Thrombopoietin Enhances Rapid Current Responses Mediated by P2X1 Receptors on Megakaryocytic Cells in Culture

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Abstract: The effect of thrombopoietin (TPO), a megakaryocyte-poitic cytokine, on the functional maturation of megakaryocytes was studied by using cell culture and patch-clamp techniques focusing on purinergic 2X1 (P2X1)-receptors, which are expressed specifically on platelets and their progenitors. Meg-01 cells, one of the typical human megakaryocytic cell lines, were cultured and studied by using a whole-cell patch electrode. In control cells cultured in RPMI1640 medium, an application of adenosine nucleotide (ADP, 40 μM) evoked transient inward currents with amplitudes of 45±19 pA (at −43 mV). Based on kinetic, ionic, and pharmacological properties as well as on previously reported findings, these currents were thought to be mediated by P2X1 receptors. When Meg-01 cells were cultured for 7–9 d in a medium to which the differentiation-inducing agent phorbol ester (PMA; 10 nM) or TPO (100 ng/ml) had been added, the responses of the cells to ADP increased to about 150% of the control with PMA and to about 200% of the control value with TPO. A combination of the two agents enhanced the response of the cells to ADP to about 570% of the control value. These results suggest that phorbol ester and TPO cause cellular differentiation of Meg-01 cells and enhance the level of expression of P2X1-receptors on cell membranes in a synergetic manner. The effect of TPO on the induction of P2X1-receptors on mouse megakaryocytes in culture was more obvious. [Japanese Journal of Physiology, 53, 287–299, 2003]

Key words: thrombopoietin, P2X1 receptors, megakaryocyte, patch clamp, cell culture.
TPO receptors on Meg-01 cells has been confirmed [24]. The induction of platelet formation from Meg-01 cells by nitric oxide and TPO has demonstrated the usefulness of the cells as a model of human megakaryocytes [25]. Also, messenger RNA of P2X1 receptors has been detected in Meg-01 cells by using PCR techniques [12, 13]. Vial et al. [12] have shown by using Ca\(^{2+}\)-sensitive dye (Fura-2/AM) that P2X1 receptors on Meg-01 cells can respond to extracellular ADP and cause an increase in the concentration of Ca\(^{2+}\) in them. Megakaryocytes can be easily isolated from the bone marrow of mammals and can be maintained in culture. The basic electrophysiological properties of rodent megakaryocytes have been described in detail [11, 26]. Using patch-electrodes and a rapid drug application system, the Y tube, P2X1 receptors that are functional on these megakaryocytic cells in culture were studied.

The results showed that phorbol ester and TPO caused a differentiation of Meg-01 cells and enhanced the expression of functional P2X1 receptors in a synergistic manner. In isolated mouse megakaryocytes, the effect of TPO, which enhances the expression of P2X1-receptors, was also determined.

**MATERIALS AND METHODS**

**Preparation of megakaryocytes.** 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 d, were anesthetized deeply with vaporized diethyl ether and then bled to death by cutting the cervical arteries in the neck. 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. The marrow was removed with a pair of forceps and placed in a plastic dish containing Ca\(^{2+}\)-free external saline. From a few pieces, megakaryocytes were dissociated mechanically with a pair of fine forceps in a recording chamber (Falcon, Primaria tissue culture dish of 35 mm in diameter, Becton Dickinson, USA). To facilitate dissociation, we washed out the bone marrow with a disposable syringe (volume, 1 ml) and needle with Ca\(^{2+}\)-free external saline. Before the experiment, the chamber was left for 10 to 15 min at room temperature until the dissociated megakaryocytes had settled on the bottom.

**Cell culture.** Meg-01 cells, a human megakaryoblastic leukemia cell line [18, 20], were obtained from the Japan Health Science Foundation (Tokyo, Japan) and maintained in RPMI 1640 medium ( Gibco-BRL, USA) with 10% heat-inactivated fetal calf serum (FCS, Gibco-BRL). CMK-11-5 cells, another human megakaryoblastic leukemia cell line [27], were also obtained from the foundation and maintained as described above. The culture dishes used (diameter, 35 mm; Falcon, Primaria, NJ, USA) were surface-modified with polystyrene. Under aseptic conditions, mouse megakaryocytes for culture were isolated from bone marrow. Surgical tools and glassware were autoclaved beforehand, and all saline was filtered through a disposable filter (Steradisc, Kurabo, Japan). Before the start of incubation, the external medium was changed from Ca\(^{2+}\)-free saline to RPMI 1640 medium with 10% FCS. Some culture media also contained phorbol ester or thrombopoietin, or both. All cells were incubated at 37°C in a fully humidified atmosphere with 5% CO\(_2\). Each culture medium was replaced by a fresh medium every 4 d during the period of incubation. The cell diameters were estimated by calculating geometrical means of the longest and shortest axes of the cells measured under a phase-contrast microscope.

**Patch clamp recordings.** The culture dish containing freshly isolated megakaryocytes, or cells after several days of culture, was mounted on the movable stage of a phase-contrast inverted microscope (final magnification, ×600; Diaphot-TMD, Nikon, Tokyo, Japan), and its field of view was continuously monitored with a TV camera (KP-140, Hitachi, Tokyo, Japan). By the use of a video-capture board (GV-VC2P2M, I.O. Data, Japan), images of the cells were stored as photo files. The culture dish used for the recording chamber was perfused with experimental saline at a rate of 1–2 ml/min, and all culture medium was washed out before the start of patch clamp recordings. The temperature of the chamber was monitored with a thermistor and maintained at 24±1°C. Whole-cell currents in Meg-01 cells or megakaryocytes were measured by a conventional patch-clamp technique [11, 26]. 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 with a vertical pipette puller (PP-83, Narishige, Tokyo, Japan). The access resistances of the patch electrodes filled with an internal solution ranged from 2 to 5 M\(\Omega\) and were monitored intermittently by subjecting the inside of the pipette to 1-mV pulses. If the access resistance increased abruptly by more than 10 M\(\Omega\), the experiment was stopped. The configuration for whole-cell recording was established in Ca\(^{2+}\)-free external saline unless stated otherwise. When a gigaohm seal with a cell had been obtained, a negative pressure of up to 300 mmH\(_2\)O was applied to
rupture the membrane at the tip of the patch-electrode. The whole-cell membrane capacity of the cell was measured immediately after obtaining whole-cell configuration by measuring the capacitative transient in response to a 10-mV voltage step [28]. Special care was taken to adjust the compensation circuit of a patch-clamp amplifier (EPC-7) for nullifying the stray capacity of the electrode before the patched membrane was ruptured. The ground electrode, which was an Ag–AgCl wire, was connected to the external solution with an agar bridge containing 3 M KCl. The correction of the liquid junction potentials (\( F_j \)) was determined by introducing 3 M KCl saline into the perfusion chamber.

**Drug application.** Drugs were applied by using a rapid application technique known as the Y tube method. The modifications used in this study (i.e., the use of a 1.4 mm delivery tube with a diameter of 300–500 \( \mu \)m and the monitoring of pressure with a digital manometer) are the same as those used previously [11]. The delivery tube outlet was usually positioned 200–300 \( \mu \)m from the cell being recorded. When ADP was applied repeatedly to a cell, the application was performed at intervals of more than 5 min to prevent a desensitization of the receptors [11].

**Data acquisition and analysis.** The signals of membrane current and potential were monitored simultaneously with a storage oscilloscope (5111, Tektronix, USA) and a pen recorder (Recticorder, Nihon Kohden, Tokyo, Japan). The electrical filters used were analog-type filters with variable corner frequencies (Model 900, Frequency Device, Massachusetts, USA). The signals were also transformed with a pulse code modulator (501-ES, SONY, Tokyo, Japan) into digital modes and stored on a videocassette recorder (VT-F55, Hitachi). The membrane currents were analyzed both on-line and off-line with a digitizer (Digidata 1320A, Axon Instruments, CA, USA) connected to a personal computer (Dimension 700cx, Dell, Japan, Tokyo, Japan). The sampling rates were 10 kHz for ADP-induced currents and 50 kHz for capacitative transient currents. The following software programs were used for analyses: AxoScope (Axon Instruments); Mini Analysis (Synaptosoft, GA, USA), Origin version 6 (Microcal Software, MA, USA), Excel 2000 (Microsoft, WA, USA), and KaleidaGraph (Synergy Software, PA, USA). All data are expressed as means \( \pm \) SEM unless otherwise indicated. An unpaired Student’s \( t \)-test and Welch’s test were used to evaluate the statistical significance of differences between the two groups being compared. For significant changes in the ratios, a test for the \( F \) distribution was used.

**Solutions and drugs.** The external saline routinely used was Ca\(^{2+}\)-free external saline containing (mM) NaCl, 140; KCl, 5; MgCl\(_2\), 1; D-glucose, 10; and N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid (HEPES), 10 (pH 7.4, adjusted with NaOH). The omission of Ca\(^{2+}\) from the external saline seemed to be effective for preventing uncontrollable deterioration of the megakaryocytes before and during the experiments [11]. The drug for external application was dissolved in the Ca\(^{2+}\)-free external saline and applied with a Y tube. For whole-cell recordings, we used the following to fill patch-pipettes: CsCl internal saline containing (mM) CsCl, 145; MgCl\(_2\), 4; ethyleneglycol-bis-(\( \beta \)-amino-ethyl-ether)N,N’-tetraacetic acid (EGTA; \( K^+\)-salt), 1; and HEPES, 10 (pH 7.2, adjusted with KOH). In an early series of experiments (Fig. 1A for the study of \( K^+\)-carried currents), KCl internal saline that contained 145 mM KCl in place of 145 mM CsCl was used. The free Ca\(^{2+}\) concentration in each of these internal salines was 61 nM (estimated as in [11]). Adenosine-5’-diphosphate (ADP; Sigma A2754, Lot #144H7820), \( \alpha,\beta\)-methylene adenosine-5’-triphosphate (\( \alpha,\beta\)-meATP), phorbol 12-myristate 13-acetate (PMA), D-glucose, bovine serum albumin, and inorganic salts were purchased from Sigma Chemical Co. (St. Louis, USA). HEPES and EGTA were purchased from Dojin Laboratories (Kumamoto, Japan). Thrombopoietin (recombinant human TPO, 100 \( \mu \)g at 0.2 mg/ml in each vial) was kindly provided by Kirin Brewery Co. (Tokyo, Japan).

**Immunohistochemistry.** Indirect fluorescence immunohistochemistry for the purinergic receptor P2X\(_1\) was performed on cultured Meg-01 cells and on mouse megakaryocytes freshly isolated from bone marrow. Megakaryocytic cells that had settled on the base of a chamber (similar to a recording chamber) were fixed with 10% formalin in a phosphate-buffered saline (PBS) for 30 min and then gently rinsed twice with PBS, followed by further rinsing with PBS containing 4% bovine serum albumin and 0.25% Triton X. The cells were covered for 120 min at room temperature with 1 ml of diluted antiserum (final dilution 1:200; containing a polyclonal antibody against P2X\(_1\) receptors raised in a rabbit, APR-001, Alomone Labs Ltd., Israel) then rinsed 3 times with PBS for 10 min each time. Each preparation was then allowed for 90 min with a second antibody solution: rhodamine-conjugated antirabbit-\( \gamma \)-globulin (AP-182R, Chemicon International Inc., CA, USA; final dilution 1:100). After rinsing in PBS three times, the cover-glass slip was mounted with 90% glycerol in PBS in the chamber, then viewed under a Zeiss epifluorescence microscope (AxioVision system, Carl Zeiss, Germany).
Co., Germany; fitted with a filter for rhodamine fluorescence, objective ×63) and photographed with an attached digital camera.

RESULTS

Electrical properties and responsiveness of Meg-01 cells to ADP

Meg-01 cells are megakaryocytic cells derived from a patient with leukemia and have been used as a model of human megakaryocytes under various experimental manipulations [18, 21, 22, 25]. In a control culture medium, about half the Meg-01 cells adhered to the base of the dish and showed various forms such as sphere, stellate, and spindle. The remaining cells were mostly spherical in shape. To characterize the electrical properties of Meg-01 cells, a whole-cell patch electrode (containing KCl internal saline) was applied to a cell, and the membrane potential was clamped at various levels. Fifty-four of 58 Meg-01 cells studied showed no obvious voltage-dependent membrane currents in response to depolarizing or hyperpolarizing voltage steps (from a holding potential of −63 mV to depolarization up to 18 mV or to hyperpolarization down to −142 mV; Fig. 1A). A typical current–voltage relationship from one Meg-01 cell is shown in the right graph of Fig. 1A. The relation in the range of membrane potentials studied was almost linear. Notably, two of the 58 Meg-01 cells showed slow outward currents at depolarization (93 pA and 133 pA evoked at 18 mV, from a holding potential of −62 mV), and two other cells showed delayed slow inward currents at hyperpolarization (240 pA and 75 pA evoked at −142 mV; from a holding potential of −42 mV). These voltage-dependent currents were not analyzed because of the small number of observations. Next, the responses of Meg-01 cells to ADP were studied. CsCl internal saline was used to stabilize the recordings of the whole-cell patch clamp [11]. An application of 40 μM ADP from a Y tube evoked transient inward currents in Meg-01 cells. The peak amplitude of the currents measured at −43 mV showed considerable intercell variation with a range from 10 to 180 pA (45 ± 9 pA, n = 10). The time course of the evoked currents in Meg-01 cells, however, showed little variation (sample traces in Fig. 1B and C).

For comparison with another human megakaryocytic cell line, CMK-11-5 cells were maintained in culture (RPMI 1640 medium with 10% FCS), and CMK-11-5 cells that had adhered to the dish were selected for measuring ADP-induced responses. Following an application of ADP (40 μM), the CMK-11-5 cells evoked transient inward currents having peak amplitudes of 60 ± 49 pA (n = 9; at a holding potential of −43 mV). The kinetic properties of the currents were similar to those of Meg-01 cells. These observations are consistent with the results of previous studies showing that messenger RNA as well as the expressed molecules of the P2X<sub>1</sub> receptor were present in CMK-11-5 cells [13, 16]. In a separate series of experiments, ADP-induced inward currents from mouse and guinea pig megakaryocytes were measured. The methods for the recording were the same as those described previously [11]. A comparison of ADP-induced inward currents from three kinds of cells (Meg-01 cells, mouse megakaryocytes, and guinea pig megakaryocytes) is shown in Fig. 1B (right columns). Megakaryocytes from the mouse and those from the guinea pig showed significantly larger amplitudes of ADP-induced currents than the Meg-01 cells did (p<0.05; measured at −43 mV; ADP 40 μM), suggesting that Meg-01 cells cultured in a control medium remain at an immature stage of differentiation.

It is notable that ADP-induced currents in Meg-01 cells showed a slow recovery after a preceding application of ADP (Fig. 1C). Even with an interval of 6 min, currents induced by the second application of ADP (40 μM for 4 s) reached only about 50% in amplitude. A slow recovery was also observed in Meg-01 cells cultured in the presence of thrombopoietin and phorbol ester. However, the time course of the ADP-induced currents when evoked repeatedly remained almost unaltered except for an occasional slight prolongation in the rising phase (Fig. 1C, right, inset traces). To avoid complexity in subsequent experiments, ADP-induced transient inward currents from each cell were measured on the first application of ADP. It has been confirmed that fast purinergic responses in megakaryocytic cells generating such rapid inward currents as those described above were mediated solely by P2X<sub>1</sub> receptors on the cells [11–13]. Notably, the presence of messenger RNA of the P2X<sub>1</sub> receptor and their expression in Meg-01 cells has been reported [12]. In the present study, the application of a specific agonist of the P2X<sub>1</sub> receptor (α,β-meATP of 10 μM [14]) induced rapid inward currents in Meg-01 cells (n = 4, sample record shown in Fig. 1D). These findings are consistent with results of an immunohistochemical study on P2X<sub>1</sub> receptors (Fig. 4A, below). Thus in the following description, it seems reasonable to refer to these receptors as P2X<sub>1</sub> receptors.
Effects of thrombopoietin and phorbol ester on the development of P2X$_1$ receptors on Meg-02 cells in culture

To investigate the effect of TPO on Meg-01 cells, the cells were cultured in a medium with TPO (100 ng/ml) for 1–14 d. The ADP-induced currents from Meg-01 cells that had settled on the bottom of the culture dish were measured with a patch-electrode (upper left traces in Fig. 2A). After 8 d of culture, the peak amplitude of the currents showed large intercell variations (from 5 to 350 pA at $-43$ mV, $n=7$), but the mean value increased to 203% of the value of control Meg-01 cells, (to 91+64 pA, from 45+19 pA; open circles in the lower right graph of Fig. 2A). This increase was significant ($p<0.05$, $t$-test). After 14 d of culture, the ADP-induced currents in Meg-01 cells were 100+632 pA ($n=11$), the mean of which had significantly increased to 220% of the control value ($p<0.05$, $t$-test). Morphological observation under a phase-contrast microscope, however, revealed no re-
Fig. 2. Effects of thrombopoietin and phorbol ester on development of P2X1 receptors on Meg-01 cells. A: Sample records of ADP-induced inward currents in Meg-01 cells cultured in the presence of TPO (100 ng/ml; left traces) and PMA (10 nM; right traces). The trace at the top of the left indicates a command pulse for operating the Y tube to apply ADP (40 µM, for 4 s). Culture time (d) was indicated on each trace. Holding potential, −43 mV; external medium, Ca²⁺-free saline. The patch-pipette contained CsCl internal saline. Lower graphs, effects of TPO and PMA on half-decay time of ADP-induced currents (left graph) and peak amplitude of ADP-induced currents (right graph) in Meg-01 cells shown as a summary of the results of experiments. In both graphs, open and filled circles represent mean amplitudes of the currents recorded from Meg-01 cells cultured in TPO (100 ng/ml) and in PMA (10 nM), respectively. The abscissa is culture time (d). Numbers of cells are shown in parentheses. Bars indicate SEM. B: Changes in whole-cell membrane capacity and in density of ADP-induced currents of Meg-01 cells during culture. Left, whole-cell membrane capacity of each Meg-01 cell studied was obtained from capacitative transient of the whole-cell membrane current that was generated in response to changes in the holding potential of the cell (from −40 to −50 mV, as shown in the sample trace). On the right, a summary of data from Meg-01 cells is shown by open squares and filled squares for cells cultured in the presence of TPO (100 ng/ml) and PMA (10 nM), respectively. Lower graph shows changes in the density of ADP-induced currents of Meg-01 cells. The current density (pA/pF) was obtained after dividing the amplitude of ADP-induced current in each cell (pA) by whole-cell capacity of the cell (pF). In all the above graphs, the abscissa is culture time (d). Numbers of cells are shown in parentheses. Bars indicate SEM. *Indicate significant difference (p<0.05, t-test or Welch’s test) from the control value (obtained before the addition of TPO or PMA).

Markable changes in Meg-01 cells during the culture period (8 to 14 d in TPO). To evaluate changes in the kinetics of the ADP-induced currents during culture, the half-decay times of the ADP-induced currents were plotted in the lower left graph of Fig. 2A. The values (open circles) showed no significant changes during culture with TPO.

The effects of phorbol ester, a differentiation-promoting substance, on Meg-01 cells in culture, are shown in the upper right figure (sample records) and the lower right graph (plotted with filled circles) in Fig. 2A. When phorbol-12-myristate-13-acetate (PMA, 10 nM) was added to the medium, the sizes of the cells decreased, and about 30% of the Meg-01 cells appeared to be degenerating on the following day (after 1 d of culture). ADP-induced currents in Meg-01 cells had also decreased to 3.5±2.5 pA (n=6). After 2 d of culture with PMA, the amplitude of ADP-...
induced currents had recovered to 42±28 pA (n=7). After 9 d of culture, ADP-induced currents had increased to 67±28 pA (n=6), indicating that the mean amplitude had increased significantly to about 150% of the control value (p<0.05, t-test). PMA-treated Meg-01 cells in these cultures (up to 9 d) were less numerous than control cells, presumably because of the inhibition of proliferation, as suggested previously [29]. The half-decay times of the ADP-induced currents showed no significant changes during culture with PMA (filled circles in the lower left graph in Fig. 2A). Thus the major changes in ADP-induced currents are presumably due more to changes in the number of P2X$_1$ receptors on the cells than to a modification of the gating properties or agonist affinity of existent receptors.

To further analyze the characteristics of changes in ADP-induced currents, whole-cell membrane capacity and current density of Meg-01 cells were measured. The upper left trace in Fig. 2B shows a sample trace for measurement of whole-cell membrane capacity of a cell in which the capacitative transient current in response to a 10 mV-step voltage was used. Changes in whole-cell membrane capacity thus obtained from Meg-01 cells during culture are shown in the upper right graph in Fig. 2B. During culture with TPO (open squares), a small increase on day 8 and a small reduction on day 14 were observed; during culture with PMA (filled squares), a transient reduction on day 1 with a rebound on day 2 and recovery afterward were observed. These changes in whole-cell membrane capacity are almost consistent with the morphological changes described above. The lower graph in Fig. 2B shows changes in the density of ADP-induced currents (pA/pF) of Meg-01 cells during culture after dividing the amplitude of ADP-induced current in each cell (pA) by whole-cell capacity of the cell (pF). During culture with TPO, the mean current density (open diamonds) increased gradually and reached 231% of the control value on day 14. During culture with PMA, the mean current density (filled diamonds) showed a transient reduction and recovery on days 1 and 2, respectively, then increased to 197% of the control value on day 9.

**Combined effects of thrombopoietin and phorbol ester on the development of P2X$_1$ receptors on Meg-01 cells**

The differentiation of megakaryocyte is regulated by multiple factors, some of which have synergic effects. Figure 3 shows the combined effects of TPO and PMA on Meg-01 cells. These cells were cultured in the presence of both TPO (100 ng/ml) and PMA (10 nM), and ADP-induced currents were measured after 3, 5, 8, and 20 d of culture. The ADP-induced currents peaked after 5 d of culture (Fig. 3A), reaching 570% (256±92 pA, n=9) of the control value. The currents then decreased and became about 280% (126±55 pA, n=7) of the control value after 20 d of culture. The inset in Fig. 3A shows sample current traces of the experiments. Most Meg-01 cells in this series of experiments settled on the bottom of the dish and had various shapes. In the right box of Fig. 3, the shapes of nine Meg-01 cells selected for measurement after 5 d of culture with TPO and PMA are shown. The order of illustration is in accordance with the amplitudes of ADP-induced currents in the cells. These drawings suggest that Meg-01 cells of larger sizes and massive processes are prone to generate larger ADP-induced currents than smaller cells are. This tendency was also observed when whole-cell membrane capacity of the cell (value in parentheses on each outline drawing) was considered as an index of cell size. Next, the possible changes in gating properties of P2X$_1$ receptors on these cells were studied by examining the half-decay times and the 10–90% rise times of the currents induced by ADP (40 μM). Figure 3B shows that the currents had almost identical time courses with similar half-decay times and similar 10–90% rise times despite a large variation in their amplitudes. This suggests that the observed variation in current amplitudes reflects differences in the number of functional P2X$_1$ receptors on these cells rather than a variation of gating properties of P2X$_1$ receptors.

The upper graph in Fig. 3C shows changes in whole-cell membrane capacity of Meg-01 cells during culture with TPO (100 ng/ml) and PMA (10 nM). The lower graph shows changes in the density of ADP-induced currents of these Meg-01 cells. The current density (pA/pF) was obtained after dividing the amplitude of ADP-induced current in each cell (pA) by whole-cell capacity of the cell (pF). The mean current density (open diamonds) of these Meg-01 cells had increased to 371% of the control value on day 6 and had reached 456% of the control value on days 7 to 9 of culture.

**Comparison with megakaryocytes isolated from the mouse**

Megakaryocytes dissociated from mouse bone marrow have been reported to express functional P2X$_1$ receptors on their surfaces [11]. The presence of P2X$_1$ receptors on mouse megakaryocytes was examined by using a specific polyclonal antibody that was raised against the peptide corresponding to residues 382–399
of the rat P2X₁ receptor (APR-001, Alamone Labs). The megakaryocytes that had settled on the bottom of the culture dish were processed for immunohistochemistry and viewed under an epifluorescent microscope. The left part of Fig. 4A shows that immunoreactions for the P2X₁ receptor were positive in the mouse megakaryocytes (23 of 24 cells with diameters of 25 μm or more that were observed), but the reaction was very faint or negative in other blood cells. When processed without using the primary polyclonal antibody (APR-001), thesemegakaryocytes were not stained, suggesting that the immunohistochemical reaction for P2X₁ receptors was specific and useful for the present analysis. Similarly, Meg-01 cells in the culture dish were processed for staining. About 98% of the Meg-01 cells were positive for the reaction (106
Unexpectedly, a close examination revealed that cellular fractions other than the surface membrane were also stained in some Meg-01 cells. This observation indicates the possibility of the presence of nonfunctional P2X<sub>1</sub> receptors within the cell or reactivity of the antibody to some unidentified molecules.

Figure 4B shows the effects of thrombopoietin on megakaryocytes isolated from the mouse. Megakaryocytes dissociated from mouse bone marrow were cultured for up to 9 d, either in a control medium (RPMI 1640 with 10% fetal calf serum; left) or in a test medium containing TPO (100 ng/ml; right). Typical megakaryocytes could easily be identified because of their large sizes and their tendency to adhere to the bottom of the dish, as shown by micrographs. Megakaryocytes cultured for 4 d with TPO were significantly larger (diameter 41±8 μm; mean±SD, n=15) than those cultured without TPO (diameter 25±5 μm; mean±SD, n=9) as shown in the upper graph.
right graph in Fig. 4B. After 8 d of culture, most of the megakaryocytes in the control medium had shrunk or died, but cells in the presence of TPO, which had a mean diameter of 113% of the value before culture, survived. The lower right graph in Fig. 4B shows changes in whole-cell membrane capacity of the megakaryocytes during culture with TPO. A reduction of whole-cell membrane capacity on day 3 and its recovery on days 8 and 9 were observed. This tendency is almost consistent with changes in cell diameter, but in the reduction on day 3 it was marked. Next, the responses of megakaryocytes to ADP were studied with a Y tube. When ADP (40 μM) was applied to the megakaryocytes that had been freshly isolated, transient inward currents were induced in the cells. The ionic mechanism and pharmacological properties of the receptors involved (purinergic P2X1 receptors) have been described in detail [11, 17]. Specimen traces in the upper part of Fig. 4C are current records from isolated megakaryocytes before culture (upper trace) and after an 8-d culture in a medium containing TPO (lower trace) measured at the membrane potential of −43 mV. The graph in the lower left part of Fig. 4C shows changes in whole-cell membrane currents measured from megakaryocytes before culture and after 3 d or 8 to 9 d of culture in a medium containing TPO. The graph shows that the amplitudes of ADP-induced inward currents of megakaryocytes cultured in the presence of TPO (100 ng/ml) for 8 to 9 d increased to 3.6-fold of the control value (from 252±42 pA, n=11 to 923±207 pA, n=10). This increase is statistically significant (p<0.05, t-test). The time course of ADP-induced currents showed no obvious changes during the culture period, again suggesting that the increase in current amplitudes reflects an increase in the number of functional P2X1 receptors on the cell. The lower right graph in Fig. 4C shows changes in current density (pA/pF) of the megakaryocytes, obtained after dividing the amplitude of ADP-induced current in each cell (pA) by the whole-cell capacity of the cell (pF). As expected, the mean current density of megakaryocytes (open diamonds) increased steadily and became 277% of the control value (i.e., before culture) after 8 to 9 d. This suggests that an increase in ADP-induced current amplitudes at this time accompanies or may be caused by an increase in the density of P2X1 receptors on the cell membrane.

**DISCUSSION**

Thrombopoietin and phorbol ester enhanced the ADP-induced inward currents in megakaryocytic cells, including Meg-01 cells of human origin and megakaryocytes isolated from mouse bone marrow, in culture. Despite marked changes in the amplitudes of ADP-induced currents of the cultured cells, there were only small changes in the time courses of the currents (Figs. 2A and 3A, B). The P2X1 receptors on Meg-01 cells showed prolonged desensitization or irreversible inactivation after a preceding activation (Fig. 1C). Such a deteriorative property may be characteristic of P2X1 receptors expressed on megakaryocytic cells [11]. This property was also observed in P2X1 receptors of Meg-01 cells under various conditions in the present study. These unaltered properties of ADP-induced currents suggest that the major mechanism of changes in the current amplitudes is a modifiable number of functional P2X1 receptors on the cell rather than a functional modification of existing P2X1 receptors on the cell (for example, changes in a single channel conductance, changes in gating properties, and/or changes in agonist properties of the receptor).

In the following analyses, it was assumed with reservation that changes in ADP-induced current amplitude observed in the present study reflect changes in the number of functional P2X1 receptors on cell membranes of megakaryocytic cells, until experiments using refined techniques (such as polymerase chain reaction, single channel recording, and immunohistochemistry with electron microscopy) were carried out.

P2X1 receptors, which have been detected on platelets, are thought to mediate rapid Ca2+ entry into platelets when activated with ADP or ATP [8, 11, 13]. Among experimental reagents, α,β-meATP is thought to be a specific agonist of the P2X1 receptor [14]. In Meg-01 cells, the application of α,β-meATP induced transient inward currents, such as those induced by ADP (Fig. 1D), suggesting the involvement of P2X1 receptors in the response. However, consideration must be given to the possibility that there exists a molecular variant of the P2X1 receptor among megakaryocytic cells that has different preferences among agonists, or to the possibility that P2X1 receptors change in agonist preference depending on their chemical state of modification in situ or on the type and species of cells expressing them [13, 16, 30]. For example, a physiological variant of the P2X1 receptor in cultured preparation showed agonist rank order of ADP>ATP>α,β-methylene-ATP [16]. In the present study, ADP (40 μM) was used as an experimental activator of the P2X1 receptor with sufficient care, as described previously [11]. Further pharmacological characterization of P2X1 receptors in Meg-01 cells will be done in the future.

**Meg-01 cells as a model for human megakaryocytes.** Meg-01 cells have several characteris-
Thrombopoietin Enhances P2X1 Response

Tics associated exclusively with megakaryocytes and have thus been considered suitable as a model for the study of human megakaryocytic maturation and differentiation [18, 20, 22, 23, 31]. The presence of P2X1 receptor mRNA and its protein in Meg-01 cells was confirmed by results of Southern blotting and Northern blotting analyses [12, 13]. So far, the detection of ADP-induced currents in leukemia cell lines or human megakaryocytes has been difficult, apparently because of the rapid desensitization of the P2X1 receptors and their slow recovery from desensitization (Fig. 1C). The present study, in which a rapid drug application technique was used, demonstrated for the first time that the current was actually inducible in human megakaryocytic cell lines (Meg-01 and CMK-11-5 cells) and that the P2X1 receptors involved have the same physiological properties as those of megakaryocytes or platelets in experimental animals and humans [8, 11, 17].

Kapural and Fein [32] found that different types of K+ channels were expressed in sequence during the maturation of human megakaryocytes. In the present study, it was found that most Meg-01 cells generated no obvious voltage-gated K+ currents or exhibited only a leakage-like current under control conditions in culture (Fig. 1A). This suggests that Meg-01 cells are arrested at a relatively early stage of development as far as their voltage-gated channels are concerned. This speculation is consistent with a finding that the expression of P2X1 receptors on Meg-01 cells was enhanced by TPO and PMA in a synergistic manner. TPO and interleukin-1 or TPO and S-nitroglutathione have been reported to induce the maturation of Meg-01 cells in a synergistic manner [25, 39]. The synergism in Meg-01 cells presumably occurs in some molecules functioning downstream of major intracellular kinases, including protein kinase C, Janus kinase, and phosphoinositol 3-kinase [4, 6, 40].

It is relevant to note that the enhanced expression of P2X1 receptors under a specific condition is very likely to have accompanied morphological changes in the cells, such as an increase in cell size and an extension of cellular processes (outline drawings in Fig. 3). In the present study, whole-cell membrane capacities of megakaryocytic cells were measured as an index for the surface area of these cells. It has recently been shown that megakaryocytes have many invaginations of the plasma membrane that are readily accessible from the external space and that the whole-cell membrane can be approximated by a simple biophysical model in which the entire membrane capacitance and parallel membrane resistance are in series with a single access resistance [28]. The results of current density of ADP-induced currents in the present study were interpreted on the basis of these findings (in Fig. 3C for Meg-01 cells and in Fig. 4C for mouse megakaryocytes). These interpretations indicate that the density of functional P2X1 receptors on the plasma membrane also increases in Meg-01 cells and in mouse megakaryocytes when the expression of P2X1 receptors is enhanced. Further study is needed to determine how P2X1 receptors are regulated in developing megakaryocytic cells in terms of their expression,
distribution, and maintenance.

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