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No requirement of P2X1 purinoceptors for platelet aggregation

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Abstract

ADP produces a series of responses in rabbit platelets such as shape changes, aggregation and intracellular Ca^{2+} mobilization. In human platelets, the P2X1 receptor mediates a rapid increase in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) upon stimulation with ADP. We investigated whether this phenomenon is also present in rabbit platelets. We found that the P2X1 receptor-mediated response was absent because there was (1) no elevation of $[Ca^{2+}]_i$ in response to α,β-methylene-ATP, a selective P2X1 receptor agonist, in fura-2-loaded platelets; (2) no change in the ADP-induced $[Ca^{2+}]_i$ increase and platelet aggregation after P2X1 receptor desensitization with α,β-methylene-ATP; (3) complete inhibition of the ADP-induced $[Ca^{2+}]_i$ elevation by the P2Y1 receptor specific antagonist, A3′P5′PS, with a similar ID_{50} value both in the presence and absence of external Ca^{2+}. These results indicate that ADP-induced $[Ca^{2+}]_i$ elevation is mainly mediated by P2Y1 receptors in rabbit platelets. We conclude that the P2X1 receptor does not play a significant role in the ADP-induced platelet shape changes and aggregation.

Keywords: ADP; Platelet aggregation; P2X1 purinoceptor; Ca^{2+}, intracellular; Platelet, rabbit

1. Introduction

ADP produces a series of responses in rabbit platelets similar to those in human platelets. The first event is a change in platelet shape, namely, a rapid disc–sphere transformation. The shape change is followed by platelet aggregation. Unlike human platelets, ADP does not induce the release of arachidonic acid in rabbit platelets. ADP causes a numbers of intracellular events in rabbit platelets, including phospholipase C activation, intracellular Ca^{2+} mobilization and adenylate cyclase inhibition (Matsuoka and Suzuki, 1983; Vickers et al., 1986; Nishio et al., 1992).

The ADP receptor on platelets is a unique ADP-selective purinocepter, termed P2T, which is recognized as a member of P2 purinoceptor family (Gordon, 1986). At P2T purinoceptor sites, ADP acts as an agonist and ATP as an antagonist. Hourani and Hall (1994) proposed a two-receptor model to explain the actions of ADP on platelets: the rapid Ca^{2+} influx is mediated by a receptor-operated Ca^{2+} channel, while the intracellular Ca^{2+} mobilization and adenylate cyclase inhibition are modulated by the P2T receptor through G-proteins. Two subtypes of purinoceptors have been recently cloned in human platelets: ionotropic (P2X types) (Clifford et al., 1998) and G-protein-coupled (P2Y types) purinoceptors (Leon et al., 1997). In 1998, Kunapuli et al. proposed a three-receptor model for the effects of ADP on platelets (Daniel et al., 1998): P2Y1 purinoceptors coupled to G_q, another receptor coupled to G_i protein-adenylate cyclase inhibition, and P2X1 purinoceptors, which induce a rapid increase in Ca^{2+} entry (Mahaut-Smith et al., 1992; MacKenzie et al., 1996). However, the role of ADP-evoked Ca^{2+} entry via P2X1 receptors in the function of blood platelets remains unclear.

This paper shows, using α,β-methylene-ATP (α,β-Me-ATP) as an agonist for P2X1 receptors (MacKenzie et al., 1996), that the P2X1 receptor is not involved in ADP-induced platelet shape changes and aggregation and that the $[Ca^{2+}]_i$ elevation in response to ADP is due to activation of P2Y1 purinoceptors in rabbit platelets.

2. Materials and methods

2.1. Rabbit washed platelets

Rabbits of either sex, weighing 3.0–3.5 kg, were anesthetized by intramuscular injection of sodium pentobarbital

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(30 mg/kg). Blood was collected from the carotid artery in an acid–citrate–dextrose (ACD) solution (v/v: 1/6), the composition of which was 85 mM sodium citrate, 65 mM citric acid and 2% dextrose. Platelet-rich plasma from whole blood was prepared by low centrifugation at $140 \times g$ for 12 min at room temperature. Platelets were pelleted by centrifugation from platelet-rich plasma at $900 \times g$ for 10 min. The platelets were washed with Tyrode-HEPES albumin buffer containing apyrase (0.1 U/ml) without Ca^{2+} at pH 6.35. Apyrase, an adenosine nucleotidase, was necessary to minimize the desensitization of purinoceptors by ATP and ADP spontaneously released from platelets during the washing procedure. The composition of the Tyrode-HEPES albumin buffer was as follows (mM): NaCl 137, KCl 2.7, $CaCl_2$ 1.8, $MgCl_2$ 1, NaH_2PO_4 0.4, glucose 5.6, HEPES (*N*-2-hydroxyethylpiperazine-*N*-2-ethansulfonic acid) 10 and 0.35% albumin.

2.2. Measurement of $[Ca^{2+}]_i$

The platelets were suspended in the same buffer pH 7.35 containing 3 μM fura-2/acetoxymethyl ester in a shaking water bath at 37°C for 20 min. The platelets were then washed twice and finally resuspended in the Tyrode-HEPES albumin buffer at $2–5 \times 10^8$ platelets/ml at pH 7.35. These platelets were kept at room temperature during the experiments. Just before determinations, aliquots of platelet suspension (1 ml) were centrifuged at $2000 \times g$ for 15 s in a microcentrifuge. The supernatant medium was discarded, and the platelets were resuspended in 2 ml of fresh buffer without apyrase that had been prewarmed to 37°C. The platelet suspension was transferred to a 10×10 mm quart cuvette placed in the thermostat-regulated sample chamber of a dual-excitation beam spectrophotometer (Hitachi, F-2000, Japan). The suspension was continuously stirred with a circular stirring bar. The excitation wavelengths were 340 and 380 nm and fura-2 fluorescence emission was measured at 510 nm. At the end of measurements, Triton X-100 was added to the platelet suspension to obtain maximal fluorescence and then excess EGTA was added to obtain minimum fluorescence. The $[Ca^{2+}]_i$ was calculated from the ratio of the fluorescence at the two excitation wavelengths, with a K_d value of 224 nM for the fura-2/Ca equilibrium, as described by Grynkiewicz et al. (1985). In experiments with A3′P5′PS, a P2Y1 receptor antagonist, the drug was incubated with the fura-2-loaded platelets for 5 min at 37°C.

2.3. Platelet aggregation

Platelet aggregation was measured by the turbidimetric method used previously (Takano, 1995) (NBC Hematracer 601; Nikou Bioscience, Tokyo, Japan). The platelet suspension (5×10^8 platelets/ml), which did not contain apyrase, was supplemented with 1.8 mM $CaCl_2$ after a 2-min preincubation, and ADP, at a final concentration of 10 μM, was added to the platelet suspension in the presence or absence of α,β-Me-ATP (10 μM).

2.4. Materials

The following drugs were used: ADP, α,β-Me-ATP, apyrase (grade 1), A3′P5′PS (adenosine 3′-phosphate 5′-phosphosulfate as a P2Y1 receptor antagonist) (Boyer et al., 1996), bovine albumin (type V) (Sigma, St. Louis MS, USA). Fura-2/acetoxymethyl ester, EGTA and HEPES (2-[4-(2-hydroxy-ethyl-1-piperazinyl)ethane sulfonic acid (Wako, Osaka, Japan).

3. Results

3.1. $[Ca^{2+}]_i$ response to ADP in the presence or absence of extracellular Ca^{2+} in fura-2-loaded platelets

In fura-2-loaded rabbit platelets, ADP (10 μM) caused a rapid and significant increase in $[Ca^{2+}]_i$ followed by a decrease to a sustained level in the presence of 1 mM external Ca^{2+} (Fig. 1, upper panel). The maximum level was obtained within 10 s and the mean value was 334.7 ± 40.0 nM ($n = 9$). In the presence of 1 mM EGTA in the external medium, ADP (10 μM) brought about a rapid but smaller increase in $[Ca^{2+}]_i$ which returned immediately to the basal level. The mean peak $[Ca^{2+}]_i$ was 81.3 ± 9.6 ($n = 9$). The experiment was finished within 5 min after the platelets were suspended without apyrase.

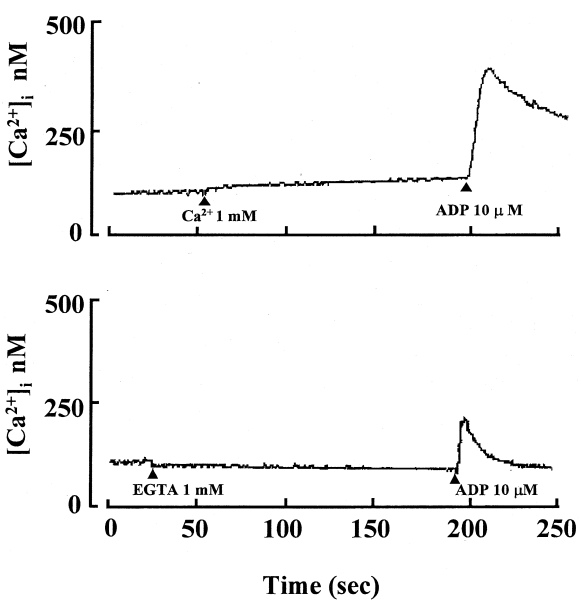

Fig. 1. Elevation of $[Ca^{2+}]_i$ by ADP in the presence or absence of extracellular Ca^{2+}. Fura-2-loaded rabbit platelets were stimulated with ADP (10 μM) and $[Ca^{2+}]_i$ was measured as described in Section 2 in the presence of 1 mM $CaCl_2$ (upper panel) and 1 mM EGTA (lower panel) in the extracellular medium.

3.2. Effect of α,β-Me-ATP on fura-2-loaded platelets

In human platelets ADP-induced activation involves not only the mobilization of intracellular Ca^{2+} stores but also the rapid influx of Ca^{2+} through P2X1 receptors (Clifford et al., 1998). So we examined the effect of a non-hydrolyzable ATP analogue, α,β-Me-ATP, a selective agonist for P2X1 purinoceptors at 10 μM, on fura-2-loaded platelets. α,β-Me-ATP had no effect on the platelets in the presence of 1 mM external Ca^{2+} (Fig. 2) or EGTA (figure not shown). The P2X1 receptors is known to desensitize following application of agonist (MacKenzie et al., 1996). We therefore examined the effect of ADP on the fura-2-loaded platelets in the presence of α,β-Me-ATP. The prior addition of α,β-Me-ATP (10 μM) to the platelets did not inhibit the ADP (10 μM)-induced increase in $[Ca^{2+}]_i$, indicating that the P2X1 receptor is not involved in the ADP-induced $[Ca^{2+}]_i$ increase (Fig. 3).

3.3. Absence of effect on ADP-induced aggregation of α,β-Me-ATP

As shown in Fig. 4, upper panel, 10 μM ADP induced a shape change followed by the reversible aggregation of rabbit platelets. α,β-Me-ATP at 10 μM did not induce a shape change or aggregation (Fig. 4, lower panel). Furthermore, α,β-Me-ATP did not affect the platelet aggregation induced by the subsequent application of ADP in rabbit platelets (Fig. 4, lower panel).

3.4. Effect of A3′P5′PS, a P2Y1 antagonist, on ADP-induced increase in $[Ca^{2+}]_i$

The P2Y1 receptor was recently demonstrated to have a functional role in ADP-induced platelet activation. The human P2Y1 receptor is coupled to the G_q protein–phospholipase C–inositol phosphate formation–Ca^{2+} mobilization system, with the Ca^{2+} being mobilized from intracellular stores (Savi et al., 1998). Therefore, we investigated the effect of A3′P5′PS, a P2Y1 selective antagonist, on the ADP-induced increase in $[Ca^{2+}]_i$. As shown in Fig. 5, A3′P5′PS inhibited the ADP induced $[Ca^{2+}]_i$ increase in a concentration-dependent manner, with ID_{50} values of

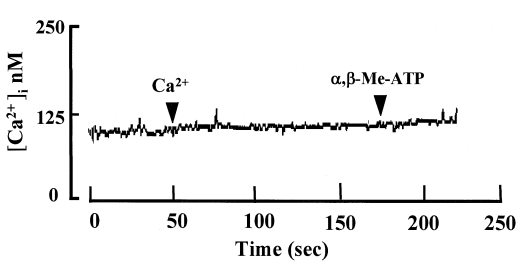

Fig. 2. No effect of α,β-Me-ATP on the $[Ca^{2+}]_i$ of rabbit platelets in the presence of extracellular Ca^{2+}. α,β-Me-ATP (10 μM) was added to fura-2-loaded platelets in the presence of 1 mM $CaCl_2$.

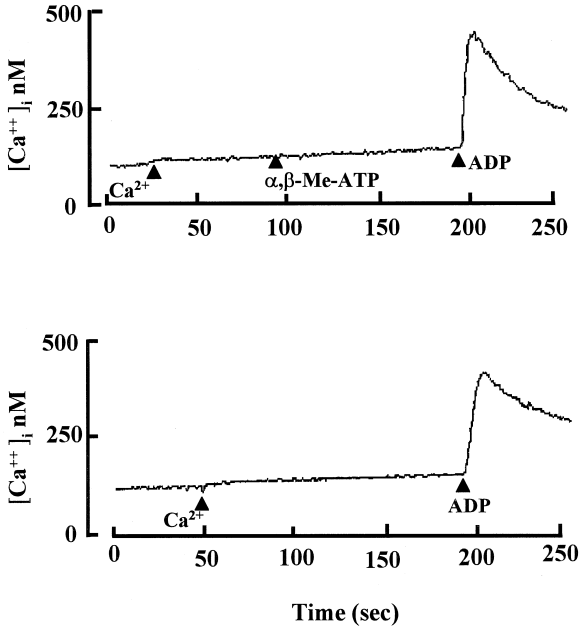

Fig. 3. No desensitization of ADP-induced elevation of $[Ca^{2+}]_i$ by α,β-Me-ATP. Fura-2-loaded rabbit platelets were incubated with ADP (10 μM) and $[Ca^{2+}]_i$ was measured as described in Section 2 in the presence (upper panel) or absence (lower panel) of α,β-Me-ATP (10 μM) in the presence of 1 mM $CaCl_2$. The trace shows a typical experiment out of experiments with platelets from three different rabbits.

112.2 ± 22.7 μM ($n = 6$) or 87.0 ± 27.6 μM ($n = 5$) in the presence or absence of external Ca^{2+}, respectively. These results suggest that the Ca^{2+} for the $[Ca^{2+}]_i$ increase induced by ADP is derived entirely from the store sites and that this increase is mediated by P2Y1 receptors

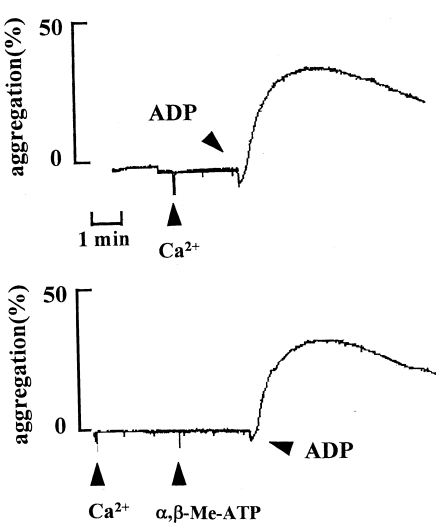

Fig. 4. No effect of α,β-Me-ATP on the ADP-induced aggregation of washed rabbit platelets. Platelet aggregation induced by 10 μM ADP (upper panel) was not different from the aggregation measured in the presence of 10 μM α,β-Me-ATP (lower panel). The trace shows a typical experiment out of experiments with platelets from three different rabbits.

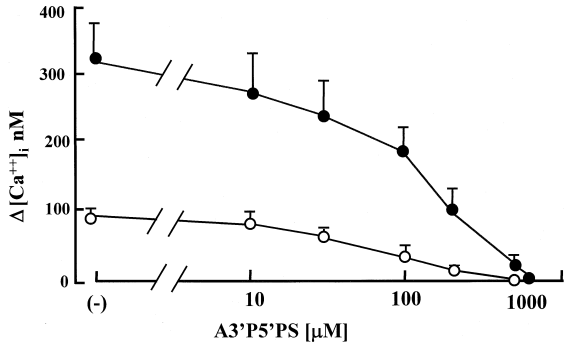

Fig. 5. Inhibition of A3′P5′PS of ADP-induced elevation of $[Ca^{2+}]_i$. Fura-2-loaded platelets were incubated with different concentrations of A3′P5′PS for 5 min before stimulation with ADP (10 μM) in the presence of 1 mM $CaCl_2$ (●) ($n = 6$) and 1 mM EGTA (○) ($n = 5$) in the external medium.

through the activation of the G_q protein–phospholipase C system in rabbit platelets.

4. Discussion

ADP induces a series of responses of blood platelets that are mediated by P2T receptors. The P2T receptor appears to have the characteristics of more than one subtype of purinoceptors. A three-receptor model has recently been proposed to explain the effects of ADP on platelet activation (Jin and Kunapuli, 1998; Jin et al., 1998). One of the receptors is the P2X1 receptor, which mediates a rapid Ca^{2+} influx, and the other two receptors are distinct G protein-coupled receptors: one is coupled to G_q to stimulate phospholipase C and the other is coupled to G_i to inhibit adenylate cyclase (MacKenzie et al., 1996; Daniel et al., 1998). The P2Y1 receptor is coupled to G_q, and the mobilization of Ca^{2+} from intracellular stores is due to phopholipase C and inositol trisphosphate accumulation. P2Y1-selective antagonists inhibited ADP-induced intracellular Ca^{2+} mobilization in a concentration-dependent manner, indicating that ADP elevates in $[Ca^{2+}]_i$ through P2Y1 activation (Jin et al., 1998; Savi et al., 1998).

The following results obtained in the study suggest that rabbit platelets lack P2X1 purinoceptors, which mediate the rapid phase of ADP-evoked Ca^{2+} entry via nonselective cation channels in human platelets (Mahaut-Smith et al., 1992). Firstly, α,β-Me-ATP, a selective P2X1 receptor agonist, induced no elevation of $[Ca^2]_i$ in rabbit platelets. Secondly, the ADP-induced $[Ca^{2+}]_i$ increase, platelet shape changes and aggregation were not affected by pretreatment of the platelets with α,β-Me-ATP. Since the P2X1 receptor is desensitized rapidly by α,β-Me-ATP (Werner et al., 1996), these results indicate that the α,β-Me-ATP-sensitive P2X1 receptor is not involved in the ADP-induced $[Ca^{2+}]_i$ increase, shape changes and aggregation of rabbit platelets. Thirdly, the ADP-induced $[Ca^{2+}]_i$ elevation, in both the presence and absence of external Ca^{2+}, was inhibited completely by the P2Y1 receptor specific antagonist, A3′P5′PS, in a dose-dependent manner. The ID_{50} values were in a similar range irrespective of the extracellular Ca^{2+} concentration. These results indicate that A3′P5′PS inhibited the P2Y1 receptor and also the Ca^{2+} mobilization in response to ADP, and that the Ca^{2+} for the ADP-induced $[Ca^{2+}]_i$ elevation was derived entirely from intracellular stores via P2Y1 receptors in rabbit platelets.

Our results also suggest that P2X1 is not essential for ADP-induced shape changes and aggregation of rabbit platelets. Daniel et al. (1998) demonstrated no effect of α,β-Me-ATP on inositol trisphosphate formation via P2Y1 receptor activation and no cyclic AMP formation by activation of adenylate cyclase. Jin and Kunapuli (1998) also showed that α,β-Me-ATP did not induce shape change, even though there was a rapid increase in $[Ca^{2+}]_i$ by Ca^{2+} influx via P2X1 receptors in human platelets.

In conclusion, P2X1 receptors are not involved in ADP-induced platelet shape changes, aggregation and $[Ca^{2+}]_i$ elevation and that the Ca^{2+} for the $[Ca^{2+}]_i$ elevation is mobilized from intracellular stores by the activation of P2Y1 purinoceptors in rabbit platelets.

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