Involvement of SLC17A9-dependent Vesicular Exocytosis in the Mechanism of ATP Release during T Cell Activation

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Recent reports have shown that T cell receptor (TCR)-dependent ATP release from T cells is involved in production of interleukin-2 (IL-2) through activation of P2 receptors. Stimulation of TCR induces ATP release from T cells through gap junction hemichannels and maxi-anion channels, at least in part. However, the mechanisms of ATP release from activated T cells are not fully understood. Here, we studied the mechanisms of ATP release during TCR-dependent T cell activation by investigating the effects of various inhibitors on TCR-dependent ATP release from murine T cells. We found that not only anion channel and gap junction hemichannel inhibitors, but also exocytosis inhibitors suppressed the ATP release. These results suggest that ATP release from murine T cells is regulated by various mechanisms, including exocytosis. An inhibitor of exocytosis, bafilomycin A, significantly blocked TCR signaling, such as Ca2+ elevation and IL-2 production. Furthermore, bafilomycin A, ectonucleotidase, and P2Y6 receptor antagonist significantly inhibited production of pro-inflammatory cytokines from external antigen-restimulated splenocytes, indicating that vesicular exocytosis-mediated purinergic signaling has a significant role in TCR-dependent cytokine production. We also detected vesicular ATP in murine T cells and human T lymphoma Jurkat cells, both of which also expressed mRNA of SLC17A9, a vesicular nucleotide transporter. Knockdown of SLC17A9 in Jurkat cells markedly reduced ATP release and cytosolic Ca2+ elevation after TCR stimulation, suggesting involvement of SLC17A9-dependent vesicular exocytosis in ATP release and T cell activation. In conclusion, vesicular exocytosis of ATP appears to play a role in T cell activation and immune responses.

Activation of T cells induces various responses required for immune functions. Interaction of T cell receptor (TCR)3/CD3 complex with major histocompatibility complex-peptide initiates an activation process of T cells (1). A key early event is the activation of the tyrosine kinase Lck, which in turn phosphorylates ITAM and ZAP-70 (2). A rise in intracellular Ca2+ ion concentration is also essential for T cell activation. The inositol 1,4,5-trisphosphate generated during T cell activation stimulates Ca2+-permeable ion channel receptors (inositol 1,4,5-trisphosphate receptor) on the endoplasmic reticulum membrane, inducing the release of Ca2+ from endoplasmic reticulum Ca2+ stores into cytoplasm (3). Depletion of endoplasmic reticulum Ca2+ stores opens Ca2+ release-activated Ca2+ channels, leading to sustained influx of extracellular Ca2+. Elevation of cytosolic Ca2+ causes activation of nuclear factor of activated T cells, which leads to the production of interleukin-2 (IL-2) (4). CD25, which is the IL-2 receptor α-chain, is also expressed during T cell activation. The production of IL-2 and expression of CD25 play critical roles in the activation and proliferation of T cells.

ATP and other nucleotides are physiologically released from various types of cells, and interact with purinergic P2 receptors in plasma membrane (5). These receptors are classified into two subfamilies: the ionotropic P2X1–7 receptors and the metabotropic P2Y1–14 receptors (5). They regulate a variety of cellular responses in immune cells, including T cells (6). We previously reported that prolonged and strong activation of P2X7 receptor by a high concentration (1 μM) of ATP induces apoptotic and necrotic cell death in lymphocytes (7, 8). On the other hand, it has been shown that ATP is released from T cells after TCR stimulation and activates P2X7 receptors (9, 10). We also reported that extracellular ATP activates P2 receptors, including P2X7 and P2Y6 receptors, and these receptors play an important role in T cell activation (11). Thus, the activation of P2 receptors by ATP is important for regulating T cell functions.

ATP is released from cells via several different efflux pathways. The main candidates suggested so far are a maxi-anion channel (12), a volume-sensitive outwardly rectifying chloride channel (13), members of the ATP-binding cassette protein family (14), a gap junction hemichannel (15), P2X7 receptor channel/pore (16, 17), and exocytosis of a pool of vesicles (18). As regards the process of T cell activation, it has been reported that ATP is released through a gap junction hemichannel, pannexin-1, and maxi-anion channel after TCR stimulation (9, 10). However, the mechanism of ATP release from activated T cells remains to be fully defined.

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3 The abbreviations used are: TCR, T cell receptor; BAPTA-AM, 1,2-bis(2-aminoethoxy)ethane-N,N,N',N'-tetraacetic acid acetoxymethyl ester; BFA, brefeldin A; IL, interleukin; mAb, monoclonal antibody; MANT-ATP, 2',3'-O-(N,N-methylene)adenosine-5'-triphosphate; BSA, bovine serum albumin; PI, phosphatidylinositol; shRNA, short hairpin RNA; VVUT, vesicular nucleotide transporter; PE, phycocerythrin; FITC, fluorescein isothiocyanate; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; RT, reverse transcription.
Recently, SLC17A9 has been identified as a vesicular nucleotide transporter (VNUT), which is required for vesicular storage of ATP (19). VNUT is responsible for exocytosis of ATP, because ATP-containing vesicles are released by exocytosis. Although VNUT is expressed in various tissues, there is no report of the expression of VNUT in T cells. It is known that vesicular exocytosis is regulated by phosphatidylinositol (PI) 3-kinase activation and cytosolic Ca\(^{2+}\) (20, 21). PI 3-kinase functions as a key regulator of vesicular trafficking, and increase of cytosolic Ca\(^{2+}\) concentration evokes exocytosis by binding to Ca\(^{2+}\)-sensing proteins. For example, PI 3-kinase is involved in insulin-induced membrane translocation of GLUT4-containing vesicles (22), Ca\(^{2+}\)-dependent exocytosis of glutamate and ATP in astrocytes (23), and exocytosis of perforin and granzymes through an increase in the cytosolic Ca\(^{2+}\) concentration in T cells (24). However, the involvement of vesicular exocytosis in TCR-dependent ATP release has not yet been established.

In this study, we investigated the mechanism of ATP release during TCR-dependent T cell activation. Our results indicate that ATP release in murine T cell is regulated by various mechanisms, including exocytosis. An inhibitor of exocytosis, bafilomycin A, significantly blocked TCR signaling, such as elevation of cytosolic Ca\(^{2+}\), expression of CD25, and production of cytokines, supporting the involvement of vesicular exocytosis of ATP in T cell activation. Furthermore, we observed a pool of vesicular ATP and expression of SLC17A9 (VNUT) in murine T cells and Jurkat cells. Knockdown of SLC17A9 mRNA by RNA interference in Jurkat cells significantly suppressed ATP release in response to TCR stimulation, supporting the involvement of VNUT in TCR-dependent ATP release. Thus, our results indicate that VNUT-dependent vesicular exocytosis is a significant contributor to ATP release during T cell activation.

**EXPERIMENTAL PROCEDURES**

Reagents—GdCl\(_{3}\), arachidonic acid, glibenclamide, flufenamic acid, carboxenolone, 18-glycyrrhetinic acid, bafilomycin A, brefeldin A (BFA), apyrase, and LY294002 were purchased from Sigma. MRS2578 and A438079 were from Tocris Bioscience (Bristol, UK). 1,2-Bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid acetoxyethyl ester (BAPTA-AM) was from Dojindo (Kumamoto, Japan). RPMI1640 medium was from Wako Pure Chemical (Osaka, Japan). Fetal bovine serum was purchased from Biowest (Nuaille, France). All other chemicals used were of the highest purity available.

**Cell Preparation and Activation of T Cells**—Pathogen-free male BALB/c mice, 5–7 weeks of age, were obtained from Sankyo Labo Service (Tokyo, Japan). Splenocytes were isolated from the spleen and purified by hemolysis. Cells were washed twice with complete RPMI 1640 medium and re-suspended in RPMI 1640-based buffer containing 102 mM NaCl, 5 mM KCl, 0.4 mM Ca\(_{\text{aq}}\), 0.4 mM MgSO\(_4\), 23.8 mM NaHCO\(_3\), 5.6 mM Na\(_2\)HPO\(_4\), 11.1 mM glucose, and 10 mM HEPES-NaOH (pH 7.4). Splenocytes were incubated with anti-CD28 monoclonal antibody (mAb) (0.5 μg/mL) (eBioscience, San Diego, CA) in a 96-well cell culture plate coated with anti-CD3ε mAb (5 μg/mL) (R&D Systems, Minneapolis, MN) in RPMI 1640 medium containing 10% heat-inactivated fetal bovine serum, 100 units/mL of penicillin, and 100 μg/mL of streptomycin in an atmosphere of 5% CO\(_2\), 95% air at 37 °C. Jurkat cells were maintained in complete RPMI 1640 medium containing 10% fetal bovine serum, 100 units/mL of penicillin, and 100 μg/mL of streptomycin.

**Measurement of Extracellular ATP**—Cells were washed and resuspended in RPMI 1640-based buffer, then 40-μl aliquots of murine splenocytes (2.0 × 10\(^6\) cells/ml) or Jurkat cells (1.0 × 10\(^6\) cells/ml) were stimulated by ligation of CD3/CD28 using Dynabeads (Invitrogen) and the extracellular ATP concentration was measured using ENLITEN Rluciferase/Luciferin Reagent (Promega, Madison, WI). For investigation of TCR/CD28-induced ATP release, conditioned medium was collected as a control sample for background ATP release. At the indicated time points, each sample was centrifuged at 600 × g for 2 min and 10 μl of the supernatant was collected for ATP determination. The concentration of ATP was determined by measuring chemiluminescence with a TR717™ Microplate Luminometer (Applied Biosystems, Foster City, CA) 1.6 s after adding 100 μl of luciferin-luciferase reagent to 10 μl of sample solution.

**Immunoblotting**—After stimulation of TCR, murine splenocytes (1.0 × 10\(^6\) cells) were dissolved in sample buffer (25% glycerin, 1% SDS, 62.5 mM Tris-Cl, 10 mM dithiothreitol) and boiled for 10 min. Aliquots of samples were analyzed by 10% SDS-PAGE and transferred onto a polyvinylidene difluoride membrane. Blots were incubated at 4 °C overnight in TBST (10 mM Tris-HCl, 100 mM NaCl, 0.1% Tween 20, pH 7.5) with 1% BSA, and then with rabbit mAbs against phospho-Lck (1:1000) or Lck (1:1000) (Cell Signaling Technology, Beverly, MA) overnight at 4 °C. After washing with TBST, blots were incubated with goat horseradish peroxidase-conjugated anti-rabbit IgG Ab (1:20,000) (Cell Signaling Technology) for 1.5 h at room temperature. The blots were further washed with TBST, and specific proteins were visualized by using ECL Western blotting detection reagents (GE Healthcare).

**Mobilization of Intracellular Calcium**—Murine splenocytes were incubated with PE-Cy5 anti-CD4 mAb (BD Biosciences) for 30 min. The cells were washed twice, then loaded with the Ca\(^{2+}\)-sensitive fluorescent dye Fluo-4 AM (10 μM) (Invitrogen) in Ca\(^{2+}\)- and Mg\(^{2+}\)-free RPMI 1640-based buffer for 30 min at 37 °C, and washed twice with RPMI 1640-based buffer. The samples were analyzed using a FACS Calibur flow cytometer (BD Biosciences) with laser excitation at 488 nm and emission at 530 nm. Cells were stimulated with anti-CD3ε mAb (10 μg/mL) and anti-CD28 mAb (0.5 μg/mL) in RPMI 1640-based buffer or Ca\(^{2+}\)- and Mg\(^{2+}\)-free RPMI 1640-based buffer. After 110 s, anti-mouse IgG Ab (18 μg/mL) (Sigma) was added. Some data were converted to dot plots using Cell Quest software (BD Biosciences). Jurkat cells were loaded with the Ca\(^{2+}\)- sensitive fluorescent dye Fluo-4 AM (10 μM) in Ca\(^{2+}\)-free RPMI 1640-based buffer for 30 min at 37 °C, and then washed twice with RPMI 1640-based buffer. Fluorescence was measured using a Wallac 1420 ARVO fluoroscan (Wallac, Turku, Finland). Cells were stimulated with anti-CD3 mAb (0.5 μg/mL) (eBioscience, clone OKT3).

**Determination of IL-2 Production**—Murine splenocytes were stimulated with anti-CD3ε mAb and anti-CD28 mAb as
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described above. After incubation for 24 h, the culture supernatant was harvested for determination of IL-2. The concentration of IL-2 was measured by an enzyme-linked immunosorbent assay as described below. A 96-well plate was coated with purified anti-mouse IL-2 mAb (1:500) (eBioscience) and incubated overnight at 4 °C. The wells were washed with phosphate-buffered saline containing 0.05% Tween 20, and nonspecific binding was blocked with phosphate-buffered saline containing 1% BSA for 1 h at room temperature. The plate was washed, and the culture supernatant was added for 2 h at room temperature.

The plate was washed again, and biotin-conjugated anti-mouse IL-2 mAb (1:500) (eBioscience) was added for 1 h at room temperature. The plate was further washed, and avidin-horseradish peroxidase (Sigma) was added. The plate was incubated for 30 min at room temperature, then washed, and 3,3′,5,5′-tetramethylbenzidine was added for a few minutes. The reaction was stopped by adding 5 N H2SO4. The absorbance at 450 nm was measured with an ImmunoReader NJ-2000 (Nihon InterMed, Tokyo, Japan). A standard curve was established with recombinant mouse IL-17 (31–4000 pg/ml) or recombinant mouse IL-6 (15–1000 pg/ml), and the concentrations of IL-17 or IL-6 were estimated from the standard curve.

Detection of CD25 Expression—Cells were washed and resuspended in RPMI 1640-based buffer, then 100-μl aliquots of murine splenocytes (2.0 × 10^6 cells/ml) were incubated with PE-Cy5-conjugated anti-mouse CD4 mAb and FITC-conjugated anti-mouse CD25 mAb (Biolegend, San Diego, CA) for 30 min on ice. The surface expressions of CD4 and CD25 were determined by flow cytometry using a FACS Calibur flow cytometer. Ten thousand cells were assessed, and the percent of CD25-positive T cells in CD4-positive T cells was determined by statistical analysis using Cell Quest and FLOW-JO software (Tree Star, San Carlos, CA).

Immunization with Methyl-BSA (mBSA) and Determination of IL-2, IL-17, and IL-6—BALB/c mice were injected subcutaneously with 100 μl of 1.25 mg/ml of mBSA (Sigma) emulsified with Complete Freund’s adjuvant (Chondrex, Redmond, WA). Ten days after immunization, splenocytes were isolated from the spleen. Splenocytes were prepared as described above. Cells were suspended in RPMI 1640 medium containing 10% heat-inactivated fetal bovine serum at a concentration of 7 × 10^6 cells/ml and cultured with mBSA (50 μg/ml) in 96-well plates. After incubation at 37 °C under an atmosphere of 5% CO2, 95% air for 24 h, the culture supernatant was harvested for determination of IL-2, IL-17, and IL-6. The concentration of IL-2 was measured as described above. The concentrations of IL-17 and IL-6 were measured by enzyme-linked immunosorbent assay as follows. A 96-well plate was coated with purified anti-mouse IL-17 (1:250) (BioLegend) or IL-6 (1:500) (eBioscience) mAb, and incubated overnight at 4 °C. The wells were washed with phosphate-buffered saline containing 0.05% Tween 20, and nonspecific binding was blocked with phosphate-buffered saline containing 1% bovine serum albumin for 1 h at room temperature. The plate was washed, and the culture supernatant was kept for 2 h at room temperature. The plate was washed again, and biotin-conjugated anti-mouse IL-17 (1:500) (BioLegend) or IL-6 (1:500) (eBioscience) mAb was added for 1 h at room temperature. The plate was further washed, and avidin-horseradish peroxidase was added. The plate was incubated for 30 min at room temperature, then washed, and 3,3′,5,5′-tetramethylbenzidine was added for a few minutes. The reaction was stopped by adding 5 N H2SO4. The absorbance at 450 nm was measured with an ImmunoReader NJ-2000. A standard curve was established with recombinant mouse IL-17 (31–4000 pg/ml) or recombinant mouse IL-6 (15–1000 pg/ml), and the concentrations of IL-17 or IL-6 were estimated from the standard curve.

Fluorescence Imaging—Murine splenocytes (1.0 × 10^6 cells) were incubated for 1 h with PE-Cy5-conjugated anti-mouse CD4 mAb, 50 μM 2′,3′-O-(N-methylanthranilloyl)-ATP (MANT-ATP) in RPMI 1640-based buffer at 37 °C. Stained cells were analyzed using a confocal laser scanning microscope (TCS SP2; Leica, Mannheim, Germany) equipped with a HCX PL/Plan ×63 1.32 NA oil objective lens. Leica confocal microscopy software (TCS SP2, version 2.6.1) was used for image acquisition and processing. Fluorescence of MANT-ATP in CD4 T cells stained by PE-Cy5-conjugated mAb was detected at 430 – 480 nm with excitation at 364 nm. Similarly, an aliquot of Jurkat cells (1.0 × 10^6 cells) was incubated for 1 h with 50 μM MANT-ATP in RPMI 1640-based buffer at 37 °C and then analyzed in the same way using a confocal laser scanning microscope.

Magnetic Cell Sorting—Splenic T cells were isolated from splenocytes of BALB/c mice by magnetic cell sorting utilizing magnetic bead-conjugated anti-FITC mAb (Miltenyi, Bergisch Gladbach, Germany). Briefly, splenocytes were washed, resuspended in cold Ca^2+ - and Mg^2+ -free RPMI 1640-based buffer, and incubated with FITC-conjugated anti-CD4 mAb. Cells were washed twice with cold Ca^2+ - and Mg^2+ -free RPMI 1640-based buffer, and incubated with magnetic bead-conjugated anti-FITC mAb for 15 min on ice, according to the manufacturer’s recommendations. The cells were washed twice with cold Ca^2+ - and Mg^2+ -free RPMI 1640-based buffer containing 0.5% (w/v) BSA and 2 mM EDTA and separated on magnetic columns in a mini-MACS separator (Miltenyi). The percentage of splenic CD4^+ T cells in the enriched fraction, as determined by flow cytometry utilizing FITC-conjugated anti-CD4 mAb, was higher than 95%.

RT-PCR—Total RNA was isolated from murine splenocytes and Jurkat T cells using a Fast Pure RNA kit (Takara Bio, Shiga, Japan). The first-strand cDNA was synthesized from total RNA with PrimerScript Reverse Transcriptase (Takara Bio). Specific primers were designed with PrimerQuestTM (Integrated DNA Technologies, Inc., Coralville, IA) and synthesized by Sigma Genosys. The sequences of specific primers for murine SLC17A9 were 5′-ATT CCT TGC TCA AGG CCT ACC TTG-3′ (sense) and 5′-GGA AGC CAA TGG AGG CTG ATG CAA-3′ (antisense) and those for human SLC17A9 were 5′-ACA CAC GAG CAG AGA GGA ACA CAA-3′ (sense) and 5′-TTT CTG GCT GTT GTC TGA CGG-3′ (antisense). GAPDH mRNA was determined as a positive control. PCR was carried out by incubating each cDNA sample with the primers (0.5 μM each), PrimeSTAR® HS DNA Polymerase (0.625 units, Takara Bio), and a deoxynucleotide mixture (0.2 mM each, Takara Bio). After the samples were incubated at 95 °C for 2 min, amplification was carried out for 35 cycles (each cycle: 95 °C for 30 s, annealing at 65 °C for 1 min) and incubated at
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72 °C for 10 min. The products were then subjected to 2% agarose gel electrophoresis. Bands were stained with ethidium bromide (Sigma) and photographed.

Real Time RT-PCR—Total RNA was extracted from murine splenocytes and Jurkat cells and the first-strand cDNA was synthesized as described above. The cDNA was used as template for real time PCR analysis; reactions were performed in an Stratagene Mx3000P® quantitative PCR system (Agilent Technologies, La Jolla, CA). Specific primers were designed with PrimerQuest and synthesized by Sigma Genosys. The sequences of specific primers for murine IL-2 were 5’-TCC AGA ACA TGC CGC AGA A-3’ (sense) and 5’-CCT GAG CAG GAT GGA GAA TTA CA-3’ (antisense), and those for human SLC17A9 were 5’-AGT CTG TGG TCT TTG CAT CAG CCT-3’ (sense) and 5’-TGT TGG CCA CAC CAA ACA GAA AGC-3’ (antisense). GAPDH mRNA was determined as a positive control. Each sample was assayed in a 20-μl amplification reaction, containing cDNA, primer mixture (0.4 μM each of sense and antisense primers), and 2× GoTaq® quantitative PCR Master Mix (Promega). The amplification program consisted of 40 cycles (each cycle: 95 °C for 15 s, annealing at 60 °C for 1 min) after 95 °C for 2 min. Fluorescent products were detected at the last step of each cycle. The obtained values were within the linear range of a standard curve and were normalized with respect to GAPDH mRNA.

Short Hairpin RNA (shRNA) Plasmid Stable Transfection—Stable transfection with shRNA was performed using the SureSilencing™ shRNA Plasmid Kit for Human SLC17A9 (SABiosciences, Frederick, MD). shRNA plasmid targeting SLC17A9 or the negative control shRNA plasmid was transfected by electroporation using the Amaxa system (Lonza, Walkersville, MD) with Nucleofector solution V and Nucleofector program X-01. The transfected cells were selected in G418-containing (2 mg/ml) culture medium for 2 weeks.

Statistics—Results are expressed as mean ± S.E. The statistical significance of differences between control and other groups was calculated using Dunnett’s test with the Instat version 3.0 statistical package (GraphPad Software, San Diego, CA). The criterion of significance was set at p < 0.05.

RESULTS

Multiple Regulation of Cellular ATP Release from Murine T Cells after TCR Stimulation—We measured the concentration of ATP released from murine T cells into culture medium in response to stimulation of TCR/CD28 by treatment with anti-CD3 and anti-CD28 mAb. As shown in Fig. 1A, extracellular ATP increased after the stimulation, reaching a maximum at 1

FIGURE 1. Effects of pharmacological inhibition on TCR/CD28 stimulation-induced ATP release in murine T cells. A, splenocytes were stimulated by ligation of CD3/CD28 using Dynabeads and incubated for the indicated times. After incubation, the concentration of ATP in the culture medium was measured (n = 12–20). B, splenocytes were pretreated with carbeneoxolone (CBX) (50 μM), 18-glycyrretinic acid (18GA) (50 μM), flufenamic acid (FFA) (50 μM), glibenclamide (100 μM), arachidonic acid (20 μM), GdCl3 (100 μM), A438079 (100 μM), and bafilomycin A (50 nM) for 30 min. At 1 min after TCR/CD28 stimulation, the supernatants were collected and ATP content was measured (n = 12–54). Each value represents the mean ± S.E. A significant difference between the control group and the indicated group is represented (*, p < 0.05 or **, p < 0.01).

FIGURE 2. Role of PI 3-kinase activation and elevation of intracellular Ca2+ in ATP release. A, splenocytes were pretreated with bafilomycin A (50 nM) and BFA (10 μM) for 30 min. At 1 min after TCR/CD28 stimulation, the supernatants were collected and ATP content was measured (n = 16–20). B, splenocytes were pretreated with LY294002 (10 μM) for 30 min. At 1 min after TCR/CD28 stimulation, the supernatants were collected and ATP content was measured (n = 12–24). Each value represents the mean ± S.E. A significant difference between the control group and the indicated group is represented (**, p < 0.01).

Instat version 3.0 statistical package (GraphPad Software, San Diego, CA). The criterion of significance was set at p < 0.05.

RESULTS

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min and returning to a near basal level within 5 min. In subsequent experiments, we measured extracellular ATP at 1 min after stimulation of TCR/CD28.

To investigate the pathway of ATP release from activated T cells, we examined the effect of several inhibitors on ATP release induced by TCR/CD28 stimulation. Fig. 1B shows that pretreatment with carbenoxolone, 18-glycyrrhetinic acid, flufenamic acid (inhibitors of gap junction hemichannels), glibenclamide (a potent inhibitor of chloride channel), arachidonic acid (an inhibitor of maxi-anion channel), and A438079 (an antagonist of P2X<sub>7</sub> receptor) significantly abrogated the release of ATP upon T cell activation, but another maxi-anion channel inhibitor, GdCl<sub>3</sub>, did not block it. Although we also examined the effect of BAPTA-AM (an intracellular Ca<sup>2+</sup> chelator) and depletion of extracellular Ca<sup>2+</sup> after stimulation of TCR/CD28.

Involvement of PI 3-Kinase and Elevation of Intracellular Ca<sup>2+</sup> in Cellular ATP Release during the Activation of Murine T Cells—We next examined the effect of another inhibitor of vesicular exocytosis on TCR-dependent ATP release. As shown in Fig. 2A, the increase of extracellular ATP was significantly suppressed by treatment with BFA (an inhibitor of vesicular trafficking), as well as bafilomycin A. This result supports the idea that TCR stimulation evokes vesicular exocytosis of ATP.

Then, we investigated the involvement of activation of PI 3-kinase and the increase of intracellular Ca<sup>2+</sup> in ATP release after TCR stimulation. First, we examined the effect of LY294002 (an inhibitor of PI3-kinase) on TCR-dependent ATP release. Cellular ATP release was significantly abrogated by treatment with LY294002 (Fig. 2B), indicating a contribution of the PI 3-kinase pathway to TCR-dependent ATP release. We also examined the effect of BAPTA-AM (an intracellular Ca<sup>2+</sup> chelator) and depletion of extracellular Ca<sup>2+</sup> on the ATP release. ATP release was completely blocked when cells were treated with BAPTA-AM or stimulated in Ca<sup>2+</sup>-free RPMI 1640-based buffer (Fig. 2C), indicating a critical role of intracellular Ca<sup>2+</sup> in TCR-dependent ATP release.

Inhibitory Effects of Bafilomycin A on Increase of Intracellular Ca<sup>2+</sup>, Expression of IL-2 mRNA and CD25, and Production of IL-2, but Not Lck Signaling, after TCR Stimulation—First, to confirm that bafilomycin A does not interfere with the activation of TCR, we examined the effect of bafilomycin A on proximal signaling in activated T cells. The activation of Lck known is to be an early event following TCR/CD28 stimulation. TCR/CD28 stimulation induced rapid phosphorylation of Lck (within 2 min), and pretreatment with bafilomycin A for 30 min had no effect on the activation of Lck (Fig. 3A), indicating that bafilomycin A does not block TCR/CD28 stimulation.

Next, to investigate the effect of bafilomycin A on the TCR-dependent increase of cellular Ca<sup>2+</sup>, cells were incubated in Ca<sup>2+</sup>-free RPMI 1640-based buffer and the TCR-dependent mobilization of cytosolic Ca<sup>2+</sup> in T cells was measured. Treatment with bafilomycin A almost completely abrogated transmembrane Ca<sup>2+</sup> influx after stimulation of TCR/CD28 in RPMI 1640-based buffer (Fig. 3B). On the other hand, bafilomycin A almost completely abrogated transmembrane Ca<sup>2+</sup> influx after stimulation of TCR/CD28 in RPMI 1640-based buffer (Fig. 3C). These results indicated that bafilomycin A inhibits extracellular Ca<sup>2+</sup> influx, rather than the mobilization of cytosolic Ca<sup>2+</sup> (release of Ca<sup>2+</sup> from endoplasmic reticulum Ca<sup>2+</sup> stores into cytoplasm).

We further examined the effect of bafilomycin A on mRNA expression and secretion of IL-2 and CD25 expression in splenic T cells after stimulation with plate-bound anti-CD3 mAb and soluble anti-CD28 mAb. The TCR-dependent increases of IL-2 mRNA and IL-2 secretion were significantly blocked by bafilomycin A (Fig. 4, A and B). The increase of CD25 expression after TCR stimulation was decreased by treatment with
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We then examined the effect of P2 receptor antagonists on mBSA-induced cytokine production. A selective P2Y6 receptor antagonist MRS2578 significantly blocked IL-2, IL-17, and IL-6 production, like apyrase (Fig. 5). A highly selective P2X7 receptor antagonist, A438079, significantly blocked IL-6 production (Fig. 5), indicating the involvement of P2Y6 and P2X7 receptors in external antigen-induced cytokine production. Furthermore, bafilomycin A significantly blocked IL-2, IL-17, and IL-6 production (Fig. 5), indicating the involvement of vesicular exocytosis of ATP in the cytokine production induced by external antigen. These results indicate that vesicular exocytosis of ATP and activation of P2Y6 and P2X7 receptors are involved in T cell activation by not only TCR stimulation, but also external antigen stimulation.

Presence of Vesicular ATP Pool and Expression of SLC17A9 mRNA in Murine CD4 T Cells—To determine the localization of cellular ATP-containing vesicles, we stained murine CD4 T cells with the fluorescent ATP indicator MANT-ATP (25). The fluorescence was observed as vesicles in the cytoplasm in murine CD4 T cells (Fig. 6A), indicating that ATP-enriched vesicles were contained in murine CD4 T cells. We also examined the expression of SLC17A9 in murine CD4 T cells, because it has been reported that SLC17A9 acts as a VNUT, which is involved in ATP storage and release (19). We detected SLC17A9 mRNA in murine CD4 T cells (Fig. 6B), suggesting that VNUT is involved in vesicular storage of ATP in murine T cells.

Blockade of TCR-dependent ATP Release from Human Jurkat Cells by Inhibitors of Vesicular Exocytosis—To further investigate the involvement of vesicular exocytosis in ATP release from activated T cells, we examined the ATP release in human T lymphoma, Jurkat cells. We first measured the concentration of ATP released from Jurkat cells into the culture medium in response to stimulation of TCR/CD28 by treatment with anti-CD3 and anti-CD28 mAb. As shown in Fig. 7A, an increase of extracellular ATP concentration was observed after the stimulation of TCR/CD28, reaching a maximum at 1 min and returning to a near basal level within 5 min. In subsequent experiments, we measured extracellular ATP at 1 min after stimulation of TCR/CD28.

We next examined the effect of inhibitors of vesicular exocytosis on ATP release induced by TCR/CD28 stimulation.

bafilomycin A (Fig. 4, C and D), indicating that bafilomycin A blocked the TCR/CD28-dependent T cell activation process.

Inhibitory Effects of Bafilomycin A on External Antigen-induced Cytokine Production by Splenocytes of mBSA-immunized Mice—We investigated the effect of bafilomycin A, apyrase (ecto-nucleotidase), MRS2578 (an antagonist of P2Y6 receptor), and A438079 (an antagonist of P2X7 receptor) on external antigen-induced cytokine production. These P2 antagonists have been reported to block TCR-dependent murine T cell activation, including IL-2 production (11). Mice were immunized with mBSA, and the splenocytes of the immunized mice were re-stimulated with mBSA in vitro. We measured the production of proinflammatory cytokines (IL-2, IL-17, and IL-6) by the splenocytes. To investigate the involvement of extracellular nucleotides in cytokine production following stimulation with preimmunized antigen (mBSA), splenocytes were incubated with apyrase, which metabolizes extracellular nucleotides, during stimulation. Apyrase significantly blocked the production of IL-2, IL-17, and IL-6 (Fig. 5), indicating the involvement of extracellular nucleotides in production of these cytokines.

Presence of Vesicular ATP Pool and Expression of SLC17A9 mRNA in Murine CD4 T Cells—To determine the localization of cellular ATP-containing vesicles, we stained murine CD4 T cells with the fluorescent ATP indicator MANT-ATP (25). The fluorescence was observed as vesicles in the cytoplasm in murine CD4 T cells (Fig. 6A), indicating that ATP-enriched vesicles were contained in murine CD4 T cells. We also examined the expression of SLC17A9 in murine CD4 T cells, because it has been reported that SLC17A9 acts as a VNUT, which is involved in ATP storage and release (19). We detected SLC17A9 mRNA in murine CD4 T cells (Fig. 6B), suggesting that VNUT is involved in vesicular storage of ATP in murine T cells.

Blockade of TCR-dependent ATP Release from Human Jurkat Cells by Inhibitors of Vesicular Exocytosis—To further investigate the involvement of vesicular exocytosis in ATP release from activated T cells, we examined the ATP release in human T lymphoma, Jurkat cells. We first measured the concentration of ATP released from Jurkat cells into the culture medium in response to stimulation of TCR/CD28 by treatment with anti-CD3 and anti-CD28 mAb. As shown in Fig. 7A, an increase of extracellular ATP concentration was observed after the stimulation of TCR/CD28, reaching a maximum at 1 min and returning to a near basal level within 5 min. In subsequent experiments, we measured extracellular ATP at 1 min after stimulation of TCR/CD28.

We next examined the effect of inhibitors of vesicular exocytosis on ATP release induced by TCR/CD28 stimulation.
Increase of extracellular ATP was significantly suppressed by both bafilomycin A and BFA (Fig. 7B). To determine whether or not vesicles enriched in ATP exist in Jurkat cells, we stained intracellular ATP with MANT-ATP, and vesicles enriched in ATP were observed (Fig. 7C). We confirmed the expression of human SLC17A9 (VNUT) mRNA in Jurkat cells by RT-PCR analysis (Fig. 7D), as was the case in murine CD4 T cells. These results indicated that vesicular exocytosis is involved in TCR-dependent ATP release from Jurkat cells.

**Involvement of VNUT in ATP Release from Jurkat Cells**—To investigate whether VNUT is involved in ATP release from activated T cells, we silenced the expression of SLC17A9 with two different shRNAs (Clone ID, green and red). In cells transfected with SLC17A9-shRNA, the mRNA expression of SLC17A9 was decreased to 46 (green) and 49% (red) of that in scramble shRNA-transfected cells (negative control) (Fig. 8A). Integration of both SLC17A9-shRNAs (green and red) and scramble shRNA into the host genome was confirmed by genomic PCR analysis (data not shown). Using these SLC17A9-knockdown cells, we examined the role of VNUT in ATP release after TCR stimulation. The decreased SLC17A9 expression resulted in suppression of ATP release in response to TCR stimulation, compared with wild-type or negative control.
remains unclear how ATP is released from activated T cells in the acute phase. In this study, we investigated in detail the mechanism of rapid (within a few minutes) release of cellular ATP from activated T cells.

We found that the concentration of extracellular ATP in culture medium increased in response to TCR stimulation and reached a peak at 1 min, then decreased to the basal level within 5 min. The ATP released from activated T cells was significantly blocked by carbamoloxone, 18-glycyrrhetinic acid, flufenamic acid (inhibitors of gap junction hemichannels), glibenclamide (an inhibitor of potent chloride channel), arachidonic acid, an inhibitor of maxi-anion channel), A438079 (an antagonist of P2X7 receptor), and bafilomycin A (an inhibitor of the vesicular H+-ATPase). These results suggest that ATP release is regulated via multiple pathways during T cell activation. It has been reported that treatment with GdCl₃ blocked TCR-dependent ATP release in Jurkat cells (10). We have also examined the effect of GdCl₃ on TCR-dependent ATP release in Jurkat cells, and the ATP release was blocked by GdCl₃ in Jurkat cells (data not shown), as described in a previous study (10). Therefore, the mechanisms of ATP release during T cell activation may differ at least in part between murine T cells and human T lymphoma cells. Although vesicular exocytosis of ATP is known to occur in various cells, including PC12 cells, astrocytes, and liver cells (19, 26, 27), there is no report of the involvement of vesicular exocytosis in ATP release from activated T cells. Therefore, in this study we focused on the question of whether or not vesicular exocytosis of ATP from T cells occurs.

To study the vesicular exocytosis of ATP in T cells, we examined the effect of the exocytosis inhibitor BFA. We found that ATP released during murine T cell activation was significantly abrogated by not only by bafilomycin A, but also BFA. Treatment with these inhibitors also inhibited ATP release in Jurkat cells, as well as murine T cells, suggesting that vesicular exocytosis is involved in ATP release in both murine T cells and human T lymphoma cells. Because activation of PI 3-kinase and increase of intracellular Ca²⁺ are known to regulate vesicular exocytosis (20, 21), we next examined the involvement of PI 3-kinase and intracellular Ca²⁺ elevation in the ATP release. Treatment with LY294002 significantly suppressed the ATP release, suggesting a contribution of the PI 3-kinase pathway to TCR-dependent ATP release. In addition, the ATP release was completely suppressed when cells were pretreated with BAPTA-AM or stimulated in Ca²⁺/H⁺-free RPMI 1640-based buffer, supporting the involvement of intracellular Ca²⁺ elevation in TCR-dependent ATP release.

It has been suggested that extracellular ATP modulates T cell activation (28) and treatment with apyrase is known to suppress TCR signaling, such as an increase of cellular Ca²⁺, expression of CD25, and production of IL-2 (10, 11). To examine the involvement of ATP released via vesicular exocytosis in T cell activation, we investigated the effect of bafilomycin A on TCR signaling. First, we confirmed that bafilomycin A did not interfere with binding of anti-CD3 antibody to TCR. Phosphorylation of Lck, which is an early event following TCR stimulation, occurred both in the presence and absence of bafilomycin A after TCR stimulation. This result suggested that bafilomycin A would not disturb TCR/CD28 activation. Because it is well
known that an increase of intracellular Ca$^{2+}$/H$^{11001}$ plays an essential role in the events of T cell activation (29), we next determined whether bafilomycin A affects the TCR-dependent elevation of intracellular Ca$^{2+}$/H$^{11001}$ in T cells. Although TCR stimulation evoked elevation of cytosolic Ca$^{2+}$/H$^{11001}$, treatment with bafilomycin A significantly suppressed the influx of extracellular Ca$^{2+}$/H$^{11001}$ rather than the intracellular Ca$^{2+}$/H$^{11001}$ mobilization. In addition, we investigated the effect of bafilomycin A on mRNA expression and production of IL-2 and expression of CD25, as markers of TCR-dependent T cell activation (30), and found that bafilomycin A significantly inhibited these events. These results suggest that vesicular exocytosis of ATP has an important role in T cell activation downstream of TCR signaling.

The involvement of P2 receptors, such as P2X$_{7}$ and P2Y$_{6}$ receptors, in TCR-dependent activation has been suggested by several researchers including us (9-11). To further examine the role of vesicular exocytosis of ATP in T cell activation, we investigated the inhibitory effect of bafilomycin A and P2 receptor antagonists on external antigen-induced cytokine production. Mice were immunized with mBSA in vivo and immune cells were re-stimulated with mBSA in vitro, then cytokine production was determined. Apyrase and MRS2578 strongly inhibited proinflammatory cytokine production, in accordance with our previous report (11). Treatment with A438079 significantly suppressed production of IL-6, but not IL-2 or IL-17. Previous studies have demonstrated that the P2X$_{7}$ receptor antagonist inhibits TCR-dependent IL-2 production in murine T cells and Jurkat cells (10, 11). However, our results imply that the effect of A438079 on IL-2 production is different between TCR stimulation and external antigen stimulation. The reason for this might be that stimulation with the external antigen activates not only T cells, but also antigen-presenting cells, such as macrophages and dendritic cells. Moreover, bafilomycin A significantly blocked IL-2, IL-17, and IL-6 production. These results suggest that ATP released via exocytosis contributes to external antigen-induced T cell activation through P2 receptors, including P2X$_{7}$ and P2Y$_{6}$ receptors, in murine T cells.

The inhibitory effect of bafilomycin A on IL-2 production was greater in mBSA re-stimulation (Fig. 5A) than in direct TCR stimulation in vitro (Fig. 4B), suggesting that vesicular exocytosis would play a more important role in IL-2 production after antigen-induced re-activation of T cells than first activation via direct TCR stimulation. On the other hand, the inhibition of IL-6 production by bafilomycin A was smaller than that by apyrase. This result suggests that other pathways of ATP release would also be involved in the production of IL-6. Thus, not only exocytosis but also other mechanisms of ATP release would be involved in the production of cytokines by stimulation with external antigen.

**FIGURE 8.** Involvement of SLC17A9 in ATP release in TCR-dependent ATP release in Jurkat cells. A, Jurkat cells were transfected with shRNA targeting SLC17A9 or the negative control shRNA, and gene expression of SLC17A9 was examined by assessing mRNA levels using real time RT-PCR ($n = 3$). B, Jurkat cells transfected with shRNA targeting SLC17A9 or the negative control shRNA were stimulated by ligation of CD3/CD28 using Dynabeads. At 1 min after TCR/CD28 stimulation, the supernatants were collected and ATP content was measured ($n = 8-12$). C, Jurkat cells loaded with Fluo-4 were stimulated with anti-CD3 mAbs. The cells were preincubated in the presence or absence of bafilomycin A (50 nM) for 15 min. D, the shRNA-transfected cells loaded with Fluo-4 were stimulated with anti-CD3 mAbs. The change in fluorescence was measured for 7 min. Each value represents the mean ± S.E. A significant difference between the control group (scramble) and the indicated group is represented (*, $p < 0.05$ and **, $p < 0.01$).
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It is known that UDP is a potent ligand of P2Y$_{16}$ receptor (31). Because it has been also reported that uptake of UTP is also caused by VNUT into vesicles (19), UTP might be released by vesicular exocytosis during T cell activation, as well as ATP. Therefore, it is possible that UDP (a metabolites of released UTP) also plays a role in activation of T cells through activation of the P2Y$_{16}$ receptor. On the other hand, Schenk et al. (9) and Yip et al. (10) have demonstrated the involvement of the P2X$_7$ receptor in activation of murine T cells and Jurkat cells, respectively. They suggest that the pericellular concentration of ATP would increase enough to activate the P2X$_7$ receptor after TCR-dependent ATP release. However, the mechanisms of activation of the P2X$_7$ and P2Y$_{16}$ receptors during T cell activation are important issues that need further investigation.

Activation of T cells plays a critical role in many human diseases, including autoimmune disease and inflammation. T cells are activated and differentiate into Th1, Th2, and Th17 cells and regulatory T cells, which induce pro- and anti-inflammatory effects (32). It is well known that Th17 cells differentiate from naive T cells in the presence of transforming growth factor-β and IL-6, and Th17 cells evolve several autoimmune diseases by producing IL-17 (33). Furthermore, differentiation of Th17 cells in the intestinal lamina propria is required for the production of extracellular ATP derived from commensal bacteria (34). These observations, together with our results, indicate that ATP release via exocytosis during T cell activation would activate P2 receptors in autocrine or paracrine manners and would be involved in the differentiation of Th17 cells. This raises the possibility that blockade of vesicular exocytosis of ATP or activation of P2 receptors might be an effective strategy to improve autoimmune diseases or inflammation by suppressing T cell activation and proinflammatory cytokine secretion.

ATP-containing vesicles in T cells were detected by using MANT-ATP, suggesting that ATP exists as a vesicular pool in both murine T cells and Jurkat cells. SLC17A9 has recently been identified as a novel vesicular nucleotide transporter, and it is involved in vesicular storage of ATP and exocytosis of ATP (19). SLC17A9 is expressed in PC12 cells and type II taste cells (19, 35). Therefore, we hypothesized that SLC17A9 contributes to storage and release of ATP through exocytosis from activated T cells. We found that SLC17A9 mRNA is expressed in both murine T cells and Jurkat cells. As well as in murine T cells, bafilomycin A suppressed TCR-dependent Ca$^{2+}$ influx in Jurkat cells. Although bafilomycin A significantly blocked ATP release and Ca$^{2+}$ influx in murine T cells and Jurkat cells, it is difficult to determine the involvement of vesicular exocytosis in TCR-dependent Ca$^{2+}$ influx only by the inhibitory effect of bafilomycin A. Therefore, we examined the effect of SLC17A9 knockdown on TCR-dependent ATP release and Ca$^{2+}$ influx in Jurkat cells. SLC17A9 knockdown Jurkat cells showed reduced TCR-dependent cellular ATP release and reduced elevation of intracellular Ca$^{2+}$ (Ca$^{2+}$ influx) compared with control cells. These results strongly support the view that SLC17A9 plays a critical role in both vesicular exocytosis of ATP and TCR signaling during T cell activation. Because our results suggest that SLC17A9 is involved as a VNUT in TCR signaling, it is possible that abnormality of VNUT function may induce immune disorder. Detailed investigations of VNUT functions seem worthwhile.

In conclusion, we studied the mechanisms of cellular ATP release from activated T cells and showed that ATP release is mediated by several pathways in murine T cells. Vesicular exocytosis is one mechanism of TCR-induced ATP release, and this exocytosis is dependent on activation of PI 3-kinase and elevation of intracellular Ca$^{2+}$. SLC17A9 is involved as a VNUT in vesicular exocytosis of ATP during the early phase of T cell activation and subsequent TCR signaling. Release of ATP and activation of P2X$_7$ and P2Y$_{16}$ receptors play critical roles in T cell activation by TCR and external antigen. This is the first study to provide evidence of the involvement of vesicular exocytosis in TCR-induced ATP release from murine T cells and human lymphoma, providing new insights into the overall mechanism of T cell activation.

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