ATP-induced Ca^{2+} response mediated by P_{2U} and P_{2Y} purinoceptors in human macrophages: signalling from dying cells to macrophages

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SUMMARY

The activation of macrophages by various stimuli leading to chemotactic migration and phago-cytosis is known to be mediated by an increase in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$). We measured changes in $[Ca^{2+}]_i$ using a Ca^{2+} imaging method in individual human macrophages differentiated from freshly prepared peripheral blood monocytes during culture of 1–2 days. A transient rise in $[Ca^{2+}]_i$ (duration 3–4 min) occurred in 10–15 macrophages in the vicinity of a single tumour cell that was attacked and permeabilized by a natural killer cell in a dish. Similar Ca^{2+} transients were produced in 90% of macrophages by application of supernatant obtained after inducing the lysis of tumour cells with hypo-osmotic treatment. Ca^{2+} transients were also evoked by ATP in a dose-dependent manner between 0·1 and 100 μM. The ATP-induced $[Ca^{2+}]_i$ rise was reduced to less than one-quarter in Ca^{2+}-free medium, indicating that it is mainly due to Ca^{2+} entry and partly due to intracellular Ca^{2+} release. UTP (P_{2U} purinoceptor agonist) was more potent than ATP or 2-chloro-ATP (P_{2Y} agonist). Oxidized ATP (P_{2Z} antagonist) had no inhibitory effect. Both cell lysate- and ATP-induced Ca^{2+} responses were inhibited by Reactive Blue 2 (P_{2Y} and P_{2U} antagonist) to the same extent, but were not affected by PPADS (P_{2X} antagonist). Sequential stimuli by cell lysate and ATP underwent long-lasting desensitization in the Ca^{2+} response to the second stimulation. The present study supports the view that macrophages respond to signal messengers discharged from damaged or dying cells to be ingested, and ATP is at least one of the messengers and causes a $[Ca^{2+}]_i$ rise via P_{2U} and P_{2Y} receptors.

INTRODUCTION

Monocytes/macrophages are categorized as a cell system that responds to inflammatory stimuli and acquires the abilities of migration, adhesion and phagocytosis. The major intracellular event that couples receptor stimulation to cell activation is an increase in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$). In macrophages and related cell lines, a transient rise in $[Ca^{2+}]_i$ occurs in response to the factors for induction of chemotaxis[1–4]

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or upon adhesion to opsonized particles by means of cross-linking between immunoglobulin G (IgG) and Fcγ receptors leading to phagocytosis.[5,6] A $[Ca^{2+}]_i$ rise also occurs in response to extracellular ATP at nanomolar/micromolar concentrations via P_2 purinoceptors, as shown in human monocyte-derived macrophages,[7] murine peritoneal macrophages,[8] human monocyte cell line U937,[9–11] mouse macrophage cell line J774[12,13] and rat macrophage cell line NR8383.[14] ATP can be released from exocytotic vesicles and/or granules of secretory cells and from the cytosol of damaged cells. As phagocytes ingest injured or dying cells as well as extrinsic micro-organisms, it is postulated that ATP could play a role of a signal messenger from damaged cells to phagocytes.[15,16] However, no evidence has been provided to date. We have observed the process of necrosis or apoptosis of cultured tumour cells attacked by human natural killer (NK) cells using a Ca^{2+} imaging method.[17–19] The target cells are more or less permeabilized through pores formed by perforin, which is released from NK cells, as detected by leakage of Ca^{2+}-indicator dye from the target cell. In the present study, we investigated whether any $[Ca^{2+}]_i$ rise occurs in macrophages in the vicinity of a dying cell that has been attacked by a NK cell. To examine the putative ATP signal, Ca^{2+} responses to

extracellular ATP in single macrophages were analysed in terms of the kinetics of the $[Ca^{2+}]_i$ rise and Ca^{2+} mobilization pathways, and responsible P_2 receptor subtypes were identified using P_2 purinoceptor agonists and antagonists. Subsequently, Ca^{2+} responses induced by application of cell lysate and of ATP were compared in those characteristics.

MATERIALS AND METHODS

Monocytes and macrophages

Fresh peripheral blood mononuclear cells (PBMCs) were separated from blood of normal volunteers by Ficoll–Conray centrifugation. PBMCs (3×10^6/ml) were incubated in glass dishes in RPMI-1640 medium (Life Technologies, Grand Island, NY) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (referred to as RPMI below) at 37° for 1–1·5 hr (5% CO_2 in air). For positive cell isolation,[2] dishes were washed twice with warm RPMI, and adherent cells were harvested with cold Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline (PBS). For negative selection, cells were treated with anti-human CD3 monoclonal antibody (mAb)-coated magnetic beads (Dynal, Oslo, Norway) (5 μm in diameter; ≈10 beads per cell) at 4° for 30 min, and non-adherent cells were harvested. About 85% of the cells were identified as monocytes by May–Grunwald–Giemsa staining. Cells were stored at −80° in 90% FBS and 10% dimethylsulphoxide (DMSO). When used, cells were rapidly thawed, and DMSO was immediately replaced by RPMI. Cells were cultured in 50% FBS/50% RPMI for 1–2 days before use. Most of monocytes adhered to the bottom of the dish and differentiated to macrophages during the 1–2-day culture.[2] Macrophages were recognized as relatively large flattened cells which contained vacuole-like structures and had irregular contour.

NK cells

PBMCs were transferred to a column of nylon wool (0·5 g in 5 ml RPMI in a syringe) and incubated at 37° for 1 hr. Non-adherent cells were eluted with warm RPMI. NK cell-rich fraction was obtained by centrifugation (650 *g* for 20 min) in Percoll density gradient solutions (Pharmacia Fine Chemicals, Uppsala, Sweden) layered at every 2·5% concentration gradient between 30 and 40%. T cells were obtained from another fraction. More details have been given previously.[17,18] About 95% of harvested cells were identified as NK cells by flow cytometry with an anti-CD16 mAb (Leu-11b; Becton Dickinson, San Jose, CA). NK cells were stored at −80° in 90% FBS and 10% DMSO. Cells were cultured in FBS overnight before use.

Preparation of cell lysate

Cell lysate was obtained by applying hypotonic shock to MOLT-4 cells (T-cell type acute lymphoblastic leukaemia cell line; Riken, Wako, Japan). About 2×10^6 cells were centrifuged (650 *g* for 10 min) to form a pellet of about 10 μl volume, resuspended in 100 μl of distilled water, and pipetted for 2 min. Then the osmotic pressure of the medium was recovered by adding 100 μl PBS of the twofold higher concentration. Supernatant was collected after centrifugation. About 10 μl of supernatant was added to a 20-μl drop of RPMI in the experimental dish. Contents of the cytosol were diluted finally to ≈1/70 in these procedures, if cells were completely lysed.

$[Ca^{2+}]_i$ measurement by Ca^{2+} imaging

Details of Ca^{2+} imaging have been described previously.[18,19] Briefly, macrophages were detached from the bottom of the culture dish with cold Ca^{2+}- and Mg^{2+}-free PBS and preloaded in a test tube with the Ca^{2+}-sensitive fluorescent dye fura-2 by incubation for 30 min in RPMI containing 2 μM fura-2-AM (acetoxymethyl derivative; Molecular Probes, Eugene, OR). Target cells for NK cells were loaded with fura-2 in the same way. After washing, cells were transferred to a 20-μl drop of RPMI in a 35-mm plastic dish which was mounted on the stage of an inverted microscope (TMD300, Nikon, Tokyo, Japan) and heated at 37°. The drop was covered with paraffin oil to avoid evaporation. Fura-2 fluorescence in the cell was activated by applying 340 nm ultraviolet (UV) light for 0·25 seconds followed 1 second later by 380 nm UV for 0·25 seconds through a ×40 objective lens (Fluor 40; Nikon). Ca^{2+} images were sampled every 20 seconds. Emission fluorescence (F) was lead to a silicon intensifier target camera through a 510 ± 10-nm bandpass filter. Data were processed to calculate the fluorescence ratio $R = F_{340} : F_{380}$. A calibration curve between R and $[Ca^{2+}]$ was obtained by measuring R for Ca^{2+}/N-(2-hydroxyethyl)ethylenedinitrilotriacetic acid (EDTAOH) buffer solutions of different Ca^{2+} concentrations. All these procedures were performed using an image processor (ARGUS-50, Hamamatsu Photonics, Hamamatsu, Japan).

Observation of dying cell–macrophage interaction

Cells from the human cell line K562 (chronic myelogenous leukaemia cells) were mainly used as targets for NK cells. K562 cells are NK cell-sensitive and undergo necrosis when attacked by NK cells.[18,19] Fura-2-loaded K562 cells together with macrophages were dispersed in the drop of RPMI in the experimental dish. After the cells were settled on the bottom of the dish, a small amount of suspension of fura-2-unloaded NK cells was gently added to the drop through a pipette. Movement of cells during this procedure was avoided, as the bottom of the dish was coated with poly L-lysin (50 μg/ml; Sigma) to facilitate adhesion of cells. $[Ca^{2+}]_i$ measurement in target cells and macrophages was started, when a NK cell approaching to a target cell was recognized in the optic field (10–20 min after NK cells arrived at the bottom of the dish).

Bright field images were obtained by illumination of red light. Transmitted images were passed through a dichroic mirror at 565 nm and separated from fura-2 fluorescence without mutual interference. Images were detected by a CCD camera (XC-77, Sony, Tokyo) and recorded on a video tape.

Observation of Ca^{2+} responses to chemicals

Ten microlitre of cell lysate or RPMI containing ATP, UTP, 2-chloro-ATP (RBI, Natick, MA), or 3′-*O*-(benzoyl)-benzoly ATP (BzATP; Sigma, St Louis, MO) was gently added to the 20-μl drop containing the cells. For examination with P_2 receptor inhibitors, cells were dispersed in RPMI containing Reactive Blue 2 (anthraquinone-sulphonic acid deraiative), pyridoxalphosphate-6-axophenyl-2′,4′-disulphonic acid (PPADS) (both from RBI). Oxidized ATP (Sigma) was administered 10 min prior to application of ATP. The number of cells that showed Ca^{2+} responses was counted in an optic field (15–30 cells). Experiments in Ca^{2+}-free medium were performed in Ca^{2+}-omitted RPMI containing

0·5 mM ethylenedioxybis(ethylamine)-N,N,N′,N′-tetraacetic acid (EGTA; Dojindo, Kumamoto, Japan).

Statistics

Experimental values were presented by mean ± standard error of the mean (n = number of cells examined). A Student's *t*-test was used for analysis between groups. A *P*-value of 0·05 or less was considered significant.

RESULTS

Signalling from a dying cell to macrophages

We found that a transient rise in $[Ca^{2+}]_i$ was generated in macrophages in the vicinity of a dying cell that was attacked by a NK cell (Fig. 1). K562 cells used as target cells have been shown to be permeabilized by NK cells and to undergo necrosis.[18,19] Figure 1(a) is a fluorescence image taken before addition of NK cells. Smaller cells of dense fura-2 fluorescence (activated by 380 nm UV light; F_{380}) are macrophages and larger cells of lesser F_{380} are K562 cells. Figure 1(b) is the digital Ca^{2+} image obtained from the image of Fig. 1(a) and the image taken 1 second ahead (F_{340}), by calculating the ratio $R = F_{340} : F_{380}$. $[Ca^{2+}]_i$ was expressed by pseudocolours (see the scale bar). NK cells were added immediately after the image of Fig. 1(a) was taken. Figure 1(c) is a Ca^{2+} image at about 20 min after the addition of NK cells. NK cells are not seen

in this image, as they were not loaded with fura-2. A K562 cell was attacked by a NK cell (recognized in bright field; see the Materials and Methods) and displayed an abrupt, large $[Ca^{2+}]_i$ rise (arrow in Fig. 1c). Figure 1(d), which was taken 1 min after the time for Fig. 1(c), shows that F_{380} of the attacked cell was remarkably reduced (see ref. 19 for the precise NK cell-to-target cell interaction). The large $[Ca^{2+}]_i$ rise and subsequent loss of fluorescence in the attacked cell indicate Ca^{2+} entry into the cell and leakage of fura-2 out of the cell due to permeabilization through perforin pores.[18,19] Subsequently, macrophages near the attacked K562 cell exhibited a transient $[Ca^{2+}]_i$ rise (14 cells in Fig. 1e, f). These phenomena were observed in seven experiments (in 10–15 macrophages in each experiment).

Figure 2(a) presents changes in F_{340} and F_{380} of the attacked cell shown in Fig. 1, and Fig. 2(b) illustrates changes in $[Ca^{2+}]_i$ obtained from $F_{340} : F_{380}$ in the attacked cell and three macrophages near the cell. In the attacked K562 cell, F_{340} increased while F_{380} decreased, resulting in an increase in the ratio; that is, a $[Ca^{2+}]_i$ rise. After $[Ca^{2+}]_i$ declined nearly to the basal level, both F_{340} and F_{380} reached a level lower than the original level, indicating leakage of fura-2 out of the cell. $[Ca^{2+}]_i$ in macrophages began to increase 30–60 seconds after the onset of the $[Ca^{2+}]_i$ rise in the attacked cell and reached the peak of 200–600 nM. The $[Ca^{2+}]_i$ rise was a little more delayed and attained a lower peak in the cells located
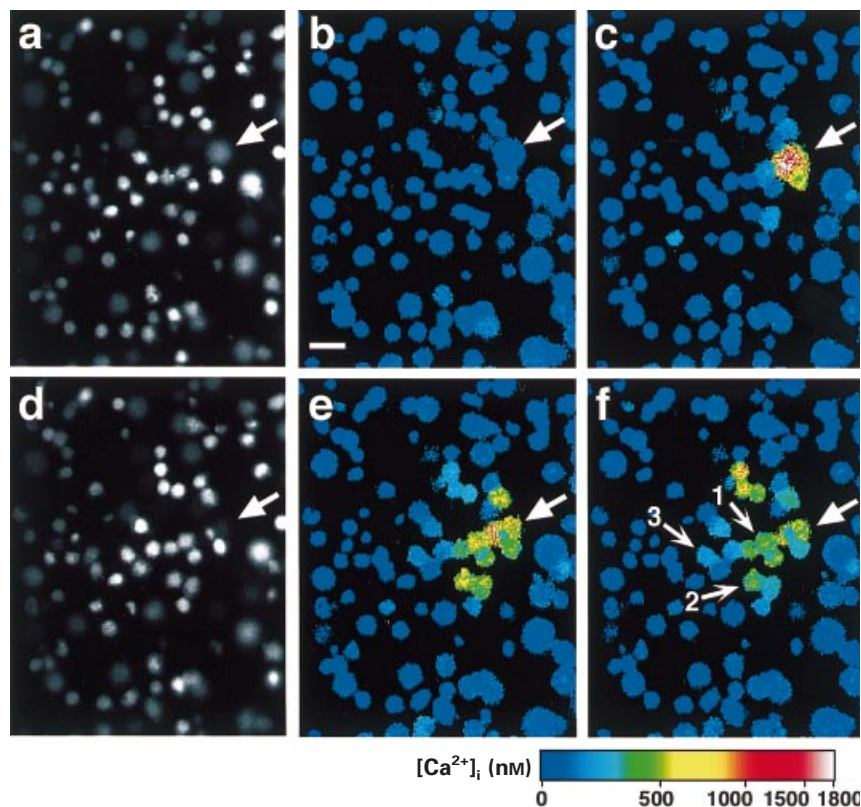

Figure 1. Increases in $[Ca^{2+}]_i$ in the macrophages near a K562 cell that was attacked and permeabilized by a NK cell. The thick arrow indicates the attacked K562 cell. (a) Fluorescence image of K562 cells and macrophages before addition of NK cells. (b) Digital Ca^{2+} image obtained from the image of (a); scale bar is 20 μm. (c) Ca^{2+} image about 20 min after addition of NK cells. (d) Fluorescence image 1 min after the time for (c). (e, f) Ca^{2+} images 1 and 2 min after the time for (c), respectively. Arrows 1, 2 and 3 indicate the three macrophages of which the Ca^{2+} responses are illustrated in Fig. 2(b).
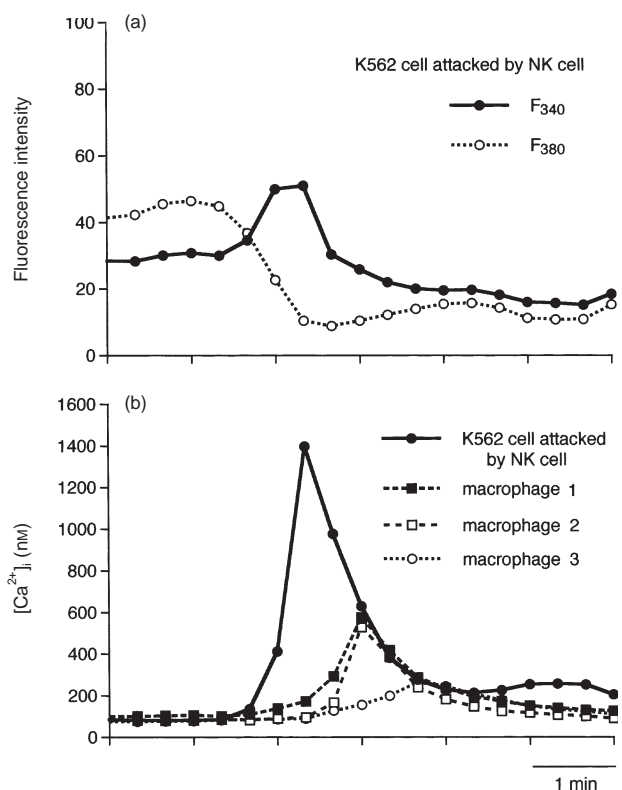
Figure 2. (a) Changes in fluorescence intensity (F_{340} and F_{380}) in the NK cell-attacked K562 cell indicated by the thick arrow in Fig. 1. (b) Changes in [Ca^{2+}]$_i$ calculated from the ratio F_{340} : F_{380} in the NK cell-attacked K562 cell and three macrophages indicated by arrows 1, 2 and 3 in Fig. 1(f). Fluorescence intensity was averaged over a cell.

farther from the attacked cell (macrophages 1–3 in Fig. 2b). All of these Ca^{2+} responses were transient, with duration of ≈ 3 min. These results indicated that a certain factor that was discharged from the permeabilized cell diffused towards macrophages and induced a Ca^{2+} response.

Using 10 cell lines that can be the target of NK cells,[17] we examined whether any Ca^{2+} response occurs in the cells surrounding a killed cell of the same cell line. The Ca^{2+} response was observed only in the monocyte cell line U937, but not in K562, MOLT-4 (see the Materials and Methods), Daudi, Namalwa, Raji (originated from Burkitt's lymphoma), Reh, KM3 (from non-T, non-B acute lymphoblastic leukaemia), Peer ($\gamma\delta$T-cell acute lymphoblastic leukaemia), and FMO (Epstein–Barr virus-induced B-cell line) (data not shown).

Ca^{2+} responses to ATP

A transient [Ca^{2+}]$_i$ rise occurred in more than 70% of macrophages in response to 10 μM ATP, as shown in Fig. 3(a) by three representative records obtained from Ca^{2+} images. The [Ca^{2+}]$_i$ before stimulation was 96 ± 2 nM ($n = 245$ cells). [Ca^{2+}]$_i$ increased immediately after application of ATP and reached a peak of 840 ± 12 nM ($n = 277$) in about 1 min. The total duration of the Ca^{2+} transient was 4–5 min. Additional Ca^{2+} transients (Ca^{2+} oscillations) were seldom observed during [Ca^{2+}]$_i$ measurement for 20 min. Figure 4 shows the dose-dependence of the percentage of responding cells and the peak

[Ca^{2+}]$_i$. The minimal effective ATP concentration was 0·1 μM or less and the efficiency was almost saturated at 100 μM. No ATP-induced Ca^{2+} response was observed in NK cells and T cells (data not shown).

One of the characteristic properties of ATP-induced Ca^{2+} responses in macrophages was desensitization. No substantial [Ca^{2+}]$_i$ rise was induced by the second application of 10 μM ATP 5 min after the previous Ca^{2+} response had ceased (Fig. 3c, dotted line) ($n = 30$). Even when 100 μM ATP was used for the second stimulation, the peak [Ca^{2+}]$_i$ was 305 ± 17 nM ($n = 85$), about one-half of the first response to 10 μM ATP (Fig. 3c, solid line). It should be noted that the desensitization persisted for a fairly long time. For example, complete recovery of the Ca^{2+} response was not obtained, when 100 μM ATP was applied twice, after an interval of 20 min.

Ca^{2+} mobilization pathway for ATP-induced [Ca^{2+}]$_i$ rise

The peak of Ca^{2+} responses to 10 μM ATP in Ca^{2+}-free medium (Ca^{2+}-omitted RPMI plus 0·5 mM EGTA) was 198 ± 6 nM ($n = 157$) (Fig. 3b, left), which was 23% of that in normal medium. A large Ca^{2+} response was restored (Fig. 3b), when 10 μl of RPMI containing 5 mM CaCl$_2$ (without ATP) was added to Ca^{2+}-free medium after the small Ca^{2+} response ceased (5 min after the application of 10 μM ATP). The estimated final concentration of free Ca^{2+} is ≈ 1 mM in the presence of 0·5 mM EGTA. The peak [Ca^{2+}]$_i$ was 593 ± 11 nM ($n = 157$), 70% of that in normal medium. Thus, the [Ca^{2+}]$_i$ rise induced by ATP is mainly due to Ca^{2+} influx from outside the cell, and the minor component that remained in Ca^{2+}-free medium is thought to be due to intracellular Ca^{2+} release from Ca^{2+} stores. Interestingly, the fact that a fairly large [Ca^{2+}]$_i$ rise is generated by delayed addition of Ca^{2+} implies that the Ca^{2+} influx pathway is not inactivated even in the continuous presence of ATP, unless Ca^{2+} influx has occurred previously.

Ca^{2+} response to UTP and other P$_2$ receptor agonists

Ca^{2+} responses to UTP and other agonists were recorded in another series of experiments using macrophages from another donor. In this series, the percentage of cells that responded to 10 μM ATP was 87% ($n = 61$), and the peak [Ca^{2+}]$_i$ was 556 ± 24 nM ($n = 52$). Ca^{2+} responses were induced by 10 μM UTP in 82% of cells ($n = 85$). The peak [Ca^{2+}]$_i$ was 623 ± 20 nM ($n = 69$), which was significantly larger than that for 10 μM ATP ($P < 0.05$). The time–course was similar to that of ATP-induced responses (Fig. 5a). Thus, ATP-induced [Ca^{2+}]$_i$ rise is, at least, mediated by the P$_{2U}$ receptor.

A Ca^{2+} transient was induced by 10 μM 2-chloro-ATP as well, a potent agonist of P$_{2Y}$ receptor,[20] in 70% of macrophages ($n = 63$) (Fig. 5b). The peak [Ca^{2+}]$_i$ was 362 ± 20 nM ($n = 42$), which was significantly smaller than that for 10 μM ATP ($P < 0.001$). BzATP (10 μM), a potent agonist of P$_{2Z}$ receptor,[21] caused a [Ca^{2+}]$_i$ rise in more than 80% of cells, although responses were variable (Fig. 5c). [Ca^{2+}]$_i$ increased to a peak (358 ± 29 μM, $n = 30$) more slowly than the Ca^{2+} transients described above. Then [Ca^{2+}]$_i$ was held at 200–300 nM in approximately two-thirds of the cells or continued to increase over 800 nM during the 10-min recording in other cells (broken line in Fig. 5c). These results suggest that both P$_{2Y}$ and P$_{2Z}$ receptors exist in macrophages.
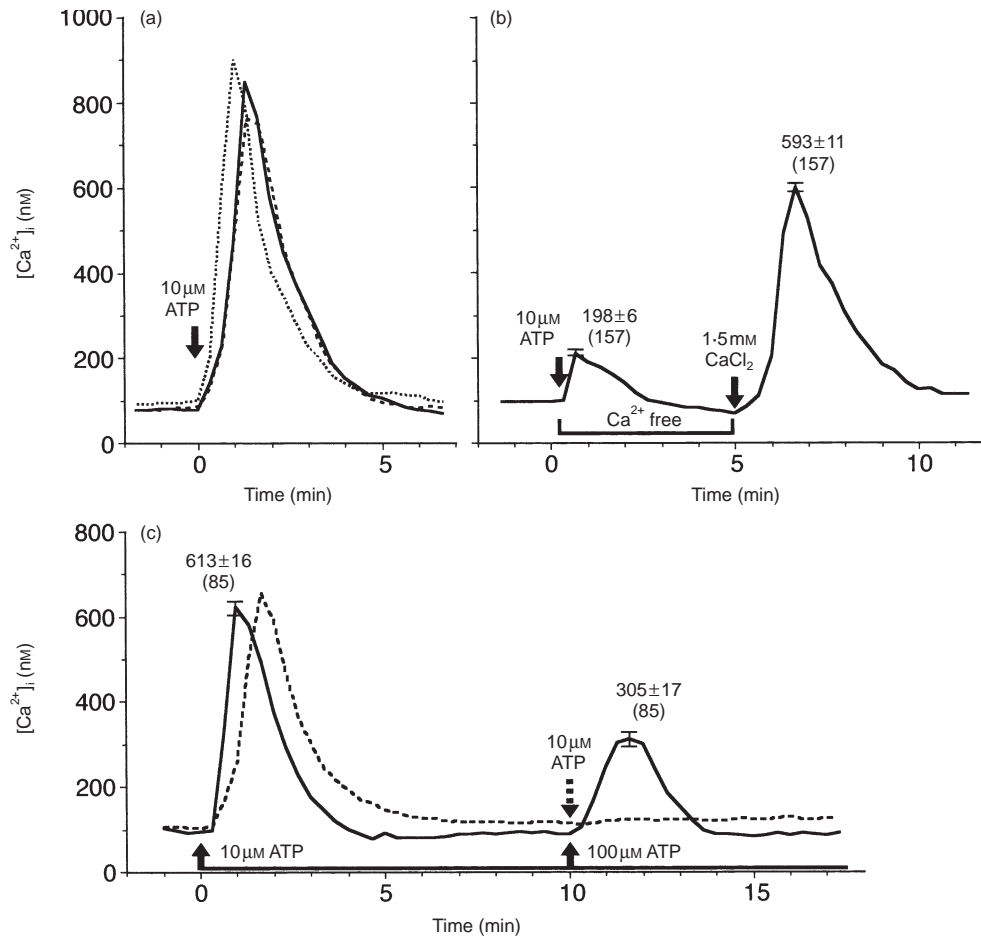
Figure 3. Ca^{2+} responses to ATP in macrophages. (a) Three representative responses to 10 μM ATP. (b) Ca^{2+} responses in Ca^{2+}-free medium and upon addition of 1·5 mM CaCl$_2$. A representative record in which the peak [Ca^{2+}]$_i$ was close to the averaged value is shown. Numbers in parentheses are the numbers of cells examined. (c) Ca^{2+} responses to repeated application of 10 μM ATP (broken line) or 100 μM (solid line) 5 min after the first application of 10 μM ATP.
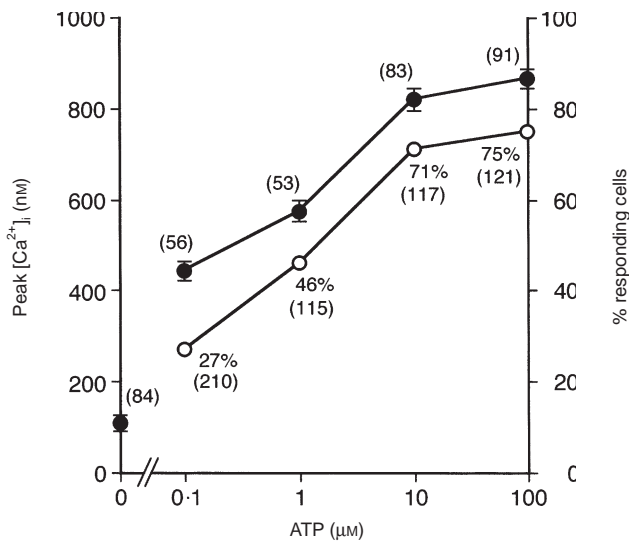

Figure 4. Dose–response relationship of ATP-induced Ca^{2+} responses in macrophages. Responses are presented in terms of the percentage of cells that responded and the average of the peak [Ca^{2+}]$_i$ in those cells.

Effects of P$_2$ receptor inhibitors

Macrophages were preincubated with P$_2$ receptor inhibitors for 1–2 hr in the experimental dish and then 10 μM ATP was gently added to the medium. In the presence of 30 μM Reactive Blue 2, an inhibitor of P$_{2Y}$ receptor,[22,23] the percentage of responding cells to 10 μM ATP was reduced to 37% (Fig. 6a), and the peak [Ca^{2+}]$_i$ was significantly smaller (57%; $P<0·001$) than the control value (Fig. 6b). ATP-induced Ca^{2+} responses were not affected by 30 μM PPADS, the P$_{2X}$ receptor inhibitor[24,25] (Fig. 6). Oxidized ATP, the P$_{2Z}$ receptor inhibitor,[26] at 100 μM did not affect Ca^{2+} responses to 10 μM ATP (Fig. 5d).

Ca^{2+} response induced by cell lysate

As expected, a transient [Ca^{2+}]$_i$ rise was induced by cell lysate in 88% of macrophages. The peak was 692 ± 10 nM ($n=120$), and the duration was 4–5 min. The time–course was similar to that of the ATP-induced response (Fig. 7). Once a large [Ca^{2+}]$_i$ rise was elicited by cell lysate, responses to the subsequent application of 10 or 100 μM ATP were much smaller (Fig. 7), compared with those evoked by the first ATP stimulation with the same concentrations (see Fig. 4). This desensitiz-
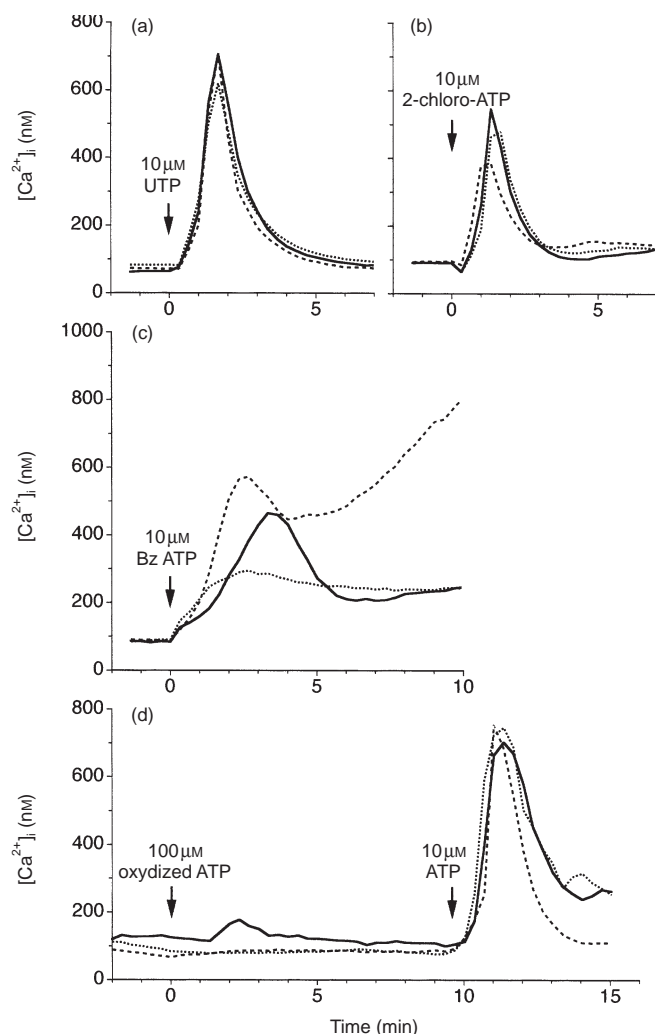
Figure 5. Ca^{2+} responses to 10 µM UTP (a), 2-chloro-ATP (b), or BzATP (c), and responses to 10 µM ATP which was added 10 min after the previous application of 100 µM oxidized ATP (d). Three representative records are shown for each stimulation.

Figure 6. Effects of Reactive Blue 2 and PPADS on the percentage of responding cells (a) and the peak $[Ca^{2+}]_i$ of Ca^{2+} responses to 10 µM ATP or cell lysate. The concentration of chemicals was 30 µM. Control was obtained in the same series of experiments. The inhibitory effect of Reactive Blue 2 was significant ($P < 0.001$).

ation was produced by the reverse order of stimuli as well (ATP followed by cell lysate; not shown). Thus, cell lysate-induced and ATP-induced Ca^{2+} responses interfered with each other, indicating a convergence of the signalling pathway activated by the two stimuli. The Ca^{2+} response induced by cell lysate was inhibited by Reactive Blue 2 (Fig. 6), but not by PPADS, to the similar extent observed in ATP-induced Ca^{2+} responses. It was very likely that the Ca^{2+} response was induced by ATP contained in the cell lysate.

DISCUSSION

The present study first demonstrated in single cells that human macrophages differentiated from monocytes *in vitro* exhibit a transient $[Ca^{2+}]_i$ rise in response to extracellular ATP. NK and T cells displayed no response to ATP. Neutrophils are known to show Ca^{2+} responses to ATP.[15,27] The responsiveness to ATP appears to be characteristic in phagocytes. The major component of the $[Ca^{2+}]_i$ rise was Ca^{2+} influx and the minor component was intracellular Ca^{2+} release, similar to
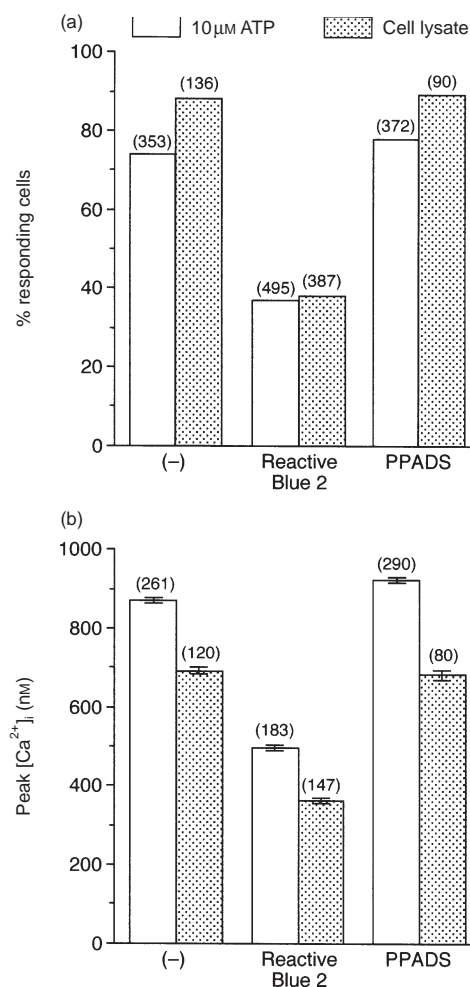
platelet-activating factor-induced $[Ca^{2+}]_i$ rise in macrophages[3] and adhesion-induced $[Ca^{2+}]_i$ rise in monocytes.[6]

As to P_2 purinoreceptors, several subtypes are distinguished by the order of agonist potency for responses.[15,16] The ATP-induced $[Ca^{2+}]_i$ rise in macrophages is thought to be mediated by P_{2U}, since UTP produced greater Ca^{2+} responses than ATP. In murine peritoneal macrophages, the $[Ca^{2+}]_i$ rise, which is due to Ca^{2+} release followed by Ca^{2+} influx, is mediated by P_{2U}.[8] On the other hand, a P_{2Y}-mediated $[Ca^{2+}]_i$ rise has been reported in human monocyte cell line U937.[10] The participation of P_{2Y} is likely in macrophages, as 2-chloro-ATP elicited a Ca^{2+} transient and ATP-induced Ca^{2+} responses were inhibited by Reactive Blue 2. It has been shown that Reactive Blue 2 inhibits the inward membrane current for which the sequence of nucleotides is consistent with P_{2Y},[22,23] but that the dye can inhibit not only P_{2Y}- but also P_{2U}-mediated endothelium-dependent vasodilatation, although to the lesser extent.[28,29] Reactive Blue 2 therefore probably acted on both P_{2U} and P_{2Y} in macrophages. The involvement of P_{2X} is negligible, as 30 µM PPADS had no significant effect. P_{2Z}, which is coupled to plasma membrane pores,[30] is expressed
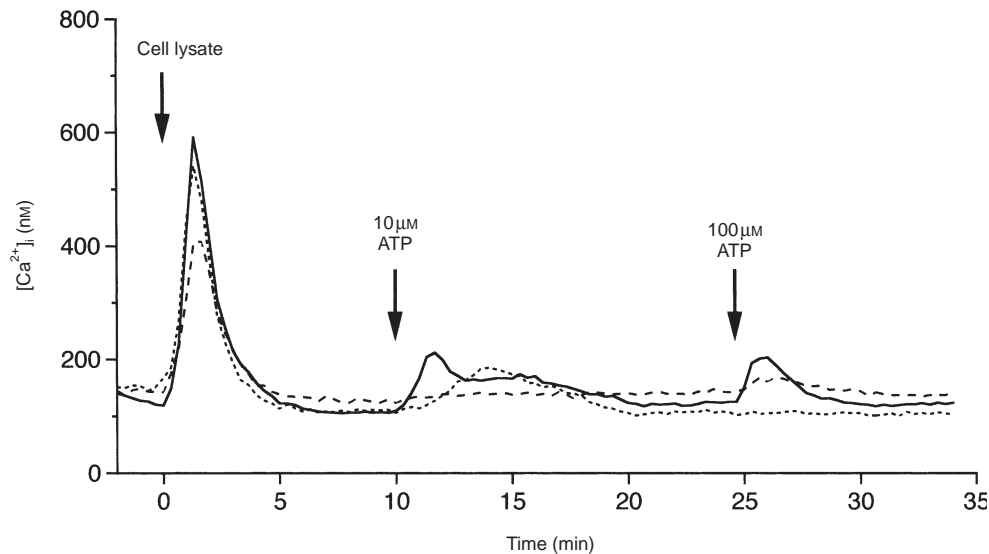
Figure 7. Three representative Ca^{2+} responses to cell lysate and to additional application of 10 and 100 μM ATP.

in macrophages and participates in the ATP-induced $[Ca^{2+}]_i$ rise[31] or Ca^{2+} inward current.[32] In our study, BzATP generated Ca^{2+} responses, but the $[Ca^{2+}]_i$ rise was sustained for a long time or augmented with time, unlike ATP-induced Ca^{2+} transient. Oxidised ATP did not affect responses to 10 μM ATP. The P_{2Z}-mediated Ca^{2+} influx, if any, is probably minor in the Ca^{2+} response on which the present study focused, since the half maximal effective concentration of ATP was ≈ 1 μM (Fig. 4) while it is over 100 μM for activation of P_{2Z} in various cells.[30] In summary, it is reasonable to consider that the ATP-induced Ca^{2+} response in human macrophages differntiated *in vitro* is mediated by P_{2U} and P_{2Y} receptors.

Both P_{2Y} and P_{2U} receptors are coupled to G protein which activates phospholipase C, leading to production of inositol 1,4,5-triphosphate ($InsP_3$) and $InsP_3$-induced Ca^{2+} release from the endoplasmic reticulum through $InsP_3$ receptors.[15,33] On the other hand, a P_{2Y}- or P_{2U}-mediated Ca^{2+} influx pathway has not been clearly identified. Candidates are Ca^{2+} channels or cation channels activated by intracellular Ca^{2+} or $InsP_3$, or store-operated Ca^{2+} channels.[33–35] The present study showed that Ca^{2+} influx can be induced by addition of Ca^{2+} to Ca^{2+}-free medium even after the $[Ca^{2+}]_i$ rise due to Ca^{2+} release has ceased (Fig. 3b); that is, Ca^{2+} influx is not directly activated by intracellular Ca^{2+} or by Ca^{2+} release. Activation of Ca^{2+} influx by depletion of Ca^{2+} stores would be possible, if Ca^{2+} channels could be continuously opened for a fairly long time (at least 5 min to explain the result of Fig. 3b). Another interesting candidate is the TRP5 Ca^{2+} channel, a homologue of *Drosophila* TRP, which mediates ATP-induced Ca^{2+} influx in the same manner as shown in Fig. 3(b) without activation via store depletion.[36] A characteristic property of the ATP-induced Ca^{2+} response in macrophages was desensitization, which took place after Ca^{2+} influx was induced (Fig. 3c) but did not occur without previous Ca^{2+} influx (Fig. 3b). The desensitization persisted for as long as 20 min or more. It is conceivable that Ca^{2+} influx itself or a large $[Ca^{2+}]_i$ rise causes desensitization in Ca^{2+} channels and/or signal transduction from the P_{2Y} or P_{2U} receptor to Ca^{2+} channels. These properties provide useful information for future studies on identification of the receptor-mediated Ca^{2+} influx.

Cell lysate induced a $[Ca^{2+}]_i$ rise in macrophages with a time–course similar to the ATP-induced rise. The response was inhibited by Reactive Blue 2 and was interfered with by the ATP-induced rise. These results indicate that ATP, which is contained in cell lysate, induces the $[Ca^{2+}]_i$ rise. Since cell contents were diluted to roughly 1/70 (see the Materials and Methods) and cytosolic ATP is usually 3–5 mM,[15] ATP in the applied cell lysate was estimated to be 40–70 μM, if cells were completely lysed by hypo-osmotic treatment. ATP in the cell lysate is enough to produce a Ca^{2+} response even if it is diluted, as the minimal effective concentration of ATP was 0·1 μM or less. ATP that is discharged from a damaged or dying cell must be capable of inducing Ca^{2+} responses in neighbouring macrophages, as demonstrated using NK cell-attacked and permeabilized K562 cells. Among 10 cell lines, only the monocye cell line U937 showed a similar Ca^{2+} response. This experimental system, however, depended on appropriate cell arrangement in the dish and on the chance of attack by the NK cell on the target, so it was difficult to examine possible inhibitory effects of Reactive Blue 2. ATP is known to activate the function of macrophages, such as phagocytosis[37] and respiratory burst (superoxide production).[38] It is interesting to examine morphological changes, such as migration or phagocytosis of the macrophages, in which $[Ca^{2+}]_i$ rise was recorded. Unfortunately, such changes could not be observed in the present experimental conditions. Despite these limitations, the present study provided evidence for ATP as a signal messenger from dying cells to macrophages.

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