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ADP is not an agonist at $P2X_1$ receptors: evidence for separate receptors stimulated by ATP and ADP on human platelets

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1 ADP, an important agonist in thrombosis and haemostasis, has been reported to activate platelets *via* three receptors, $P2X_1$, $P2Y_1$ and $P2T_{AC}$. Given the low potency of ADP at $P2X_1$ receptors and recognized contamination of commercial samples of adenosine nucleotides, we have re-examined the activation of $P2X_1$ receptors by ADP following HPLC and enzymatic purification.

2 Native $P2X_1$ receptor currents in megakaryocytes were activated by α,β-meATP (10 μM) and commercial samples of ADP (10 μM), but not by purified ADP (10–100 μM).

3 Purified ADP (up to 1 mM) was also inactive at recombinant human $P2X_1$ receptors expressed in *Xenopus* oocytes. Purification did not modify the ability of ADP to activate P2Y receptors coupled to Ca^{2+} mobilization in rat megakaryocytes.

4 In human platelets, $P2X_1$ and P2Y receptor-mediated $[Ca^{2+}]_i$ responses were distinguished by their different kinetics at 13°C. In 1 mM Ca^{2+} saline, α,β-meATP (10 μM) and commercial ADP (40 μM) activated a rapid $[Ca^{2+}]_i$ increase (lag time $\leqslant$0.5 s) through the activation of $P2X_1$ receptors. Hexokinase treatment of ADP shifted the lag time by $\approx$2 s, indicating loss of the $P2X_1$ receptor-mediated response.

5 A revised scheme is proposed for physiological activation of P2 receptors in human platelets. ATP stimulates $P2X_1$ receptors, whereas ADP is a selective agonist at metabotropic ($P2Y_1$ and $P2T_{AC}$) receptors.

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Abbreviations: α,β-meATP, α,β-methyleneadenosine 5′-triphosphate; Ap_nA, diadenosine polyphosphates; EGTA, ethylene glycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid; HEPES, N-[2-hydroxyethyl]piperazine-N′-[2-ethane-sulphonic acid]; HPLC, high performance liquid chromatography

Introduction

P2 receptors for nucleotides are located on the extracellular surface of a variety of mammalian cell types including neurons, smooth muscle cells and a range of haematopoietic cells including megakaryocytes and platelets (Kunapuli & Daniel, 1998; Ralevic & Burnstock, 1998). They can be divided into two main classes according to their signalling mechanism (Fredholm *et al*., 1997); P2X receptors are ligand-gated cation channels, whereas metabotropic P2Y receptors are G-protein coupled receptors. Recombinant P2Y receptors couple through the activation of phospholipase-C. In addition, native P2Y receptors have been described that couple to adenylyl cyclase and ion channels (North & Barnard, 1997). To date, seven types of P2X receptor ($P2X_{1-7}$) and at least five types of P2Y receptor ($P2Y_{1,2,4,6,11}$) have been identified at the molecular level (Ralevic & Burnstock, 1998; Kunapuli & Daniel, 1998; Evans *et al*., 1999; Boeynaems *et al*., 2000). Frequently, multiple purinoceptor subtypes co-exist in the same cell, however their relative importance to physiological function is only just beginning to be elucidated (Boarder & Hourani, 1998).

$P2X_1$ receptors are the major ionotropic purinoceptor in blood platelets and smooth muscle (Vulchanova *et al*., 1996; Mackenzie *et al*., 1996; Vial *et al*., 1997; Mulryan *et al*., 2000). They are characterized by rapid desensitization and a pharmacological profile of 2-methylthio-ATP$\geqslant$ATP $>\alpha,\beta$Me-ATP$>$L-β,γ-me-ATP$>$ADP (Valera *et al*., 1994; Evans *et al*., 1995). ATP is co-stored and co-released with a number of 'classical' neurotransmitters (e.g. noradrenaline and acetylcholine) and leads to the activation of P2X receptors during synaptic transmission (Burnstock, 1990). However, during haemostasis, both ADP and ATP are released at high concentrations from platelet dense granules (Holmsen & Weiss, 1979) and ADP levels may initially rise whilst ATP levels are falling due to the action of ectonucleotidases (Boeynaems & Pearson, 1990). Thus, following vascular damage, both ADP and ATP are potentially important stimuli of smooth muscle, endothelial and blood cell purinoceptors. ADP has long been recognized as an important activator of human platelets (Gaarder *et al*., 1961; Born, 1962; Kunapuli & Daniel, 1998), whereas ATP is normally regarded as an inhibitor of this cell type, *via* its antagonistic action at platelet metabotropic purinoceptors (Hourani & Hall, 1994; Kunapuli & Daniel, 1998). The discovery of $P2X_1$ receptors on platelets has brought into question the purely antagonistic view of ATP during haemostasis, especially considering the large difference in EC_{50} values reported for activation of recombinant $P2X_1$ receptors by ATP and ADP (0.8 and 34 μM, respectively; Evans *et al*., 1995). Furthermore, $P2X_1$ receptors have recently been suggested to couple to a functional response in platelets (Rolf & Mahaut-Smith, 2000) and ATP will be the principal nucleotide directly released from the cytoplasm following injury. In view of the concerns that commercial samples of nucleotide diphosphates can be contaminated by nucleotide triphsophates (Nicholas *et al*., 1996; Hechler *et al*., 1998b), we have re-examined the relative activity of ADP and ATP on recombinant $P2X_1$ receptors and native $P2X_1$-like receptors in megakaryocytes and platelets. We find that purified ADP is

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completely inactive at $P2X_1$ receptors and that previous reports of ADP-evoked activation of this receptor can be entirely accounted for by contamination of commercial samples by ATP. A revised scheme for activation of different platelet P2 receptor subtypes is proposed.

Methods

Materials

Fura-2AM was obtained from Molecular Probes, Europe (Leiden, The Netherlands). Hexokinase (EC 2.7.1.1, from yeast) was purchased from Boehringer Mannheim (Lewes, East Sussex, U.K.). 'Commercial' ADP, type V or VII apyrase, α,β-meATP and all other chemicals were obtained from Sigma (Poole, Dorset, U.K.). HPLC-purified ADP was a kind gift of Prof M.R. Boarder, De Montfort University, Leicester.

Solutions

Two types of external or patch pipette salines were used during patch clamp and $[Ca^{2+}]_i$ recordings, designated 'A' or 'B'. External solution 'A' contained (in mM): NaCl 150, HEPES 10, KCl 2.5, $MgCl_2$ 1, pH 7.3 (NaOH), with or without 2.5 mM $CaCl_2$. External solution 'B' contained (in mM): NaCl 145, KCl 5, $MgCl_2$ 1, $CaCl_2$ 1, HEPES 10, glucose 10, pH 7.35 (NaOH). $CaCl_2$ was replaced by 1 mM $MgCl_2$ for Ca^{2+}-free solution 'B'. Patch pipette saline 'A' contained (in mM): Kgluconate 140, NaCl 5, EGTA 9, HEPES 10, pH 7.3 (KOH). Patch pipette saline 'B' contained (in mM): KCl 150, $MgCl_2$, 2 EGTA 0.1, Na_2EGTA 0.05, K_5fura-2 0.05, HEPES 10, pH 7.2 (KOH). 'A' salines were used for all recordings of $P2X_1$ receptor currents in megakaryocytes; the external saline was Ca^{2+}-free since the $P2X_1$ response was more stable in the absence of Ca^{2+}, as described in an earlier study by Kawa (1996). 'B' salines were used for all $[Ca^{2+}]_i$ recordings from rat megakaryocytes and human platelets, with or without external Ca^{2+} as indicated. Oocyte voltage clamp recordings used an extracellular solution of the following composition (mM): NaCl 96, KCl 2, HEPES 5, sodium pyruvate 5, $MgCl_2$ 1, $BaCl_2$ 1.8 and both electrodes were filled with 3 M KCl.

Enzymatic degradation of contaminating ATP within commercial ADP solutions was achieved by incubation of a stock of 10 mM ADP in Ca^{2+}-containing external solution 'A' at pH 8, with 22 mM glucose and 3 u ml^{-1} hexokinase for 1 h at 37°C. A similar procedure has been used to remove UTP contaminations from within commercial stocks of UDP (Nicholas *et al*., 1996).

Cell preparation

Megakaryocytes were prepared from the femoral and tibial marrow of MF1 mice or adult male Wistar rats using the protocol previously described for the rat (Mahaut-Smith *et al*., 1999). The saline used for the megakaryocyte isolation was either external saline 'A' or 'B' (see above) with Ca^{2+} and 0.16–0.32 u ml^{-1} apyrase. Human blood was drawn from the antecubetal vein of informed donors and platelets isolated and loaded with fura-2 as described previously (Mackenzie *et al*., 1996; Rolf & Mahaut-Smith, 2000). All platelets used in the present study were treated with aspirin (100 μM) and exposed to apyrase (0.16 or 0.32 u ml^{-1}) in order to reduce the risk of spontaneous activation by endoperoxide metabolites and adenosine nucleotides, respectively.

Heterologous expression

Stage V oocytes from *Xenopus laevis* were isolated and human $P2X_1$ receptor cRNA injected into each oocyte as described previously (Evans *et al*., 1995).

$[Ca^{2+}]_i$ measurements

Cuvette fluorescence measurements of $[Ca^{2+}]_i$ in fura-2-loaded human platelets were performed essentially as described previously (Mackenzie *et al*., 1996; Rolf & Mahaut-Smith, 2000). The cuvette temperature was lowered to 13°C using a combination of a reduced ambient temperature and Peltier effect devices (RS Components Ltd, Corby, U.K.). Standard methods to calibrate the fura-2 fluorescence were complicated by a temperature-dependent shift in the properties of the dye in the cytoplasm compared to free solution. The effect could not be accounted for by a simple shift in minimum and maximal ratio, as shown for viscosity, therefore background-corrected 340/380 nm ratios are presented as an indication of $[Ca^{2+}]_i$. Measurements of $[Ca^{2+}]_i$ in single rat megakaryocytes were conducted following introduction of fura-2 free acid into the cell from a patch pipette under whole-cell patch clamp as described in detail elsewhere (Mahaut-Smith *et al*., 1999).

Electrophysiological recordings and agonist application

Conventional whole-cell patch clamp recordings from megakaryocytes were performed using an Axopatch 200B or 200A with CV-202 headstage (Axon Instruments, CA, U.S.A.). Correction for liquid : liquid junction potentials was applied *a priori* for experiments with pipette saline 'A'. Two electrode voltage clamp recordings of *Xenopus* oocytes were conducted using a Geneclamp 500B amplifier (Axon Instruments). Membrane currents were acquired using a Digidata 1200 analogue to digital converter in combination with pClamp 7 acquisition software (Axon Instruments). For measurements of ionotropic purinoceptor currents, agonists were applied from a nearby U-tube perfusion system (Evans & Kennedy, 1994). Repeated agonist exposures were separated by 5 min in order to allow maximal recovery of P2X receptors from desensitization. During measurement of $[Ca^{2+}]_i$ responses in rat megakaryocytes, agonists were applied through a gravity-driven perfusion system. For cuvette fluorimetric measurements of $[Ca^{2+}]_i$ in human platelets; agonists were injected from Hamilton syringes inserted into a custom lid (Rolf & Mahaut-Smith, 2000). The timing of agonist application was recorded electronically to allow analysis of response times off-line. Lag times for agonist-evoked $[Ca^{2+}]_i$ responses were measured as the first detectable increase above the resting level.

Statistical values are shown as the means $\pm$ s.e.mean. Differences between means were tested by Student's unpaired *t*-test for significance at $P<0.05$ and $P<0.01$. Concentration response data were fitted with the equation response $= \alpha(A)^H/([A]^H + [A_{50}]^H)$ where a is the asymptote, H is the hill coefficient, A is the agonist concentration and A_{50} is the concentration of agonist evoking 50% of the maximum response.

Results

Patch clamp studies of megakaryocytes have shown that the initial rapid response to application of extracellular adenosine nucleotides is the activation of a transient inward cation current (Somasundaram & Mahaut-Smith, 1994; Kawa, 1996). The rapid onset kinetics, permeability to both Ca^{2+} and Na^+

and sensitivity to α,β-meATP, together with molecular studies from platelets and megakaryocytic cell lines, indicate that the response is mediated by $P2X_1$ receptors (Valera *et al.*, 1994; Mackenzie *et al.*, 1996; Vial *et al.*, 1997). Using whole-cell patch clamped mouse megakaryocytes, we have compared $P2X_1$ receptor currents activated by α,β-meATP, ADP from a commercial source and ADP immediately after purification by HPLC (Figure 1a). At a holding potential of -60 mV, 10 μM α,β-meATP rapidly activated an inward current with a peak of 531 ± 112 pA (range 98–1119 pA, $n=9$) that started to desensitize during the 200 ms application. ADP from a commercial source evoked an inward current with similar kinetics but smaller amplitude (233.7 ± 42 and 333.3 ± 80 for 10 and 100 μM ADP respectively $n=3$ and 4). After HPLC purification, ADP (10 μM) had only a small effect (24.3 ± 4.8 $n=4$) (Figure 1a). In these cells, current responses to ADP mediated *via* P2Y receptors (Uneyama *et al.*, 1993; Somasundaram & Mahaut-Smith, 1994) were not observed due to the brief exposure to agonist, in addition to the Ca^{2+}-free external saline and high concentration of EGTA in the pipette saline. Based on HPLC analysis ATP is the most likely contaminant of ADP responsible for $P2X_1$ receptor activation, therefore we used hexokinase to convert ATP to ADP in the 'commercial' stock (Nicholas *et al.*, 1996; see Methods for conditions). Little activation of $P2X_1$ receptors was observed with hexokinase-purified ADP up to concentrations of 100 μM (peak inward current 23.7 ± 11.8 pA, $n=4$) (see Figure 1b). These data suggest that ATP contamination is responsible for the apparent activity of commercial samples of ADP at the megakaryocyte $P2X_1$ receptor.

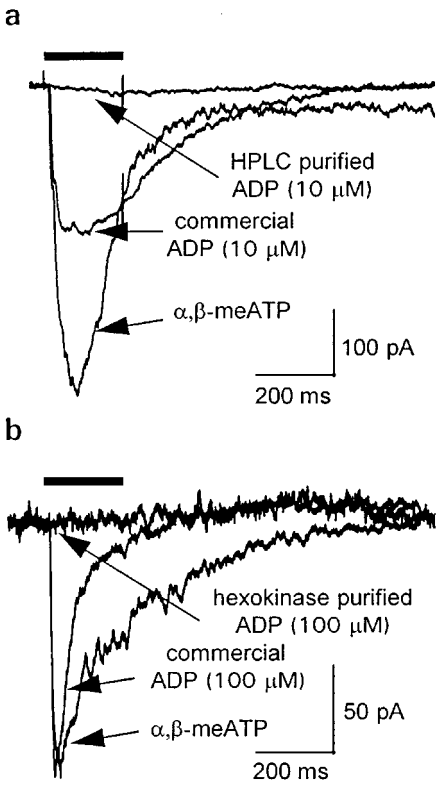

Figure 1 $P2X_1$ receptor currents in mouse megakaryocytes: responses to commercial grade and purified ADP. Whole-cell currents at a holding potential of -60 mV. Agonist applications (bar) were separated by 5 min to allow recovery of $P2X_1$ receptors from desensitization. (a) Representative traces during application of α,β-meATP, commercial ADP or HPLC-purified ADP, each at 10 μM. (b) Effect of commercially available and hexokinase-treated ADP (100 μM) compared to 10 μM α,β-meATP.

Commercial grade ADP also activated human $P2X_1$ receptors expressed in *Xenopus* oocytes. Typical responses to 100 μM commercial ADP and 100 μM ATP are shown in Figure 2a. Figure 2b shows the concentration-response relationships for ATP and commercial ADP. The EC_{50} for ATP was ~ 0.6 μM, and maximal responses were recorded at concentrations $\geqslant 10$ μM. Commercial ADP had an apparent EC_{50} value of ~ 40 μM. HPLC-purified ADP (10 μM) and hexokinase-treated ADP (up to 1 mM) were completely ineffective at evoking $P2X_1$ responses in oocytes (Figure 2a,b). This confirms the result obtained in the megakaryocytes (Figure 1) that contaminating ATP is most likely responsible for activation of $P2X_1$ receptors by commercially available ADP.

To assess the possibility that the lack of $P2X_1$ stimulation by HPLC-purified and hexokinase-treated ADP samples could result from reduced ADP activity, we conducted control experiments by measurement of $[Ca^{2+}]_i$ responses mediated

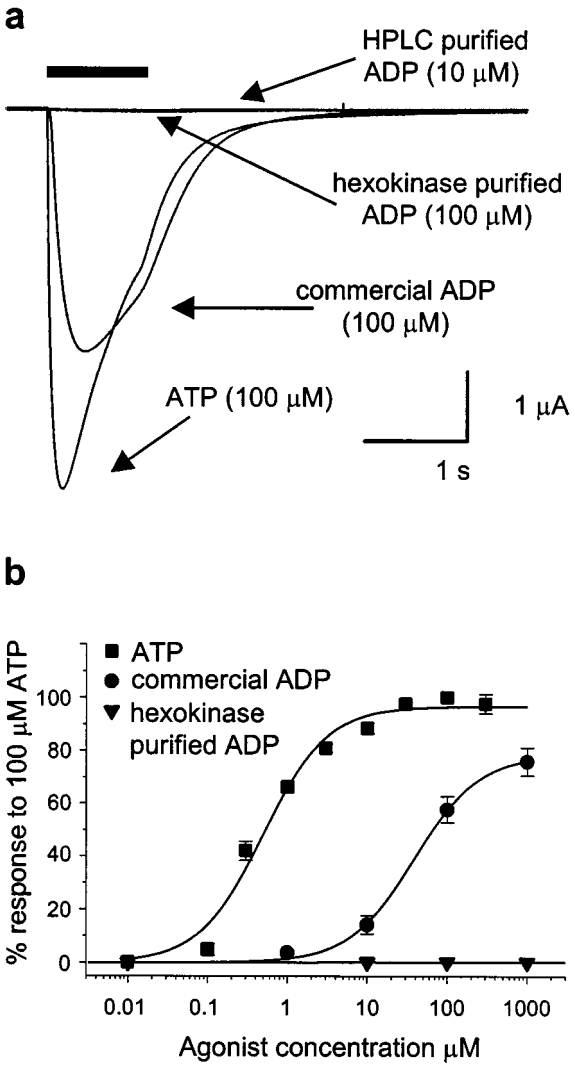

Figure 2 Human $P2X_1$ receptors expressed in *Xenopus* oocytes: responses to ATP, commercial grade ADP and purified ADP. $P2X_1$ receptor currents recorded by two-electrode voltage clamp at a holding potential of -60 mV. Agonist applications (bar) were separated by 5 min to allow recovery from desensitization. (a) Responses to ATP, commercial grade ADP, HPLC-purified ADP and hexokinase-treated ADP, each at 100 μM. (b) Average concentration:response relationships for ATP, commercial ADP and hexokinase-treated ADP, constructed from 4–6 oocytes.

through P2Y receptors in rat megakaryocytes. ADP activates this receptor at concentrations almost 30 fold lower than ATP (Uneyama *et al*., 1993; Somasundaram & Mahaut-Smith, 1994). Figure 3 shows that hexokinase-treated ADP (1 μM) evoked a robust $[Ca^{2+}]_i$ increase in the form of one or more spikes followed by a raised plateau level of $[Ca^{2+}]_i$, as reported previously (Mahaut-Smith *et al*., 1999). After wash and recovery of the $[Ca^{2+}]_i$ to resting levels, 1 μM unpurified ADP stimulated a response of similar magnitude to that evoked by hexokinase-treated ADP. Owing to receptor desensitization and/or effects of whole-cell dialysis, repeated responses to the same agonist displayed variations in amplitude and pattern of oscillations, as demonstrated by the reduced response following a second exposure to hexokinase-treated ADP. However, in a total of nine cells, 1 μM unpurified (commercial) ADP, evoked a response which was qualitatively indistinguishable from that observed with hexokinase-treated ADP (Figure 3). Similar results were obtained with HPLC-purified ADP (1 μM) on the megakaryocyte P2Y receptor-mediated $[Ca^{2+}]_i$ response (data not shown, $n=6$). These data confirm that the loss of activity at $P2X_1$ receptors following HPLC purification or hexokinase treatment results from removal of a contaminant rather than a loss of ADP-dependent activity.

The above data indicate that earlier reports of ADP-evoked currents through $P2X_1$ receptors (Valera *et al*., 1994; Evans *et al*., 1995; Kawa, 1996) result from ATP contamination of commercial ADP samples. Studies on human platelets have also reported that ADP stimulates $P2X_1$ receptors (Sage & Rink, 1987; Mahaut-Smith *et al*., 1990, 1992; Sage *et al*., 1991; Mackenzie *et al*., 1996). To investigate whether this response also results from ATP contamination, we conducted cuvette fluorescence measurements of $[Ca^{2+}]_i$ from suspensions of fura-2-loaded platelets. A reduced temperature was used to slow the kinetics of the response so that $P2X_1$ and metabotropic P2Y receptor-mediated rises in Ca^{2+} could be distinguished (Sage *et al*., 1990). Figure 4a compares the $[Ca^{2+}]_i$ responses at 13°C following selective activation of either $P2X_1$ or P2Y receptors in freshly isolated human platelets. At 13°C, the $[Ca^{2+}]_i$ increase evoked by 40 μM ADP in Ca^{2+}-free medium, which is due to activation of $P2Y_1$ receptors (Hechler *et al*., 1998b; Daniel *et al*., 1998), showed a delay to initial increase of 2.52 ± 0.06 s ($n=15$). On the other hand, the α,β-meATP-evoked Ca^{2+} influx was activated with faster kinetics. For 10 μM α,β-meATP, the time to initial $[Ca^{2+}]_i$ increase was 0.30 ± 0.02 s ($n=19$) and the response had significantly recovered prior to the start of the P2Y-mediated Ca^{2+} release. In Figure 4b, we compare $[Ca^{2+}]_i$ responses to commercial and hexokinase-purified ADP in the presence and absence of external Ca^{2+}. A sample trace for each condition is shown and the average lag times are shown in the bar chart in Figure 4c. In Ca^{2+}-free medium, the $[Ca^{2+}]_i$ responses to commercial and hexokinase-treated ADP had lag times of 2.52 ± 0.06 s ($n=15$) and 2.71 ± 0.06 s ($n=15$), respectively. This difference was significant at $P<0.05$ (Student's *t*-test), thus removal of ATP has only a small influence on the delay of the ADP-evoked Ca^{2+} release. In the presence of 1 mM external Ca^{2+}, commercial grade ADP evoked a $[Ca^{2+}]_i$ increase with a lag time of 0.50 ± 0.03 s ($n=16$), which was only slightly slower than the response to α,β-meATP (see above), suggesting a significant contribution from $P2X_1$ receptor-mediated Ca^{2+} influx. Hexokinase purification of the ADP resulted in a large shift in the lag time of the agonist-evoked $[Ca^{2+}]_i$ increase in Ca^{2+}-containing medium to 2.32 ± 0.05 s ($n=16$), which can be explained by a loss of $P2X_1$ receptor stimulation. The delay for hexokinase-treated ADP was slightly faster in the presence compared to the absence of external Ca^{2+} (2.32 ± 0.05 s versus 2.71 ± 0.06 s, respectively, significance at $P<0.01$). This small acceleration of the response by external Ca^{2+} has been reported previously for other agonists (Sage & Rink, 1987) and therefore could result from a non-specific effect of Ca^{2+} on the platelet membrane or on all metabotropic receptors rather than remaining receptor-operated Ca^{2+} influx. Taken together, these data indicate that removal of ATP contamination from ADP samples abolishes $P2X_1$ receptor stimulation in human

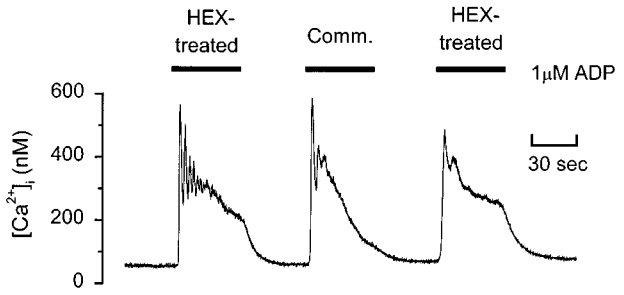

Figure 3 P2Y receptor-mediated $[Ca^{2+}]_i$ responses to commercial grade ADP and hexokinase-treated ADP in a rat megakaryocyte. Ratiometric fluorescence measurements of fura-2 were used to monitor $[Ca^{2+}]_i$ following introduction of the dye under whole-cell patch clamp conditions. Hexokinase-treated (Hex-treated) ADP or commercial (comm.) ADP were applied for the periods indicated by the bars. Holding potential was −75 mV.

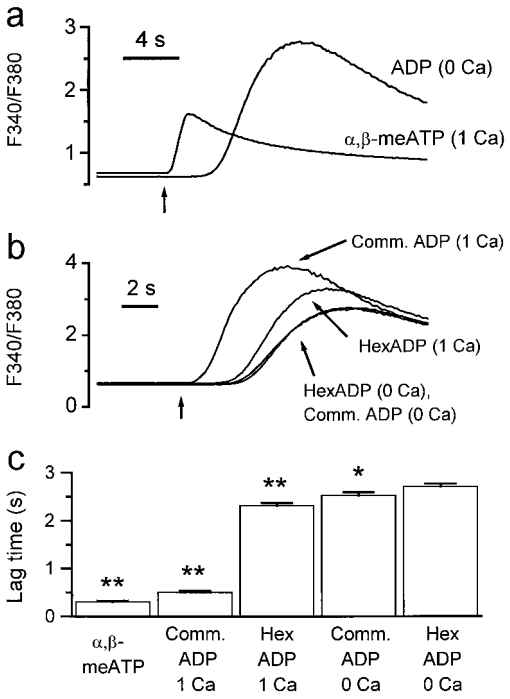

Figure 4 Effects of hexokinase purification on the ADP-evoked $[Ca^{2+}]_i$ responses of human platelets. $[Ca^{2+}]_i$ was monitored using the background-corrected 340/380 nm fluorescence ratio from stirred suspensions of fura-2-loaded human platelets at 13°C. The vertical arrow below the traces in (a) and (b), indicates injection of agonist. (a) Responses following selective activation of either $P2X_1$ receptors (α,β-meATP in 1 mM Ca^{2+}-containing saline) or P2Y receptors (ADP in nominally Ca^{2+}-free saline). (b) Responses to commercial grade ADP (comm. ADP) and hexokinase-treated ADP (hexADP) in the presence or nominal absence of external Ca^{2+} (1 mM). (c) Average lag times from 15–19 experiments for each of the conditions in (a) and (b). *$P<0.05$, **$P<0.01$, (Student's *t*-test) when compared to hexokinase-treated ADP in Ca^{2+}-free saline.

platelets, as described above for mouse megakaryocytes and human $P2X_1$ receptors expressed in *Xenopus* oocytes.

Discussion

Previous studies on ionotropic $P2X_1$ receptors have reported EC_{50} values for activation by ATP and ADP of 0.8 μM and 34 μM, respectively (Valera *et al*., 1994; Evans *et al*., 1995). Despite the low estimated activity of ADP at $P2X_1$ receptors, ADP and ATP are both released at high concentrations from platelet dense granules and the actions of nucleotidases can lead to a high ratio of ADP to ATP (Boeynaems & Pearson, 1990). However, we now show that apparent ADP activity at $P2X_1$ receptors is completely lost following removal of contaminants by HPLC purification or incubation with hexokinase to convert any ATP to ADP. The contaminant in the commercial ADP activated $P2X_1$ receptors expressed in *Xenopus* oocytes with an effective EC_{50} of $\sim$40 μM (Figure 2b), compared to 0.6 μM for ATP. Thus, ATP would only need to be present at 1–2% of the predicted ADP concentration in order to account for the actions of the contaminant. Indeed previous reports have indicated that commercial sources of nucleotide diphosphates are $\leqslant$99% pure and are contaminated by nucleotide triphosphates (Nicholas *et al*., 1996; Hechler *et al*., 1998b). Thus, ATP contamination is likely to account entirely for previous reports that unpurified/commercially available ADP activates native and cloned $P2X_1$ receptors (Valera *et al*., 1994; Evans *et al*., 1995). In addition, ATP rather than ADP accounts for the previous reports that ADP can activate rapid Ca^{2+} and Na^+ influx *via* non-selective cation channels in platelets and megakaryocytes (Sage & Rink, 1987; Sage *et al*., 1991; Mahaut-Smith *et al*., 1990; 1992; Mackenzie *et al*., 1996; Kawa, 1996). Differing levels of ATP contamination of ADP stocks may account for apparent discrepancies in ADP sensitivity at megakaryocytes reported previously (Somasundaram & Mahaut-Smith, 1994; Kawa, 1996).

ADP has been reported to be a weak agonist at other P2X receptors, which should be re-examined in light of the present study. The EC_{50} for ADP-dependent activation of P2X receptors is typically 1–3% of that reported for ATP which can be entirely accounted for by ATP contamination (Evans *et al*., 1995; Chen *et al*., 1995; Lewis *et al*., 1995; Buell *et al*., 1996; Surprenant *et al*., 1996).

ADP is an important platelet agonist (Gaarder *et al*., 1961; Born, 1962; Kunapuli & Daniel, 1998). The current model for the action of ADP on platelets proposes the involvement of three purinoceptors; the ionotropic $P2X_1$ receptor and two metabotropic purinoceptors, $P2Y_1$ and $P2T_{AC}$ (Kunapuli & Daniel, 1998). The $P2X_1$ receptor protein expressed by platelets is a ligand-gated ion channel and ATP binding opens the non-selective cation channel (Mackenzie *et al*., 1996; Vial *et al*., 1997). $P2Y_1$ receptors are coupled to Ca^{2+} mobilization *via* the heterotrimeric G-protein G_q, and to Rho/Rho kinase *via* other G-proteins, most likely $G_{12/13}$ (Leon *et al*., 1997; Jin *et al*., 1998; Paul *et al*., 1999). Selective activation of $P2Y_1$ receptors can account for ADP-dependent shape change, however co-activation of $P2Y_1$ and $P2T_{AC}$ is required for ADP-induced aggregation at all but high concentrations of ADP (Jin & Kunapuli, 1998; Hechler *et al*., 1998a; Savi *et al*., 1998; Fabre *et al*., 1999; Leon *et al*., 1999). The latter receptor has yet to be cloned and is termed $P2T_{AC}$ since it is coupled to inhibition of adenylyl cyclase *via* the heterotrimeric G-protein, G_i (Ohlmann *et al*. 1995). Whether the reduction in adenylyl cyclase activity is essential for the aggregation response is currently controversial (Daniel *et al*., 1999).

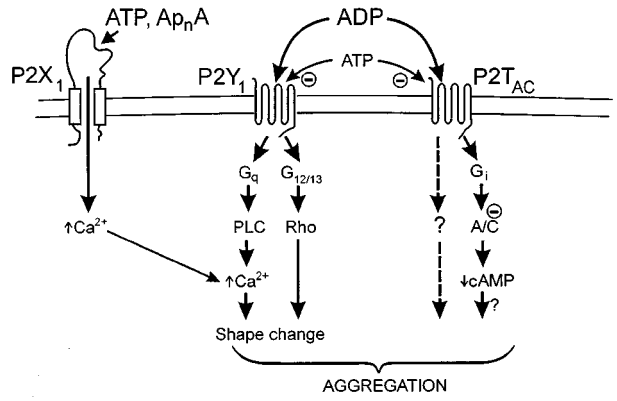

Figure 5 Model for activation of platelets *via* P2 receptors. A/C: adenylyl cyclase; PLC: phospholipase-C; G: G-protein. Ap_nA, diadenosine polyphosphates. See text for full explanation.

The findings of the present study that purified ADP is ineffective as an agonist at $P2X_1$ receptors at concentrations up to 1 mM, requires a redefinition of the platelet ADP receptors. ADP should be considered to stimulate platelets only *via* two receptors, $P2Y_1$ and $P2T_{AC}$, whereas ATP is an agonist at $P2X_1$ receptors and an antagonist at $P2T_{AC}$ receptors (Figure 5). ATP has low intrinsic efficacy at $P2Y_1$ receptors (Palmer *et al*., 1998) and its actions are dependent on the level of receptor expression. ATP is an antagonist of the ADP response at low levels of receptor expression (Leon *et al*., 1997; Palmer *et al*., 1998) whereas at higher levels ATP is an agonist (30 fold less potent than ADP, Palmer *et al*., 1998). As ATP is an antagonist at platelet $P2Y_1$ receptors this infers a low receptor density. The diadenosine polyphosphates, Ap_4A, Ap_5A and Ap_6A, are also $P2X_1$ receptor agonists (Sage *et al*., 1997; Wildman *et al*., 1999; Lewis *et al*., 2000) and are released from platelet dense granules (Flodgaard & Klenow, 1982; Jankowski *et al*., 1999), therefore may be important physiological activators of platelet ionotropic purinoceptors.

The role of $P2X_1$ receptors during platelet activation following vascular injury is presently unclear. Some groups have reported that α,β-meATP fails to stimulate shape change or aggregation in platelet suspensions and also does not affect the ADP-evoked functional responses, when measured turbidimetrically (Savi *et al*., 1997; Jin *et al*., 1998). However, this may be a consequence of receptor desensitization since a recent study reports that $P2X_1$-mediated shape changes are observed if steps are taken to reduce spontaneous activation during platelet isolation (Rolf & Mahaut-Smith, 2000). ATP is present in the cytoplasm at high concentrations (several mM) and will be one of the first agonists to stimulate platelets at the site of vascular injury. In support of this hypothesis, Born & Kratzer (1984) have shown that the initial increase in ATP levels detected at the site of vascular injury is due to release from damaged cells in the injured vessel wall. Activation of $P2X_1$ receptors may also potentiate the action of agonists at metabotropic receptors, since the resultant Ca^{2+} influx occurs more rapidly than release from intracellular stores (reviewed in Sage *et al*., 1997). The rapid Ca^{2+} influx could accelerate phospholipase-C activity (Eberhard & Holz, 1988) or IP_3-dependent Ca^{2+} release (Bezprozvanny & Ehrlich, 1995). Further studies are required to resolve the relative physiological and pathophysiological importance of ATP as a platelet agonist or antagonist.

In conclusion, the present study clearly demonstrates that ADP is not an agonist at $P2X_1$ receptors as had been previously thought. Activation of $P2X_1$ receptors during

haemostasis depends upon ATP, released from damaged cells or ATP and diadenosine polyphosphates secreted by platelets and other cells.

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