Three cation influx currents activated by purinergic receptor stimulation in rat megakaryocytes

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1. Simultaneous patch clamp and fura-2 fluorescence measurements were used to study ATP-evoked membrane currents and intracellular $[Ca^{2+}]$ ($[Ca^{2+}]_i$) changes in rat megakaryocytes.

2. At negative potentials, under conditions that blocked K^+ currents, 20 μM ATP activated a biphasic inward current and a concurrent biphasic increase in $[Ca^{2+}]_i$. The initial $[Ca^{2+}]_i$ increase was due to Ca^{2+} influx whereas the delayed ($1{\cdot}70 \pm 0{\cdot}13$ s, mean $\pm$ S.D.) increase was at least partly due to the release of internal Ca^{2+} stores.

3. The initial current was activated within 100 ms, inactivated within 1–4 s and was carried by both Na^+ and Ca^{2+}.

4. The delayed current was also transient and carried mainly by Na^+ when Ca^{2+} buffering in the pipette was low. This Na^+ conductance did not require an increase in $[Ca^{2+}]_i$ for activation, but was triggered by inositol 1,4,5-trisphosphate (IP_3), or a metabolite of IP_3.

5. Buffering of $[Ca^{2+}]_i$ changes with BAPTA revealed a third current activated by Ca^{2+} release from internal stores. This channel was selective for divalent cations with the permeability sequence $Ca^{2+} \gg Ba^{2+} > Mn^{2+}, Mg^{2+}$.

6. Adenosine-5′-*O*-3-thiotriphosphate (ATPγS), like ATP, evoked all three influx currents, whereas ADP only stimulated Ca^{2+} release and the two currents associated with it. Increasing the external divalent cation concentration abolished the ATP-evoked Ca^{2+} release and delayed currents but not the initial transient current.

7. We conclude that rat megakaryocytes express two types of purinergic receptor. One type, activated by ATP, is closely coupled to a non-selective cation channel. The second type, which recognizes ATP^{4-} and ADP^{3-}, activates Ca^{2+} release and two types of membrane conductance, one more selective for monovalent cations, the other highly selective for Ca^{2+}.

Platelets and their progenitor cells, megakaryocytes, share several properties including the ability to secrete endogenous nucleotides and the expression of purinergic receptors (Miller, 1983; Gordon, 1986; Hourani & Hall, 1994). The P_{2T}-type purinergic receptor of platelets is unique in that it has a higher affinity for ADP than for ATP (Gordon, 1986) and, interestingly, ATP acts as an antagonist for the ADP response. However, in the megakaryocyte both ADP and ATP are potent agonists (Uneyama, Uneyama & Akaike, 1993). This apparent difference in the ATP response may play a significant physiological role in megakaryocytic function. Calcium-activated potassium currents have been used to indirectly study the effects of ATP on intracellular $[Ca^{2+}]$ ($[Ca^{2+}]_i$) in megakaryocytes (Uneyama *et al.* 1993). However, little is known about ATP-induced calcium entry and its regulation in megakaryocytes. The present study has used patch clamp and simultaneous measurement of $[Ca^{2+}]_i$ to study ATP-evoked transmembrane currents and Ca^{2+} mobilization in rat megakaryocytes.

METHODS

Cell preparation

Adult male rats (Wistar), weighing 200–300 g, were killed by cervical dislocation. Bone marrow from the femoral and tibial bones was gently washed out using a standard external solution containing 20 μg ml^{-1} apyrase and 0·1% bovine serum albumin (BSA). After filtration through a fine cotton mesh the suspension was spun and washed twice before storage in the same standard solution. Megakaryocytes were clearly distinguished from other bone marrow cells under the

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microscope by their distinctive size (30–60 μm) and multi-lobular nucleus. Recordings were made at room temperature (20–23 °C) within 9 h of isolation.

Solutions and reagents

The standard external solution contained (mM): NaCl, 140; KCl, 5; $MgCl_2$, 1; $CaCl_2$, 2; glucose, 10; Hepes, 10 (pH 7·4, Tris). For K^+-, Na^+- and Ca^{2+}-free external media these ions were replaced by Cs^+, *N*-methyl-D-glucamine ($NMDG^+$) and Mg^{2+}, respectively. $CaCl_2$ was increased without substitution for other ions to obtain 10 mM Ca^{2+} saline. Isotonic divalent cation solution contained (mM): $CaCl_2$, 110 (or $BaCl_2$, 110); Hepes, 10; glucose, 10 (pH 7·4, Tris). The patch pipette solution contained (mM): caesium gluconate, 140; NaCl, 5; $MgCl_2$, 2; Hepes, 10; Na_2GTP, 0·1; fura-2, 0·05 (pH 7·4, Tris). For experiments using internal inositol 1,4,5-trisphosphate (IP_3), the pipette tip was dipped in IP_3-free saline and then backfilled with saline containing 10 μM IP_3. For a highly calcium-buffered pipette solution, 60 mM caesium gluconate was replaced by 20 mM Cs_4BAPTA. Cs_4BAPTA and K_5fura-2 were from Molecular Probes (Eugene, OR, USA). IP_3 was a gift of Dr R. F. Irvine, AFRC Babraham Institute, Cambridge, UK. All other reagents were from Sigma Chemical Co. (UK). A pressure injector was used to administer agonists from a patch pipette placed 150 μm from the cell. The time delay for arrival of agonists at the cell was measured and subtracted.

Electrophysiology

Patch clamp experiments were performed in tight-seal whole-cell configuration by means of an Axopatch 200A patch clamp amplifier (Axon Instruments, Foster City, CA, USA). Capacitative currents and series resistances (3–15 MΩ) were electronically compensated. Membrane currents during voltage ramps were filtered at 2 kHz and sampled at 100 ms using Axon Instruments hardware and pCLAMP software (Axon Instruments). Voltage ramps prior to agonist application were used to subtract linear leak currents from ATP-evoked currents. Currents were also continuously acquired at a rate of 60 Hz (filtered at approximately 30 Hz) by the Cairn Spectrophotometer (see below) for simultaneous display alongside the fura-2 fluoresence and agonist injection recordings. Liquid junction potentials were measured by reference to a 3 M KCl bridge and membrane potentials adjusted accordingly.

Fluorescence recordings

Fura-2 fluoresence measurements were made by single cell photometry using a Cairn Spectrophotometer system (Cairn Research Ltd, Kent, UK), coupled to a Nikon Diaphot inverted microscope (Nikon, UK). Excitation light passed through a spinning filter wheel assembly containing four 340 nm and two 380 nm bandpass excitation filters. Emitted light was selected by two (400–600 nm) dichroic filters and further filtered by a 485 nm long-pass filter. The combined output from all 340 and 380 nm excitation filters provided a 340/380 nm ratio for each revolution of the filter wheel. The signal was then averaged to give a ratio value every 67 ms. Background and cell autofluorescence was measured in the cell-attached recording mode and subtracted to give fura-2 fluorescence. $[Ca^{2+}]_i$ was calculated according to the following equation (Grynkiewicz, Poenie & Tsien, 1985):

$$[Ca^{2+}]_i = K_d \beta (R - R_{min}) / (R - R_{max}),$$

where K_d is the dissociation constant for fura-2 (250 nM, taken from Williams & Fay, 1990), β is the 380 nm fluorescence intensity at R_{min}/380 nm fluorescence intensity at R_{max}; R is the measured ratio; R_{min} is the ratio under Ca^{2+}-free conditions (pipette solution containing 20 mM Cs_4BAPTA; external solution containing 10 mM EGTA), and R_{max} is the ratio when fura-2 is saturated with Ca^{2+} (pipette solution containing 1 mM Ca^{2+}). Results are expressed as means ± standard deviation.

RESULTS

Characterization and time course of ATP-evoked $[Ca^{2+}]_i$ changes and inward currents

In order to permit measurement of Ca^{2+} and other inward currents, potassium currents, known to exist in megakaryocytes (Kawa, 1990; Uneyama *et al.* 1993), were eliminated using external and internal solutions in which K^+ was replaced by Cs^+. To minimize possible Cl^- currents we also held the cells close to the calculated Cl^- reversal potential of −73 mV. At a holding potential of −80 mV, 20 μM ATP evoked a biphasic inward current and a concurrent biphasic increase in $[Ca^{2+}]_i$ (Fig. 1*A*). At a holding potential of −80 mV, the first current was activated within 100 ms, had an amplitude of 2·8 ± 1·2 pA pF^{-1} ($n = 16$) and inactivated within 1–4 s, even in the continued presence of ATP. The second current was activated at the same time (1·7 ± 0·1 s, $n = 16$) as the second rise in $[Ca^{2+}]_i$, had an amplitude of 2 ± 1 pA pF^{-1} ($n = 16$) and inactivated within 2–5 s. In the absence of external calcium 20 μM ATP still evoked a biphasic inward current; however the first phase of the $[Ca^{2+}]_i$ increase was abolished (Fig. 1*B*). This suggested that the initial $[Ca^{2+}]_i$ increase was due entirely to an influx of calcium whereas the second rise in $[Ca^{2+}]_i$ was at least partly due to calcium mobilized from internal stores. Variability in the size of the secondary $[Ca^{2+}]_i$ increase prevented us from determining the extent to which Ca^{2+} entry contributed to this delayed increase. The close association of the delayed transient current with the second phase of $[Ca^{2+}]_i$ increase suggested that this current may be activated directly by $[Ca^{2+}]_i$. However, under conditions where $[Ca^{2+}]_i$ was strongly buffered using 20 mM Cs_4BAPTA in the pipette, the delayed current was not blocked (Fig. 1*C*). Instead, an additional non-inactivating current was observed which developed at the time corresponding to release of Ca^{2+} from internal stores. Repeated applications of ATP reactivated both the initial transient current and the delayed transient current on top of the sustained current. This suggested the existence of three inward currents at these negative potentials.

Ionic selectivity of the ATP-evoked currents

To examine the three different ATP-evoked inward currents further, current–voltage (I–V) relationships were obtained by applying voltage ramps in the range of −100 to +60 mV (0·6 mV ms^{-1}; 233 ms) during activation of each

current. In NaCl external solution and with 140 mM caesium gluconate internal solution, the initial transient current showed an inwardly rectifying $I-V$ relationship, reversing near +10 mV (Fig. 2*A a*). The rectification was not due to rapid inactivation of this current during the ramp since when cells were voltage clamped at positive voltages (+40 to +70 mV), ATP evoked a much smaller initial outward current in comparison with results at negative potentials (not shown). When external Na^+ was replaced by $NMDG^+$ the inward current was reduced and the reversal potential shifted to a more negative potential (−40 mV; Fig. 2*A b*). In 110 mM Ca^{2+} solution, ATP evoked a large inward current reversing at +40 mV (Fig. 2*A c*). A similar result was obtained using 110 mM $BaCl_2$ solution (not shown), except that little or no outward current was observed. This suggested that in 110 mM Ca^{2+} saline a large Ca^{2+} leak was increasing $[Ca^{2+}]_i$ and activating a Ca^{2+}-dependent outward current. Overall, the above results show that the channel carrying the rapid, transient ATP-evoked inward current is permeable to both monovalent and divalent cations.

The second current observed in 140 mM NaCl external solution and with 140 mM caesium gluconate internal solution displayed a fairly linear $I-V$ relationship which reversed near −10 mV (Fig. 2*B a*). Replacing the external Na^+ by $NMDG^+$ shifted the $I-V$ relationship such that only an outward current was observed in the range of potentials −80 to +60 mV (Fig. 2*B b*). The $NMDG^+$ solution contained 2 mM Ca^{2+}, suggesting that Ca^{2+} has a much lower permeability than monovalent cations or that Ca^{2+} is impermeant through this pathway. Alternatively, any small inward Ca^{2+} current may have been obscured by a contaminating outward Cl^- current. In an attempt to detect a possible Ca^{2+} current, we increased the external

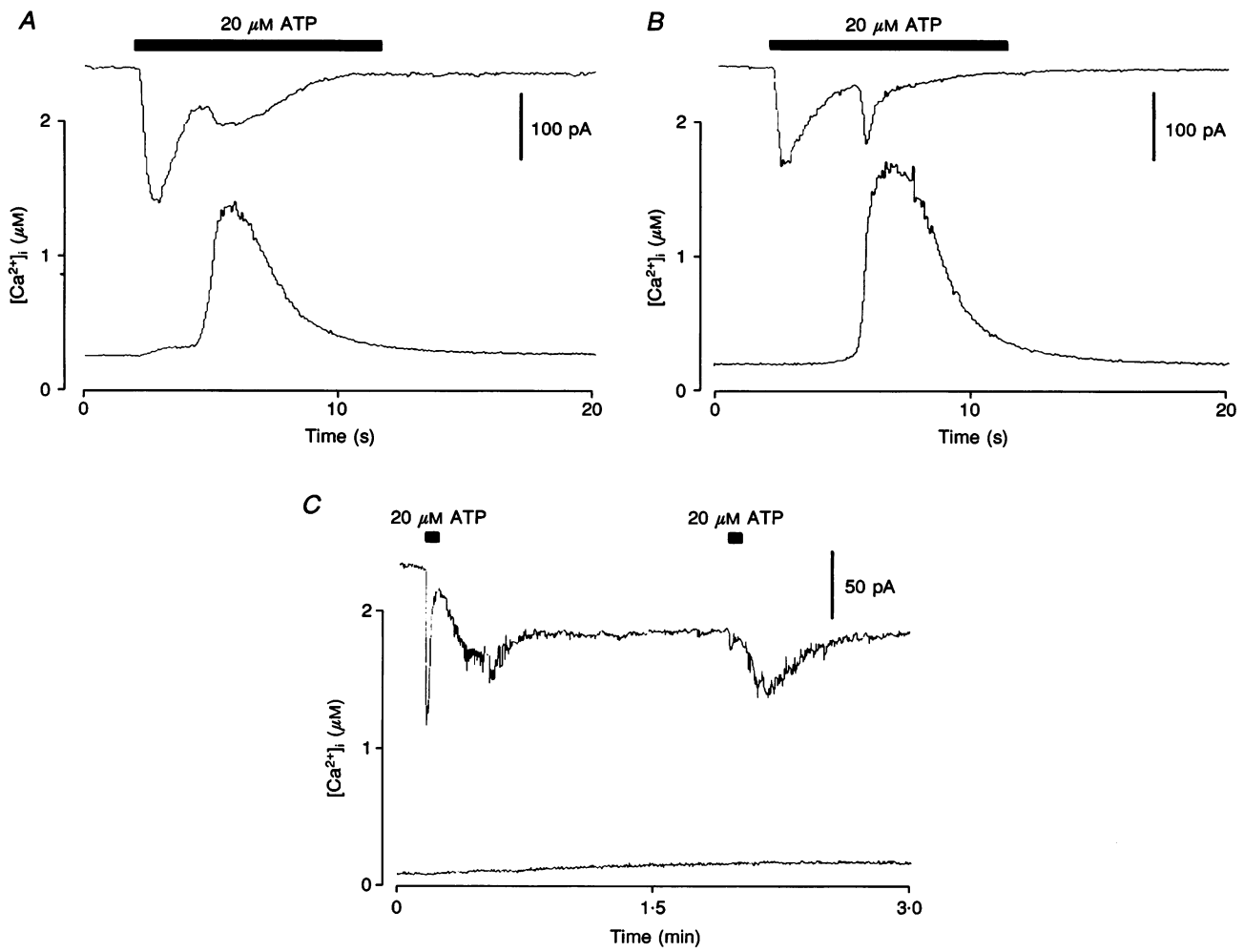

Figure 1. ATP-evoked inward currents and $[Ca^{2+}]_i$ changes
Effect of 20 μM ATP (10 s applications) on whole-cell voltage clamp currents (upper trace) and intracellular Ca^{2+} (lower trace) in the presence (*A* and *C*) or nominal absence (*B*) of 2 mM external Ca^{2+}. The bath contained 140 mM NaCl, 5 mM CsCl saline in all three experiments. The pipette saline in (*A*) and (*B*) was 140 mM caesium gluconate saline with 0·05 mM fura-2 as the only added Ca^{2+} buffer; in (*C*) the pipette saline contained 80 mM caesium gluconate with 0·05 mM fura-2 and 20 mM Cs_4BAPTA. See Methods for full details of the saline solutions. Holding potential was −80 mV in *A* and *B* and −60 mV in *C*.

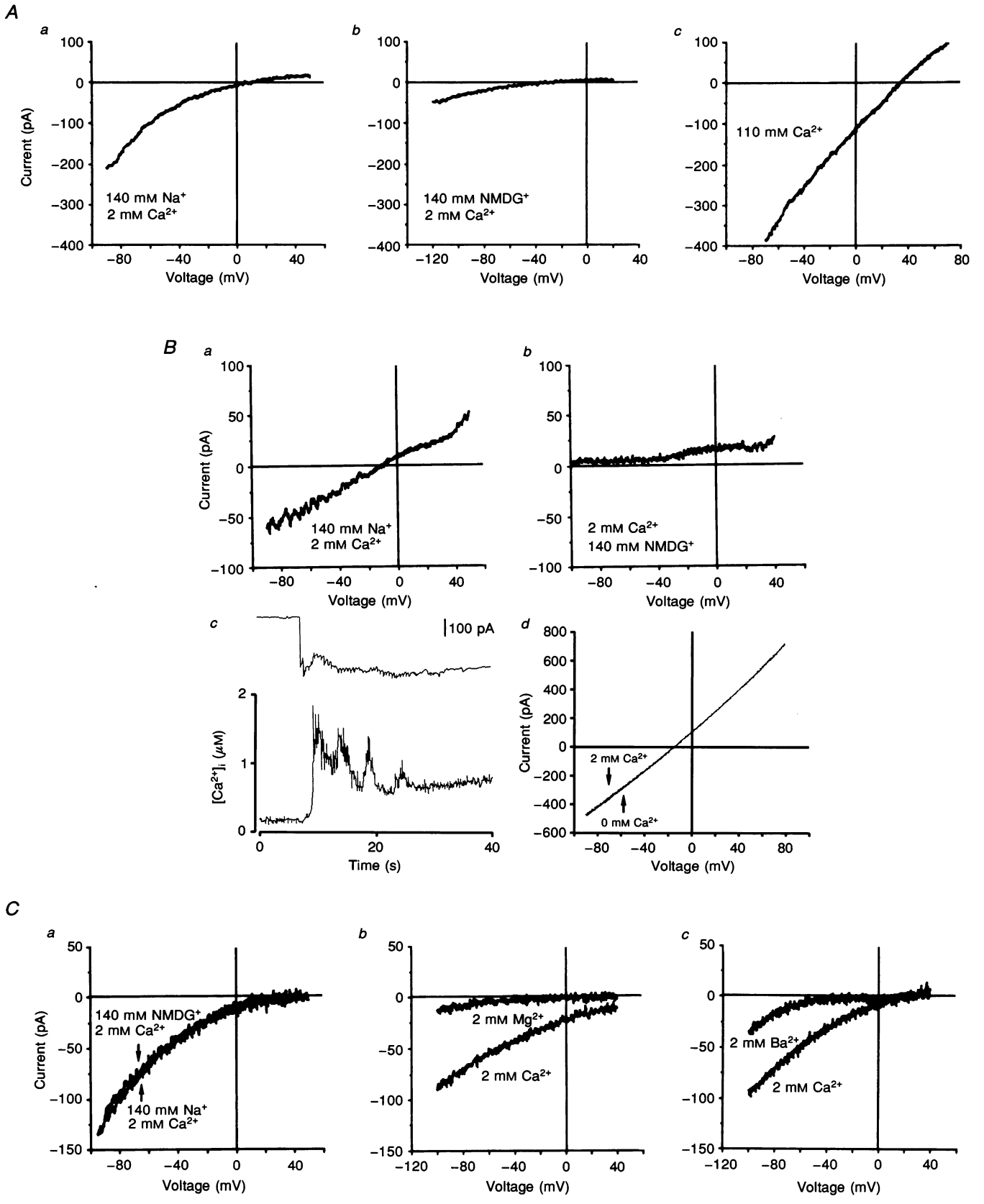

Figure 2. For legend see facing page.

calcium to 10 mM in the absence of any Na^+ (replaced by $NMDG^+$) or used isotonic calcium (110 mM Ca^{2+}) but under both conditions the release of internal Ca^{2+} and the second transient current evoked by 20 μM ATP were much reduced or absent (see below). Thus we were unable to clarify the Ca^{2+} permeability by this protocol. An increase in $[Ca^{2+}]_i$ was not required for activation of this second transient current, since it was still observed when $[Ca^{2+}]_i$ changes were prevented using 20 mM BAPTA in the pipette (see above and Fig. 2*C*). The fact that this current coincided with the release of stored Ca^{2+} suggested that IP_3 or another metabolite of inositol lipid metabolism was involved. To test this, pipettes were backfilled with 10 μM IP_3 (see Methods), and $[Ca^{2+}]_i$ and membrane current at −70 mV were monitored as IP_3 diffused into the cell. Using 140 mM caesium gluconate pipette saline and NaCl external saline, an inward current was activated which peaked 1–2 s before the initial increase in $[Ca^{2+}]_i$ and was sustained throughout the recording (Fig. 2*Bc*). The *I–V* relationship of the IP_3-dependent inward current was linear, reversed at about −15 mV, and was not affected by removal of external Ca^{2+} (Fig. 2*Bd*). The similarity of the IP_3-dependent current to the delayed ATP-evoked current suggests that they are mediated by the same pathway. The data also confirm that an increase in $[Ca^{2+}]_i$ is not required for activation of the current and that little or no Ca^{2+} is carried by the channel in normal (2 mM Ca^{2+}) saline.

Voltage ramps applied at the time of the sustained current activated by ATP in the presence of high internal Ca^{2+} buffering, revealed an inwardly rectifying *I–V* relationship reversing near +20 mV (Fig. 2*Ca*). Replacing the external Na^+ by $NMDG^+$ did not alter this current (Fig. 2*Ca*), whereas removal of external Ca^{2+} (replaced by Mg^{2+}, Fig. 2*Cb*) abolished the inward current. This third pathway also appeared to be more selective for Ca^{2+} over Ba^{2+} (Fig. 2*Cc*) and Mn^{2+} (not shown, relationship similar to that obtained for Mg^{2+} in Fig. 2*Cb*). The high selectivity of this channel for Ca^{2+} over other divalent cations and monovalent cations is similar to that reported for the channel activated by the release of Ca^{2+} from internal pools (termed I_{crac}; Hoth & Penner, 1992). The ATP-evoked Ca^{2+}-selective current in the megakaryocyte was similarly activated by store depletion since it could be induced by thapsigargin (1 μM) and by passive depletion of stores with high internal BAPTA (not shown).

Effects of ADP and ATPγS

Recently, Uneyama *et al.* (1993) have shown that the purinergic receptor of rat megakaryocytes, which evokes Ca^{2+} oscillations, has a higher affinity for ADP than ATP. We were thus concerned that, under our experimental conditions, the responses could be due to ADP produced from ATP by ectonucleotidases. However, this was unlikely since adenosine-5′-*O*-thiotriphosphate (ATPγS) (20 μM), a non-hydrolysable analogue of ATP, evoked a response similar to that of ATP (Fig. 3*A*). ADP, on the other hand, activated only the Ca^{2+} release and delayed current (Fig. 3*B*), even at supramaximal doses (50 μM, not shown). This implies the presence of two types of purinergic receptor: one activated by both ATP and ADP, the other selective for ATP. In high divalent cation saline solutions (10 or 110 mM $CaCl_2$ or 110 mM $BaCl_2$), the initial transient current was still activated by ATP, whereas the Ca^{2+} release and second current were very much reduced or abolished (Fig. 3*C*). Increasing the divalent cation concentration reduces the ATP^{4-} concentration (Uneyama *et al.* 1993) and this result provides further evidence for two types of purinergic receptor on megakaryocytes. One recognizes ATP and is closely coupled to a non-selective cation channel. The other receptor recognizes ATP^{4-} and ADP^{3-}, which activates internal Ca^{2+} release and two cation currents, one carried by Na^+, the other by Ca^{2+}.

DISCUSSION

We present evidence for three separate cation currents in rat megakaryocytes activated by ATP under conditions which blocked K^+ channels. The first is a non-selective cation current, activated rapidly (within 100 ms) and

Figure 2. Ionic selectivity of ATP- and IP_3-evoked currents
Current–voltage relationships were obtained by voltage ramps (0·6 mV ms^{-1}) from −100 to +60 mV following activation by 20 μM external ATP or 10 μM internal IP_3. *A*, ramp currents for the initial transient ATP-evoked current in external solution containing 140 mM Na^+ and 2 mM Ca^{2+} (*a*), 140 mM $NMDG^+$ and 2 mM Ca^{2+} (*b*), and 110 mM Ca^{2+} (*c*). Pipette contained 140 mM caesium gluconate saline. *Ba* and *b*, ramp currents during the second transient ATP-evoked current with 140 mM caesium gluconate pipette saline and an external solution containing 140 mM Na^+ and 2 mM Ca^{2+} (*a*), and 140 mM $NMDG^+$ and 2 mM Ca^{2+} (*b*). *Bc*, effect of 10 μM IP_3 backfilled in the patch pipette (140 mM caesium gluconate saline) on membrane current (upper trace) and $[Ca^{2+}]_i$ (lower trace) at a holding potential of −70 mV. Record begins 10 s after obtaining the whole-cell configuration. *Bd*, ramp currents during IP_3-activated current in 140 mM Na^+ saline in the presence and absence of 2 mM Ca^{2+}. *C*, current–voltage relationship of the third sustained ATP-evoked current obtained with high internal Ca^{2+} buffering (20 mM BAPTA) and external solutions containing 140 mM Na^+, 2 mM Ca^{2+} or 140 mM $NMDG^+$, 2 mM Ca^{2+} (*a*), 2 mM Ca^{2+} or 2 mM Mg^{2+} (*b*), and 2 mM Ca^{2+} or 2 mM Ba^{2+} (*c*).

inactivated within a further 1–4 s. Under physiological conditions, at negative potentials, this current will be carried by a combination of Na^+ and Ca^{2+}, and in fact, as shown by Fig. 1*A* and *B*, can produce a significant increase in $[Ca^{2+}]_i$. The properties of this rapidly activated transient current are similar to the receptor-operated channel (ROC) of smooth muscle (Benham & Tsien, 1987) and that of the human platelet (Mahaut-Smith, Sage & Rink, 1992) except that ADP is an agonist in the platelet but not the megakaryocyte. The second current evoked by ATP coincided with the release of internally stored Ca^{2+} but did not require a rise in $[Ca^{2+}]_i$ for activation. IP_3, or another product of inositol lipid metabolism such as IP_4, appeared to be triggering this current. This second, transient conductance was permeable to monovalent ions and at negative membrane potentials would principally carry Na^+. We failed to detect a significant Ca^{2+} current through this conductance because high divalent cation saline solutions reduced the response, probably by lowering the concentration of ATP^{4-}. We were also unable to trace divalent cation influx through this pathway using Mn^{2+} influx and quench of fura-2 (B. Somasundaram & M. Mahaut-Smith, unpublished observations). Thus we are at present unable to estimate the exact Ca^{2+} permeability of this second ATP-evoked conductance, but it is likely to be small or negligible. The third ATP-evoked current was carried by a channel highly selective for Ca^{2+} and could be clearly distinguished when the pipette saline contained a high concentration of the Ca^{2+} chelator, BAPTA. This current was activated by the depletion of internal stores and will therefore also be activated during ATP-induced Ca^{2+} release. Under normal Ca^{2+} buffering, however, the contribution of this store-depletion-activated current to the delayed inward current was small since inward currents were no longer detected following removal of Na^+, but not Ca^{2+}, from the external medium (Fig. 2*Bb*).

Uneyama *et al.* (1993) have recently described a unique purinergic receptor in rat megakaryocytes recognizing

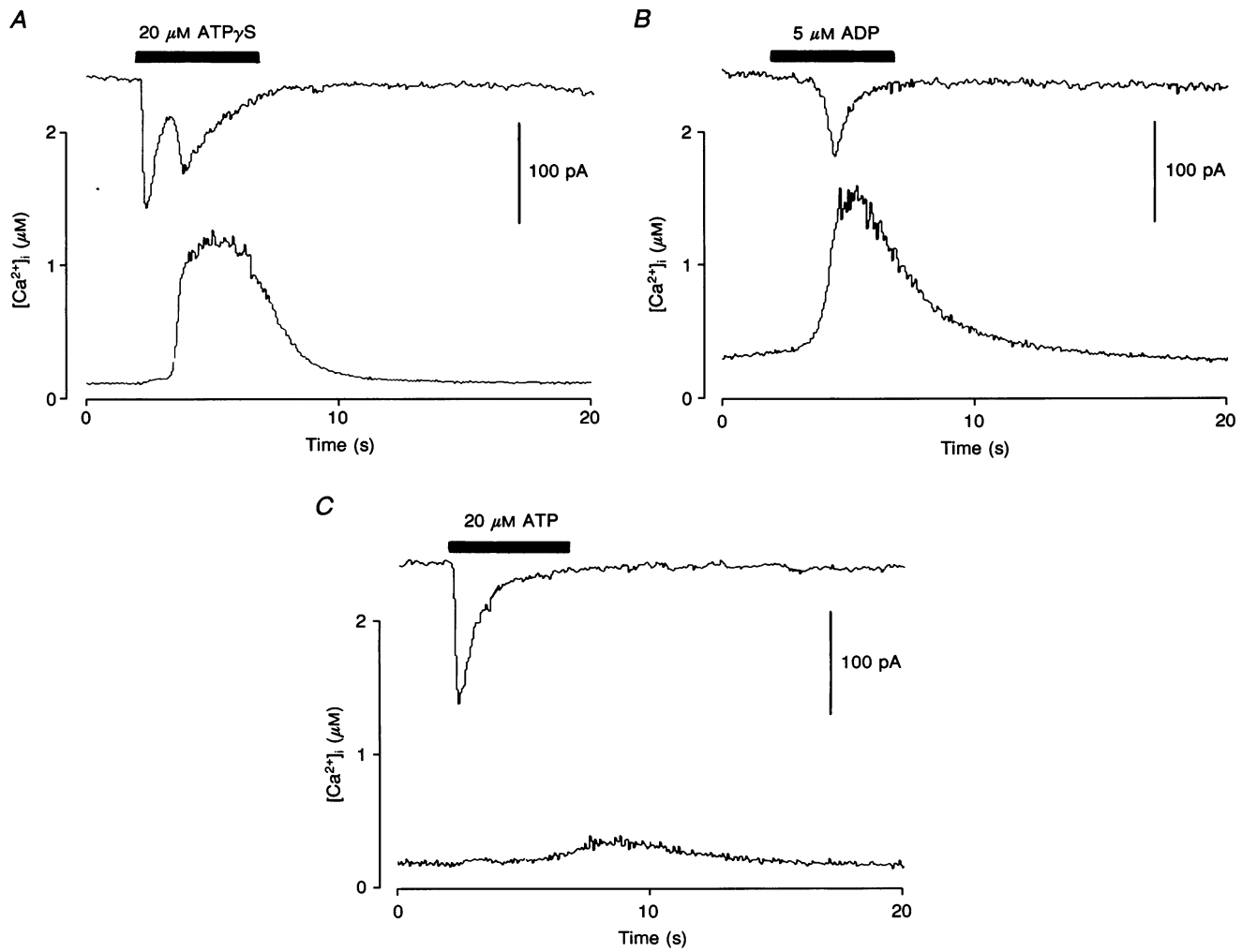

Figure 3. Inward currents and $[Ca^{2+}]_i$ rises evoked by ATPγS, ADP and ATP
Whole-cell voltage clamp currents (upper traces) and intracellular Ca^{2+} (lower traces) evoked by a 5 s application of 20 μM ATPγS (*A*) or 5 μM ADP in external saline with 2 mM Ca^{2+} (*B*), and 20 μM ATP in external saline with 10 mM Ca^{2+} (*C*). Membrane potential was −80 mV and internal saline was 140 mM caesium gluconate, low Ca^{2+} buffering (0·05 mM fura-2).

ATP^{4-} and ADP^{3-}, with a higher affinity for ADP^{3-}. This receptor mobilizes internally stored Ca^{2+}, presumably by stimulating phospholipase C and releasing Ca^{2+} from IP_3-dependent pools. The same receptor is probably responsible for the activation of Ca^{2+} release together with the delayed Na^+ and Ca^{2+} currents in our experiments because we also found that ADP and ATP-evoked Ca^{2+} release was absent in high divalent cation saline solutions, which reduce the ionized content of these nucleotides (Uneyama *et al.* 1993). The rapidly activated non-selective cation current was coupled to a different receptor since ADP was not an agonist and ATP could act in isotonic divalent cation saline solutions. Uneyama *et al.* (1993) do not describe this first current, which may be explained by a difference in their isolation procedure. We used apyrase in all media to break down ATP and ADP released from damaged cells and reduce receptor desensitization, whereas Uneyama *et al.* (1993) appear not to have used this enzyme. At present, we cannot explain why a rapidly activated non-selective cation current is activated by ATP, but not ADP, in the rat megakaryocyte and yet is activated by ADP in the human platelet (Mahaut-Smith *et al.* 1992). There may be a species difference, although the rat platelet also shows a rapid Ca^{2+} increase in response to ADP (Heemskerk, Feijge, Sage & Walter, 1994), indicative of an ion channel similar to that of the human platelet. However, patch clamp studies have not been performed on the rat platelet and ATP has not been tested on human platelet membrane currents.

As for the relative roles of these currents in purinergic receptor signalling, we can only speculate at this stage. The rapidly activated non-selective cation channel may act as a primer for Ca^{2+} release as the IP_3 receptor is somewhat Ca^{2+} sensitive (Finch, Turner & Goldin, 1991). Both this first current and the second, transient current will carry a significant amount of Na^+ into the cytoplasm. It has been suggested that Na^+ has an important role in the spreading reaction of the megakaryocyte (Leven, Mullikin & Nachmias, 1983), which may be a functional megakaryocyte response leading to platelet production. The third current, activated by the release of Ca^{2+} from internal stores, is a major component of the Ca^{2+} influx in a variety of cell types lacking voltage-gated Ca^{2+} channels. We have yet to test the contribution of this pathway to the overall $[Ca^{2+}]_i$ response in the rat megakaryocyte, but with its high selectivity for Ca^{2+}, this current should allow significant Ca^{2+} influx for Ca^{2+}-dependent signalling either directly, or indirectly by maintaining a charged internal pool of Ca^{2+}. The megakaryocyte is a developing cell which must eventually express most, if not all, of the membrane conductances of platelets, which bud off directly from the megakaryocyte membrane. It is likely that the three conductances we describe here are incorporated into the platelet membrane and have specific roles in haemostasis. With the much larger surface area to volume ratio of the platelet, the cation conductances reported here will be capable of rapidly altering the Na^+ and/or Ca^{2+} content of the platelet cytosol.

In conclusion, we have demonstrated the existence of three cation conductances activated by ATP in rat megakaryocytes. These currents will carry Na^+ and Ca^{2+} into the cell at negative potentials and may have a functional role in megakaryocyte signalling or be expressed only for later use in platelet responses. The data also suggest that megakaryocytes express two types of receptors, one selective for ATP over ADP, the other recognizing both ADP^{3-} and ATP^{4-}.

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