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GUINEA-PIG MEGAKARYOCYTES CAN RESPOND TO EXTERNAL ADP BY ACTIVATING Ca^{2+}-DEPENDENT POTASSIUM CONDUCTANCE

BY KAZUYOSHI KAWA

From the Department of Pharmacology, Gunma University School of Medicine, Maebashi 371, Japan

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SUMMARY

1. The responses of megakaryocytes to adenosine diphosphate (ADP) were studied using whole-cell patch electrodes and a Ca^{2+}-sensitive fluorescent dye, Fura-2. Megakaryocytes (diameter, 17–42 μm) were mechanically dissociated from the bone marrow of adult guinea-pigs and ADP (1–10 μM) was pressure-applied to megakaryocytes under recording.

2. In megakaryocytes immersed in standard saline, ADP evoked an obvious outward current at a membrane potential of −63 mV. The current was identified as a K^+-carried current, since the reversal potential depended distinctly on the external K^+ concentration, but it showed no changes after removal of external Na^+. The amplitude of evoked K^+ currents showed considerable intercell variation, which is presumably due to differences of current density in the membrane.

3. During application of ADP, the evoked K^+ current was not sustained but slowly decayed to become negligible within 10–20 s, suggesting the appearance of desensitization. The response of the megakaryocyte to ADP recovered slowly and returned to an original level after 4–5 min of continuous washing.

4. When the intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) was measured using the Ca^{2+}-sensitive fluorescent dye, Fura-2, application of 10 μM-ADP induced an increase of $[Ca^{2+}]_i$ by about 5-fold, which was followed by a gradual decay to the original level within 30–50 s. Roles of internal Ca^{2+} for activating the K^+ current were confirmed by observing (1) enhancement of evoked currents by the use of internal saline containing no Ca^{2+} chelators and (2) generation of prolonged K^+ current by application of a Ca^{2+} ionophore, A23187, to the megakaryocyte.

5. In a fraction of the megakaryocytes, spontaneous hyperpolarization of the resting membrane potential was observed. The hyperpolarization seemed to result from the activation of K^+ channels in the membrane, which was caused by spontaneous release of Ca^{2+} from the internal storage site.

6. It was concluded that megakaryocytes of the guinea-pig can respond to external ADP by increasing $[Ca^{2+}]_i$ and consequently by activating Ca^{2+}-dependent K^+ channels in the membrane.

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INTRODUCTION

Adenosine diphosphate (ADP) is one of the most potent activators of mammalian platelets, inducing aggregation as well as transformation of platelets and release of their granule contents to facilitate plug formation (Born, 1962; Born, Dearnley, Foulks & Sharp, 1978; Zucker, 1980; Frojmovic & Milton, 1982; Siess, 1989). The molecular mechanisms underlying these activation phenomena have been studied extensively and intracellular free Ca^{2+} has been recognized as one of the key substances involved (Hallam & Rink, 1985; Siess, 1989; Kroll & Schafer, 1989). Recently, using a Ca^{2+}-sensitive fluorescent dye, Fura-2, on platelets, an increase in the intracellular concentration of free Ca^{2+} ($[Ca^{2+}]_i$) was demonstrated upon exposure of the platelets to ADP (Sage & Rink, 1987; Pedersen & Reichelt, 1988). However, detailed physiological analyses of the ADP response in the platelet membrane have been limited, presumably due to their small size and fragility. Under whole-cell patch clamping in the platelets, attempts to detect obvious responses on exposure to ADP have been unsuccessful (Maruyama, 1987).

In the present study, we employed megakaryocytes of the guinea-pig as a primary model of the platelet membrane and studied ADP-induced responses in the membrane using the patch-clamp technique. These cells are known to have basically similar characteristics to those of platelets and thus have often been used as functional models of platelets (Fedorko, 1977*a*, *b*; Fedorko, 1978; Miller, 1983; Schick, Walsh & Jenkins-West, 1988). The responsiveness of isolated megakaryocytes to ADP has been studied morphologically including process formation and cellular spreading, showing similarities to those in platelets (Leven & Nachmias, 1982; Leven, Mullikin & Nachmias, 1983).

The freshly isolated megakaryocytes from guinea-pig bone marrow had diameters of 17–42 μm and the cells permitted stable recordings with whole-cell patch electrodes (Kawa, 1990). In these cells, ADP induced obvious membrane currents which were carried by K^+. The K^+ channels in the membrane responsible for these K^+ currents seemed to be activated by an increase of $[Ca^{2+}]_i$ in the megakaryocyte. The properties of the ADP response were further analysed using the Ca^{2+} ionophore A23187, or the Ca^{2+}-sensitive fluorescent dye, Fura-2.

METHODS

Preparation and electrical recordings

Megakaryocytes isolated from the bone marrow of adult guinea-pigs (Hartley strain, male) were used and the whole-cell variation of the patch-electrode voltage-clamp technique was applied. Procedures for cell preparation and whole-cell recording were the same as those described in the previous paper (Kawa, 1990). The resistance of patch electrodes ranged between 2 and 10 MΩ when filled with the internal solution. No compensation for leakage at the tip of the patch electrode was made in the present study. When ADP (adenosine-5′-diphosphate) was applied to a megakaryocyte under recording, a glass micropipette (diameter of orifice, 4–8 μm) filled with external saline containing ADP was positioned within a distance of 100 μm from the cell (Fig. 1*A*). To the other end of this micropipette, pneumatic pressure of about 3 kPA was applied to perfuse the cell locally with the saline containing ADP. Up to three micropipettes for local perfusion were operated independently with hydraulic three-dimensional manipulators (Narishige, Tokyo, Japan). The regulator for pneumatic pressure was a home-made apparatus using magnetic valves (3N-3X-12D;

General Valve Corp., NJ, USA). In some cases, external salines (listed in Table 1) were also applied by switching the perfusion media in the chamber.

Analysis of intracellular Ca^{2+} with Fura-2

Procedures used for isolation of megakaryocytes in the recording chamber were the same as those for electrical recordings. Adhesive polypeptide (Cell-Tak; Collaborative Research Inc., MA, USA),

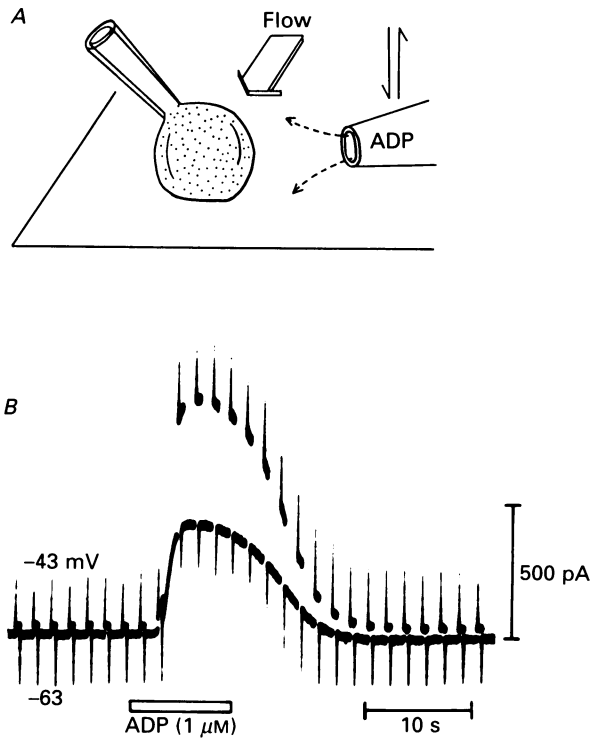

Fig. 1. *A*, schematic drawing of experimental arrangement. The patch electrode was applied to a megakaryocyte settled on the base of the recording chamber. The chamber was perfused constantly with external standard saline (thick arrow marked 'flow'), ADP was dissolved in the external saline and applied from a glass micropipette (tip diameter, 4–8 μm; pneumatic pressure 3 kPa) to the megakaryocyte. The glass micropipette was positioned within 100 μm of the cell with a hydraulic manipulator (thin dashed arrows). *B*, outward currents evoked by application of 1 μM-ADP. The membrane potential was voltage clamped at −63 mV and depolarizing pulses to −43 mV (duration, 420 ms) were superimposed every 1·5 s. Open bar under the trace indicates the duration of ADP application. Cell diameter, 19 μm.

which was coated on the base of the chamber, greatly assisted the stable settlement of megakaryocytes on the base. Fura-2 acetoxymethylester (Fura-2 AM) was first dissolved in dimethyl sulphoxide (DMSO) at a concentration of 1 mM, and 50 μl of this Fura-2 solution was mixed with 20 μl of a synthetic surfactant, cremophor EL (Sigma, MO, USA). Seventy microlitres of the mixture was then added to 10 ml of the external standard saline, and thoroughly dissolved by application of an ultrasonicator for 10–20 min; the final concentration of Fura-2 AM was 5 μM assuming complete dissolution of the added dose. Intracellular loading with Fura-2 was

accomplished by incubating megakaryocytes at 37 °C for 90–120 min in the chamber containing the external standard saline with 5 μM-Fura-2 AM. The measurement of Fura-2 fluorescence was carried out according to the method of Kudo & Ogura (1986) and Tsuzuki, Iino & Ozawa (1989). In brief, the chamber containing megakaryocytes after Fura-2 loading was mounted on the stage of an inverted epifluorescence microscope (objective ×40, Diaphot; Nikon, Japan). Fluorescence

TABLE 1. Composition of solutions (mM)

External solutions*	NaCl	KCl	$CaCl_2$	EGTA (sodium salt)	$MgCl_2$
Standard saline	120	5	10	—	1
0 mM-Ca^{2+} saline	135	5	—	—	1
1 mM-EGTA saline	135	5	—	1	1
25 mM-K^+ saline	100	25	10	—	1
125 mM-K^+ saline	—	125	10	—	1

Internal solutions†	KCl	CsCl	$CaCl_2$	EGTA (potassium salt)	$MgCl_2$
Simple KCl saline	150	—	—	—	1
Ca/EG-0·1 KCl saline	150	—	0·05	0·1	1
Ca/EG-0·5 KCl saline	150	—	0·25	0·5	1
Ca/EG-5 KCl saline	150	—	2·5	5	1
EG-5 KCl saline	150	—	—	5	1
Ca/EG-0·1 CsCl saline	—	150	0·05	0·1	1
EG-5 CsCl saline	—	150	—	5	1

* In external media, each saline also contained 10 mM-D-glucose and 10 mM-HEPES (sodium salt) to give a final pH of 7·3±0·1 at 25±1 °C.

† In internal media, each saline also contained 10 mM-HEPES (potassium salt) to give a final pH of 7·2±0·1.

images of Fura-2-loaded megakaryocytes were obtained by illumination with 340 and 380 nm light at 10 s intervals (light source, 100 W high-pressure mercury lamp equipped with neutral filters). The images were displayed on a video screen with the aid of highly sensitive video cameras (C2400-08H; Hamamatsu Photonic Co., Japan). The brightness of the video image was converted to a digital signal by a video frame memory with an A–D converter and fed into a computer-equipped image-analysis unit (Argus-100/CA, Hamamatsu Photonic Co., Japan). Calculation of the ratio of fluorescence intensity at 340 nm (F340) to that at 380 nm (F380), and reference of the value to a calibration curve, yielded the absolute value of intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$). The calibration curve for $[Ca^{2+}]_i$ was obtained using solutions of known $[Ca^{2+}]_i$, which contained 10 μM-Fura-2 (potassium salt), 150 mM-KCl and 20 mM-PIPES (piperazine-*N*,*N*′-bis(2-ethanesulphonic acid)-KOH (pH 7·0), and EGTA (ethyleneglycol-bis-(β-amino-ethylether)-*N*,*N*′-tetraacetic acid) and $CaCl_2$ in a calculated ratio (Kudo & Ogura, 1986).

Solutions

The compositions of the external and internal (inside the patch electrode) solutions are listed in Table 1. The osmolalities were measured as described previously (Kawa, 1990) and were 280±5 and 290±5 mosmol/kg H_2O for the external and internal salines, respectively. The free Ca^{2+} concentrations in internal media were estimated by calculation as described (Kawa, 1987) and were 61, 61, 59 nM and less than 1 nM for Ca/EG-0·1, Ca/EG-0·5, Ca/EG-5 and EG-5 KCl salines, respectively. For Ca/EG-0·1 and EG-5 CsCl salines, the calculated value was 61 nM and less than 1 nM, respectively. ADP had been dissolved in external media and stored in small vials (0·5–1 ml) at −70 °C. These media were thawed immediately before use. They were kept at 4 °C and used within 4 h after thawing. Calcium ionophore A23187 was dissolved in dimethyl sulphoxide (DMSO)

at a concentration of 1 mM and further dissolved in external media at a final concentration of 10 μM before use. ADP (sodium salt from equine muscle), EGTA and A23187 were obtained from Sigma Co. (St Louis, MO, USA). Fura-2 and Fura-2 AM were obtained from Molecular Probes Inc. (Eugene, OR, USA).

RESULTS

Outward currents induced by externally applied ADP

Time course of currents

Figure 1*B* shows a typical example of ADP-evoked currents recorded in a dissociated megakaryocyte using the whole-cell configuration. The pipette was filled with Ca/EG-0·1 KCl saline (free Ca^{2+} concentration, 61 nM). The cell was voltage clamped at −63 and −43 mV alternatively. Under the conditions used, activation of voltage-dependent membrane currents (see Kawa, 1990) as well as linear leakage currents at the tip of the patch electrode were relatively small, thus making the ADP-induced currents observable without serious disturbance. After application of ADP (open bar in Fig. 1*B*), the megakaryocyte showed obvious outward currents. Although the amplitude of the evoked currents was larger at −43 mV than at −63 mV, the time course of the currents was almost the same at −43 and at −63 mV. The currents reached a peak within several seconds and declined slowly to the original level in 10–20 s. Even when ADP was applied for a prolonged period (30 s), the decline of the currents was similar. In the above experiment, the micropipette used for delivering ADP was positioned at a distance of about 60 μm from the cell. The latency for the ADP response was 2–5 s. The real latency containing no time for diffusion of ADP to the cell would have been shorter, because the latency decreased to less than 2 s when the delivery micropipette was moved to the vicinity of the cell (within 20 μm).

Recovery from desensitization

One feature of the ADP-induced outward currents in megakaryocytes was decline of the current during ADP application. As the reversal potential did not seem to change during the decline of the current (see below, Fig. 4), the decline was unlikely to be caused by depletion of ions carrying the current, but probably due to reduction of the responsiveness to ADP in the megakaryocyte because of desensitization of the receptor site and/or diminution of transduction efficacy.

Figure 2 shows the time course of recovery from desensitization. At various intervals, ADP (1 μM) was applied repetitively to a megakaryocyte under investigation, and the maximum amplitude of the ADP-induced current was plotted against the time of recording (Fig. 2*A*). Within an interval of 60 s from the preceding application, ADP evoked barely detectable currents (inset *b* in Fig. 2*A*), whereas with longer intervals ADP at the same dose evoked larger currents (insets *c* and *d* for intervals at 210 and 130 s, respectively). It was often noted that the maximum amplitude of ADP-induced currents did not recover fully to the control value, even though a sufficient interval for recovery was allowed; the amplitude showed a tendency to decrease during the experiments. It was also recognized that the latency of the ADP-induced currents seemed to be prolonged after repeated ADP application. Such deteriorative changes in ADP responses may result from exchange of the

intracellular medium with the solution in the electrode. Such exchange might cause loss of relatively larger molecules from the cytoplasm and/or changes in molecular conformation in the membrane (Marty & Neher, 1983; Neher, 1988). Thus, in Fig. 2, amplitudes of ADP-induced currents were corrected by normalizing each of the

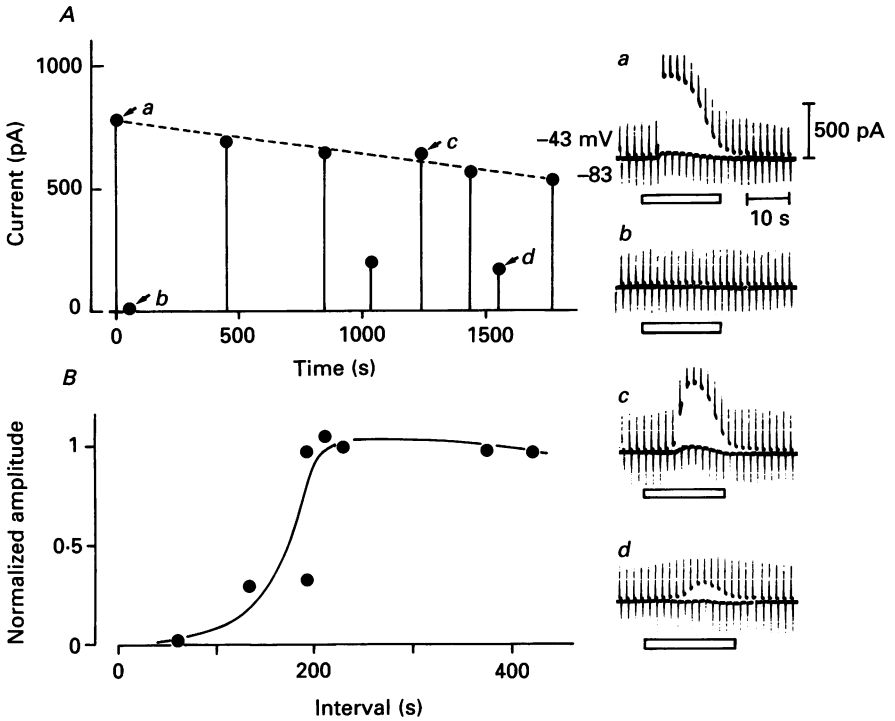

Fig. 2. Recovery of responsiveness to ADP in megakaryocytes. *A*, changes in evoked currents during successive application of 1 μM-ADP of various intervals. Peak amplitudes of ADP-induced outward currents were measured at −43 mV and plotted against the time of ADP application. Cell diameter, 19 μm. A dashed line connecting the first and the last data points may indicate the degree of spontaneous decrease in cellular responsiveness to ADP. On the right are specimen current traces corresponding to the plots marked *a*, *b*, *c* and *d*. The membrane potential was clamped at −83 mV and depolarizing pulses to −43 mV (duration, 420 ms) were superimposed every 1·5 s. Open bars below each trace indicate the duration of 1 μM-ADP application. Calibrations in *a* apply to all traces. *B*, time course of recovery after desensitization. The current amplitude in *A* was normalized at each time of measurement to the value indicated by the dashed line. The normalized value was plotted along the ordinate against the interval between the preceding application and the present application along the abscissa. The curve was drawn by eye.

obtained values to the corresponding value of presumptive full recovery (dashed line in Fig. 2*A*). In Fig. 2*B*, the corrected values are plotted on the ordinate against the interval on the abscissa. The plots in Fig. 2*B* show that recovery from desensitization was slow, and that more than 200 s was required for full recovery. In the following experiments where full recovery was essential, ADP was applied to the megakaryocyte at intervals of about 220 s or longer and experiments were discontinued

after five or fewer ADP applications. Occasionally, megakaryocytes exposed to ADP had extended filopodia-like processes of a few micrometres long. These processes, however, retracted into the cells after washing with the control saline for 4–10 min.

Dose–response relationship and intercell variation

The dose–response relation of ADP-induced currents shown in Fig. 3 was obtained by applying various doses of ADP at intervals of about 300 s. At ADP concentrations

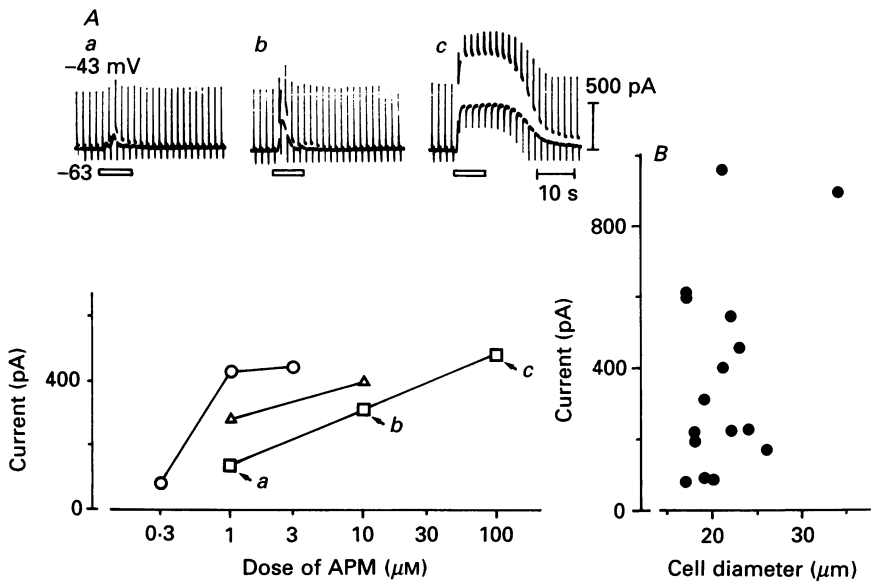

Fig. 3. *A*, dose–effect relation for ADP. Upper traces, example of ADP-induced currents at three different doses from one megakaryocyte. In each trace, the membrane potential was held at −63 mV and depolarizing pulses to −43 mV (duration, 420 ms) were applied every 1·5 s. Open bars below each trace indicate duration of ADP application. Doses are 1 (*a*), 10 (*b*) and 100 μM (*c*), respectively. Cell diameter, 19 μm. Lower plot, peak amplitude of ADP-induced currents measured at −63 mV plotted against the dose of ADP. Three megakaryocytes were plotted with different symbols. The marked data are those illustrated above. Diameters of the other two cells, plotted with ○ and △, are 19 and 21 μm, respectively. *B*, variation of responsiveness to ADP among cells. In each megakaryocyte, responsiveness to ADP was assessed by the amplitude of the outward current at −63 mV after application of 10 μM-ADP to the cell. The peak amplitudes of the ADP-induced current were plotted along the ordinate against the diameter of the cell along the abscissa. A total of sixteen cells are shown.

of less than 0·1 μM, the outward currents were scarcely detectable. As the dose of ADP was increased, the amplitude and duration of the ADP-induced currents increased remarkably. In part *A* of Fig. 3, typical examples obtained in three megakaryocytes with similar diameters (19–21 μm) are shown. In the case of the illustrated cell, the peak of the outward current evoked with 100 μM-ADP exceeded 400 pA (at −63 mV) and was sustained for more than 10 s (inset *c*). However, at the second application of 100 μM-ADP, the amplitude and duration of the evoked

current were 290 pA (at −63 mV) and 5 s, respectively, which were significantly smaller and shorter than those at the first application. In other megakaryocytes, similar phenomena were also observed after application of 100 μM-ADP. This probably indicates that at a high dose of ADP, the desensitization of the megakaryocyte to ADP was enhanced or became partly irreversible. Thus, in the following study, we usually employed an ADP concentration of 10 μM or less.

It was noticed that the responsiveness in megakaryocytes to ADP showed considerable intercell variation. In Fig. 3*B*, the peak amplitudes of evoked currents in sixteen cells are plotted on the ordinate against cell diameter on the abscissa. The applied dose of ADP was 10 μM and the holding potential of the cells was −63 mV. Although the cells with larger diameters tended to generate larger currents, the variation did not seem to be totally explained by differences in the cell surface area. For example, in megakaryocytes with a diameter range of 19–21 μm, the difference in current amplitude was about 10-fold (i.e. 85–960 pA). Thus, it is likely that the predominant cause of intercell variation seemed to be differences in the membrane current amplitude *per se*. The detailed mechanisms or factors influencing this variation remain to be clarified.

Characteristics of ADP-induced currents

Effects of external K^+ concentration

To identify the ions which carry these ADP-induced currents, the reversal potential of the currents in various concentrations of external K^+ ($[K^+]_o$) were investigated. During application of 10 μM-ADP to the megakaryocyte under study, the membrane potential of the cell was changed to two or three different levels repeatedly (Fig. 4*A* and *B*) and the magnitudes of the ADP-induced currents were observed. The external media used for these experiments were standard saline (containing 5 mM-K^+), 25 mM-K^+ saline and 125 mM-K^+ saline (Table 1), and the mean values of the reversal potential thus determined were −87, −46 and −6 mV for the above salines, respectively. The reversal potential of the currents strongly depended on $[K^+]_o$; the slope of the change in potential coincided fairly well with the value expected from the Nernst equation for K^+ equilibrium potential (59 mV/decade change in $[K^+]_o$, dashed line in Fig. 4*C*). Thus, it seems safe to conclude that the currents are carried predominantly by K^+.

Effects of blockers

Megakaryocytes used in the present study were able to generate K^+ currents upon depolarization (Kawa, 1990). The K^+ currents were sensitive to K^+ channel blockers such as quinine, tetraethylammonium (TEA) and 4-aminopyridine, and seemed to permeate through the delayed rectifier K^+ channels in the membrane (Kawa, 1990).

By contrast, the K^+ currents induced by ADP showed little sensitivity to these K^+ channel blockers. When 30 μM-quinine was added to the experimental saline containing 10 μM-ADP, the membrane currents induced were similar to those induced with the control saline containing 10 μM-ADP but no quinine. The same was true when 30 mM-TEA was added to the 10 μM-ADP saline (TEA replacing equimolar NaCl in the external standard saline) and applied to megakaryocytes. It

is thus suggested that the pharmacological properties of the K^+ channels involved in the ADP response are quite different from those of the delayed rectifier K^+ channels. Apamin at a concentration of 10 nM showed no significant effects on ADP-induced currents. A higher dose of apamin induced an irreversible increase in the leakage, and made it difficult to obtain consistent results.

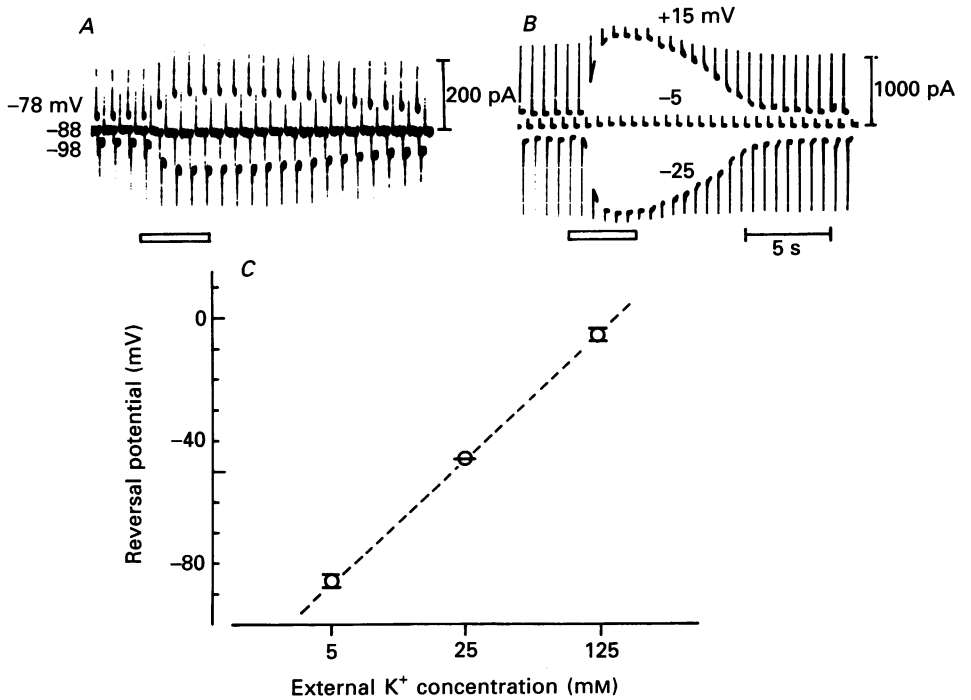

Fig. 4. Reversal potential of ADP-induced currents and its dependence on K^+ concentration. *A* and *B*, specimen records showing reversal of ADP-induced currents recorded in external standard saline (containing 5 mM-K^+; *A*) and in 125 mM-K^+ saline (*B*). In *A*, holding potential was −88 mV and depolarizing pulses to −78 mV (duration, 120 ms) and hyperpolarizing pulses to −98 mV (duration, 200 ms) were superimposed alternatively every 800 ms. In *B*, holding potential was −5 mV and depolarizing pulses to +15 mV (duration, 200 ms) and hyperpolarizing pulses to −25 mV (duration, 200 ms) were superimposed alternatively every 600 ms. Open bars indicate the duration of application of 10 μM-ADP, which was dissolved in respective external media. Cell diameter, 18 μm for *A* and 24 μm for *B*. *C*, relationship between reversal potential and external K^+ concentration. ○ and bars indicate average potential and the range of measured values. Number of measurements was four, two and three for 5, 25 and 125 mM-K^+, respectively. The dashed line represents a slope of 59 mV for a 10-fold change in external K^+ concentration.

Voltage dependence

The traces of ADP-induced currents measured at −78 and −98 mV in the standard saline were almost symmetrical (Fig. 4*A*), suggesting that the gatings of the K^+ channels depended little on the membrane potential. To further evaluate this

possibility, the amplitude and time course of the ADP-induced currents were studied in 125 mM-K^+ saline where, with almost similar intra- and extracellular K^+ concentrations, the K^+ currents *vs.* membrane potential relation predicted by the constant-field equation becomes linear (Hodgkin & Katz, 1949; Hille, 1984). As illustrated in Fig. 4*B*, the time course of ADP-induced currents showed no obvious dependence on membrane potential between −25 mV and +15 mV. The peak amplitudes of the evoked currents were nearly proportional to the potential difference from the reversal potential within a range of potential of −25 to +15 mV (not shown). Also in 25 mM-K^+ saline, the gating of the K^+ channels showed no dependence on membrane potential at −36 to −56 mV. These results are in contrast to those for the delayed rectifier K^+ channels in the same membrane, which showed distinct potential dependence for both activation and inactivation within a range of potential of −50 to 0 mV (Kawa, 1990).

Intracellular Ca^{2+} may mediate ADP-induced K^+ currents

Effects of changing internal Ca^{2+} concentration

In an early series of experiments, it was noted that the ADP response of megakaryocytes seemed to be influenced by the type of internal saline used. When Ca/EG-5 KCl saline containing 5 mM-EGTA and 2·5 mM-Ca^{2+} (Table 1) was used for filling the patch electrode, the membrane currents induced by 10 μM-ADP were generally small, being less than 100 pA at −63 mV, and often disappeared after two or three ADP applications. Use of Ca/EG-0·1 KCl saline containing 100 μM-EGTA and 50 μM-Ca^{2+} improved this situation; as shown in Figs 1–4, experiments using Ca/EG-0·1 KCl saline enabled repeated measurements of ADP-induced currents to be made without remarkable deterioration. This observation suggests that the internal free Ca^{2+} may play some role in the process of K^+ channel activation.

The experiment in Fig. 5*A* illustrates a typical phenomenon obtained in one megakaryocyte showing different responsiveness to ADP between two internal salines used for the same cell. In *a* of Fig. 5*A*, the membrane currents induced by 10 μM-ADP were first recorded using internal Ca/EG-0·5 KCl saline. Then the patch electrode was gently removed from the cell to render the patched area of the cell closed and sealed. Then, the other patch electrode filled with simple KCl saline containing no EGTA was applied to the same megakaryocyte to establish again a whole-cell patch-clamp configuration. As shown in *b* of Fig. 5*A*, the application of 10 μM-ADP induced significantly larger outward currents than previously; the ADP-induced current in the figure was measured at −73 mV because at −63 mV the evoked current was off the scale. Although non-specific leakage seemed to increase during the change of the patch electrodes, the above results clearly indicate that reduction of buffering capacity for Ca^{2+} in the internal saline may enhance the responsiveness of the megakaryocyte to ADP.

Application of Ca^{2+} ionophore mimics the ADP response

When the external standard saline containing Ca^{2+} ionophore A23187 (10 μM) was pressure applied from a micropipette to the megakaryocyte under recording, large outward currents were induced in the cell (Fig. 5*B*). The currents decayed slowly to

the original level in about 300 s. With an interval of 10 min, a second application of 10 μM-A23187 induced smaller and shorter outward currents in the megakaryocytes (peak amplitude at −63 mV, 93 pA; duration, about 250 s) than those at the first application. The third application of 10 μM-A23187 induced even smaller outward

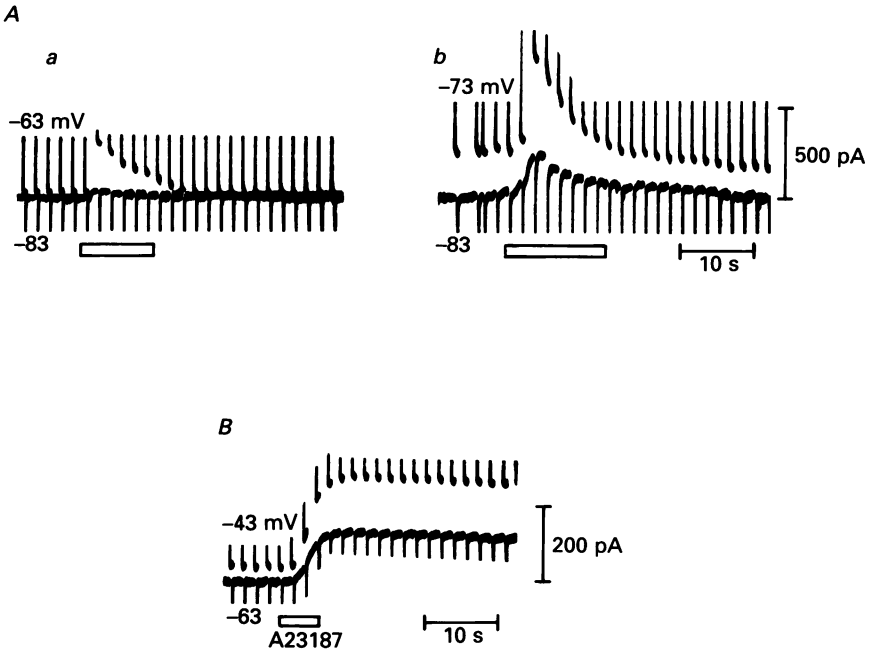

Fig. 5. Roles of intracellular Ca^{2+} in inducing outward currents. *A*, effects of low internal Ca^{2+} concentration on ADP-induced currents. When the patch electrode contained Ca/EG-0·5 KCl saline, outward currents induced by 10 μM-ADP were small (*a*). However, when the same cell was examined with a patch electrode containing simple KCl saline, the outward currents induced by the same dose of ADP were remarkably enhanced (*b*). For both traces, the holding potential was −83 mV. The levels of superimposed depolarizing pulses (duration 420 ms, applied every 1·5 s) were, however, reduced from −63 mV in *a* to −73 mV in *b* to avoid overscaling of current amplitude in the latter. Open bars indicate the duration of ADP application. Scales apply to both traces. Cell diameter, 34 μm. External medium, standard saline. *B*, application of calcium ionophore A23187 also induced outward currents. Open bar indicates the duration of application of 10 μM-A23187. Membrane potential was held at −63 mV and depolarizing pulses to −43 mV (duration, 420 ms) were applied every 1·5 s. Cell diameter, 19 μm. Patch electrode contained Ca/EG-0·1 KCl saline. External medium, standard saline. Currents of capacitative transient were truncated.

currents, which decayed completely in 100 s. In two other megakaryocytes, similar results were obtained. Reduction of the responsiveness to A23187 is presumably associated with an unphysiologically intense and prolonged rise in $[Ca^{2+}]_i$, which probably deteriorates the function of the K^+ channels.

The current induced by A23187 also depended on the external K^+ concentration. When the megakaryocyte was immersed in 125 mM-K^+, application of 10 μM-A23187

dissolved in this saline evoked obvious inward currents at −10 mV. The direction of the A23187-induced currents became clearly outward at +10 mV.

Rise of internal Ca^{2+} concentration demonstrated by Fura-2

From the above results, it is conceivable that the ADP-induced membrane currents were mediated by an increase in the intracellular free Ca^{2+} concentration

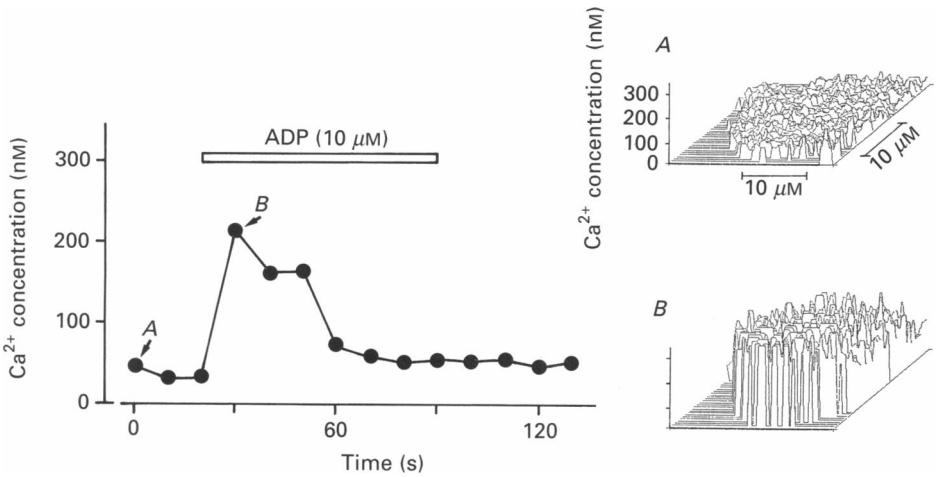

Fig. 6. Changes in the intracellular Ca^{2+} concentration after ADP application. Megakaryocytes were loaded with Fura-2 AM and their intensities of fluorescence were recorded every 10 s during excitation with 340 and 380 nm light. On the digitized image of the cell, the fluorescence ratios obtained were transformed into free Ca^{2+} concentrations after comparison with calibration data. On the left, a typical example of the changes in free Ca^{2+} concentration in a megakaryocyte is shown. The ordinate of each plot represents the spatial average of Ca^{2+} concentration in the megakaryocyte, while the abscissa represents the time of measurements. Open bar indicates the duration of 10 μM-ADP application. Cell diameter, 23 μm. On the right, specimen records for intracellular Ca^{2+} concentration in the cell are illustrated in two-dimensional figures. *A* and *B* correspond to the plots on the left, marked with arrows. *X*, *Y* and *Z* axis values in *A* also apply to *B*.

($[Ca^{2+}]_i$). To confirm this, we measured changes in $[Ca^{2+}]_i$ using a Ca^{2+}-sensitive fluorescent dye, Fura-2. ADP was pressure-applied from a micropipette to a megakaryocyte for 50–100 s and fluorescence intensities were sampled with an interval of 10 s (Fig. 6). The resting value of $[Ca^{2+}]_i$ measured at 10–20 s before ADP application was 51 ± 32 nM (mean ± S.D., $n = 8$). Within 10 s after the start of ADP application, $[Ca^{2+}]_i$ reached a maximum value of 218 ± 76 nM (S.D., $n = 8$) and declined to the original level in about 50 s. The average ratio of the maximum $[Ca^{2+}]_i$ to the resting $[Ca^{2+}]_i$ was $4{\cdot}8 \pm 1{\cdot}4$ (S.D., $n = 8$), when the concentration of ADP was 10 μM. The time course of change in $[Ca^{2+}]_i$ and that of the K^+ currents induced by ADP were apparently similar, but upon close comparison, the former tended to be slower than the latter. This discrepancy may be due to the difference in disturbance of physiological buffering capacity for cytoplasmic Ca^{2+} between the two methods and/or to the uneven distribution of $[Ca^{2+}]_i$ throughout the cytoplasm.

When the second application of 10 μM-ADP was tried after washing the cell for about 20 min with the standard saline, changes in $[Ca^{2+}]_i$ were mostly identical to those at the first application. In a few cases, return of $[Ca^{2+}]_i$ to the original level was slow during the washing interval. In such cases, $[Ca^{2+}]_i$ often remained elevated by

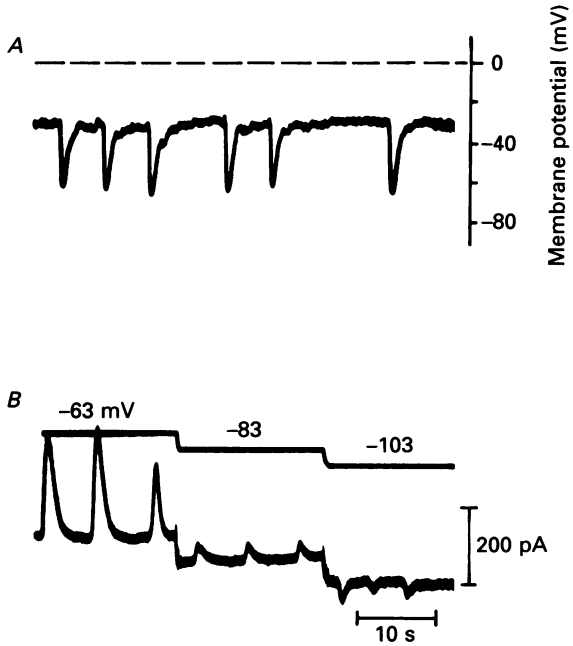

Fig. 7. Spontaneous hyperpolarization of a megakaryocyte. *A*, typical example of spontaneous hyperpolarization under current clamping at the null holding current. Dashed line indicates zero potential level. Patch electrode contained Ca/EG-0·1 KCl saline. Cell diameter, 24 μm. *B*, membrane currents occurring spontaneously under voltage clamping. Same cell as in *A*. At a membrane potential of −63 mV, spontaneously occurring currents were outward (left-hand part of lower trace). Their amplitudes decreased at −83 mV (middle part) and reversed their direction at −103 mV (right-hand part). Potential levels were monitored simultaneously and are shown in the upper part of the figure.

50–80 % of the original value even after washing for 20 min. Due to bleaching of the fluorescent dye, the measurements of fluorescence in one megakaryocyte were discontinued after a maximum of four ADP applications.

In summary, it seems safe to conclude that during application of 10 μM-ADP to megakaryocytes, $[Ca^{2+}]_i$ increases by about 5-fold. The reasons for the intercell variation in the magnitude and time course of $[Ca^{2+}]_i$ are open to future study.

Spontaneous electrical activities in megakaryocytes

Spontaneous hyperpolarization

During measurement of the resting potential in megakaryocytes, it was occasionally recognized that the membrane potential shifted abruptly to a negative direction (Fig. 7*A*). Such hyperpolarization occurred spontaneously and often

attained a value more negative than −60 mV, returning to the original level in 3–5 s. The frequency of spontaneous hyperpolarization varied from cell to cell. In fifteen of the twenty-two cells examined, no obvious hyperpolarization events were observed during the first 120 s after establishment of the whole-cell configuration. In the other cells, four to twenty-two hyperpolarization events were observed during 120 s. The involvement of changes in $[Ca^{2+}]_i$ was suggested from the preliminary observation that the frequency of hyperpolarization seemed to increase when internal saline containing no EGTA (simple KCl saline in Table 1) was used instead of the regularly used Ca/EG-0·1 KCl saline, whereas the frequency became almost unobservable when EG-5 KCl saline (containing 5 mM-EGTA) was employed.

Ionic mechanism

Figure 7*B* illustrates typical examples of membrane currents recorded from a megakaryocyte showing spontaneous hyperpolarization. Obvious outward currents with a duration of 3–4 s were observed when the membrane potential was held at −63 mV. When the membrane potential was made more negative, the spontaneous currents decreased in amplitude and finally changed their direction to inward (far right in Fig. 7*B*). The reversal potential of the spontaneous currents estimated by interpolation was −88 mV (mean of two cells), which almost coincides with the equilibrium potential for K^+. When the concentration of external K^+ was raised by immersing the cells in 125 mM-K^+ saline, the reversal potential of the spontaneous currents shifted to −5 mV (mean of two cells). This reversal potential again coincides with the value expected for the K^+ equilibrium potential under the present experimental conditions. The above results suggest that the spontaneous hyperpolarization was caused by activation of K^+ channels in the membrane. As the frequency of spontaneous activation of the channels showed no distinct dependence on membrane potential but seemed to be modulated by the buffering capacity for Ca^{2+} in the internal saline, the K^+ channels involved are very probably Ca^{2+}-dependent K^+ channels.

Relevance to ADP-activated K^+ currents

The Ca^{2+} which appeared in the interior of megakaryocytes on exposure to ADP could have been due to influx of Ca^{2+} through the cell membrane and/or release from the intracellular storage site (Siess, 1989; Williamson & Monck, 1989). Occurrence of spontaneous hyperpolarization in the megakaryocyte lead to speculation that an intracellular storage site for Ca^{2+} might exist, and that Ca^{2+} could be released either spontaneously or in response to ADP application.

To elucidate the possible roles of the Ca^{2+} storage site in the megakaryocyte, ADP-induced K^+ currents were measured by minimizing the contribution of Ca^{2+} influx through the membrane. The megakaryocytes were continuously perfused with 0 mM-Ca^{2+} saline (Table 1), and 10 μM-ADP, which had been dissolved in either the 1 mM-EGTA saline or the standard saline (Table 1), was applied from micropipettes. When the cell was perfused with the 1 mM-EGTA saline, the non-specific leakage current appeared to increase. However, the cell was able to generate definite K^+ currents in response to 10 μM-ADP. In the absence of extracellular Ca^{2+} (i.e. in 1 mM-EGTA saline), the ADP-induced K^+ currents could be reproduced at least three times when intervals of 300 s were interposed. The average amplitude of the ADP-induced

currents, however, was significantly smaller than those for the same cell evoked in standard saline; the former was 45% of the latter (measured at −63 mV, $n = 3$). The duration of ADP-induced currents in 1 mM-EGTA saline was remarkably reduced, becoming 3–5 s (measured at half-width).

The above results indicate two possibilities: first that on exposure to ADP, the Ca^{2+} storage site in the megakaryocyte releases Ca^{2+}, and second that the presence of extracellular Ca^{2+} might promote the increase of $[Ca^{2+}]_i$, presumably by permeating the cell (cf. Hallam & Rink, 1985; Sage & Rink, 1987), or by stabilizing the binding of ADP to its receptor site (cf. Pletscher, Erne, Bürgisser & Ferracin, 1985) or both. Other possible ways in which Ca^{2+} could affect the membrane, such as through fluidity or surface change (Ohmori & Yoshii, 1977), together with more quantitative analyses of Ca^{2+} mobilization, remain to be studied.

DISCUSSION

In megakaryocytes dissociated from bone marrow of the guinea-pig externally applied ADP evoked obvious K^+ currents in the membrane. The activities of the K^+ channels involved are strongly dependent on the intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$). Using a fluorescent dye, Fura-2, an approximately 5-fold increase in $[Ca^{2+}]_i$ was observed in the megakaryocyte when the cell was exposed to 10 μM-ADP.

One characteristic of the ADP response in the megakaryocyte is that an intense desensitization was recognized during and after application of ADP (Fig. 2). The rise of $[Ca^{2+}]_i$ monitored with Fura-2 was also transient and could not be sustained during the period of ADP application (Fig. 6). In contrast, when a Ca^{2+} ionophore, A23187 (10 μM), was applied to the megakaryocyte to elevate $[Ca^{2+}]_i$, the evoked K^+ currents could be maintained for about 300 s (Fig. 5*B*). These observations lead to speculation that the desensitization might not be due mainly to inactivation or a decrease in Ca^{2+} sensitivity of the K^+ channels, but rather might result from reduced effectiveness of the receptor sites and/or the molecular machinery for signal transduction increasing the $[Ca^{2+}]_i$ (Sugiya & Furuyama, 1989; Williamson & Monck, 1989). The molecular mechanisms of desensitization and its recovery might, however, be more intricate and might require detailed studies (e.g. Karaki, 1989; Kennedy, 1989).

The accompanying paper on electrical properties of the guinea-pig megakaryocyte revealed that this cell has two kinds of voltage-gated ion channels in the membrane, namely Ca^{2+} channels of the transient type and K^+ channels of the delayed rectifier type (Kawa, 1990). The present study clarified the presence of another kind of membrane K^+ channel, the activity of which is regulated by intracellular Ca^{2+}. Although subtle differences in physiological or pharmacological properties among tissues could be present, this type of K^+ channel has been found in many tissues including exocrine glands, muscles and neurones (Petersen & Maruyama, 1984; Blatz & Magleby, 1987; Marty, 1989).

Using conventional microelectrodes, Miller, Sheridan & White (1978) showed that the resting membrane potential of the guinea-pig megakaryocyte became more negative when the extracellular Ca^{2+} concentration was increased. It is conceivable that when conventional microelectrodes are employed, leakage at the cell penetration site might not be negligible and that $[Ca^{2+}]_i$ in the cell might increase according to

the rate of Ca^{2+} influx from the external medium. The observation by Miller *et al.* (1978) could be explained if we assume that at a high concentration of external Ca^{2+}, enhanced activation of the Ca^{2+}-dependent K^+ channel might occur due to accelerated influx of Ca^{2+}. Other factors such as sealing effects of external Ca^{2+} might make an additional contribution.

The physiological roles of Ca^{2+}-dependent K^+ channels in megakaryocytes remain to be clarified. Since intracellular Ca^{2+} is considered to be one of the key intracellular messengers regulating metabolic, motile and secretory activities of the cell (e.g. Karaki, 1989; Kennedy, 1989; Siess, 1989), the K^+ channels regulated by internal Ca^{2+} might presumably be involved in these activities (Rasmussen & Goodman, 1977; Lewis & Cahalan, 1988; Marty, 1989). In several tissues including haematocytes, volume or pH regulation seems to be coupled with the activities of K^+ channels (Hoffmann & Simonsen, 1989).

In mammalian platelets, external ADP is a potent activator, causing rapid reversible as well as slow irreversible changes in the platelets (Born, 1962; Born *et al.* 1978; Frojmovic & Milton, 1982; Siess, 1989). Intracellular Ca^{2+} has been considered to be one of the key messengers involved in the regulation of these changes (Hallam & Rink, 1985; Kroll & Schafer, 1989; Siess, 1989). It is of interest to note that between mammalian platelets and megakaryocytes, a striking similarity in responsiveness to ADP seems to exist with regard to the effective dose of ADP within the range of 1–20 μM (Figs 1–3; Hallam & Rink, 1985) and the functional involvement of Ca^{2+} storage sites in the cell (Fig. 7; Hallam & Rink, 1985; Sage & Rink, 1987). It seems conceivable that in megakaryocytes, the receptor and signal transduction mechanisms for the ADP response may be identical or very similar to those in platelets. More speculatively, a cellular mechanism of reaction with ADP might be complete in the megakaryocytes before it starts to release platelets into the circulation.

In previous studies on guinea-pig megakaryocytes, it was revealed that prolonged exposure of megakaryocytes to ADP (10 μM) induced cell spreading with changes in the cytoskeleton and membrane depolarization (Leven & Nachmias, 1982; Leven *et al.* 1983). These changes occurred slowly, taking more than 10 min, and were almost irreversible. The depolarization depended on external Na^+. These phenomena are in marked contrast to those observed in the present study. The responses to ADP, which were evaluated in terms of the magnitude of the K^+ current, were completed within tens of seconds, independent of external Na^+ and repeatable. If further speculation about the functional homology between megakaryocytes and platelets could be taken, it is possible to consider that in megakaryocytes the ADP response might also consist of two phases; one involving rapid and reversible changes and the other slow and irreversible changes. Our present observations may thus be related to the rapid phase, whereas those of Leven & Nachmias (1982) and Leven *et al.* (1983) may have a relationship to the slow phase. If this speculation is acceptable, the following issues as to whether the ADP receptors and signal transduction mechanisms involved are separate and whether the two putative phases in megakaryocytes have further substantial relevance to those operating in platelets appear to be the next key concern.

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