Discrete but simultaneous release of adenine nucleotides and serotonin from mouse megakaryocytes as detected with patch- and carbon-fiber electrodes

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Kawa, Kazuyoshi. Discrete but simultaneous release of adenine nucleotides and serotonin from mouse megakaryocytes as detected with patch- and carbon-fiber electrodes. Am J Physiol Cell Physiol 286: C119–C128, 2004. First published September 10, 2003; 10.1152/ajpcell.00014.2003.—Using patch- and carbon-fiber electrodes, we studied release phenomena of adenine nucleotides and serotonin from megakaryocytes isolated from the bone marrow of the mouse. Megakaryocytes express ionotropic purinergic receptors on their surfaces. Under the condition of whole cell recording, the cells showed spikelike spontaneous inward currents. The spontaneous currents were carried by cations and had amplitudes of 30–800 pA at −43 mV and durations of 0.1–0.3 s. Pyridoxalphosphate-6-azophenyl-2,4'-disulfonic acid (PPADS; 100 µM) and suramin (100 µM), purinoceptor-blocking agents, depressed the currents reversibly. It is thought that the receptor involved was the P2X1 subtype on the cell and that the currents were due to activation of the P2X1 receptor by adenine nucleotides released from the cell. The currents showed a skewed amplitude distribution, suggesting variation of vesicular contents and/or distinct localization or varied density of receptors on the cell. Frequency of the spontaneous inward currents was enhanced by external application of platelet-activating substances, thrombin (0.4 U/ml), phorbol ester (100 nM), and ADP (2 µM), at low concentrations. With a carbon-fiber electrode, which can detect oxidizable substances including serotonin, spikelike oxidation currents from the external surface of the megakaryocyte were detected. The frequency of the oxidation currents increased remarkably after the application of thrombin (10 U/ml). The majority of the oxidation currents coincided with the rising phase of the whole cell currents, suggesting corelease of serotonin and adenine nucleotide from the same vesicle. We concluded that megakaryocytes store adenine nucleotides and serotonin in the same vesicle and release them simultaneously in a discrete manner.

ADP; ATP; serotonin; patch clamp; P2X1 receptor

PLATELETS AND THEIR PROGENITOR CELLS, megakaryocytes, contain three kinds of releasable vesicles. Among them, dense bodies (namely, δ-vesicles) have interested interest because congenital defects in dense bodies are known to cause a severe bleeding disorder, “storage pool disease,” in humans and animals (3, 25). Release of vesicular contents of adenine nucleotides and serotonin from the cytoplasm is presumably carried out under specific regulation, as in other secretory cells (4, 25, 32). However, direct evidence of vesicular exocytosis from platelets and information on functional characteristics of these exocytotic processes are quite limited (22, 29), probably due to the small size and fragility of the preparation.

Megakaryocytes appear to provide a suitable model for the study of release phenomena in platelets because these progenitor cells express most of the functional properties of platelets and have large diameters suitable for easy identification and stable analyses (18, 19). Specific receptors for adenine nucleotide (both P2X1 receptor of an ionotropic subtype and P2Y1 receptor of a metabotropic subtype) are already functional in megakaryocytes and show similar properties to those of mature platelets (18, 19, 35). Thrombin receptors are also expressed on megakaryocytes and have functional properties similar to those in platelets (40). In isolated megakaryocytes, uptake and storage of serotonin have been morphologically and biochemically demonstrated (39, 43, 44). Furthermore, in a mass preparation of megakaryocytes, releasability of serotonin and adenine nucleotides from the cytoplasm has been confirmed (10, 26, 42, 43).

In the present study, the release phenomena of adenine nucleotides and serotonin from megakaryocytes were studied using the combined techniques of patch clamping and amperometry (2, 5, 13). Serotonin is a suitable substrate for amperometric detection with a carbon-fiber electrode (CFE) and has been used for analyzing exocytotic processes in neural and nonneural cells (5, 38). Activation of purinergic receptors and the consequent generation of transient inward currents in megakaryocytes have been characterized (6, 7, 19, 35). Thus it is conceivable that adenine nucleotides released from a megakaryocyte activate purinergic receptors on the same megakaryocyte and generate definite cationic currents in the cell, which can be detected by using a patch electrode. This phenomenon has been briefly described in mammalian platelets (22). Using these techniques and characteristics, we demonstrated that release of adenine nucleotides and serotonin from megakaryocytes can physiologically occur in a discrete manner and that these substances are released simultaneously, presumably from the same vesicle. We also show that platelet-activating substances, such as thrombin or phorbol esters or even adenine nucleotide, can facilitate the discrete release from megakaryocytes. The present study has revealed unique characteristics of release activities in the megakaryocyte/platelet system.

METHODS

Preparation. All experiments were carried out in accordance with the Guiding Principles of the Physiological Society of Japan. Young male mice of the BALB/c strain, aged 28 to 35 days, were anaesthetized deeply with vaporized diethyl ether and then bled to death by cutting the cervical arteries in the neck. Procedures for cell preparation were the same as those described in the previous paper (19). In brief, the bilateral femoral bones of each animal were excised and
placed under a dissecting microscope (×8–40), and the osseous tissues were then gently cut to expose the bone marrow, which was removed with a pair of forceps, placed in a plastic dish containing Ca²⁺-free external saline, and stored at 24–25°C until required. Megakaryocytes in a recording chamber (Falcon, Primaria 35-mm diameter tissue culture dish) were dissociated mechanically from a few pieces of bone marrow using a pair of fine forceps. To facilitate dissociation, the bone marrow was further washed out with a disposable syringe (volume, 1 ml) and needle with Ca²⁺-free external saline.

The chamber was then mounted on the movable stage of a phase-contrast inverted microscope (final magnification, ×600; Diaphot-TMD, Nikon, Tokyo, Japan), and its field view was continuously monitored with a TV camera (KP-140, Hitachi, Tokyo). The chamber was perfused with experimental saline at a rate of 1–2 ml/min, whereas the temperature of the chamber was monitored with a thermistor and maintained at 24 ± 1°C.

**Patch-clamp recordings.** Whole cell currents in megakaryocytes were measured using a conventional patch-clamp technique (19). The patch-clamp amplifier used was an EPC-7 (List, Germany), and the pipettes were pulled from a 1.5-mm glass capillary tube in two stages using a vertical pipette puller (PP-83; Narishige, Tokyo, Japan). The access resistance of the patch clamped cells was maintained until the access resistance to the cell interior fell below 10 MΩ. The experiment was discontinued in Ca²⁺-free external saline unless stated otherwise. When a gigaohm seal with a cell had been obtained, negative pressure of up to 3 × 10⁴ Pa was applied to rupture the membrane at the tip of the patch electrode. To minimize metabolic disturbance of the recorded megakaryocytes, a nystatin-perforated-patch technique was used in some experiments (40). Nystatin was dissolved in acidic methanol (adjusted to pH 0–2 with 1 N HCl), and then the pH was adjusted to 7.2 with 1 N KOH to produce a nystatin stock solution (10 mg/ml), which was dissolved in Ca²⁺-free internal saline to produce a final concentration of 150 µg/ml immediately before the experiment and used within 60 min. A patch electrode containing internal saline supplemented with nystatin was pressed gently onto the cell, and negative pressure of 500–1,000 Pa was applied inside the pipette and maintained until the access resistance to the cell interior fell below 10 MΩ. The ground electrode, which was an Ag-AgCl wire, was connected to the external solution with an agar bridge containing 3 M KCl. Correction of the liquid junction potentials (Vj) was determined by introducing 3 M KCl saline into the perfusion chamber.

**Drug application and data acquisition.** Drugs were applied using a rapid application technique known as the “Y tube” method. The modifications used in this study (i.e., the use of a delivery tube with a diameter of 300–500 µm and length of 1.4 mm, and monitoring of pressure with digital manometers) are the same as those used previously (19). The delivery tube outlet was usually positioned 200–300 µm from the cell being recorded. In some experiments, drugs were applied by puffing. A glass micropipette (tip diameter, 0.2–5 µm) was filled with external saline containing the drug, and the micropipette was positioned on the megakaryocytes or within 50 µm downstream from the constantly perfused megakaryocyte. To expose the cell to the drug-containing saline, pneumatic pressure (50–2 × 10⁴ Pa) was applied to the other end of the micropipette. Signals of membrane current and potential were monitored simultaneously using a storage oscilloscope (5111; Tektronix) and a pen recorder (Recticorder, Nihon-Koden, Tokyo, Japan). The signals were also transformed with a pulse code modulator (501-ES; SONY, Tokyo, Japan) into digital mode and recorded on a videocassette recorder (VT-435; Hitachi, Tokyo, Japan). The electrical filters used were analog-type filters with variable corner frequency (model 900, Frequency Devices). Some data were digitized at 10 kHz using a Digidata 1320A interface and its software (Axoscope, Axon Instruments, CA) and were stored on a hard disk. Software programs were used for analyses of amplitude distribution, time course of the inward currents, and dose-response relations for the agonist (Mini Analysis; Synaptosoft, CA; and Origin version 6; Micrcal Software). One-hundred to ten percent decay time of the inward current was defined as a period between the time when the amplitude of the current showed its peak and the time when the amplitude decayed to 10% of the peak value. Student’s t-test and Welch’s test were employed to evaluate the statistical significance of differences between two groups being compared. Significance test for F-distribution was used for intergroup changes in ratios.**

**Amperometry with CFEs.** To load megakaryocytes with exogenous serotonin, the megakaryocytes dissociated in experimental dishes were incubated for 20 min at 37°C in Ca²⁺-free external saline containing 100 µM serotonin. Throughout the incubation period, the dishes were kept under 100% oxygen gas and shaken horizontally at low velocity. The incubation medium in the dish was then rapidly changed to control saline using a peristaltic micropump (MP-3; Tokyo Rikakikai, Tokyo, Japan). The patch-clamp recordings; Nihon Koden, Tokyo, Japan). The ground electrode was the same as that used for patch-clamp recordings. Before each experiment, the sensitivity of the CFE tip to serotonin was calibrated by applying 1 µM serotonin to the tip from a glass pipette (orifice diameter, 30–50 µm). The CFE was then pressed gently onto the surface of an identified megakaryocyte and held in place with a hydraulic manipulator (MO-103, Narishige, Tokyo). Signals of the amperometric currents and the applied potential to the CFE were monitored with an oscilloscope, and digitized and stored on a videocassette recorder as described for the patch-clamp recordings. The applied voltage was also monitored with a digital potentiometer (CD-720C; Sanwa, Tokyo, Japan). After the experiment, the sensitivity of the electrode to serotonin usually showed some deterioration, presumably due to attachment of cellular debris on the tip. When the tip was slightly beveled with a diamond disk rotor (EG-6; Narishige, Tokyo, Japan), however, the sensitivity of the electrode could recover for another use (total use, 3–4 times). In a series of experiments in which corelease of serotonin and adenine nucleotide was studied, simultaneous recordings for both the whole cell currents and the oxidation currents from one megakaryocyte were made.

**Solutions and drugs.** The external saline used as routine was Ca²⁺-free external saline containing (in mM) 140 NaCl, 5 KCl, 1 MgCl₂, 10 d-glucose, and 10 HEPES (pH 7.4, adjusted with NaOH). Omission of Ca²⁺ from the external saline seemed to be effective for preventing uncontrollable deterioration of the megakaryocytes during and after the experiments (19). The drug for external application was dissolved in Ca²⁺-free external saline and applied with a Y tube or through a puffing pipette. The magnetic valves of these application systems were controlled by a programmable pulse generator (SEN-3201; Nihon-Koden, Japan). For whole cell recordings, CsCl internal saline, containing (in mM) 145 CsCl, 4 MgCl₂, 1 EGTA (K⁺-salt), and 10 HEPES (pH 7.2, adjusted with KOH), was used to fill patch pipettes. The free-Ca²⁺ concentration in this saline was 61 nM (estimated as in Ref. 19). Immediately before use, guanosine-5’-O-(3-thiotriphosphate) (GTPγS, 400 µM) was added to the CsCl internal saline unless otherwise stated. The following chemicals were purchased from Sigma Chemical: adenosine-5’-diphosphate sodium salt (ADP, Sigma-A-2754), adenosine-5’-triphosphate disodium salt (ATP, Sigma-A-2383, lot no. 113H7821), adenosine-5’-triphosphate sodium salt (α,β-methyleneadenosine-5’-triphosphate sodium salt (α,β-meATP, Sigma M-8386), phorbol 12-myristate 13-acetate (PMA), PPADS, suramin,
RESULTS

Spontaneous inward currents in megakaryocytes. Megakaryocytes of the mouse, dissociated from the bone marrow, were placed in a recording chamber and perfused with the external saline (containing nominally free Ca^{2+}). With a whole cell patch electrode applied to the megakaryocyte, generation of spontaneous inward currents having rapid time courses was observed occasionally. Figure 1 shows a typical example of the currents. The frequency of the spontaneous currents was low in an early series of experiments in which a whole cell patch electrode containing CsCl-internal saline without the addition of GTPγS was used. The frequency was within the range of 0.2–1.6 events/min (measured from 2 to 12 min after the establishment of whole cell recordings) with a mean of 0.7 ± 0.4 events/min (mean ± SD, n = 13). When the internal saline contained GTPγS (400 μM), the frequency of the currents was higher (7.4 ± 5.6 events/min, n = 11) than that in the case of no GTPγS when compared under otherwise similar conditions. Thus, in the present study on whole cell recordings, GTPγS (400 μM) was added to the internal saline unless otherwise stated, and megakaryocytes showing a high frequency of spontaneous currents were selected for detailed analyses.

The time courses of the spontaneous inward currents were rather fast: the average rising time of the currents was in the range of 16–31 ms with a mean of 22 ± 6 ms (SD; from 5 cells, at −43 mV). Most of the rapid inward currents (30 of 33 events analyzed) showed a single-exponential time course for the decay, the half decay time of which was 35 ± 4 ms (average

**Fig. 1.** Basic properties of spontaneous inward currents recorded from megakaryocytes. A: spontaneous inward currents recorded from a representative cell (holding potential, −43 mV). The patch pipette contained CsCl internal saline. Holding potential, −43 mV. A total of 114 events was recorded during a period of 90 s. The distribution of peak amplitudes is shown in the histogram at right. Lower scatter plots show 10–90% rise time vs. peak amplitude (left) and 10–90% rise time vs. 100–10% decay time (right) of the currents. B: 2 kinds of inward currents induced in megakaryocytes by external application of adenine nucleotides (mixture of 20 μM ADP and 20 μM ATP). The upper trace at left shows duration of the application from a Y tube. Middle and lower traces show representative results for whole cell currents obtained from 17 cells. Only fast inward currents were detected in 10 cells (middle trace), whereas both fast and delayed inward currents were detected in the other 7 cells (lower trace). Time courses of the current generation (10–90% rise time for the fast inward current and the time to peak for the delayed inward current) are summarized in histograms at right. Bars indicate SDs. External medium, Ca^{2+}-free saline. The patch pipette contained CsCl internal saline. Holding potential, −43 mV. C: example of spontaneous inward currents showing rapid as well as slow time courses (indicated by downward open arrows). Recordings were obtained in another megakaryocyte under the same experimental conditions as those described in A.
of means from 5 cells measured at −43 mV). The amplitudes of the inward currents showed large variation even when recorded from one cell (as shown in Fig. 1A, at −43 mV). It was further noted that mean amplitude of the currents showed intercell variation; mean amplitude ranged from 23 to 105 pA with a mean of 65 ± 34 pA (SD, from 5 cells). The external saline in the present study usually contained nominally free Ca\(^{2+}\), which is suitable for obtaining a stable sealing and preventing the deterioration of purinergic receptors (19). In one series of experiments, external saline containing 2 mM Ca\(^{2+}\) or containing 10 mM Ca\(^{2+}\) was applied to the recorded cell through a Y tube, but there were no immediate changes observed in current frequency or in current amplitude (n = 3).

The findings of rapid time courses of currents and dependence of the frequency on intracellular milieu strongly suggest that the currents were due to spontaneous release of adenine nucleotides from the cell and subsequent activation of purinergic receptors on the same cell. To confirm this possibility, external saline that contained a mixture of 20 μM ADP and 20 μM ATP, mimicking the contents of adenine nucleotides from the dense bodies of a megakaryocyte, was applied to the cell under the condition of whole cell patch clamping. Figure 1B shows typical results of experiments showing generation of a rapid inward current alone (Fig. 1B, middle trace, representative of 10 cells) or generation of a rapid inward current followed by a delayed inward current (Fig. 1B, lower trace, representative of 7 cells). Rapid inward currents were generated in all of the megakaryocytes studied in response to the application of adenine nucleotides. These observations are consistent with results of previous studies showing that application of adenine nucleotide could induce two kinds of inward currents in megakaryocytes that were different in their time courses and receptors involved (19, 35). The 10–90% rise time of the rapid currents and the time to peak of the delayed currents showed distinct ranges of mean values, as shown in histograms in Fig. 1B. During whole cell recordings from megakaryocytes, it was also noted that spontaneous inward currents showing slow rise time (with time to peak of >500 ms) and slow decay time appeared (as indicated by downward open arrows in Fig. 1C). However, the frequencies of these slow spontaneous inward currents were low (2.9 ± 2.0 events/min; means ± SD, from 15 cells) and their peak amplitudes were small (8.1 ± 4.5 pA, means ± SD, from a total of 64 events in 15 cells) under the present experimental conditions. The mechanism by which the slow inward currents were generated [namely, by inositol trisphosphate (IP\(_3\))-dependent cation channels; Refs 14, 35, 36] seems different from the mechanism mediated by ionotropic purinergic receptors. In the following study, major analyses focused on the rapid inward currents of megakaryocytes, and the criterion for a 10–90% rise time less than 200 ms was principally adopted for the selection of rapid inward currents. As described later, some of the spontaneous inward currents in a specific series of experiments (after incubation of megakaryocytes in serotonin-containing saline) showed a 10–90% rise time of an intermediate range (200–500 ms). These spontaneous currents were regarded as variants of the rapid inward currents (See Fig. 5 and discussion).

**Current-voltage relationship of the inward currents.** The current-voltage (I-V) relationship of the spontaneous rapid currents in megakaryocytes was examined by measuring the amplitude of the currents at various holding potentials (Fig. 2A). Voltage-dependent K\(^+\) currents seemed to be depressed by Cs\(^+\) in the internal CsCl saline. Left traces in Fig. 2A show representative spontaneous current recordings from one megakaryocyte. As the holding potential was shifted from −43 mV to more negative values, the average amplitude of spont-

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**Fig. 2.** Current-voltage (I-V) relationships and sensitivity to purinergic receptor agonists of spontaneous inward currents. **A:** I-V relationships of spontaneous currents in megakaryocytes. Traces at left represent specimen records from a single cell. Figures on each trace indicate the holding potentials (mV). At right, mean amplitudes of the currents are plotted against holding potentials. Data from 4 megakaryocytes after normalization at −43 mV (double circles) are shown. Bars indicate SDs. **B:** effects of suramin (100 μM), a purinergic antagonist, on spontaneous inward currents. The figure at left shows the superimposed current trace from a megakaryocyte, obtained before (top) and during (bottom) suramin application, respectively. Histograms at right show average current amplitudes obtained before, during, and after suramin application (from 4 megakaryocytes). In each cell, currents were normalized to the value before suramin application. Bars indicate SDs. Reduction of the amplitude in the presence of suramin is statistically significant (P < 0.05). **C:** effects of α,β-methyleneadenosine-5'-triphosphate sodium salt (α,β-meATP; 40 μM for 4 s), a specific agonist of the P2X\(_1\) receptor, on spontaneous inward currents. The figure at left shows a current trace from a megakaryocyte obtained before (top) and after (bottom) α,β-meATP application, respectively. Histograms at right show frequency of the spontaneous currents obtained before application (control) and after α,β-meATP application (during a period of 20–140 s after application). Bars indicate SDs (from 4 megakaryocytes). The reduction of frequency after application of α,β-meATP is statistically significant (P < 0.05). In all of the experiments for which the results are shown in Fig 2, the external medium used was Ca\(^{2+}\)-free saline. The patch pipette contained CsCl internal saline. Holding potential, −43 mV.
taneous currents increased, whereas it decreased at less negative potentials and became outward at more positive potentials than 0 mV. In all five megakaryocytes, the current amplitudes at various holding potentials were measured and normalized to the value obtained at the holding potential of −43 mV. In Fig. 2A, right, means and SDs thus obtained from the five cells are plotted against the holding potentials. The I-V relationship for the spontaneous currents had a reversal potential of −3 ± 1 mV (means ± SD, n = 5).

**Blockage of currents by suramin, PPADS, or α,β-meATP.** The above-described properties of spontaneous currents showing a rapid time course and reversal potential near 0 mV suggest the involvement of purinergic receptor P2X1 subtype expressed on megakaryocytes (6, 19, 33). When suramin (100 μM), an antagonist to purinergic receptors (11), was applied from a Y tube to a megakaryocyte under the condition of recording for 20 s, amplitude of the spontaneous current immediately decreased (Fig. 2B). After the cell was perfused with control external saline, amplitude of the current recovered. Almost full recovery was observed after 5 min. The blocking effects of suramin (100 μM) are summarized in Fig. 2B, right. As shown, suramin caused a significant decrease in the current amplitude (P < 0.05). Involvement of other ionotropic receptors was unlikely. When acetylcholine (ACH; 100 μM), glutamate (1,000 μM), GABA (100 μM), glycine (100 μM), GTP (100 μM), UTP (100 μM), or adenosine (100 μM) was applied from a Y tube to a megakaryocyte, no recognizable rapid currents were observed under the present recording conditions (n = 3, each).

In other series of experiments, PPADS, a more specific antagonist of the P2X1 receptor, was tested. When PPADS (100 μM) was applied from a Y tube to a megakaryocyte under the condition of recording for 3 min, the frequency of the spontaneous inward current immediately decreased from 7.0 ± 2.0 events/min (SD, n = 4) to 0.8 ± 0.2 events/min (SD, n = 4). After the cell was perfused with control external saline, the frequency of the current recovered to 5.0 ± 1.7 events/min (SD, n = 4, measured after 5 to 10 min). The effects of α,β-meATP, a specific agonist of P2X1 receptor, on megakaryocytes are shown in Fig. 2C. When α,β-meATP was applied to the cell (40 μM for 4 s), rapid inward currents were evoked (with peak amplitude of 1,870 ± 740 pA at −43 mV; means ± SD, n = 4). After the current generation, spontaneous inward currents as observed in control saline (Fig. 2, upper trace) were totally blocked (lower trace), presumably due to prolonged desensitization of P2X1 receptors. The blockage persisted for up to 120 s after the washout of α,β-meATP. In the histograms in Fig. 2C, right, results from four cells are summarized.

**Effects of platelet-activating substances on spontaneous inward currents.** In this series of experiments, whole cell currents of a megakaryocyte were measured using nystatin-perforated-patch recording, a recording technique that is suitable for maintenance of intracellular signal transduction. In the control saline, megakaryocytes under the condition of perforated-patch recording showed spontaneous rapid currents at 3.1 ± 3.2 events/min (means ± SD, n = 15). When thrombin, the most commonly used activator for platelets, was applied to a cell, the

![Fig. 3: Facilitation of the occurrence of spontaneous currents by application of thrombin, phorbol ester, or ADP. A–C: the current records from different megakaryocytes to which thrombin (0.4 U/ml for 40 s), PMA (100 nM for 60 s), or ADP (2 μM for 2 s) were applied from a Y tube. The upper figures show pen-recorder displays of current responses, and the lower figures show expanded and superimposed current traces in each illustration above (corresponding to the marked duration with arrows). Holding potential, −43 mV. External medium, Ca2+-free external saline. The patch pipette contained CsCl internal saline and nystatin (150 μg/ml) but no GTPγS. D: summary of the effects of thrombin, PMA, and ADP on the generation of spontaneous inward currents. In each cell, frequencies of spontaneous currents obtained in the presence of an activator were normalized to the value obtained in the absence of an activator, and the means and SDs of the normalized values are plotted. Numbers of cells are indicated in parentheses.](http://ajpcell.physiology.org/Downloadedfrom)
frequency of the spontaneous currents increased (Fig. 3A, spikelike deflection in the trace). Expanded and superimposed current records (Fig. 3, lower traces) confirmed that the inward currents have rapid rise times and exponential decay times. Frequency of the currents increased by 4.9 ± 2.2-fold (means ± SD, n = 5) during application of thrombin (0.4 U/ml, measured between 30 and 90 s), whereas the holding membrane currents showed no observable changes under this condition. The second application of thrombin (0.4 U/ml), after an interval of 5 min, failed to elicit these responses (n = 5), suggesting that desensitization or an irreversible mechanism of signal transduction is involved in the response.

Phorbol ester has also been shown to facilitate release of vesicles from platelets by activating protein C kinase in the cell (8). Figure 3B shows that phorbol ester (PMA) also enhances the release of adenine nucleotides from a megakaryocyte. About 30 s after the application of PMA (100 nM), the frequency of spikelike inward currents increased considerably (Fig. 3B, bottom). After an application of PMA, the frequency was increased by 3.8 ± 2.3-fold on average (means ± SD, n = 4). Adenine nucleotides, such as ADP, are ubiquitous stimulators of platelets, activating various kinds of purinoceptors on the cell (6, 20). When ADP was applied from a Y tube to a megakaryocyte (2 μM for 2 s; Fig. 3C), multiple spikelike inward currents appeared on top of the slow inward current. The spikelike currents showed characteristics similar to those occurring spontaneously or those induced by phorbol ester or thrombin. The slow component of the inward currents looked the same as those shown in Fig. 1B, although the duration of the slow current in the sample record appeared to be longer, probably due to the use of nystatin-perforated-patch recording. The effects of thrombin (0.4 U/ml), PMA (100 nM), and ADP (2 μM) on the frequency of rapid inward currents under the condition of nystatin-perforated-patch recording are summarized in Fig. 3D. All of the increases in frequency (%control) were statistically significant.

Detection of release activity with a CFE. In this series of experiments, the intracellular milieu of the megakaryocyte seemed to be maintained intact because no whole cell patch electrode was applied to the cell. By the use of a CFE (tip diameter, 5 μm; holding potential, 820–850 mV) in contact with on the megakaryocyte, generation of oxidation currents caused by release of oxidizable substances from the cell (e.g., 5-HT) was measured. In the control megakaryocyte, the rate of detection of oxidation currents (Fig. 4A, upper current trace) was 1.0 ± 0.9 events/min (means ± SD, n = 4). When thrombin was applied at a concentration of 10 U/ml to the cell, rate of detection increased to 13.0 ± 9.5 events/min (means ± SD, n = 4; Fig. 4A, lower current trace). The amplitude of the oxidation currents thus detected, however, was generally small, being 10 pA or less. The most likely reason for this was a low concentration of 5-HT in the storage site of the megakaryocyte rather than insufficient stimulation for release from the storage site, because the dose of thrombin used (10 U/ml) was confirmed to be sufficient for the megakaryocyte to induce release (Fig. 4A, inset).

Thus, in the following experiments, megakaryocytes were incubated beforehand for 20 min at 30°C in saline containing 100 μM 5-HT to load the cells. Also, the sensitivity of a CFE to serotonin was calibrated assiduously by puffing 1 μM serotonin to its tip for 800 ms before each experiment (example shown in a box in Fig. 4B). CFE showing oxidation currents with amplitudes of >30 pA and with a 10–90% rise time of <200 ms were selected for subsequent use. Figure 4B shows results of measurements from a megakaryocyte thus loaded...
with exogenous serotonin to which thrombin was applied from a Y tube for activation. After a delay of 5–10 s, numerous spikelike currents were detected with the CFE (upward flexion on the current trace). Before application of thrombin, the frequency of the spikelike currents was 1.3 ± 0.8 events/min, but it was increased to 23 ± 16 events/min by thrombin (means ± SD of n = 5; measured during 1–2 min after the onset of application). Over a period of 2–3 min, the frequency then decreased gradually to the resting level. Even in the same cell, the current responses showed large variation of peak amplitudes, as well as of rising and decaying time courses (Fig. 4B, a–c). These variations may have been the result of various factors, including difference in distances between the release site of the cell and the detection site of the CFE, inhomogeneous sensitivities of the electrode spots, and/or diversities in the serotonin content of the vesicles. Further study is needed to determine the reasons for the variations.

Simultaneous detection of release activity with a CFE and a whole cell electrode. Simultaneous recordings of whole cell membrane currents and oxidation currents were obtained from the same cell by using a patch electrode (holding potential, −43 mV) and a CFE (tip diameter, 5 μm; holding potential, 820–850 mV), respectively (Fig. 5A). The internal saline in the patch electrode was supplemented with 400 μM GTPγS as before. To prevent contamination from noise, thresholds for the detection of the currents in following analyses were set at 1 and 10 pA for oxidation currents and for whole cell currents, respectively. Furthermore, to eliminate the possibility of con-
tamination of slow types of inward currents, possibly generated by mechanisms other than a mechanism via P2X1 receptors, inward currents having prolonged 10–90% rising times (>500 ms) were omitted from the analysis. Sample traces in Fig. 5, A and B, show that the time courses of oxidation currents were much faster than those of whole cell inward currents and that the oxidation currents, if detected, always occurred at the rising phases of the whole cell inward currents. In the case of the cell for which results are shown in Fig. 5B, 40 events of oxidative currents and 147 events of whole cell currents were detected during a recording period of 360 s. A scatter plot of the whole cell and oxidation currents from these recordings is shown in Fig. 5B, bottom left. Among the plots, 25 pairs of events were detected simultaneously (i.e., with a time difference of 100 ms or less) and are plotted with open circles in the scatter plot. The amplitude of the oxidation currents definitely depends on the distance from the release site to the recording site because the CFE (tip diameter, 5 μm) can cover only a fraction of the surface of the megakaryocyte under study (diameter, 20–30 μm). It is likely that release of vesicles, if occurring very close to the CFE, can evoke large oxidation currents (such as the first current event in the inset trace in Fig. 5A). The scatter plot in Fig. 5B, bottom left, shows a poor correlation and supports the above-stated interpretation. Results similar to those described above were obtained in five other megakaryocytes. Histograms of the 10–90% rise times of whole cell currents for 25 events are shown in Fig. 5B, bottom right. The distribution skewed to longer values than those of megakaryocytes shown in Fig. 1. One possible explanation for this is that metabolic states of the megakaryocytes during incubation with serotonin may be affected in such a way as to cause slowing of the gating of inward currents. The mean of 10–90% rise times still remained <100 ms (i.e., 94 ± 81 ms; means ± SD, n = 25). These observations were confirmed in five other megakaryocytes studied. As an additional analysis, using a software program (Mini Analysis), a cross-correlation histogram of oxidative currents against whole cell membrane currents was calculated for three records, including those shown in Fig. 5B (duration, 300–360 s). All of the histograms showed a sharp peak at time zero (bin width, 100 ms), confirming that occurrence of both of the events was highly coincidental. It thus seems reasonable to conclude that serotonin and adenine nucleotides are simultaneously released from a storage site; that is, they are coreleased from the same dense body.

**DISCUSSION**

The present study has shown that megakaryocytes can release adenine nucleotides and serotonin in a discrete but simultaneous manner, presumably by exocytosis of dense bodies. Release phenomena of dense bodies or δ-vesicles have attracted interest because a defect of δ-vesicles causes severe clinical symptoms (3, 25) and because molecules regulating the vesicular release show close similarity to those in the nervous system (4, 32, 43). Specific accumulation of serotonin and ATP (and also ADP) into dense bodies and their later release from megakaryocytes and platelets were confirmed by biochemical methods (8, 26, 42, 43, 44). The observations in the present study are consistent with those findings.

There are three kinds of cation channels functioning on megakaryocytes that could be activated by extracellular adenine nucleotides; namely, ionotropic P2X1 receptor, “IP3 (or its metabolite)-dependent cation channel,” and “store-operated Ca2+ channel” (14, 35, 36). Functional properties of these cation channels seem to be distinct in their activation time course, cation permeability, gating mechanisms, and sensitivity to blocking agents. Actually in the present preparation, application of adenine nucleotides (mixture of 20 μM ADP and 20 μM ATP) to the megakaryocyte induced fast inward current, which was followed by delayed inward current in one third of the cells (Fig. 1B). The former and the latter were thought to be due to activation of P2X1 receptors and activation of IP3-dependent channels on the plasma membrane, respectively (schema in Fig. 6). In the present study, inward currents via the third mechanism, namely mediated by store-operated Ca2+ channel, seemed negligible because external Ca2+ was omitted from all of the salines perfusing the megakaryocytes. Addition of GTPγS to the intracellular solution may have further reduced the store-operated Ca2+ channel, as described in the megakaryocytes (37). Distinction between the two currents having fast time course (via P2X1 receptor) and delayed time course (via IP3-dependent channel) were clear in most of the measurements. One might, however, wonder that if local con-

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![Fig. 6. Model for generation of spontaneous inward currents in megakaryocytes. **Top** shows candidate molecules on the plasma membrane of a megakaryocyte being involved in the generation of spontaneous inward currents. The thick horizontal line represents the plasma membrane, connected with a demarcation membrane system (curved line at right). The area above the line corresponds to extracellular space. **Bottom**, 1 and 2, illustrate 2 possible mechanisms of the generation of inward currents. 1: activation of P2X1 receptors by ATP released from a dense body; 2: activation of IP3-dependent cation channels by intracellular IP3 produced in the cell (details unknown as indicated by question mark). On the demarcation membrane, some of these molecules may also be expressed. P2X1, P2X1 receptor; P2Y1, P2Y1 receptor; P2Y12, P2Y12 receptor; and IP3, inositol trisphosphate. Na+ represents permeant cations, including Na+ and K+ (also, Ca2+ for P2X1 receptors). See text for further explanation.](http://ajpcell.physiology.org/)
centration of IP3 (or its active metabolite) in the megakaryocyte should increase and decrease rapidly, then IP3-dependent cation channels could be activated transiently to induce fast inward currents (Fig. 6, bottom). This possibility seems minor, if any, in the present study because almost all the spontaneous rapid inward currents (with rise time <200 ms) were blocked by specific purinergic antagonist PPADS or by desensitizing agonist α,β-meATP (Fig. 2C). In this regard, it should be noted that time course of the inward currents mediated by P2X1 receptors can be slowed if induced by low concentration of the agonist or induced in cells having a modified cytoskeleton (31). This possibility becomes likely when P2X1 receptors on the demarcation membrane were involved in the generation of the currents (upper schema in Fig. 6). Furthermore, it was thought that some reagents (for example, suramin, Fig. 2B; serotonin, Fig. 5) might modify the time course of the rapid inward currents, which showed variability in kinetics of the receptor involved. Presence of serotonin receptors of 5-HT2 subtype has been shown in the megakaryocytes and the platelets (1, 45). Errors due to these factors or variability affecting the time courses and also the peak amplitudes of the currents may be included in the results of the present study, particularly in the results of scatter plot analysis shown in Fig. 1A and Fig. 5B.

The frequency of the spontaneous rapid inward currents showed considerable intercell variation; some megakaryocytes generated more than 20 events/min, whereas other megakaryocytes generated <1 event/min. This variation is presumably due to varied maturational stages or different regulatory activities of the megakaryocytes isolated from bone marrow (21, 39). It seems possible that release phenomena detected under the condition of whole cell patch clamping might be caused artificially by some disturbances in the intracellular milieu via the patch electrode. This possibility, however, seems unlikely because spontaneous release was detected also by using nystatin-perforated whole cell recordings, in which disturbance of the metabolic state of the cell seems minor (Fig. 3). Furthermore, similar thrombin-induced release phenomena from megakaryocytes were detected by amperometry without patch clamping (Fig. 4).

It is relevant to note that there is controversy regarding agonist specificity of the P2X1 receptor. Mahaut-Smith et al. (23) and Vial et al. (41) suggested that the P2X1 receptor can be activated by ATP but not by ADP and that the observed response evoked by ADP was due to contamination of ATP. On the other hand, Greco et al. (12) have found a variant of P2X1 receptors, which showed an agonist rank order of ADP ≫ ATP ≫ α,β-meATP. Thus, when referring to the active substance in the present study being released from dense bodies and generating rapid inward currents, the term adenine nucleotides was principally used instead of ATP or ADP. Involvement of other transmitter substances and their receptors in the generation of rapid inward currents was unlikely. Other nucleotides (such as adenosine, adenosine monophosphate, and uridine triphosphate) or major ionotropic neurotransmitters (such as glutamate, GABA, glycine and, ACh) failed to generate measurable responses in the present preparations even at a dose of 100 μM (applied from a Y tube, data not shown).

An amplitude histogram of spontaneous rapid inward currents obtained in the present study showed a skewed distribution rather than a simple Gaussian distribution (Fig. 1A). Possible reasons for the skewed distribution include varied sizes of the released vesicles or their contents (17), uneven densities of the corresponding receptors on the cell surface (28), including the demarcation membrane (24), and simultaneous release of multiple vesicles (multivesicular release; Ref. 2). In human platelets, multivesicular release has been suggested theoretically (10). Whether multivesicular release also occurs in megakaryocytes, particularly from δ-vesicles, is an interesting but unresolved question. The present study also showed that serotonin is coreleased from the same vesicle with adenine nucleotides (Fig. 5). In the nervous system, corelease of bioactive substances has been documented (15, 16). In isolated chromaffin cells with heterologous expression of P2X receptors, corelease of adenine nucleotides and catecholamines from the same vesicles was demonstrated (13).

As for the physiological roles of vesicular exocytosis in megakaryocytes, it is relevant to note that cell-to-cell signaling in mast cells and in mammary epithelial cells occurs through release of ATP or pyrophosphorylated nucleotides from mast cells (30) or from adjacent epithelial cells (9, 34), respectively. The actions of these released substances are mediated by their specific receptors and transformed into the spread of intracellular calcium signals or other pathways. Such a novel type of signal transmission among cells may also occur in megakaryocytes in vivo and may underlie refined regulation of cellular differentiation or maturation in concert with other trophic factors (27).

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