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Both the ADP receptors $P2Y_1$ and $P2Y_{12}$, play a role in controlling shape change in human platelets

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Two types of ADP receptors, $P2Y_1$ and $P2Y_{12}$ are involved in platelet aggregation. The $P2X_1$ receptor is also present but its role, in terms of platelet function, is not yet defined. The aim of this study was to establish if the ADP receptors, $P2Y_1$, $P2Y_{12}$ and $P2X_1$ play a role in controlling platelet shape change (PSC) in human platelets. PSC is an early phase of platelet activation that precedes aggregation.

Using a high-resolution channelyzer, PSC was assessed by measuring the median platelet volume (MPV). The $P2Y_1$ receptor antagonist MRS 2179 ($1.06 - 10.25\,\mu mol/l$) blocked ADP-induced PSC (by 100%). The median IC_{50} was $3.16\,\mu mol/l$. MRS 2179 also significantly ($P = 0.01$) inhibited PSC induced by the combination of ADP + serotonin (5HT). The $P2Y_{12}$ receptor antagonist AR-C69931MX significantly inhibited (at 10s, $P = 0.009$; 15 s, $P = 0.001$ and 30 s, $P = 0.015$) ADP-induced PSC. The $P2X_1$ receptor antagonist TNP-ATP had no significant effect on ADP- or ADP + 5HT-induced PSC.

We conclude that the IC_{50} of a $P2Y_1$-blocker can be derived because of the high-resolution and reproducibility of the channelyzer technique. In addition to the $P2Y_1$ purinoceptor, the $P2Y_{12}$ receptor appears to be involved in ADP-induced PSC since this process was significantly inhibited by AR-C69931MX. The channelyzer technique may be more reliable than optical aggregometry to assess PSC.

Introduction

Purinergic receptors[1] are potential targets for antithrombotic drugs, since ADP plays a crucial role in platelet activation. Two types of ADP receptors, $P2y_1$ and $P2Y_{12}$, contribute to platelet aggregation.[2,3] The $P2y_1$ receptor is involved in the initiation of aggregation by mobilizing calcium stores.[2] The $P2Y_{12}$ receptor is necessary for the completion and amplification of aggregation by inhibiting adenylyl cyclase activity.[4,5] The $P2X_1$ receptor is also present but its role, in terms of platelet function, is not yet defined.[4]

The initial functional response of platelets to ADP is the shape change. These cells undergo cytoskeletal reorganisation resulting in spheration, contraction and generation of pseudopodia.[6] Platelet shape change (PSC) precedes aggregation and, *in vitro*, this process is essentially aspirin resistant.[7–18] Several studies that assessed PSC used light transmission aggregometry.[19–21] This technique is not as sensitive as using a high-resolution channelyzer.[7–18] Therefore, aggregometry may not have detected small variations in platelet size.

More deficiencies can be attributed to the optical method used to assess PSC. Indeed, Maurer–Spurej (2001) and others[22–24] proposed that a decrease in light transmission actually represents the formation of platelet-microaggregates rather than PSC. We have shown (EM paper 2000), using a high-resolution channelyzer, that an increase in median platelet volume (MPV) as an indication of PSC occurs concomitantly with morphological changes seen by electron microscopy.[15]

Since our channelyzer method is reproducible and sensitive,[7–18] we used it to evaluate the role of the $P2y_1$, $P2Y_{12}$ and $P2X_1$ receptors in mediating human PSC.

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In order to achieve this objective we also used specific purinergic receptor antagonists, MRS 2179 for $P2y_1$[25] receptors, AR-C69931MX for $P2Y_{12}$[26] receptors and TNP-ATP for $P2X_1$[27] receptors.

Methods

Blood collection and MPV measurement

Venous blood was collected in 3.8% citrate (1 part citrate to 9 parts blood) from healthy volunteers. All the volunteers denied taking any drugs for at least two weeks prior to sampling. The citrated blood was centrifuged at 160 g for 15 min at room temperature. The platelet rich plasma (PRP) was then carefully collected with a Pasteur pipette. Aliquots of PRP (450 µl), in siliconised glass cuvettes, were placed in a Chronolog dual channel optical aggregometer (Coulter Electronics, Luton, Beds, UK) and kept at 37°C for 15 min prior to the start of the experiments detailed below.[7–18] The specific times for adding agonists and antagonists are described in the appropriate methods/results section below. These times were based on preliminary experiments and our previous publications.[7–18]

PRP was stirred using a Teflon-coated metal stir bar, at 1000 rpm and 100 µl aliquots were removed and mixed with 400 µl of fixative, (4% aqueous glutaraldehyde). We adjusted the concentration of agonists and sampling times to obtain a middle range response that could be either inhibited or enhanced as determined in previous studies.[7–18] This sample was further diluted 400 fold in Isoton II (Coulter Electronics, Luton, Beds, UK). These samples were then assessed using a Coulter ZM counter (electrical impedance method with a 70 µm diameter sampling tube orifice) coupled to a C-256 channelyzer (Coulter Electronics, Luton, Beds, UK). The resolution of the channelyzer is 0.07 fl. The analyzer sample "window" was set between 2.67 and 19.12 fl.[7–18] The counter was calibrated using 9 fl latex-particles (Coulter Electronics, Luton, Beds, UK). For particle analysis, data were accumulated to a maximum of 500 platelets in each of the 256 channels. The MPV is the volume of the channel on each side of which 50% of the platelet population is distributed.[7–18] The increase in MPV was taken as representing the PSC.[7–18] Saline (control) was added to samples to match those of the agonist (as 10–50 µl volumes).[7–18] The platelet count was monitored throughout the experiment to exclude PRP samples where significant (> 5%) aggregation occurred.

[A] (i) *Obtaining an IC_{50} for the $P2Y_1$ ADP receptor antagonist (MRS 2179)*

PRP was prepared from seven healthy volunteers (6 men and 1 woman; mean age: 38 years, range: 26–57) using the above method. PRP was stimulated with ADP at a final concentration (fc) of 0.2 or 0.4 µmol/l for 30 sec and the MPV assessed. This dose was selected to cause a 5–15% increase in MPV when compared with the saline control. This range was selected to provide a sub-maximal increase that is within the detection capacity of the high-resolution channelyzer and can still allow for any inhibitory responses with antagonists. In order to obtain an IC_{50} for MRS 2179, PRP was incubated for 1 min with increasing doses of MRS 2179 (fc ranging from 0.62 to 20 µmol/l in each individual) before adding ADP. These increasing doses of MRS 2179 were used until the measured MPV value returned to that observed for the saline control (see Figure 1).

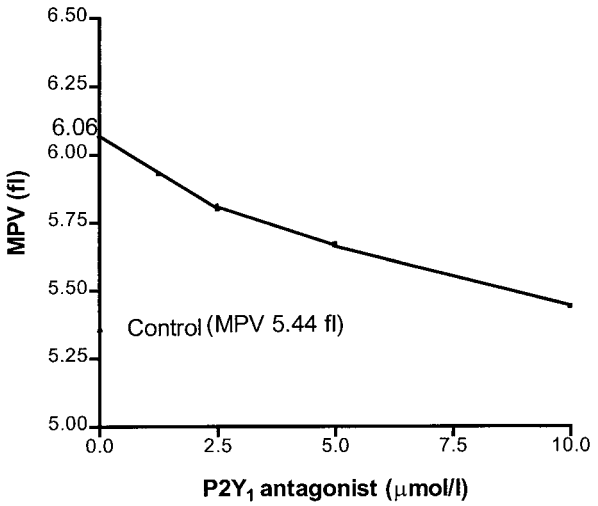

Figure 1. ADP-induced PSC. A representative curve showing that the IC_{50} of MRS 2179 (was 3.33 µmol/l) in this subject.

[A] (ii) *The effect of the $P2Y_1$ receptor antagonist (MRS 2179) on a combination of ADP and serotonin (5HT) – induced PSC*

PRP was prepared from seven healthy volunteers (6 men and 1 woman; mean age of 38 years, range: 26–57) using the above method. PSC was stimulated with ADP only (fc 0.2 µmol/l), 5HT only (fc 0.03–0.25 µmol/l), and then ADP combined with 5HT. The increase in MPV induced by either ADP or 5HT alone was of the order of 1–3%. This range was selected to provide a sub-maximal increase that is within the detection capacity of the high-resolution channelyzer and can still allow for an additive of synergistic effect. In each individual we determined the dose of the MRS 2179 that resulted in 100% inhibition of ADP-induced PSC (as described in [A] (i), above). This maximum dose of MRS 2179 ranged from (fc) 5 to 20 µmol/l. The selected dose was then incubated with PRP for 1 min before adding ADP combined with 5HT; the MPV was assessed 30 s later.

[B] *The effect of the $P2Y_{12}$ ADP receptor antagonist (AR-C69931MX) on PSC*

PRP was prepared using the above method from 12 healthy volunteers (7 men and 5 women; mean age

of 32 years, range: 23–54). ADP alone (fc. 0.2–0.4 μmol/l) then ADP together with AR-C69931MX (10 μmol/l) was added to PRP. The MPV was analyzed as described above 10 s, 15 s and 30 s after adding these agents.

[C] (i) *The effect of a $P2X_1$ ADP receptor antagonist (TNP-ATP) on PSC*

PRP was prepared from seven healthy volunteers (6 men and 1 woman; mean age of 38 years, range: 26–57) using the above method. PRP was stimulated with ADP (fc) 0.2 or 0.4 μmol/l for 30 s and the MPV assessed. This dose was selected to cause a 4–12% increase in MPV. This range was selected to provide a sub-maximal increase that is within the detection capacity of the high-resolution channelyzer and can still allow for any inhibitory responses with antagonists. To determine if there were any responses with TNP-ATP, PRP was incubated for various times (30 s–3 min) with a range of doses of TNP-ATP (26–102 μmol/l) before the addition of ADP ($n=7$), or a combination of ADP and 5HT ($n=5$).

[C] (ii) *The effect of a $P2X_1$ ADP receptor agonist α,β-methylene ATP (α,βmeATP) on MPV*

PRP was prepared from seven healthy volunteers (3 men and 4 women; mean age of 36 years, range: 28–56) using the above method. PRP was stimulated with α,βmeATP in increasing doses ranging from 0.5 μmol/l to 20 μmol/l. PRP was incubated with α,βmeATP for various times (10 s, 15 s and 30 s) and the MPV was assessed. These times were selected on the basis of previous research showing that the response of this receptor is very rapid.

Materials

TNP-ATP was obtained from Molecular Probes, (Eugene Oregon, USA); MRS 2179 was from Tocris Cookson Ltd. (Bristol, UK). AR-C69931MX was a gift from AstraZeneca R&D, (Charnwood, UK) and α,βmeATP was purchased from Sigma (Poole, UK).

Statistical analysis and presentation of the results

The results are presented as median and range. Statistical comparisons are by Wilcoxon paired test.

Results

[A] (i) *Obtaining an IC_{50} for the $P2Y_1$ ADP receptor antagonist (mRs 2179)*

MRS 2179 inhibited ADP-induced PSC by 100% in each of the seven subjects tested. The median (and range) IC_{50} for MRS 2179 was 3.16 (1.06–10.25 μmol/l). Therefore the range for IC_{50} of MRS 2179 showed a ten-fold variation when all seven subjects were considered. A representative dose response curve is shown in Figure 1.

[A] (ii) *The effect of the $P2Y_1$ ADP receptor antagonist (MRS 2179) on a combination of ADP and 5HT-induced PSC*

The ADP-induced increase in MPV was of the order of 2% greater than that for the saline control. For 5HT, the increase was of the order of 1%. In combination, ADP + 5HT caused a synergistic increase in MPV of 8%. This increase was significantly ($P=0.01$) inhibited by MRS 2179 at a dose that abolished PSC induced by ADP alone (Table 1).

[B] *The effect of the $P2Y_{12}$ ADP receptor antagonist (AR-C69931MX) on PSC*

ADP-induced PSC showed a 4–6% increase in MPV (fl) when compared with the saline controls. The ADP (0.2 μmol/l)-induced increase in MPV was2 significantly inhibited by AR-C69931MX (10 μmol/l) (Table 2). AR-C69931MX also inhibited ADP-induced PSC up to 5 min but with increasing time the inhibition was less (Figure 2.) This same trend in inhibition was seen for 0.4 μmol/l.

Although AR-C69931MX inhibited ADP-induced PSC 30 s after its initiation, the inhibitory effect gradually decreased so that it was no longer evident at 5 min.

[C] (i) *The effect of the $P2X_1$ receptor antagonist (TNP-ATP) on PSC*

There was no significant effect ($n=7$) on ADP-induced shape change, (6.31; 6.12–6.88 fl) after

Table 1. Effect of MRS 2179 on a combination of 5HT + ADP induced PSC ($n=7$).

	MPV (fl), median (range)
Saline	6.18 (5.36 – 6.44)
ADP	6.31 (5.6 – 6.88)
5HT	6.25 (5.55 – 6.82)
ADP + 5HT	6.69 (6.18 – 7.14)
MRS 2179 plus ADP + 5HT	6.44 (5.93 – 6.69)

ADP + 5HT vs ADP + 5HT plus MRS 2179, $P=0.01$.

Table 2. The effect of AR-C69931MX on ADP-induced PSC ($n=12$).

Time (sec)	10 s	15 s	30 s
Saline	5.88 (4.78 – 6.95)	5.85 (4.95 – 6.76)	5.90 (4.78 – 6.69)
ADP	6.12 (5.48 – 7.01)	6.19 (5.48 – 7.07)	6.24 (5.55 – 7.20)
AR-C69931MX + ADP	5.94 (5.42 – 6.76)	6.08 (5.47 – 6.84)	6.10 (5.48 – 7.01)
P value	$P=0.009$	$P=0.001$	$P=0.010$

The *P* values are a comparison of ADP vs ADP + AR-C69931MX at 10, 15 and 30 s.

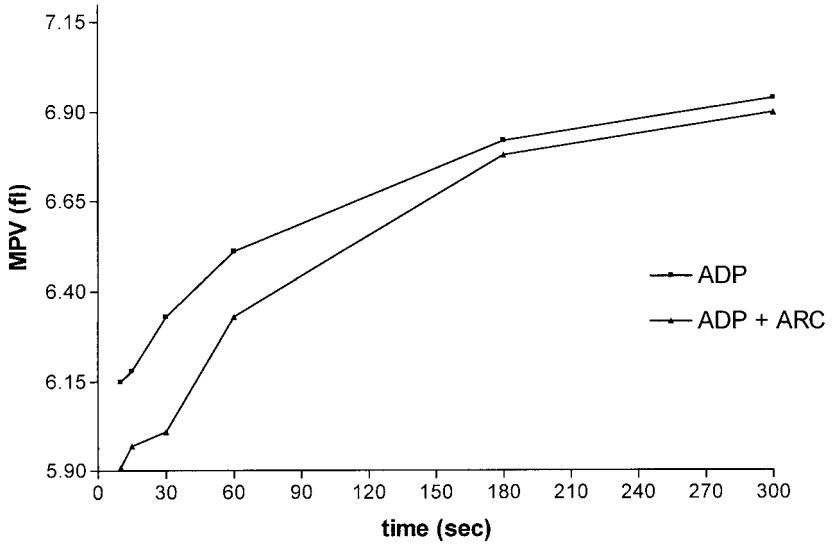

Figure 2. A representative curve ($n = 2$) of the effect of AR-C69931MX (ARC; 10 μmol/l) on ADP (0.2 μmol/l)-induced PSC.

adding TNP-ATP (6.36; 6.18–7.01 fl). There was also no significant effect ($n = 5$) on PSC induced by the combination of ADP + 5HT (6.57; 6.18–7.07 fl) after adding TNP-ATP (6.61; 6.12–6.95 fl).

[C] (ii) *The effect of a P2X$_1$ ADP receptor agonist (α,βmeATP) on MPV*

There was no effect of α,βmeATP on MPV at any of the time points, or concentrations assessed (e.g. saline control at 10 s, 5.87; 5.36–6.66 fl vs α,βmeATP at 10 s, 5.87; 5.36–6.63 fl).

Discussion

Using a P2Y$_1$ receptor-blocker (MRS 2179) we achieved 100% inhibition of PSC induced by ADP. Others have also shown that ADP-induced shape change occurs via the P2Y$_1$ receptor.[2] However, these authors measured PSC using light transmission aggregometry and it has been suggested that the aggregometer tracing taken to represent PSC rather reflects the formation of platelet-microaggregates.[19] The P2Y$_1$ receptor is also essential for platelet aggregation.[28,29] Therefore, antagonists to this receptor may become useful antiplatelet agents.[29]

The IC$_{50}$ for MRS 2179 varied between 1.06 and 10.25 μmol/l (i.e. a ten-fold variation in IC$_{50}$ when all seven subjects were considered); the median IC$_{50}$ was 3.16 μmol/l. To our knowledge, an IC$_{50}$ has not been derived before for a P2Y$_1$ receptor-blocker using the human PSC phenomenon. An IC$_{50}$ for P2Y$_1$ receptor-blockers can now be derived using a channelyzer because of the high-resolution (0.07 fl) and reproducibility of this technique.[7–18]

AR-C69931MX inhibited ADP-induced PSC, in a time-dependent manner, in samples collected from healthy volunteers. This is the first study that actually quantifies PSC inhibition by this or any other P2Y$_{12}$ blocker. Others[20] have shown, using an optical aggregometer and washed platelets, that another P2Y$_{12}$ receptor antagonist (AR-C66096) caused a more rapid reversion of platelets to the discoid form. There was also evidence that PSC was inhibited by cAMP.[20] This finding may explain why blocking the P2Y$_{12}$ receptor results in inhibition of PSC since stimulating the P2Y$_{12}$ receptor inhibits cAMP synthesis.[20]

When using two agonists (i.e. ADP + 5HT) in combination in the presence of the maximal dose of the P2Y$_1$-blocker (MRS 2179), the mean MPV was greater than that with 5HT alone. This finding suggests that the unblocked P2Y$_{12}$ purinergic receptor can enhance the 5HT-mediated PSC response. Therefore, it would seem that the P2Y$_{12}$ receptor is involved when ADP acts as a co-stimulus in the presence of low concentrations of other agonists, as previously suggested.[4]

The P2X$_1$ receptor could be implicated in PSC.[30] However, many investigators failed to find any functional activity for that purinergic receptor other than calcium influx.[6,31,32] We did not find an effect of the P2X$_1$ receptor on PSC (after using both a specific antagonist and agonist). However, this receptor is involved with very rapid actions (1–2 s).[32,33] The P2X$_1$ receptor also becomes rapidly desensitized.[4] Therefore, it is difficult to assess its effect on platelets *in vitro*[4] because our sampling time is limited by the capacity of human operators. We also used citrated PRP and this may have masked any calcium-mediated effect.

It was important to exclude an effect of the P2X$_1$ receptor on PSC within our experimental design because the P2Y$_1$-blocker (MRS 2179) we used may also exert some action on the P2X$_1$ receptor.[34]

However, any such interference is unlikely to have occurred because the $P2X_1$ receptor showed no effect on PSC (after using both a specific antagonist and agonist). Furthermore, in another experiment (rat vas deferens), MRS 2179 at concentrations of 100 μmol/l did not exert any action on the $P2X_1$ receptor.[34] The maximal concentration of MRS 2179 we used was 20 μmol/l.

We previously used the channelyzer method to demonstrate the action of drugs (added *in vitro* at therapeutic concentrations) on platelets[7,8,10–14,16–18] when other methods only detected an effect at unrealistically high concentrations. These findings are of clinical relevance because PSC is an early phase of platelet activation that precedes aggregation and it is essentially aspirin resistant,[7–18] at least *in vitro*.

Our findings suggest that $P2Y_{12}$ antagonists are clinically useful since they inhibit both early and late platelet activation.[35] This concept is supported by clinical trials.[35] For example, there is extensive trial-based evidence showing that clopidogrel (a $P2Y_{12}$ antagonist) significantly decreases the risk of vascular events when administered alone or in combination with aspirin.[36–38] Unfortunately, the inhibitory effect of clopidogrel cannot be assessed *in vitro* because it requires to be metabolized before exerting its actions.[38] Whether $P2Y_1$ or $P2Y_{12}$ receptor antagonists will prove superior to other antiplatelet agents in certain disease states remains to be proven.[39,40] In addition, there is a need to establish whether the contribution of different platelet purinergic receptors is altered in various types of vascular disease.

Assessing the effect of clopidogrel on the $P2Y_{12}$ receptor is limited because this antiplatelet agent is inactive *in vitro*.[41] Clopidogrel needs to be metabolized by the liver before becoming active.[41] At least one of the active metabolites of clopidogrel has been purified and shown to be active *in vitro*.[41] However, the production of this metabolite requires incubation with human liver microsomes followed by a purification process.[41] In addition, this metabolite is highly unstable.[41] It follows that the use of this metabolite is limited.

In conclusion, platelet receptor antagonists will exert different actions on human platelets. Both the $P2Y_1$ and $P2Y_{12}$ receptors appear to be involved in mediating PSC in human platelets.[42] The channelyzer method provides a useful tool to assess the platelet inhibitory action of these drugs.[7–18]

The preliminary results of this study were presented[42] at the 'Nottingham Platelet Conference', University of Nottingham, UK, July 28–31, 2002.

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