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ADP can induce aggregation of human platelets *via* both $P2Y_1$ and P_{2T} receptors

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1 In the present study we have investigated the roles of $P2Y_1$ and P_{2T} receptor subtypes in adenosine 5′-diphosphate (ADP)-induced aggregation of human platelets in heparinized platelet rich plasma.

2 The response to ADP can be characterized as the initial rate or the maximum or final extent of aggregation. The response profile is determined by the concentration of ADP used, being transient at lower and sustained at higher concentrations.

3 The $P2Y_1$ receptor antagonist, adenosine-3′-phosphate-5′-phosphate (A3P5P) competitively antagonized the initial rate of aggregation (pK_B 5.47) and transformed the response profile to a slowly developing but sustained response. Both maximum and final extents were also inhibited by A3P5P although not in a competitive manner (Schild slope <1).

4 The P_{2T} receptor antagonist, AR-C67085, competitively antagonized the final extent of aggregation (pK_B 8.54), transforming the response profile to one of rapid, transient aggregation. Its effect on maximum extent (the most widely used index of aggregation) was complex, and further supported the involvement of both receptor subtypes in the aggregation response.

5 ADP-induced aggregation is a complex phenomenon, the nature of which is determined by the relative occupancy of the two receptor subtypes. While $P2Y_1$ receptor activation causes a rapid and transient aggregation, the extent of sustained aggregation is determined by the level of P_{2T} receptor occupancy. Hence, detailed analysis of the aggregation response is essential to correctly define the purinergic pharmacology of the platelet and interpretation of results is critically dependent on the response index chosen.

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Abbreviations: ADP, adenosine 5′-diphosphate; AMP, adenosine 5′-monophosphate; A3P5P, adenosine-3′-phosphate-5′-phosphate; AR-C67085, 2-propylthio-D-β,γ-dichloromethylene ATP; ATP, adenosine 5′-triphosphate; PRP, platelet rich plasma; TxA_2, thromboxane A_2

Introduction

Receptors for adenine nucleotides and nucleosides were originally divided into two groups: P1 receptors for which adenosine and adenosine 5′-monophosphate (AMP) were agonists and P2 receptors for which adenosine 5′-diphosphate (ADP) and adenosine 5′-triphosphate (ATP) were agonists (Burnstock, 1978). P2 receptors have been further subdivided into two groups, P2X and P2Y. This classification reflects the secondary messenger system responsible for mediating the intracellular consequences of receptor activation; P2X receptors are intrinsic ligand-gated cation channels, whereas P2Y subtypes belong to the family of G-protein coupled receptors (Fredholm *et al*., 1994). P2 receptors with other designations have subsequently been re-named in order to harmonize the nomenclature for this receptor class and to reflect their known coupling mechanisms; for example, P2U and P2Z receptors are now referred to as $P2Y_2$ and $P2X_7$ receptors respectively (Fredholm *et al*., 1997).

As early as the 1960s ADP was recognized as a platelet activating agent (Born, 1962) and was implicated as a significant mediator in haemostasis and thrombosis (Gaarder *et al*., 1961). However, the nature of the platelet receptor(s) for ADP has remained a subject of much discussion. The designation P2T was introduced (Gordon, 1986) to uniquely identify the platelet ADP receptor, in recognition of the fact that, in contrast to other nucleotide (P2) receptors described at that time (Burnstock, 1978), while ADP was an agonist, ATP was a competitive antagonist (Macfarlane & Mills, 1975). The designation 'T' was used to indicate its unique location on the thrombocyte. In this paper we will use the name P_{2T} (TiPS nomenclature supplement, 1999), italicised in order to indicate that, until definitive cloning of the receptor provides a structural basis for inclusion in the P2Y family, the nomenclature remains provisional.

In recent years, evidence has accumulated to support the view that there is more than one subtype of ADP receptor on the platelet. MacKenzie *et al*. (1996), using single platelet patch clamp techniques, demonstrated that platelets had $P2X_1$ receptors on their surface, a finding that has subsequently been confirmed by other investigators (Vial *et al*., 1997; Scase *et al*., 1998). Activation of the $P2X_1$ receptor results in a rapid influx of cations, although the functional significance of this response remains undetermined. The discovery of mRNA for $P2Y_1$ receptors in platelets in combination with the realization that, contrary to what was previously thought, ATP is in fact an antagonist at this subtype, led certain investigators to claim that the so-called elusive 'P_{2T}' receptor was in fact the $P2Y_1$ receptor (Léon *et al*., 1997; Gachet *et al*., 1997). However, further evidence suggests that there remains still a third subtype (P_{2T}) in addition to the $P2X_1$ and $P2Y_1$ receptors (Daniel *et al*., 1998; Geiger *et al*., 1998).

The identification of competitive antagonists at the $P2Y_1$ and P_{2T} receptors has greatly facilitated the investigation of the

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purinergic pharmacology of the platelet. Adenosine-3′-phosphate-5′-phosphate (A3P5P) was shown to be a selective and competitive antagonist at the human $P2Y_1$ receptor ($pK_B = 6.05$) (Boyer *et al*., 1996), and 2-propylthio-D-β,γ-dichloromethylene ATP (AR-C67085, formerly FPL 67085), an ATP analogue developed for therapeutic use in acute coronary syndromes, has been reported as a potent and selective competitive antagonist at the P_{2T} receptor ($pK_B = 8.9$) (Humphries *et al*., 1995). In the present study we have used these compounds to investigate the roles of $P2Y_1$ and P_{2T} receptors in ADP-induced platelet aggregation.

Optical aggregometry techniques have been in use for as long as ADP has been recognized as a platelet agonist (Born, 1962). However, interpretation of aggregometry data has not been uniform between laboratories, a situation that may have given rise to irreconcilable conclusions and hence contributed to the confusion surrounding the pharmacology of the platelet. The majority of studies have used citrated platelet rich plasma with the aggregometry response defined as the maximum degree of aggregation obtained following addition of the platelet activating agent or the degree of aggregation at 6 min, whichever is the greater. Other investigators have preferred to measure and report the initial rate of aggregation (Hourani *et al*., 1992; Cusack & Hourani, 1982). In this study, we analysed both of these measures of aggregation as well as another which has not previously been used. The need for a third measure of the response arises as a consequence of the phenomenon of reversible aggregation. This is observed in platelet rich plasma at low concentrations of agonists and is manifest as a change in the direction of the aggregometry trace as a result of disaggregation of the platelets. The consequence of this is that the eventual final extent of aggregation is different to the maximum extent when there is reversibility in the response. Final extent therefore is the third index of aggregation.

The terms reversible/irreversible and primary/secondary aggregation have been used frequently to describe phenomena associated with ADP-induced platelet aggregation in which thromboxane A_2 (TxA_2) generation was involved (MacMillan, 1966; O'Brien, 1968). In this paper we have chosen to use the terms transient and sustained aggregation to prevent confusion with previous work.

A preliminary description of this work was previously presented at a meeting of the British Pharmacological Society (Jarvis *et al*., 1998).

Methods

Drugs

Adenosine 5′-diphosphate (sodium salt; ADP) and adenosine 3′-phosphate-5′-phosphate (sodium salt; A3P5P) were obtained from the Sigma Chemical Co. (Poole, U.K.). Heparin sodium (Monoparin®) was obtained from CP Pharmaceuticals. 2-propylthio-D-β,γ-dichloromethylene ATP (tetrasodium salt; AR-C67085) was synthesized in the Department of Medicinal Chemistry, AstraZeneca R&D Charnwood, U.K. Stock solutions of ADP, A3P5P and AR-C67085 were made up and subsequently diluted in saline to the required concentrations.

Preparation of platelet rich plasma

Blood (108 ml) was collected from each of six donors (four male, two female) into two syringes, each containing 6 ml heparin sodium (100 U ml^{-1}) to give a final concentration of heparin of 10 U ml^{-1}. The blood was centrifuged at $240 \times g$ for 15 min and the platelet rich plasma pipetted off. A small sample of blood was centrifuged for 5 min at 13,000 r.p.m. in a microfuge to produce platelet poor plasma.

Platelet aggregometry

Platelet aggregometry was carried out according to the method of Born & Cross (1963) using two BioData PAP-4 aggregometers (Alpha Laboratories, Eastleigh, U.K.). The assay was performed at 37°C with a sample stir speed of 900 r.p.m. Each sample consisted of 390 μl platelet rich plasma, 50 μl of either A3P5P or saline and 50 μl of either AR-C67085 or saline, to make a total volume of 490 μl in a cuvette. Following incubation of the sample for approximately 10 min, 10 μl ADP was added to the cuvette to activate the platelets and the response recorded.

As previously noted in the introduction, three indices of aggregation were quantified: rate, maximum extent and final extent of aggregation (see Figure 1). The rate and final extent values were electronically generated by the PAP-4 aggregometers. Rate of aggregation is equal to the maximum slope in the aggregometry trace over the period of the aggregometry assay which is expressed in arbitrary units (personal communication, BioData Corporation) and the final extent is the percentage extent of aggregation when the assay was terminated. Maximum extent was determined by visual examination of the traces. Aggregation responses were permitted to continue until there was no more substantial change in the level of response. This usually occurred by 10 min, and all traces were allowed to run for at least 8 min.

ADP concentration-response data were collected in the absence or presence of A3P5P (3, 10, 30, 100 μM) and AR-C67085 (3, 10, 30, 100 nM). These concentrations of antagonist were selected on the basis of their reported pK_B values at $P2Y_1$ and P_{2T} receptors respectively (A3P5P, 6.05 (Boyer *et al*., 1996); AR-C67085, 8.9 (Humphries *et al*., 1995)).

Data analysis

The data were fitted to a four parameter logistic equation (De Lean *et al*., 1978) incorporating a model of competitive antagonism of the form:

$$R = \frac{(Min - Max)}{\left(1 + \left(\frac{A}{(10^{-pA_{50}}) \times (1 + (B/10^{-pK_B}))}\right)^{n_H}\right)} + Max \qquad (1)$$

where R = response (dependent variable); A = concentration of ADP in mol l^{-1} (independent variable); B = concentration of antagonist in mol l^{-1} (independent variable); . . . and . . . Min = response when $A = 0$; Max = response when $A = \infty$; $pA_{50} = -\log_{10}$ of concentration of A that gives a response equal to $(Min + Max)/2$; $pK_B = -\log_{10}$ dissociation constant of the antagonist; and n_H = Hill coefficient.

The agonist location parameter (pA_{50}) and antagonist potency parameter (pK_B) were estimated as negative logarithms as the error associated with these parameters is logarithmically distributed (De Lean *et al*., 1982; Hancock *et al*., 1988).

Antagonist-induced parallel shifts were tested by fitting the data from individual concentration-response curves to a four parameter model (i.e., equation 1, where $B = 0$), followed by refitting of the data for all concentration-response curves simultaneously whilst constraining the Min, Max and n_H to be equal for each set of antagonist data and performing an extra

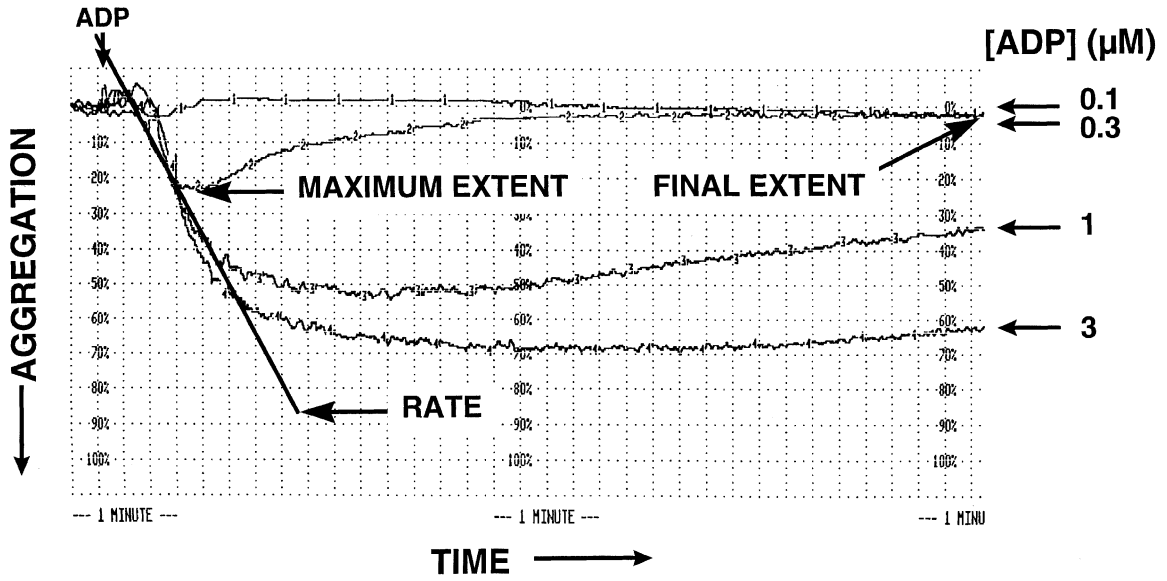

Figure 1 Typical example of a trace recording of ADP-induced aggregation generated by a PAP-4 aggregometer, illustrating the indices of aggregation used to characterize the response. The concentration-dependency of the response is clearly evident. At the lower concentrations of ADP, the response is transient, resulting in different values for the maximum and final extents of aggregation. The rate of aggregation is equivalent to the maximum slope of the trace over the period of the aggregation assay measured in arbitrary units. The final extent and rate values are calculated automatically by the aggregometer and the maximum extent is determined by visual inspection. The results for the response to 0.3 μM ADP in this case were: rate = 27; final extent = 2%; maximum extent = 22%.

sum of squares analysis for nested models (Bates & Watts, 1988).

For each combination of antagonist and index of aggregation, where there was no significant deviation from a pattern of parallel shifts, data were fitted simultaneously to equation (1) with the following modifications (Lew & Angus, 1995):

$$B = B^{n_1} \quad (2)$$

. . . and . . .

$$B = B \times \left(1 + \frac{n_2 \times B}{10^{-pK_B}}\right) \quad (3)$$

where n_1 = the Schild slope, which is equal to unity for competitive antagonism; and n_2 = a quadratic function, which is equal to zero for competitive antagonism.

An extra sum of squares analysis (Bates & Watts, 1988) following these two modifications allowed an assessment to be made as to whether the Schild slope was firstly, equal to unity (equation 2), and secondly, linear (equation 3). Where the Schild slope was significantly different to unity, a pA_2 value was calculated, equal to the estimated pK_B divided by the Schild slope.

Data were analysed using Microsoft Excel for Windows 95. P values of >0.10 were considered non-significant; P values of <0.01 were considered significant. Intermediate values were considered of borderline significance. Under these circumstances, actual P values are reported. Results are given as the mean $\pm$ s.e.mean.

Results

Transient and sustained aggregation

Figure 1 illustrates the normal response to a range of ADP concentrations in the absence of either antagonist. At the lower ADP concentrations the transience of the response can easily be appreciated. Figure 2 illustrates a set of typical traces in the presence of A3P5P (100 μM) or AR-C67085 (100 nM). Both of these concentrations are approximately 100-fold higher than the pK_B values at $P2Y_1$ and P_{2T} receptors respectively. It can clearly be seen that the nature of the two sets of traces is different: in the presence of the P_{2T} antagonist, the response is predominantly transient, whereas in the presence of the $P2Y_1$ antagonist, the response is slower to develop but sustained, showing no evidence of transience even at low concentrations of ADP.

Final extent of aggregation

The control pA_{50} for the final extent of aggregation produced by ADP was 5.95 ± 0.07 and the Hill coefficient was 2.3 ± 0.4 ($n = 6$).

Figure 3a illustrates the effect of AR-C67085 on the final extent of aggregation. In 5/6 subjects AR-C67085 caused a parallel rightward displacement of the ADP concentration-response curve. In the remaining subject, there was a borderline deviation from parallelism ($P = 0.015$). The Schild slope was 0.95 ± 0.02 ($n = 5$) and differed significantly from unity in only one case (0.86, $P = 0.005$). The pK_B was 8.54 ± 0.06 ($n = 5$).

Figure 3b illustrates the effect of A3P5P on the final extent of aggregation. In 2/6 subjects A3P5P caused a parallel rightward displacement of the ADP concentration-response curve. In two other subjects there was a borderline deviation from parallelism ($P = 0.037$ and 0.013). In the two remaining subjects there was a significant deviation from parallelism. The Schild slope was 0.70 ± 0.11 ($n = 4$) and was significantly less than unity in 4/6 subjects. The pA_2 was 5.19 ± 0.33 ($n = 4$).

Maximum extent of aggregation

The control pA_{50} for the maximum extent of aggregation produced by ADP was 6.23 ± 0.07 and the Hill coefficient was 1.5 ± 0.1 ($n = 6$).

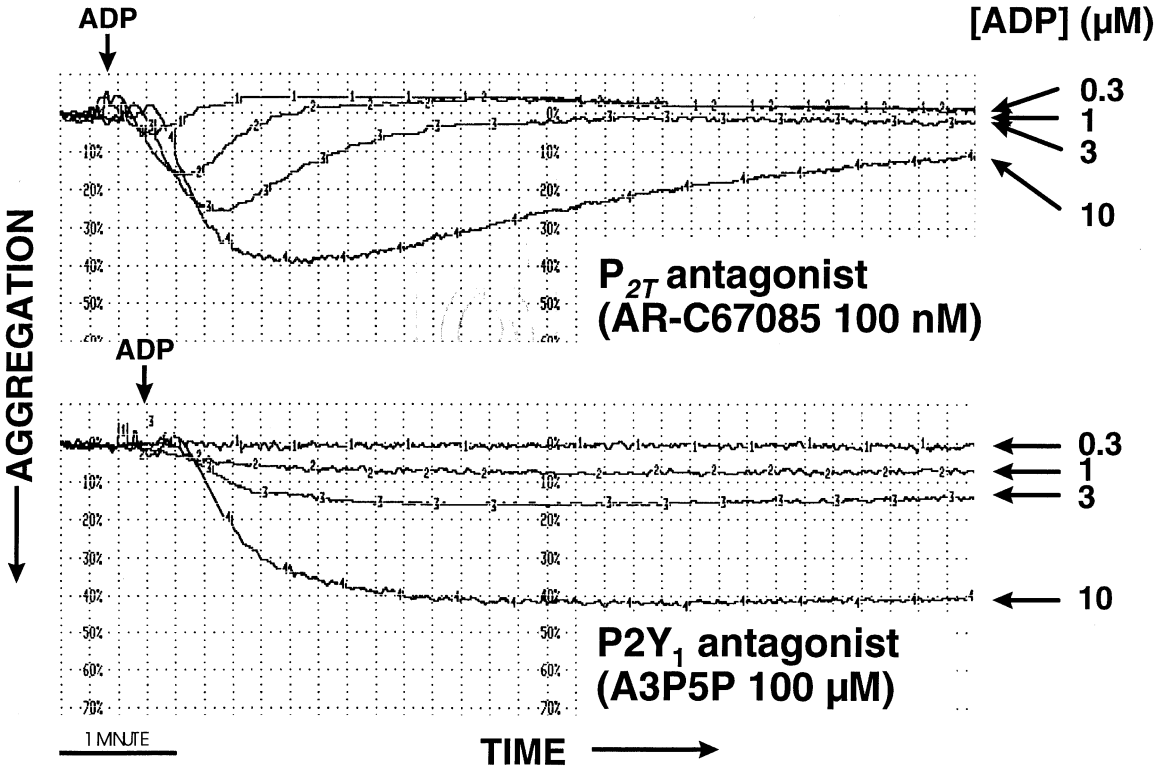

Figure 2 Trace recordings illustrating the effects of the P_{2T} antagonist AR-C67085 (100 nM) and the $P2Y_1$ antagonist A3P5P (100 μM) on the aggregation response to ADP. Both antagonists (at concentrations approximately 100 fold greater than their respective pK_B values) inhibit the response (*c.f.*, response to ADP in Figure 1) although it is clear that the nature of the inhibition is different in the two cases. In the presence of the P_{2T} antagonist, the response to ADP becomes characterized by a greater degree of transience apparent even at the higher concentrations of ADP, whereas the $P2Y_1$ antagonist abolishes all signs of transience and even the lowest concentrations of ADP produce a sustained response.

Figure 4a illustrates the effect of AR-C67085 on the maximum extent of aggregation. In no subjects did AR-C67085 cause a parallel shift in the ADP concentration-response curve. This deviation from parallelism was characterized by a gradual reduction in the Hill coefficient. In one donor, there was sufficient platelet rich plasma to investigate the effects of two higher concentrations of AR-C67085 (300 nM and 1 μM). These data (see Figure 5) suggest that the reduction in the Hill coefficient was due to a selective AR-C67085-induced rightward displacement of the upper part of the concentration-response curve combined with a more resistant lower portion, thereby resulting in a bi-phasic response at sufficiently high concentrations of antagonist.

Figure 4b illustrates the effect of A3P5P on the maximum extent of aggregation. In 3/6 subjects A3P5P caused a parallel rightward displacement of the ADP concentration-response curve. In two other subjects there was a borderline deviation from parallelism ($P=0.052$ and 0.094). In the remaining subject there was a significant deviation from parallelism. The Schild slope was 0.75 ± 0.03 ($n=5$) and was significantly less than unity in 4/6 subjects. The pA_2 was 5.32 ± 0.25 ($n=5$).

Rate of aggregation

The control pA_{50} for the rate of aggregation produced by ADP was 6.46 ± 0.13 and the Hill coefficient was 1.5 ± 0.2 ($n=6$).

Figure 6a shows that AR-C67085 had a minimal effect on the rate of aggregation, characterized by a small decrease in the maximum response. In 4/6 subjects, there was a significant deviation from parallelism.

Figure 6b illustrates the effect of A3P5P on the rate of aggregation. In 3/6 subjects A3P5P caused a parallel rightward displacement of the ADP concentration-response curve. In two other subjects there was a borderline deviation from parallelism ($P=0.076$ and 0.046). In the remaining subject there was a significant deviation from parallelism. The Schild slope was 1.04 ± 0.10 ($n=4$) and was significantly different from unity in one subject (1.21, $P=0.004$). The pK_B was 5.47 ± 0.16 ($n=4$).

Discussion

ADP-induced platelet aggregation

It is clear from the data presented herein that ADP can induce aggregation of human platelets *via* both $P2Y_1$ and P_{2T} receptors as previously demonstrated (Jin & Kunapuli, 1998). However, the claim that co-activation of both receptors is required for aggregation is an oversimplification of a complex phenomenon, the nature of which is critically dependent upon the relative degree of occupancy of the two receptors. Hence, at low concentrations of ADP, the $P2Y_1$ receptor is capable of mediating a rapid and transient aggregation response without any activation of the P_{2T} receptor. This is demonstrated by the observation that transient aggregation remains even in the presence of high concentrations of AR-C67085 which preclude the possibility of ADP-induced activation of the P_{2T} receptor. The observation that A3P5P competitively antagonizes the rate of aggregation with a potency consistent with its activity at the $P2Y_1$ receptor ($pK_B=5.47$) further supports the view that the $P2Y_1$ receptor mediates the initial rapid response to ADP which predominantly determines the maximum rate of aggregation induced by ADP.

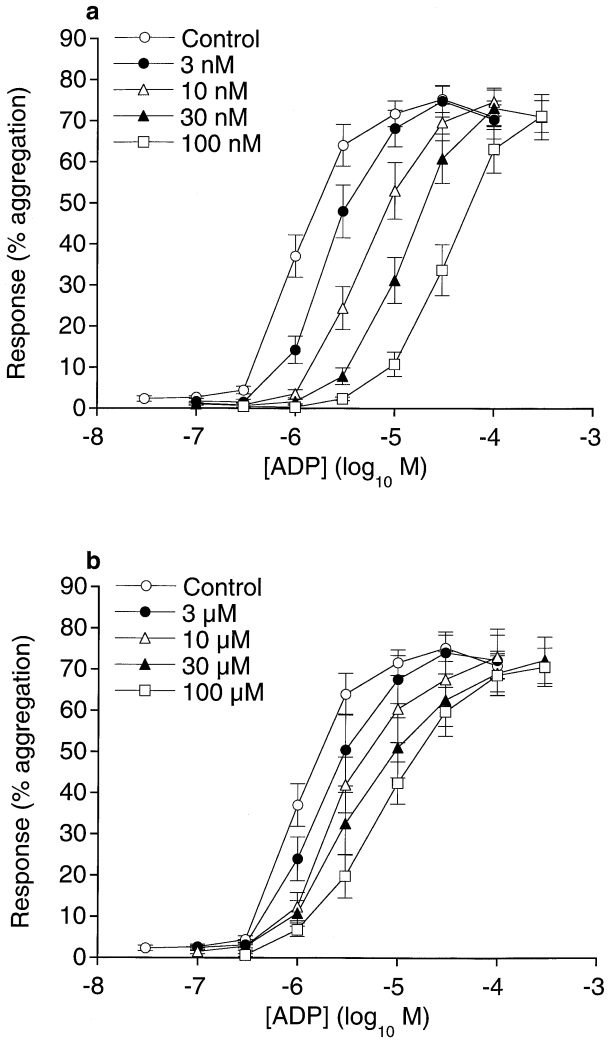

Figure 3 Antagonist effects of (a) AR-C67085 and (b) A3P5P on the final extent of ADP-induced platelet aggregation. The pA_{50} of the control response was 5.95 ± 0.07 and the Hill coefficient 2.3 ± 0.4 ($n=6$). The P_{2T} antagonist AR-C67085 caused a parallel rightward displacement of the curve in a manner consistent with competitive antagonism. The pK_B was estimated to be 8.54 ± 0.06 ($n=5$). The $P2Y_1$ antagonist A3P5P also caused rightward shifts of the curve although the Schild slope was significantly lower than unity (0.70 ± 0.11; $n=4$). The pA_2 was estimated as 5.19 ± 0.33 ($n=4$). The inhibition of the response is not consistent with competitive antagonism. Data points represent the mean and the standard error of the mean for all six sets of experimental data.

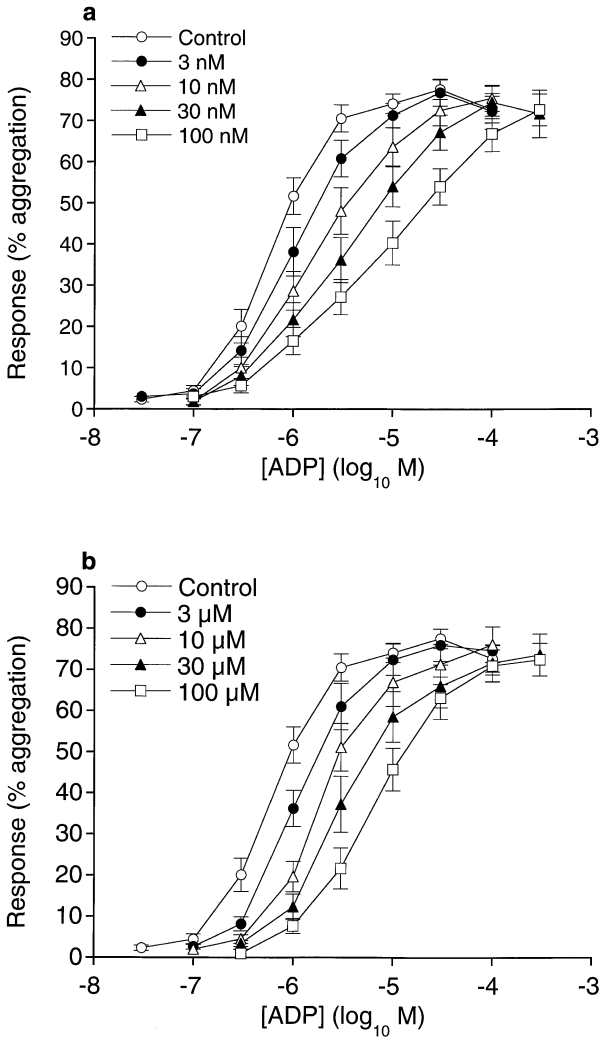

Figure 4 Antagonist effects of (a) AR-C67085 and (b) A3P5P on the maximum extent of ADP-induced platelet aggregation. The pA_{50} of the control response was 6.23 ± 0.07 and the Hill coefficient 1.5 ± 0.1 ($n=6$). The P_{2T} antagonist AR-C67085 caused a rightward displacement of the curve characterized by a concentration-dependent reduction in the Hill coefficient. The $P2Y_1$ antagonist A3P5P caused parallel rightward shifts of the curve although the Schild slope was significantly lower than unity (0.75 ± 0.03; $n=5$). The pA_2 was estimated as 5.32 ± 0.25 ($n=5$). Data points represent the mean and the standard error of the mean for all six sets of experimental data.

The potency estimate for the inhibition of the final extent of aggregation by AR-C67085 ($pK_B=8.54$) is similar to that obtained previously (Humphries *et al*., 1995; $pK_B=8.9$). In this latter study, a washed platelet preparation was used in which no transient aggregation was observed, making maximum and final extents identical. The competitive nature of the antagonism in both of these systems, indicates that within the concentration range of ADP used, the degree of sustained aggregation is determined by the level of occupancy by ADP of the P_{2T} receptor under circumstances where the $P2Y_1$ receptor is activated. The requirement for $P2Y_1$ activation to facilitate this response is suggested by the ability of A3P5P also to inhibit the final extent of aggregation. However, in common with the findings of other investigators (Hechler *et al*., 1998a,b) (who measured maximum extent of aggregation) we have shown that this inhibition is not competitive (Schild slope = 0.70). It is likely that at higher concentrations of a $P2Y_1$ antagonist, there would either be no further antagonism observed, indicating that at sufficiently high concentrations of ADP, activation of the $P2Y_1$ receptor was not necessary for a sustained response, or else the concentration-response relationship would shift in a competitive manner, indicating that there is a necessary level of $P2Y_1$ receptor activation below which a sustained response would decrease to zero. To date, it has not been possible to test these hypotheses, owing to the high concentrations of ADP that would be required.

P2 receptors on platelets and their functions

The distinctive patterns of inhibition of ADP-induced aggregation seen in the presence of either A3P5P or AR-C67085 also provide further evidence for the existence of a third P2 receptor subtype (P_{2T}) on platelets pharmacologically distinct from the $P2X_1$ (MacKenzie *et al*., 1996) and the $P2Y_1$ subtypes (Fagura *et al*., 1998; Daniel *et al*., 1998; Geiger *et al*., 1998). Activation of the $P2X_1$ receptor results in a rapid influx of cations (MacKenzie *et al*., 1996), although attempts

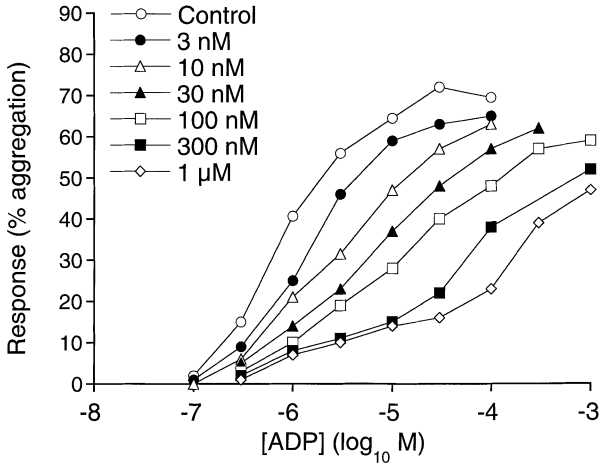

Figure 5 Data from a single experiment using high concentrations of the P_{2T} antagonist AR-C67085 (300 nM and 1 μM) showing the effect on the maximum extent of ADP-induced platelet aggregation. At the high concentrations of antagonist it can be appreciated that the curve has become bi-phasic, suggesting that the response measured at the lower part of the curve is resistant to the effects of the antagonist.

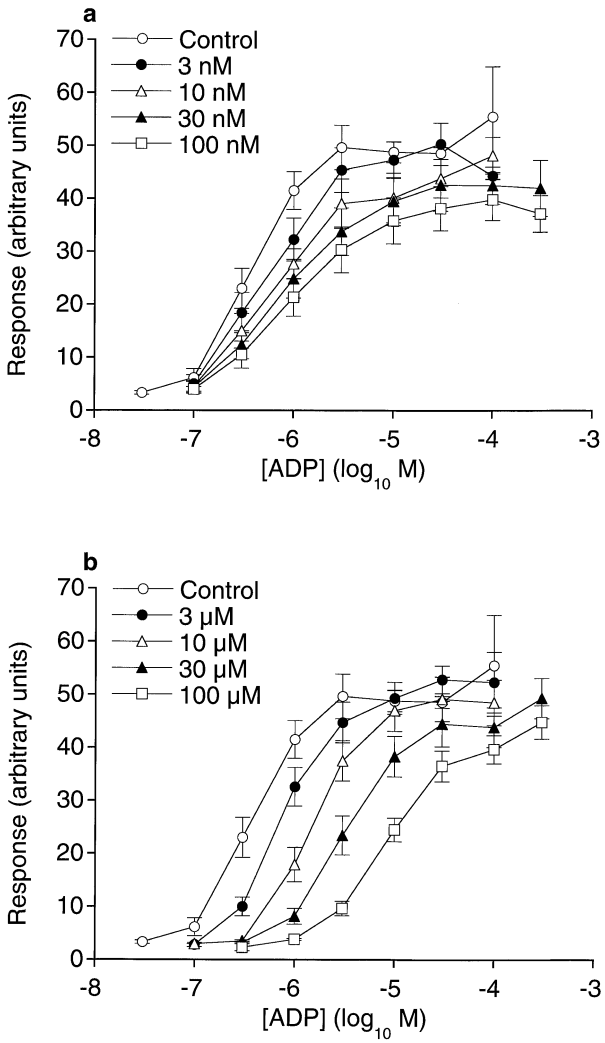

Figure 6 Antagonist effects of (a) AR-C67085 and (b) A3P5P on the rate of ADP-induced platelet aggregation. The pA_{50} of the control response was 6.46 ± 0.13 and the Hill coefficient 1.5 ± 0.2 ($n=6$). The effect of the P_{2T} antagonist AR-C67085 was characterized by a small decrease in the maximum response parameter. The $P2Y_1$ antagonist A3P5P caused parallel rightward shifts of the curve consistent with competitive antagonism. The pK_B was estimated as 5.47 ± 0.16 ($n=4$). The response is measured in arbitrary units equivalent to the maximum slope of the aggregation trace. Data points represent the mean and the standard error of the mean for all six sets of experimental data.

to identify the functional significance of this response have to date been unsuccessful; no evidence for its involvement in ADP-induced shape change or aggregation has been identified either in rat (Savi *et al*., 1997) or human platelets (Jin *et al*., 1998; Jin & Kunapuli, 1998). The $P2Y_1$ receptor is a G-protein coupled receptor which mediates ADP-induced increases in intracellular Ca^{2+} concentrations *via* the phospholipase C pathway (Fagura *et al*., 1998; Jin *et al*., 1998). It has also been reported that this receptor is responsible for mediating ADP-induced platelet shape change (Jin *et al*., 1998) and it has been critically implicated in the ADP-induced aggregation response (Hechler *et al*., 1998a; Savi *et al*., 1998). The ability of ADP to inhibit stimulated adenylate cyclase activity in the platelet has been well reported previously (Cusack & Hourani, 1982), although only recently has this property been ascribed to the third receptor subtype on the platelet, distinct from $P2X_1$ and $P2Y_1$ (Jin *et al*., 1998). As a consequence of this property, the receptor has been given a variety of names: $P2T_{AC}$ has been used to indicate that it is coupled to inhibition of adenylate cyclase (Jin *et al*., 1998) and P2Ycyc has been used for the same reason, although this name also contains the presumption that the receptor is coupled to a G-protein (Hechler *et al*., 1998a,b). G-protein coupling is also implied by the name $P2Y_{ADP}$ which was suggested (Fredholm *et al*., 1997) in order to indicate that, unlike in the case of other P2 receptors, ADP and not ATP is an agonist. However, with the demonstration that ATP is not an agonist but a competitive antagonist at the $P2Y_1$ receptor (Léon *et al*., 1997), this nomenclature is potentially misleading and prone to generate confusion, given that the $P2Y_1$ receptor is clearly present on the platelet. The original designation P2T was introduced (Gordon, 1986) in order to recognize the unique distribution of the receptor on the thrombocyte. It is this third receptor for which AR-C67085 is a potent and selective competitive antagonist and it is this pharmacological property that clearly distinguishes this receptor from both the $P2Y_1$ and $P2X_1$ subtypes (Humphries *et al*., 1995). We have used the nomenclature P_{2T} in this paper (in a manner consistent with the preliminary presentation of these results to the British Pharmacological Society (Jarvis *et al*., 1998)) for the identification of the receptor for which AR-C67085 is an antagonist until such time as the receptor is cloned and a definitive name can be ascribed.

Transient and sustained aggregation

Previous research has distinguished between so-called primary reversible aggregation and secondary irreversible aggregation (MacMillan, 1966), the latter which could be abolished by aspirin pre-treatment (O'Brien, 1968), indicating the involvement of thromboxane A_2 (TxA_2) in the response. It is important to recognize that these are not the same phenomena as those being discussed here. It has been shown that ADP-induced TxA_2 generation is an artefact seen only in platelet preparations with abnormally low concentrations of calcium, such as citrated platelet rich plasma. In heparinized platelet rich plasma this response is not seen (Packham *et al*., 1989). These conclusions are in accordance with our own observa-

tions that aspirin has no effect upon ADP-induced aggregation in heparinized platelet rich plasma but is capable of partially inhibiting ADP-induced aggregation in citrated platelet rich plasma (data not shown). The work described herein was all conducted in heparinized platelet rich plasma, thereby removing the additional factor of TxA_2 generation. Hence, the transient and sustained aggregations observed in this study can be clearly ascribed to receptor-mediated phenomena and are not due to generation of other platelet activating agents such as TxA_2.

Nevertheless, it is of interest to speculate as to why the response mediated *via* the $P2Y_1$ receptor alone is transient whereas activation of the P_{2T} receptor generates a sustained response. Possible explanations may relate either to receptor-specific phenomena or to functional interactions between the two receptors. Hence, it may be that the transience of the $P2Y_1$-mediated aggregation is caused by receptor desensitization, caused by persistent occupancy of the receptor by the agonist ADP. Stimulation of inositol-1,4,5-trisphosphate production *via* bovine P2Y receptors has previously been shown to desensitize over a period of seconds to minutes (Wilkinson *et al*., 1994). Such a time course of desensitization would be consistent with the phenomenon of transient aggregation reported here. In contrast, the observed sustained response to ADP in the presence of 100 μM A3P5P (Figure 2) suggests that the response mediated *via* the P_{2T} receptor is not affected by desensitization.

Alternatively, the simultaneous activation of $P2Y_1$ and P_{2T} receptors may result in a functional interaction between the increase in intracellular Ca^{2+} and the inhibition of adenylate cyclase in such a way as to convert a fundamentally transient response following activation of $P2Y_1$ alone into a sustained response. The functional significance of inhibition of adenylate cyclase for ADP-induced aggregation remains uncertain (Savi *et al*., 1996), but such an interaction may provide an explanation for its role in this phenomenon.

Indices of aggregation

For the maximum extent of aggregation, the selective inhibition of the upper part of the concentration-response curve by AR-C67085 and the parallel displacement of the whole curve by A3P5P reflects the fact that the lower portion of these curves (resistant to P_{2T} antagonism) is a measure of the transient aggregation which is mediated by the $P2Y_1$ receptor. Hence, a further implication of these results is that the choice of index to measure the platelet response is capable of significantly altering the interpretation given to that data. Although in the past, certain investigators have measured several different indices of aggregation (O'Brien, 1968), more recently it has become usual to measure only one, with most investigators using maximum extent (Boneu & Destelle, 1996; Hechler *et al*., 1998a; Gachet *et al*., 1995) while others have chosen to use rate of aggregation (Hourani *et al*., 1992; Cusack & Hourani, 1982). Separate use of these two indices may give rise to different conclusions about the effects of the two antagonists used in this study and hence the purinergic pharmacology of the platelet. A P_{2T} antagonist might be considered as only a partial inhibitor owing to its inability to completely block maximum extent, unlike a $P2Y_1$ antagonist, or even dismissed as virtually insignificant by comparison on the basis of their effects on rate of aggregation. Further consideration of these data confirms the importance of characterizing responses over a range of agonist and antagonist concentrations rather than just measuring the effect of antagonists on the response to a single agonist concentration.

Conclusions

Platelet aggregation is a complex biological phenomenon which requires detailed analysis for correct pharmacological characterization. By distinguishing between different indices of aggregation, we have demonstrated that ADP-induced platelet aggregation is mediated *via* the activation of $P2Y_1$ and P_{2T} receptors, and that the nature of this response depends critically upon the relative contributions to the activation of the platelet of each of these two receptor subtypes.

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