the receptor in the presence of EGTA. When the ADP concentration is presented in terms of free ADP^{3-} (Fig. 3b) the pD_2 values are no longer significantly different, the values being 6.98 ± 0.10 and 7.21 ± 0.24 in the presence of calcium and magnesium or EGTA, respectively. When the pA_2 values for ATP were calculated in terms of ATP^{4-} these were again not significantly different, being 6.64 ± 0.03 in the presence of divalent cations and 6.59 ± 0.26 in the presence of EGTA.

Interestingly, along with its inhibition of the effect of ADP, ATP also seemed to enhance the ability of PGE_1 to stimulate adenylate cyclase in the presence of EGTA (Fig. 3). However, incubation of platelets with 0.5 U/mL apyrase (a concentration which completely inhibited aggregation induced by 1 μM ADP in PRP) could also enhance the PGE_1-stimulated accumulation of cyclic AMP. In the presence of EGTA both ATP (100 μM) and apyrase caused a slight increase in the cyclic AMP accumulation induced by PGE_1. The levels of cyclic AMP were 4854.9 ± 195 dpm (N = 3) for PGE_1 alone, 5318.8 ± 354.1 dpm (N = 3) in the presence of apyrase and 5216.7 ± 101 dpm (N = 3) in the presence of ATP; however, neither of these increases was significant. Similarly, in the presence of divalent cations there was a slight, but not quite significant, increase in the amount of cyclic AMP accumulated in the presence of apyrase (6076.5 ± 285.4 dpm, N = 3) compared with its absence (5578 ± 54 dpm, N = 4). In the presence of divalent cations the accumulation of cyclic AMP induced by PGE_1 was similar in the presence or absence of ATP (100 μM) (5578.8 ± 54 dpm, N = 4, in its absence; 5522.5 ± 193 dpm, N = 3, in its presence). Also, in the presence of EGTA, ATP (100 μM) caused no significant accumulation of cyclic AMP in the absence of PGE_1, the basal level of cyclic AMP in platelets was 562 ± 136.4 dpm (N = 4) in the absence of ATP, and 547.9 ± 110 dpm (N = 3) in the presence of ATP (100 μM).

In HPLC studies, platelets released significantly more ADP, 1.39 ± 0.11 μM (N = 3), in the presence of EGTA than in the presence of divalent cations, 1.12 ± 0.11 μM ADP (N = 3), ($P < 0.05$).

DISCUSSION

These data suggest that the platelet ADP receptor does not recognize all of the species of ADP present in solution as agonists but that predominantly ADP^{3-}, the free ionic form of ADP, is able to act as an agonist at this receptor. Similarly, it would appear that it is predominantly ATP^{4-} which acts as the antagonist at this receptor rather than other ATP species.

In the presence of 1 mM calcium and 1 mM magnesium the concentration of ATP^{4-} present in solution is approximately 4% of the total ATP concentration. This is, therefore, approximately 25-fold less than the concentration present in the absence of these divalent cations, which has been assumed to be 100% of the total ATP present. Thus if ATP^{4-}, rather than all forms of ATP, were the active antagonist at the ADP receptor a 25-fold increase in the affinity of ATP would be expected in the absence of divalent cations. This is, however, an over-estimate as, for example, the second ionizable hydroxyl of the γ-phosphate will be protonated to some extent at physiological pH. The true concentration of ATP^{4-} in the absence of divalent cations will therefore be slightly less than 25-fold lower than that in the presence of divalent cations. The observed increases in the affinity of ATP for the ADP receptor in the absence of divalent cations, of 18.2 and 22.3 in the shape change and adenylate cyclase inhibition experiments, respectively, compare very well, therefore, with this predicted value.

In a solution of ADP containing 1 mM of these two divalent cations, however, approximately 30% of the ADP is present as ADP^{3-}, and therefore only a 3.3-fold leftward shift in the log concentration–response curve to ADP would be expected in the absence of divalent cations. In the experiments measuring inhibition of PGE_1-stimulated adenylate cyclase by ADP, there was indeed a small, but significant, leftward shift in the log concentration–response curve, giving a potency ratio of 5.5, which is also close to that predicted from the change in the concentration of ADP^{3-} present in the solution in the absence and presence of divalent cations. In the case of shape change the EC_{50} values for ADP in the presence and absence of divalent cations were not significantly different and the observed potency ratio of 1.5 is only about half of the expected value of 3.3. However, the log concentration–response curves in the presence and absence of divalent cations are somewhat differently shaped. The curves in the absence of divalent cations show a lower maximal response and are rather flatter than those in their presence, suggesting that the removal of extracellular divalent cations interferes with this response in some way. Interestingly, it is only in the absence of both divalent cations that this depression of the maximal response is seen rather than simply in the absence of calcium, suggesting that calcium influx is not required for shape change but that mobilization of intracellular calcium is sufficient to produce a maximal response. This observation also suggests that some extracellular factor which requires divalent cations (either calcium or magnesium) for functional integrity is necessary to produce a maximal shape change in response to ADP.

The differing maxima of the log concentration–response curves for induction of shape change in the presence and absence of divalent cations means that these results are not as clear-cut as those from the adenylate cyclase experiments, and makes the

comparison of the EC_{50} values from these curves unreliable as an index of changing potency. However, when the shape change data for ADP in the presence of divalent cations and in the presence of EGTA are normalized (Fig. 2) there is a 2.2-fold increase in the potency of ADP in the absence of divalent cations compared with in their presence, which again agrees well with the ratio of 3.3 expected if ADP^{3-} is in fact the agonist. Indeed, when the data are presented in terms of the concentration of ADP^{3-} the log concentration–response curves are almost superimposed and this difference in potency disappears, further suggesting that the ADP receptor is in fact an ADP^{3-} receptor.

In the results from the studies on the inhibition by ADP of PGE_1-stimulated adenylate cyclase it was noticeable that, in the absence of divalent cations, ATP not only inhibited the effects of ADP but also appeared to enhance the ability of PGE_1 to stimulate adenylate cyclase. There has recently been a suggestion that ATP, as well as acting as a competitive antagonist at the ADP receptor, is able to stimulate platelet adenylate cyclase via a P_{2X}-like receptor [24, 25]. However, washed platelets are known to release adenine nucleotides into the suspending medium [26] and this apparent stimulation of adenylate cyclase by ATP may, therefore, represent the reversal of the inhibitory effects of released ADP rather than a separate effect of ATP mediated by a distinct purinoceptor subtype on these cells. In the present study washed platelets did indeed release ADP and, in fact, released slightly more ADP in the presence of EGTA than they did in the presence of divalent cations. Thus in the absence of divalent cations, when the apparent stimulation of adenylate cyclase by ATP is most pronounced, not only do platelets release more ADP, which will cause a greater basal level of inhibition of this enzyme, but a greater proportion of this ADP will be in the active form (ADP^{3-}) and a greater proportion of the added ATP will be present as the active antagonist (ATP^{4-}). Thus if the apparent stimulation of adenylate cyclase by ATP is actually due to its inhibition of the effects of released ADP, the ATP would be expected to show the most marked effect in the presence of EGTA, as indeed it does. When platelets were exposed to PGE_1 in the presence of apyrase, which will break down the released ADP to AMP, the increase in cyclic AMP was slightly greater than that in the absence of apyrase. Although the increase with either apyrase or ATP did not quite reach significance at the 5% level there was a trend in the effect of both towards a potentiation of the effect of PGE_1. Thus, the breakdown of released ADP has the same effect as the presence of ATP on the response to PGE_1, suggesting that this effect of ATP is simply due to reversal of the inhibition by released ADP of the response to PGE_1. More importantly, when platelets were exposed to ATP in the absence of PGE_1 (in the presence of EGTA, when ATP had its greatest apparent stimulatory effect) the basal level of cyclic AMP was not significantly different from that in the presence of IBMX alone, further suggesting that the effect of ATP is not to increase cyclic AMP itself but simply to antagonize competitively the effects of released ADP.

In the study by Soslau *et al.* [25] it was suggested that the ability of ATP to inhibit aggregation by the thromboxane mimetic U46619 and by collagen was due to this apparent ability of ATP to increase platelet cyclic AMP levels. However, both of these aggregating agents are known to cause the release of ADP from platelet-dense granules [3], this released ADP potentiating the response to the primary agonist. The inhibition of aggregation induced by U46619 and collagen is, therefore, attributable to the ability of ATP to inhibit the effects of released ADP. The putative ATP receptor in this study was apparently desensitized by pre-incubation in the presence of a stable analogue of ATP for up to 120 min, the ability of ATP to inhibit aggregation induced by U46619 and collagen decreasing with increasing time of incubation. The inhibition of ADP-induced aggregation by ATP remained relatively constant over this period. However, these authors did not show the effect of this incubation period on their control responses, thus it was not possible to see whether there was a reduction in the responses to U46619 or collagen simply due to the incubation period. Platelets are known to become refractory to ADP when stored in the absence of ADP scavenging systems (e.g. apyrase or creatine phosphate/creatine kinase) due to the release of ADP, whilst they are still able to respond to other aggregating agents. Because of this gradual reduction in the platelet response to ADP, the relative contribution that released ADP will make to the response to these other aggregating agents will also decrease with time. Thus ATP would be expected to show a reduced level of non-competitive inhibition of the response to other agonists simply due to the reduced contribution to the response made by released ADP. It appears therefore that the results of this study may be explicable in terms of previously characterized responses of platelets and that a separate ATP receptor need not be postulated.

In conclusion, the data presented here suggest that the platelet ADP receptor is in fact an ADP^{3-} receptor, binding predominantly this form of ADP as its agonist. Similarly, the receptor appears to recognize predominantly ATP^{4-} as an antagonist. Thus, in common with other P_2-purinoceptors, the platelet ADP receptor appears to recognize the free ionic form of its ligands rather than the divalent cation complexes. This is true for both responses studied, induction of shape change and inhibition of adenylate cyclase, and supports the contention that these responses to ADP are mediated by one [10, 11] rather than two [9] ADP receptors on human platelets. ATP also appears to potentiate the accumulation of cyclic AMP by platelets induced by PGE_1. However, this potentiation is probably due to the reversal of the inhibitory effects of ADP released by platelets when suspended in buffer rather than to any independent effect of ATP.

Acknowledgement—DAH held an MRC collaborative studentship supported by Ciba-Geigy Pharmaceuticals.

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