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P2X$_7$ Receptor-Dependent Cell Death Is Modulated during Murine T Cell Maturation and Mediated by Dual Signaling Pathways$^1$

Mitsutoshi Tsukimoto,* Machiko Maehata,* Hitoshi Harada,²* Akira Ikari,* Kuniaki Takagi,* and Masakuni Degawa*†

Extracellular ATP causes apoptosis and/or necrosis of the hemopoietic lineage through the activation of P2X$_7$, receptors. In this study, we investigated P2X$_7$, receptor-mediated cell death during murine T cell maturation. The expression level and activity of P2X$_7$, receptors, as measured by induction of cell death and pore formation, were higher in splenocytes than thymocytes. Flow cytometric analysis revealed that cell shrinkage was induced by activation of the P2X$_7$, receptor in murine lymphocytes and the responding cells were T cells. Splenic T cells were more responsive than their thymic counterpart. These observations indicate that the system of P2X$_7$, receptor-mediated cell death in T cells could be modulated during T cell maturation. Furthermore, decreased extracellular Cl$^-$ suppressed ATP-induced cell shrinkage in splenocytes without inhibiting ERK1/2 phosphorylation, which is reported to mediate necrotic cell death. Treatment with U0126 (a MEK inhibitor) suppressed ATP-induced ERK1/2 phosphorylation without inhibiting cell shrinkage. Moreover, decreased extracellular Cl$^-$ and treatment with U0126 suppressed ATP-induced cell death. These observations indicate that the activation of P2X$_7$, receptor leads to T cell death by two independent pathways, one of which is cell shrinkage dependent and the other of which involves the phosphorylation of ERK1/2. In conclusion, we demonstrate increasing P2X$_7$, receptor activity during T cell maturation and the existence of two essential pathways in P2X$_7$, receptor-mediated T cell death. Our findings suggest that ATP-induced cell death of peripheral T lymphocytes is important in P2X$_7$, receptor-regulated immune responses. The Journal of Immunology, 2006, 177: 2842–2850.

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$^2$ Abbreviations used in this paper: BzATP, O-(4-benzoyl)benzoyl-ATP; oATP, oxidized ATP; HMBG1, high mobility group box 1; LDH, lactate dehydrogenase; EtBr, ethidium bromide; PI, propidium iodide.
manner unrelated to apoptosis via P2Y receptors (17), cell shrinkage and its significance in cell death mediated by P2X7 receptor have not been studied. Recently, we founded that activation of the P2X7 receptor induces cell shrinkage. Because the cell death and cell shrinkage were abolished by decreased extracellular Cl–, we proposed the involvement of extracellular Cl– in P2X7 receptor-mediated apoptotic cell death (18).

Most studies on P2X7 receptor-mediated cell death have examined different cells of hemopoietic origin such as lymphocytes, macrophages, and dendritic cells, and activation of the P2X7 receptor induces several cellular events related to immune responses, including cytokine release and L-selectin shedding (19). Although earlier research suggested a possible role for P2Z/P2X7 receptors in programmed cell death underlying the positive and negative selection of immature thymocytes during thymus development (5), there is now growing consensus that major purinoceptors may be P2X1 receptors and/or A3 receptors (20, 21). Thus, the potential role of P2X7 receptor in the thymus and peripheral T lymphocytes remains elusive. Chused et al. (22) reported that T cells at different developmental stages differ in the activity of P2Z/P2X7 receptor. Recently, Seman et al. (23) demonstrated that ADP ribosylation of cell surface proteins by NAD and ecto-ADP-ribosyltransferase 2 activates P2X7 receptor and induces T cell death. The sensitivity of mature T cells to NAD is higher than immature T cells. These findings strongly suggest that the system of P2X7 receptor-mediated cell death should mainly function in peripheral T cells. A large number of studies have been made on P2X7-mediated thymocyte death, but little attention has been given to this point. In this study, we analyzed cell shrinkage to investigate the modulation of P2X7 receptor-mediated cell death during T cell maturation. We demonstrated increasing activity of the P2X7 receptor during T cell maturation, and the contribution of two different signaling pathways originating from the P2X7 receptor and leading to cell death.

Materials and Methods

Materials

RPMI 1640 medium, FBS, ATP, ADP, AMP, adenosine, UTP, α,β-methylene-ATP, 2-methylthio-ATP, BzATP, oxidized ATP (oATP), DTT, propidium iodide (PI), protease inhibitor mixture (104 mM 4-(2-aminoethyl)-ethylamine-ATP, 2-methylthio-ATP, BzATP, oxidized ATP (oATP), DTT, protease inhibitors), aprotinin, 2.1 mM leupeptin, 3.6 mM bestatin, 1.5 mM pepstatin A, and 1.4 mM E-64), and rabbit anti-rat P2X7 receptor Ab were purchased from Sigma-Aldrich. U0126 was from Cellbiochem. Rat anti-mouse CD4 mAb-FITC, rat anti-mouse CD8 mAb-FITC or PE, rat anti-mouse B220 mAb-FITC, and hamster anti-mouse TCR αβ mAb-FITC were from Beckman Coulter. Rabbit anti-high mobility group box 1 (HMGB1) Ab was from BD Biosciences. Rabbit anti-rat phospho-ERK1/2 (Thr202/Tyr204) Ab and rabbit anti-rat ERK1/2 Ab were from Cell Signaling Technology. All other chemicals used were of the highest purity available.

Mice and cell preparation

Pathogen-free male BALB/c and C57BL/6 mice, 4–6 wk of age were obtained from Japan SLC. Thymocytes and splenocytes were isolated from the thymus and spleen. Erythrocytes in tissue suspensions were lysed with 0.17 M NH4Cl. Cells were washed twice with complete RPMI 1640 medium and resuspended in RPMI 1640 medium containing 10% FBS. To remove adherent cells such as macrophages and dendritic cells, the cells were incubated in a plastic culture plate for 2 h in an atmosphere of 5% CO2/95% air at 37°C. Nonadherent cells were collected and washed once with RPMI 1640-based buffer containing 102 mM NaCl, 5 mM KCl, 0.4 mM CaCl2, 0.4 mM MgSO4, 23.8 mM NaHCO3, 5.6 mM Na2HPO4, 11.1 mM glucose, and 10 mM HEPES-NaOH (pH 7.4). The cells were immediately used in the assay. In some experiments, chloride was replaced by gluconate.

Quantification of lactate dehydrogenase (LDH) release

The release of LDH into cell culture supernatant was quantified using a Cytotoxicity Detection kit (Roche Applied Science), following the instructions. In a 96-well plate, 2 × 104 cells/well were incubated at 37°C for the indicated time with 1 mM ATP. At the end of incubation, supernatants were collected and the LDH content was measured. LDH release is expressed as the percentage of total content determined by lysing an equal amount of cells with 1% Triton X-100.

Determination of ethidium bromide (EtBr) or PI uptake

Cells were washed with and resuspended in RPMI 1640-based buffer at 2 × 106 cells/ml, and then incubated with EtBr (25 μM) or PI (25 μM) and 1 mM ATP at 37°C for 10 min. After incubation, the samples were analyzed using a flow cytometer (Coulter EPICS XL, version 3.0; Beckman Coulter) with laser excitation at 488 nm and examined at 620 nm. Light scattered in the forward direction is proportional to cell size, whereas light scattered at a 90° angle (side scatter) is proportional to density (24). With cellular debris and aggregates gated out by forward and side scatter, the fluorescence signals from ethidium or propidium were analyzed. Some data were converted to dot plots using FlowJo software (Tree Star).

Measurement of changes in cell size

Cell size was determined by the change in forward light scattering properties on a flow cytometer as described previously (18). Briefly, cells were washed and reseeded in RPMI 1640-based buffer at 2 × 106 cells/ml, and then incubated with ATP at 37°C. After incubation, 10,000 cells for each sample were examined by flow cytometry by exciting the cells with a 488-nm argon laser and determining their distribution on a forward-scatter vs side-scatter dot plots. After cellular debris and aggregates were gated out by forward- and side-scatter dot plot, a gate was set up based on the shrunk cells to determine the percentage of cells that had a decrease in forward light cell size compared to the entire population of cells, and remained constant throughout the analysis. The percentage of shrunk cells was determined by statistical analysis using FlowJo software. Some data were converted to dot plots and histograms.

Detection of surface markers

After cells were washed and resuspended in RPMI 1640-based buffer, 100–150 μl of thymocytes (2 × 105 cells/ml) were incubated with FITC-conjugated anti-mouse CD4 mAb and PE-conjugated anti-mouse CD8 mAb for 30 min on ice. As with thymocytes, splenocytes were incubated with FITC-conjugated anti-mouse CD4, CD8, or B220 mAb. After incubation, cells were resuspended to a final volume of 1 ml and incubated at 37°C. After the addition of 1 mM ATP, thymocytes and splenocytes were incubated for 10 min. The surface expressions of CD4, CD8, or B220 were determined, and the cell size change was measured by flow cytometry. Twenty thousand particles of each cell subset were assessed. The distribution of thymocyte and splenocyte population, and the percentage of shrunk cells in each subset were determined by statistical analysis using FlowJo software. To determine which subset is alive after treatment with P2X7 receptor agonists, splenocytes were incubated with FITC-conjugated anti-mouse TCR αβ mAb and PE-conjugated anti-B220 mAb for 30 min on ice. Cells were incubated for 3 h with vehicle, 1 mM ATP, or 100 μM BzATP, and then with 25 μM PI for 10 min. Percentages of T cells (TCR+) and B cells (B220+) in living cells (PI-negative cells) were analyzed by flow cytometry.

MACS

Splenic T cells and splenic B cells were isolated from spleenocytes of BALB/c mice by MACS using magnetic bead-conjugated anti-IgG mAb (Miltenyi Biotec). Briefly, splenocytes were washed and resuspended with cold Ca2+- and Mg2+-free RPMI 1640-based buffer, and incubated with FITC-conjugated anti-CD4 and anti-CD8 mAbs or FITC-conjugated anti-B220 mAb. Cells were washed twice with cold Ca2+- and Mg2+-free RPMI 1640-based buffer, and incubated for 15 min on ice with magnetic bead-conjugated anti-IgG mAb (Miltenyi Biotec), according to the manufacturer’s recommendations. The cells were washed twice with cold Ca2+- and Mg2+-free RPMI 1640-based buffer containing 0.5% (w/v) BSA and 2 mM EDTA and separated on magnetic columns in a miniMACS separator (Miltenyi Biotec). The percentages of positively enriched splenic T cells (CD4+ or CD8+) and splenic B cells (B220+) were determined by flow cytometry using FITC-conjugated anti-CD4 and anti-CD8 mAbs and FITC-conjugated anti-B220 mAb, respectively. The percentage of splenic T cells (CD4+ or CD8+) was >85% in the population of enriched CD4+ or CD8+ cells. The percentage of splenic B cells (B220+) was >90% in the population of enriched B220+ cells.
Detection of P2X<sub>7</sub> receptor expression

Cells were dissolved in sample buffer (25% glycerin, 1% SDS, 62.5 mM Tris-Cl, 10 mM DTT) and boiled for 5 min. The protein content in each sample was measured by Bio-Rad Protein Assay kit according to the manufacturer’s instructions. Aliquots of samples containing 25 μg of protein were analyzed by 10% SDS-PAGE and transferred onto a nitrocellulose membrane. Blots were incubated for 1 h in TBST with 5% skim milk at room temperature, and then with rabbit anti-rat P2X<sub>7</sub> receptor Ab (1/200) or rabbit anti-HMGB1 (1/200) to show equal loading at 4°C overnight. After washing with TBST, blots were incubated with donkey HRP-conjugated anti-rabbit IgG (1/2000) (Amersham Biosciences) at room temperature. After washing with TBST, specific proteins were visualized by an ECL method using ECL Western blotting detection reagents (Amersham Biosciences) according to the manufacturer’s instructions. The anti-P2X<sub>7</sub> receptor Ab was raised against a synthetic peptide corresponding to residues 576–595 of rat P2X<sub>7</sub> receptor with an additional N-terminal cysteine.

Detection of ERK1/2 phosphorylation

Cells were lysed in 0.1 ml of PBS containing 10 mM HEPES-NaOH (pH 7.4), 1% Triton X-100, 5 mM EDTA, 30 mM sodium pyrophosphate, 50 mM sodium fluoride, 1 mM sodium pervanadate, 1.04 mM 4-(2-aminoethyl)-benzenesulfonyl fluoride, 0.8 μM aprotinin, 21 μM leupeptin, 36 μM bestatin, 15 μM pepstatin A, and 14 μM E-64, at 4°C for 30 min. Lysates were centrifuged at 12,000 × g for 15 min. After removing cellular debris, the protein content in each sample was measured by Bio-Rad Protein Assay kit according to the manufacturer’s instructions. Equal amounts of cell lysate were dissolved in 2× sample buffer (50% glycerin, 2% SDS, 125 mM Tris, 10 mM DTT) and boiled for 5 min. Aliquots of samples containing 5 μg of protein were analyzed by 12% SDS-PAGE and transferred to a nitrocellulose membrane. Blots were blocked in TBST with 1% BSA at 4°C overnight and incubated with rabbit anti-rat phospho-ERK1/2 (Thr202/Tyr204) Ab (1/1000) or rabbit anti-rat P2X<sub>7</sub> receptor Ab (1/200) at 4°C overnight. After washing with TBST, specific proteins were visualized by an ECL method.

Statistics

Values are given as the mean ± SE. Comparison between two values was performed by unpaired Student’s t test. Multiple groups were compared using ANOVA followed by pairwise comparisons with Bonferroni’s post hoc analysis (25). Significance was defined at p < 0.05 using the Instat, version 3.0, statistical package (GraphPad Software).

Results

P2X<sub>7</sub> receptor activity in thymocytes and splenocytes

ATP-treated thymocytes and splenocytes undergo necrotic and/or apoptotic cell death as assessed by the release of cytosolic LDH, and cell death by ATP stimulation was detected early and intensely in splenocytes compared with thymocytes (Fig. 1). Activation of the P2X<sub>7</sub> receptor rapidly induces the opening of nonselective pores with a smaller molecular cut-off of slightly over 300 Da; ethidium (molecular mass, 314) is admitted, propidium (molecular mass, 414) is excluded (12). To investigate the activity of pore formation in thymocytes and splenocytes, we examined the uptake of EtBr and PI into cells by flow cytometry. A few dead cells were observed by the uptake of EtBr and PI in normal conditions, and treatment with ATP caused the uptake of EtBr, but not PI (Fig. 2, A and B). These results indicate that EtBr uptake in splenocytes is due to the formation of nonselective pores, not to cell damage. Moreover, the activity of pore formation was higher in splenocytes than thymocytes (Fig. 2C). These results confirm that functional P2X<sub>7</sub> receptors are expressed in murine thymocytes and splenocytes, and the activity of P2X<sub>7</sub> receptors is higher in splenocytes than thymocytes.

P2X<sub>7</sub> receptor-mediated cell shrinkage in lymphocytes

We have demonstrated P2X<sub>7</sub> receptor-mediated cell shrinkage (18), which is an early detectable event in the apoptotic program, using a stable transfectant of the P2X<sub>7</sub> receptor (13). To investigate P2X<sub>7</sub> receptor-mediated cell death in murine lymphocytes, we measured the change in cell size by treatment with ATP using a flow cytometer. ATP stimulation led to cell shrinkage in both thymocytes and splenocytes, and splenocytes shrank more rapidly.
than thymocytes (Fig. 3). Therefore, in subsequent pharmacological experiments, we analyzed cell shrinkage in thymocytes at 90 min and in splenocytes at 10 min after ATP stimulation. Cell shrinkage by ATP stimulation was observed at 1 mM in both thymocytes and splenocytes (Fig. 4, A and C). This supports that cell shrinkage is induced by activation of the P2X7 receptor, because P2X7 receptors are activated by concentrations of ATP > 0.1 mM and activity peaked at 1 mM ATP (14). Moreover, adenosine (a P1 receptor agonist), ADP (a P2Y1, P2Y12, and P2Y13 receptor agonist) (26), UTP (a P2Y2, P2Y4, and P2Y6 receptor agonist) (27), α,β-methylene-ATP (a P2X1, and P2X7 receptor agonist) (28) and 2-methylthio-ATP (a P2Y1 and P2Y11 receptor agonist) (27) were unable to induce cell shrinkage in both thymocytes and splenocytes. In contrast, BzATP (a P2X1, P2X7 receptor agonist) (100 μM) (14) promoted cell shrinkage (Fig. 4, A and C), and oATP (a P2X7 receptor antagonist) (14) significantly inhibited ATP-induced cell shrinkage in both thymocytes and splenocytes (Fig. 4, B and D). Together, these data provide evidence for the involvement of P2X7 receptor in ATP-induced cell shrinkage of murine lymphocytes.

Virginio et al. (29) reported that divalent cations act as allosteric modulators to alter the affinity of ATP binding to the P2X7 receptor. The rank order of potency of divalent cations was consistent with the previous report, BzATP. At the end of incubation, the percentage of shrunk cells in total cells was measured by flow cytometry as described in Materials and Methods. B and D, Thymocytes or splenocytes were preincubated for 1 h with vehicle or 500 μM oATP, and then thymocytes were incubated with vehicle (□) or 1000 μM ATP (▲) for 90 min (B) or splenocytes were incubated with vehicle (□) or 1000 μM ATP (▲) for 10 min (D). At the end of incubation, the percentage of shrunk cells in total cells was measured by flow cytometry. The data represent the mean ± SE of four experiments performed. **, p < 0.01, significant differences compared with vehicle-treated controls.

**FIGURE 4.** Characterization of the purinoceptor involved in ATP-induced cell shrinkage in murine thymocytes and splenocytes. A and C, Thymocytes were incubated for 90 min (A) or splenocytes were incubated for 10 min (C) with vehicle (control), various concentrations of ATP, 100 μM adenosine, 100 μM ADP, 100 μM UTP, 100 μM 2-methylthio-ATP (2-MeSATP), 100 μM α,β-methylene-ATP (α,β-meATP), and 100 μM BzATP. At the end of incubation, the percentage of shrunk cells in total cells was measured by flow cytometry as described in Materials and Methods. B and D, Thymocytes or splenocytes were preincubated for 1 h with vehicle or 500 μM oATP, and then thymocytes were incubated with vehicle (□) or 1000 μM ATP (▲) for 90 min (B) or splenocytes were incubated with vehicle (□) or 1000 μM ATP (▲) for 10 min (D). At the end of incubation, the percentage of shrunk cells in total cells was measured by flow cytometry. The data represent the mean ± SE of four experiments performed. **, p < 0.01, significant differences compared with vehicle-treated controls.

**FIGURE 3.** Flow cytometric analysis of ATP-induced cell shrinkage in murine thymocytes and splenocytes. A, Splenocytes were incubated with vehicle (left panel) or 1 mM ATP (right panel) for 10 min. At the end of incubation, the percentage of shrunk cells in total cells was measured by flow cytometry as described in Materials and Methods. With cellular debris and aggregates gated out by forward- and side-scatter dot plots, a gate was set up based on the shrinkage of cells to determine the percentage of cells with a decrease in forward scatter light (cell size) compared with the entire population of cells. The gate remained constant throughout the analysis. B, Thymocytes (○) and splenocytes (□) were incubated with vehicle (○, □) or 1 mM ATP (●, ■) for the indicated time. At the end of incubation, the percentage of shrunk cells in total cells was measured by flow cytometry.

showed cytotoxicity, we measured the effect of divalent cations on pore formation and cell shrinkage induced by BzATP (10 μM for 2.5 min) in Ca2+- and Mg2+-free RPMI 1640-based buffer. Pore formation and cell shrinkage were completely inhibited by 10 μM Cu2+, and partially inhibited by 10 μM Zn2+. Magnesium ion inhibited at 100 μM but not 10 μM (Fig. 5). Because this rank order of potency of divalent cations was consistent with the previous report, BzATP-induced cell shrinkage should be dependent on the activation of P2X7 receptors.

Furthermore, to address the issue of a link between the activation of P2X7 receptors and cell shrinkage, we investigated a difference in responses to ATP between splenocytes from BALB/c and C57BL/6 mice. The P2X7 receptor C-terminal region of C57BL/6 mice bears a Pro-451 to Leu mutation that impairs non-selective pore formation (30), but there are no differences between the two mouse strains in terms of P2X7 receptor mRNA and protein expressions, and ATP-induced phosphorylation of ERK1/2 (9). Reduced sensitivity to ATP-induced pore formation, cell shrinkage, and cell death were observed in splenocytes from C57BL/6 mice compared with BALB/c mice (Fig. 6). These results indicate that cell shrinkage is an early detectable event of P2X7 receptor-mediated cell death in murine lymphocytes, and
strongly suggest that the C terminus of P2X7 receptors is essential for the induction of cell shrinkage.

Lymphocyte subsets differ in sensitivity to ATP-induced cell shrinkage

Because the activity of ecto-ATPase is lower in murine immature thymocytes than in mature thymocytes (31) and BzATP was more potent than ATP at the same dose (100 μM), we examined the contribution of ATPase on slow cell shrinkage and cell death in thymocytes by experiments performed with BzATP, which resists breakdown by ATPase and hydrolyzes more slowly than ATP. Treatment with BzATP did not induce rapid cell shrinkage (Fig. 7A), as with ATP treatment, in thymocytes. These results suggest that the lower responsiveness to ATP of thymocytes is mainly due to lower P2X7 receptor expression.

Chused et al. (22) reported that the activity of elevating intracellular free Ca2+ concentration of the P2Z/P2X7 receptor is modulated during T cell differentiation and maturation. CD4+CD8− double-positive thymocytes were the least responsive. CD4+CD8+ single-positive thymocytes, CD8+ splenic T cells, CD4+8− single-positive thymocytes, and CD4−8− splenic T cells showed increasing reactivity. Therefore, to determine which subset responds to ATP stimulation, changes in the cell size of thymic and splenic subsets, defined by CD4, CD8, and B220 expression, were measured 10 min after treatment with ATP (1 mM) (Fig. 8, Table I). CD4+8− double-positive thymocytes, which accounted for ~80% of the population, were the weakest and CD4+8− splenocytes were the strongest responders in T cell subsets. B220+ positive B cells were less responsive and CD48−CD8+8− triple-negative splenocytes shrunk slightly. The percentage of shrunk CD4+8− thymocytes was higher than that of shrunk CD4−8− thymocytes, and the percentage of shrunk CD4+8− splenocytes was also higher than that of CD8+ splenocytes. Of note, considerably more splenic T cells were responsive than their thymic counterpart. These results show that the shrinking activity of the P2X7 receptor depends on T cell maturation.

FIGURE 5. Inhibition of BzATP-induced cell shrinkage by divalent cations in murine splenocytes. Splenocytes were incubated for 2.5 min with 25 μM EtBr and vehicle (☐) or 10 μM BzATP (■) in Ca2+- and Mg2+-free RPMI 1640-based buffer containing Cu2+(10 μM), Zn2+(10 μM), or Mg2+(10 or 100 μM). At the end of incubation, EtBr fluorescence (A) and the percentage of shrunk cells in total cells (B) were measured by flow cytometry as described in Materials and Methods. The data represent the mean ± SE of four experiments performed. *, p < 0.05; **, p < 0.01, significant differences compared with vehicle-treated controls.

FIGURE 6. The level of cell shrinkage and cell death correlates with nonselective pore formation in murine splenocytes. A and B, Splenocytes from BALB/c and C57BL/6 mice were incubated with 25 μM EtBr and vehicle (☐) or 1 mM ATP (▪) for 10 min. At the end of incubation, the fluorescence intensity of EtBr (A) and the percentage of shrunk cells in total cells (B) were analyzed by flow cytometry as described in Materials and Methods. C, Splenocytes from BALB/c and C57BL/6 mice were incubated with vehicle (☐) or 1 mM ATP (▪) for 6 h. At the end of incubation, supernatants were collected and LDH content was measured. LDH release is expressed as the percentage of total content determined by lysing an equal amount of cells with 1% Triton X-100. The data represent the mean ± SE of four experiments performed. **, p < 0.01, significant differences compared with vehicle-treated controls.

FIGURE 7. Cell shrinkage and cell death induced by BzATP in murine thymocytes. A, Thymocytes were incubated with vehicle (control), ATP (100 or 1000 μM), or BzATP (100 μM) for 10 min. At the end of incubation, the percentage of shrunk cells in total cells was analyzed by flow cytometry as described in Materials and Methods. The data represent the mean ± SE of four experiments performed. **, p < 0.01, significant differences compared with vehicle-treated control. B, Thymocytes were incubated with vehicle (☐) or 100 μM BzATP (▪) for the indicated time. At the end of incubation, supernatants were collected and LDH content was measured. LDH release is expressed as the percentage of total content determined by lysing an equal amount of cells with 1% Triton X-100.
Because expression levels of P2X<sub>7</sub> receptor protein in the thymus and spleen have not yet been compared, immunoblot analysis was performed (Fig. 9). The expression level of P2X<sub>7</sub> receptor protein in splenocytes was higher than that in thymocytes and higher in splenic T cells than splenic B cells. These results indicate that the increasing activity of P2X<sub>7</sub> receptors corresponds well with the expression level of P2X<sub>7</sub> receptors. Moreover, we analyzed the cell population in living cells (PI-negative cells) 3 h after ATP treatment by flow cytometry (Fig. 10). The T cell population (TCR<sup>+</sup>B220<sup>−</sup>) disappeared by treatment with ATP (1 mM) or BzATP (100 μM). This observation also indicates that splenic T cells respond to the activation of P2X<sub>7</sub> receptors.

**Dual signaling pathways of P2X<sub>7</sub> receptor-mediated cell death**

We have revealed that extracellular Cl<sup>−</sup> influx following pore formation plays a critical role in P2X<sub>7</sub>-receptor-mediated cell shrinkage and cell death (18). In contrast, Auger et al. (9) reported that ERK1/2 phosphorylation plays a role in P2X<sub>7</sub>-receptor-mediated necrotic cell death, and these events are independent of pore formation. Thus, we investigated the contributions of cell shrinkage and the ERK1/2 pathway to P2X<sub>7</sub>-receptor-mediated splenocyte death. Decreased extracellular Cl<sup>−</sup> influx suppressed ATP-induced cell shrinkage in a concentration-dependent manner (Fig. 11A), but not ATP-induced ERK1/2 phosphorylation (B). In contrast, pretreatment with a specific MEK1/2 inhibitor, U0126 (32) blocked ATP-induced ERK1/2 phosphorylation (Fig. 12A), but not ATP-induced cell shrinkage (B). Moreover, decreased extracellular Cl<sup>−</sup> (54 mM) and pretreatment with U0126 (10 μM) suppressed LDH release significantly. These results indicate that both cell shrinkage and ERK1/2 phosphorylation play essential roles in P2X<sub>7</sub>-receptor-mediated cell death through independent pathways.

**Discussion**

This study focused on the modulation and mechanism of P2X<sub>7</sub>-receptor-mediated cell death during maturation in murine T lymphocytes. We verified that ATP-induced cell death (Fig. 1), pore formation (Fig. 2), and the expression level of P2X<sub>7</sub> receptors (Fig. 9) were greater in splenocytes than in thymocytes. Because we have already reported a good correlation between pore formation and cell shrinkage in ATP-P2X<sub>7</sub>-receptor-induced apoptotic cell death using a stable transfectant of P2X<sub>7</sub> receptor (18), we characterized ATP-induced cell shrinkage in thymocytes and splenocytes. Cell shrinkage is a major hallmark of apoptosis and starts before cell fragmentation (16). Treatment with ATP induced cell shrinkage in both thymocytes and splenocytes, and splenocytes showed higher reactivity than thymocytes (Fig. 3). Although the expression of P2X<sub>1</sub>, P2X<sub>2</sub>, P2X<sub>3</sub>, P2X<sub>6</sub>, P2X<sub>7</sub>, P2Y<sub>1</sub>, P2Y<sub>2</sub>, and A<sub>2a</sub> receptors in murine thymocytes has been reported (33–36),

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**Table I. ATP-induced cell shrinkage in murine lymphocytes**

| Cell Population | Shrunken Cells (percentage of each subset) | Shrunken Cells (percentage of total cells) |
|-----------------|------------------------------------------|-------------------------------------------|
|                 | Control | ATP (1 mM) | Control | ATP (1 mM) |
| Thymocytes      |         |            |         |            |
| CD<sup>4</sup>CD<sup>8</sup> cells | 5.2 ± 0.11 | 38.9 ± 1.40 | 52.0 ± 1.17** | 1.6 ± 0.03 | 2.6 ± 0.10** |
| CD<sup>4</sup>CD<sup>8</sup> cells | 78.3 ± 0.84 | 2.4 ± 0.13 | 5.4 ± 0.69** | 1.9 ± 0.09 | 4.2 ± 0.58* |
| CD<sup>4</sup> CD<sup>8</sup> cells | 12.4 ± 0.65 | 8.7 ± 0.53 | 32.9 ± 1.35** | 1.1 ± 0.03 | 4.5 ± 0.25** |
| CD<sup>4</sup> CD<sup>8</sup> cells | 4.1 ± 0.11 | 9.8 ± 0.95 | 26.0 ± 1.96** | 0.5 ± 0.03 | 1.3 ± 0.09** |
| Splenocytes     |         |            |         |            |
| CD<sup>4</sup> cells | 26.1 ± 0.33 | 10.7 ± 2.4 | 72.2 ± 0.58** | 2.8 ± 0.66 | 19.0 ± 0.2** |
| CD<sup>8</sup> cells | 13.4 ± 0.21 | 26.1 ± 3.05 | 58.9 ± 1.42** | 3.6 ± 0.5 | 7.8 ± 0.19** |
| B220<sup>+</sup> cells | 39.2 ± 0.50 | 7.8 ± 0.88 | 7.0 ± 0.88 | 3.1 ± 0.34 | 2.7 ± 0.30 |
| CD<sup>4</sup> CD<sup>8</sup> B220<sup>+</sup> cells | 15.1 ± 0.33 | 21.4 ± 0.90 | 26.7 ± 0.82** | 3.3 ± 0.05 | 3.9 ± 0.07** |

*Thymocyte subsets (CD<sup>4</sup><sup>+</sup>, CD<sup>4</sup><sup>+</sup>CD<sup>8</sup><sup>+</sup>, CD<sup>8</sup><sup>+</sup>, and CD<sup>8</sup><sup>+</sup>) were characterized by staining with anti-CD4-FITC and CD8-PE. Splenocyte subsets (CD<sup>4</sup><sup>+</sup>, CD<sup>8</sup><sup>+</sup>, B220<sup>+</sup>, and CD<sup>4</sup> CD<sup>8</sup> B220<sup>+</sup>) were characterized by staining with anti-CD4, CD8, or B220-FITC. After incubation for 10 min with 1 mM ATP, shrunk cells in each subset were detected by flow cytometry. Results represent the percentage of distribution of thymocytes or splenocytes, and the percentage of shrunk cells in each subset or total cells. The data represent the mean ± SE of four experiments performed.

*<sup*p < 0.05; **<sup*p < 0.01, significant differences compared with vehicle-treated controls.
cell shrinkage was induced by only P2X7 receptor agonist (ATP and BzATP) within the limits used for agonists in this study, and was inhibited by P2X7 antagonist, αATP (Fig. 4). Treatment with divalent cations such as Cu2+, Zn2+, and Mg2+ inhibited BzATP-induced pore formation and cell shrinkage with the rank order potency, Cu2+ > Zn2+ > Mg2+ (Fig. 5), similar to a previous report that divalent cations act as allosteric modulators to alter the affinity of ATP binding to the P2X7 receptor (29). Moreover, decreases in ATP-induced pore formation, cell shrinkage, and cell death were observed in splenocytes from C57BL/6 mice, which have decreases in ATP-induced pore formation, cell shrinkage, and cell death were observed in splenocytes from C57BL/6 mice, which have

FIGURE 9. Expression of P2X7 receptors in murine lymphocytes. Expression of P2X7 receptors was analyzed by Western immunoblotting as described in Materials and Methods. Splenic T cells (CD4+ and CD8+) and splenic B cells (B220+) were isolated by magnetic cell sorting. Whole-cell lysates of thymocytes, splenocytes, splenic T cells, and splenic B cells were separated on 10% SDS-PAGE and immunoblotted for anti-P2X7 receptor Ab (top panel) or anti-HMGB1 (bottom panel). The result shown is typical of those obtained in three independent experiments.

FIGURE 10. Demonstration of ATP-induced splenic T cell death. Splenocytes were stained by anti-TCRαβ-FITC and anti-B220-PE, and then incubated with vehicle (A) or 1 mM ATP (B) or 100 μM BzATP (C) for 3 h. Living cells (PI-negative cells) were analyzed by flow cytometry. Data were converted to FITC fluorescence vs PE fluorescence dot plots.

FIGURE 11. Decreased extracellular Cl− suppressed ATP-induced cell shrinkage, but not ATP-induced phosphorylation of ERK1/2. A, Splenocytes were incubated with vehicle (○) or 1 mM ATP (●) for 10 min in RPMI 1640-based buffer containing various concentrations of Cl−. At the end of incubation, the percentage of shrunk cells in total cells was measured by flow cytometry as described in Materials and Methods. B, Splenocytes were incubated with vehicle (control) or 1 nM ATP for 5 min in RPMI 1640-based buffer containing various concentrations of Cl−. Proteins from lysates of splenocytes were separated on 12% SDS-PAGE and immunoblotted for phospho-threonine and phospho-tyrosine with anti-ERK1/2 (pTpY 202/204) Ab or total ERK1/2 Ab. The results are typical of those obtained in three independent experiments.

and proceeds via an ordered sequence of developmental steps characterized notably by the variable expressions of CD4 and CD8. Early precursor cells are initially double-negative CD4−8− and go through a double-positive CD4+8+ intermediate stage before differentiating into single-positive CD4+8− or CD4−8+. Splenocytes consist of mature T cells (CD4+ or CD8+), B cells (B220+), and others (CD4−CD8−B220−). Flow cytometric analysis by staining specific cell surface Ags confirmed that the responding cells for ATP were T cells (Figs. 8 and 10; Table I), CD4+CD8+ double-positive thymocytes were slightly responsive. Peripheral T lymphocytes (splenic CD4+ and CD8+) showed higher reactivity than the most mature thymocytes (CD4+8− single-positive and CD4−8+ single-positive thymocytes). We also revealed that the expression level of P2X7 receptors was higher in splenic T cells than in splenic B cells (Fig. 9). These findings suggest that the cell-shrinking activity of ATP-P2X7 receptor increases during T cell maturation, and corresponds well with the expression level of P2X7 receptors. Therefore, we propose that there should be an essential role for P2X7 receptor in peripheral T cells rather than in the thymus. Seman et al. (23) have reported that ADP ribosylation of P2X7 receptor by NAD and ecto-ADP-ribosyltransferase-2 induces T cell death and the sensitivity of mature T cells to NAD is higher than immature T cells. These observations coincide with our results. Splenic B cells did not respond to ATP, whereas there are some reports suggesting the expression of P2X7 receptors on B cells (37, 38). There is room for further investigation into the role of P2X7 receptor on B cells, but it was focused on P2X7 receptor-mediated cell death in this paper.

Decreased extracellular Cl− suppressed ATP-induced cell shrinkage of splenocytes in a concentration-dependent manner (Fig. 11A). The reported value of intracellular Cl− concentration in

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lymphocytes is in the range of 50–85 mM (39, 40), and the extracellular Cl\(^{-}\) concentration was \(\sim 108\) mM under our experimental conditions, suggesting that extracellular Cl\(^{-}\) influx also plays a significant role in the regulation of P2X\(_7\) receptor-mediated splenic T cell shrinkage. In contrast, Auger et al. (9) reported that the mode of P2X\(_7\) receptor-mediated thymocyte death is necrosis/lysis via the phosphorylation of ERK1/2, rather than apoptosis. It was also reported that activation of the ERK pathway is induced by the activation of \(\Delta\lambda\) and P2Y\(_2\) receptors among the purinoceptors expressed by murine T cells; however, \(\Delta\lambda\) receptor-mediated ERK activation protects sympathetic neurons against nerve growth factor withdrawal (41) and P2Y\(_2\) receptor-mediated ERK activation leads to cell proliferation of HeLa cells (42). Thus, only P2X\(_7\) receptor-mediated ERK1/2 activation plays a significant role in T cell death. Therefore, we investigated the contributions of cell shrinkage and the ERK1/2 pathway to P2X\(_7\) receptor-mediated splenocyte death; the role of ATP-induced cell shrinkage in cell death was defined by decreased extracellular Cl\(^{-}\) (54 mM) and the role of the ERK1/2 pathway was defined by pretreatment with a specific MEK1/2 inhibitor, U0126 (10 \(\mu\)M). Decreased extracellular Cl\(^{-}\) (54 mM) suppressed ATP-induced cell shrinkage completely but did not affect ATP-induced phosphorylation of ERK1/2 (Fig. 11), whereas pretreatment with U0126 (10 \(\mu\)M) suppressed ATP-induced ERK1/2 phosphorylation completely, but did not affect ATP-induced cell shrinkage (Fig. 12). These results indicate that decreased extracellular Cl\(^{-}\) (54 mM) and pretreatment with U0126 (10 \(\mu\)M) do not prevent ATP from binding to P2X\(_7\) receptors, and cell shrinkage and the phosphorylation of ERK1/2 are independent of each other. Moreover, decreased extracellular Cl\(^{-}\) (54 mM) and pretreatment with U0126 (10 \(\mu\)M) suppressed ATP-induced cell death significantly (Fig. 13). These results demonstrate the existence and importance of two different pathways, one involving pore formation and rapid cell shrinkage, and a second involving the phosphorylation of ERK1/2, in P2X\(_7\) receptor-mediated splenocyte death. The cytoplasmic C-terminal region of P2X\(_7\) receptor is essential for opening of a large pore (2), which correlates with apoptotic cell shrinkage (18), and the importance of the N-terminal region in the phosphorylation of ERK1/2 has already been demonstrated using mutants of P2X\(_7\)-receptor C and N termini (8). Together, these results suggest that the C-terminal region of P2X\(_7\) receptor initiates apoptotic-like signaling and the N-terminal region initiates necrotic signaling, which could explain the so-called “P2X\(_7\) receptor-mediated apoptotic and/or necrotic cell death.”

In conclusion, we clearly demonstrated the increasing activity of the P2X\(_7\) receptor during T cell maturation, and the contribution of two different signaling pathways originating from the P2X\(_7\) receptor and leading to cell death. Because cell death in peripheral T lymphocytes is an important mechanism of negative feedback during immune response, ATP may act as a terminator of excessive immune response via the P2X\(_7\) receptor. Until now, the involvement of the P2X\(_7\) receptor in regulating factors for inflammation has suggested that the inhibition of function of this receptor might lead to the amelioration of diseases of an inflammatory nature. Consistent with this possibility are the observations in P2X\(_7\) receptor-deficient mice (38, 43); however, more recently, it was reported that the loss of apoptotic activity in lymphocytes in P2X\(_7\) receptor-deficient mice contributes to the exacerbation of experimental autoimmune encephalomyelitis (44). These data suggest the complex function of P2X\(_7\) receptors in immune responses. It is of particular interest to note that these models were tested using the same C57BL/6 mouse strain that expresses a receptor with low affinity for ATP (30). Therefore, our observations with BALB/c mice provide significant information to elucidate the complex role of P2X\(_7\) receptor in immune responses. Earlier studies have revealed that nerve cells, endothelia, platelets, red cells, and epithelia can release nucleotides in a nonlytic and physiological manner (45). However, the sources of such large amounts of extracellular ATP to activate P2X\(_7\) receptors on T lymphocytes is not fully understood except for the possibility of release by stressed or dying cells. Recently, the release of large amounts of ATP (100–200

**FIGURE 11.** Treatment with MEK1/2 inhibitor suppressed ATP-induced phosphorylation of ERK1/2 but not ATP-induced cell shrinkage. A. Splenocytes were preincubated with various concentrations of U0126 for 1 h and incubated with vehicle (control) or 1 mM ATP for 5 min. Proteins from lysates of splenocytes were separated on 12% SDS-PAGE and immunoblotted for phospho-threonine and phospho-tyrosine with anti-ERK1/2 (pTpY 202/204) Ab or total ERK1/2 with anti-ERK1/2 Ab. The results are typical of those obtained in three independent experiments. B. Splenocytes were preincubated with vehicle (control) or 10 \(\mu\)M U0126 for 1 h, and incubated with vehicle (□) or 1 mM ATP (■) for 10 min. At the end of incubation, the percentage of shrunk cells in total cells was measured by flow cytometry as described in Materials and Methods. The data represent the mean \(\pm\) SE of four experiments performed. **, \(p < 0.01\), indicates that there are significant differences. **FIGURE 12.** Effect of ATP-induced cell death by decreased extracellular Cl\(^{-}\) and MEK1/2 inhibitor. Splenocytes were preincubated with or without 10 \(\mu\)M U0126 for 1 h, and incubated with vehicle (□) or 1 mM ATP (■) in RPMI 1640-based buffer containing 108 or 54 mM Cl\(^{-}\) for 6 h. At the end of incubation, supernatants were collected and LDH content was measured. LDH release is expressed as the percentage of total content determined by lysing an equal amount of cells with 1% Triton X-100. The data represent the mean \(\pm\) SE of four experiments performed. **, \(p < 0.01\), indicates that there are significant differences.
μM) in response to P2X2 receptor activation has been reported using a novel recombinant plasma membrane-targeted luciferase (46). This novel pathway might unveil a hitherto unsuspected non-lytic and physiological release of large amounts of ATP. Under physiological conditions, P2X2 receptors are maintained in a re-strained state that coordinate limits ATP binding, channel gating, and coupling of the receptor to signaling pathways (47). Moreover, the augmentation of extracellular ATP levels by down-regulation of ectoATPase could also enhance the functional contribution of P2X2 receptors in the diseased tissue. Thus, studying the factors or environmental conditions that maintain P2X2 receptors in a re- strained state will require novel approaches for analyzing P2X2 receptor function in normal and diseased states.

Disclosures

The authors have no financial conflict of interest.

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