A P2X\textsubscript{7} receptor stimulates plasma membrane trafficking in the FRTL rat thyrocyte cell line

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Kochukov, M. Y., and A. K. Ritchie. A P2X\textsubscript{7} receptor stimulates plasma membrane trafficking in the FRTL rat thyrocyte cell line. Am J Physiol Cell Physiol 287: C992–C1002, 2004. First published June 9, 2004; 10.1152/ajpcell.00538.2003.—Thyroid cells express a variety of P2Y and P2X purinergic receptor subtypes. G protein-coupled P2Y receptors influence a wide variety of thyrocyte-specific functions; however, functional P2X receptor-gated channels have not been observed. In this study, we used whole cell patch-clamp recording and fluorescence imaging of the plasma membrane marker FM1-43 to examine the effects of extracellular ATP on membrane permeability and trafficking in the Fisher rat thyroid cell line FRTL. We found a cation-selective current that was gated by ATP and 2',3'-O-(4-benzoylbenzoyl)-ATP but not by UTP. The ATP-evoked currents were inhibited by pyridoxal phosphate 6-azophenyl-2',4'-disulfonic acid, adenosine 5’-triphosphate-2',3'-dialdehyde, 100 \mu M \text{Zn}^{2+}, and 50 \mu M \text{Cu}^{2+}. Fluorescence imaging revealed pronounced, temperature-sensitive stimulation of exocytosis and membrane internalization by ATP with the same pharmacological profile as observed for activation of current. The EC\textsubscript{50} for ATP stimulation of internalization was 440 \mu M in saline containing 2 mM \text{Ca}^{2+} and 2 mM \text{Mg}^{2+}, and 33 \mu M in low-Mg\textsubscript{2}-free, nominally \text{Ca}^{2+}-free saline. Overall, the results are most consistent with activation of a P2X\textsubscript{7} receptor by ATP. However, low permeability to N-methyl-D-glucamine and the propidium cation YO-PRO-1 indicates absence of the cytolytic pore that often accompanies P2X receptor activation. ATP stimulation of internalization occurs in Na\textsuperscript{+}-free, Ca\textsuperscript{2+}-free, or low-Mg\textsuperscript{2+} saline and therefore does not depend on cation influx through the ATP-gated channel. We conclude that ATP activation of a P2X\textsubscript{7} receptor stimulates membrane internalization in FRTL cells via a transduction pathway that does not depend on cation influx.

purinergic receptor; internalization; patch clamp

IN THE THYROID GLAND, thyroid epithelial cells secrete immature thyroglobulin by exocytosis into a follicular lumen, where the protein is iodinated and stored. Thyroid hormone release occurs when mature iodinated thyroglobulin is internalized by the thyrocyte and cleaved by lysosomal proteases. TSH, the primary regulator of thyroid cell differentiation and thyroid hormone secretion, stimulates the exocytosis and endocytosis of thyroglobulin primarily via activation of adenyl cyclase, with additional activation of PLC (8, 35). Another regulator of thyroid function is extracellular ATP. ATP could be released from the rich, autonomic innervation of the thyroid gland (19), from capillary endothelial cells (49), or possibly from the thyrocytes themselves. Some of the actions of ATP in cultured human (48), dog (32), and porcine thyrocytes (5) and in the Fisher rat thyroid cell line FRTL-5 (16, 17, 50) include stimulation of thyroglobulin secretion (14, 15), mobilization of Ca\textsuperscript{2+}, mitogenesis, production of H\textsubscript{2}O\textsubscript{2}, increased efflux of I\textsuperscript{-} and Cl\textsuperscript{-}, and inhibition of forskolin-stimulated Na\textsuperscript{+} absorption (5). Most of these effects are mimicked by UTP and occur via G protein-coupled P2Y purinergic receptors that activate PLC and PLA\textsubscript{2}.

Another class of purinergic receptors is the P2X family of ATP-gated ion channels. Heterologous expression of six of the seven mammalian family members results in homomeric channels that are permeable to small monovalent and divalent cations, whereas one member (P2X\textsubscript{6}) contributes to functional cation channels mainly when coexpressed with other (P2X\textsubscript{2} or P2X\textsubscript{4}) P2X subunits (38). In chicks, the P2X\textsubscript{6} receptor appears to be permeable to both cations and Cl\textsuperscript{-} (38), and recent reports indicate that a P2X receptor may directly gate a Cl\textsuperscript{-} current in mouse parotid acinar cells (4). In a subset of the P2X family (homomeric P2X\textsubscript{2}, P2X\textsubscript{4}, and P2X\textsubscript{7} and heteromeric P2X\textsubscript{2,3}), repeated or prolonged exposure to ATP leads to gradual opening of a pore that is highly permeable to large organic cations (up to 900 Da) such as N-methyl-D-glucamine (NMDG\textsuperscript{+}) and YO-PRO-1 (25, 53, 54). In macrophages (20) and in cells transfected with P2X\textsubscript{7} receptors, prolonged activation of the pore may lead to apoptosis or necrosis (58). The mRNA transcripts for three (P2X\textsubscript{3}, P2X\textsubscript{4}, and P2X\textsubscript{5}) of the P2X receptor subunits are present in FRTL-5 (17), and immunoreactivity for these same P2X subunits has been observed in rat thyroid follicular cells (18). At present, however, there are no reports of functional P2X receptor activity in thyrocyte-derived cells. In this study on FRTL cells, the parent cell line of FRTL-5, we show that ATP activates a plasma membrane cationic current and stimulates pronounced plasma membrane trafficking via activation of a P2X\textsubscript{7} receptor that does not undergo pore formation.

MATERIALS AND METHODS

Cell culture. FRTL, the Fisher rat thyroid cell line (2), was obtained from American Type Culture Collection (ATCC; Rockville, MD). The cells were grown in Coon’s F-12 medium containing 0.5% calf serum, 10 \mu g/ml insulin, 10 nM hydrocortisone, 5 \mu g/ml transferrin, 10 ng/ml glycyrrhetinic acid, 10 ng/ml somatostatin, and 10 nM/ml TSH. Jurkat clone E6 (human acute T-cell leukemia), also from ATCC, was grown in RPMI 1640 medium containing 10% fetal bovine serum. The cells were maintained in a humidified 5% CO\textsubscript{2} incubator at 37°C. For electrophysiological and fluorescence studies, FRTL cells were plated on glass coverslips.

Whole cell voltage clamp. The cells were voltage clamped using an EPC7 patch-clamp amplifier (Adams List, Great Neck, NY) and the ruptured patch technique of whole cell recording (21). Data acquisition and analyses were performed using Pulse-HEKA software and

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the ITC-18 computer interface (Instrutech, Port Washington, NY). The bath was grounded with a Ag-AgCl pellet via a Ag-AgCl lagar the computer interface (Instrutech, Port Washington, NY).

Solutions. The pipette solution contained (in mM) 25 CsCl, 65 Cs2SO4, 2 EGTA, 0.6 mM CaCl2 (100 mM free Ca2+ at 36°C), 3 MgCl2, 10 NaCl, 10 glucose, 60 sucrose, and 10 HEPES (pH 7.4 at 36°C) and had an osmolality of 305 mosmol/l. Cs+ was used as the major cation to block K+ currents in the cell. Standard bath solution consisted of (in mM) 150 NaCl, 5.6 KCl, 2 CaCl2, 2 MgCl2, 5 glucose, 30 sucrose, and 10 HEPES (pH 7.4 at 36°C) and had an osmolality of 335 mosmol/l. Additions or modifications to the solutions were made by isosmotic ion substitutions and by using sucrose to make small changes in osmolality. Concentrated stock solutions of 50 mM ATP or 5 mM UTP were prepared in Ca2+-free bath solution with reduced NaCl, subsequently adjusted for pH and osmolarity, and stored at −80°C. For experiments with no external Na+, the ATP stock solution was prepared with NMDG+ as the major cation. All experiments were performed at 36–37°C. The concentrations of free Ca2+, Mg2+, and/or ATP4– were calculated with the software WEBMAXCLITE version 1.15 (available at http://www.stanford.edu/~epatron/maxcl.html).

Confocal and epifluorescence imaging. Plasma membrane trafficking was assessed by continuously monitoring the fluorescence of FM1-43. The coverslip containing the cells was mounted in a chamber, rinsed with standard bath saline, and then maintained in 1 ml of bath saline containing 5 μM FM1-43 (added from a 5 mM stock solution prepared in DMSO). Agonists (ATP, UTP, or vehicle) were applied to the edge of the chamber either from a concentrated stock solution that was isosmotic in total solute concentration or by continuous perfusion of a small chamber with the indicated concentration of agonist. The solution temperature was maintained at 37°C by heating the chamber and the perfusion solution. To monitor plasma membrane permeability to YO-PRO-1, we continuously monitored fluorescence at 37°C in the presence of 2 or 5 μM YO-PRO-1.

To obtain measurements from only internalized membrane, the coverslip attached cells were pretreated four times with standard saline at 37°C, incubated for 10 min at 37°C in saline containing 5 μM FM1-43 with or without agonists, and then washed four times at 37°C. The washes remove agonist, external dye, and dye bound to the plasma membrane. The cells were then examined for internalized fluorescence in standard saline at room temperature. For incubations performed at 4 and 23°C, the prerinse and wash were done at the same temperature as the incubation, but the fluorescence was measured at room temperature. When the effects of antagonists (except adenosine 5′-triphosphate-2,3′-dialdehyde (oxoATP)) were studied, the antagonists were present in the prerinse and during the incubation with FM1-43. The effects of oxoATP were studied by preincubating the cells with the drug for 2 h at 37°C. 5% CO2 in Coon’s F-12 medium without serum or additives. The oxoATP was then removed and was not present during the incubation with agonist.

To measure the response to multiple applications of ATP, cells were rinsed, incubated for 10 min with or without 500 μM ATP, rinsed, allowed to recover for 10 min, incubated for 10 min in 5 μM FM1-43 in the presence or absence of ATP, rinsed free of ATP and FM1-43, and then monitored for internalized fluorescence. All manipulations were performed at 37°C except for the measurement of fluorescence, which was done at room temperature.

The confocal and epifluorescence measurements were performed on Nikon Eclipse 200 inverted microscopes (Nikon Instruments, Melville, NY) equipped with Photometrics CoolSnap HQ charge-coupled device (CCD) cameras (Roper Scientific, Tucson, AZ), Nikon fluor ×40 and ×60 oil objectives (1.3 and 1.4 NA, respectively), and optical filters from Chroma Technology (Brattleboro, VT). Confocal images were obtained using the Noran OZ video-rate laser scanner (Noran Instruments, Middleton, WI), argon-krypton laser, 488LP dichroic mirror, 500 LP emission filter, and Intervision acquisition software. Epifluorescence images were obtained using a xenon lamp, HQ475/30× excitation filter, Q495LP dichroic mirror, HQ515/30m emission filter, and MetaFluor or MetaMorph software (Universal Imaging, Downington, PA). The same filter combinations and excitation wavelengths were used to monitor FM1-43 and YO-PRO-1 fluorescence. Image analysis was performed off-line using MetaMorph software. The cell boundary was drawn from the transmission image, and then the mean pixel density of the inscribed area in the fluorescence image was determined. The pixel density per cell, after subtraction of the pixel density from regions devoid of cells, is reported as the relative fluorescence intensity per cell. Measurements were made in the linear range of the CCD camera by making adjustments in the acquisition time (25, 50, or 100 ms) and final scaling of the signal to 50 ms.

Cell surface biotinylation and Western blot. Jurkat cells in suspension culture and FRTL cells suspended by trituration were rinsed with cold phosphate-buffered saline containing 0.1 mM CaCl2 and 1 mM MgCl2, pH 9 (PBS-CM). Cell surface proteins were biotinylated at room temperature for 30 min with 1.5 mg/ml EZ-Link sulfo-NHS-LC-biotin (Pierce, Rockford, IL). The cells were rinsed, incubated at 4°C for 20 min with quench buffer (PBS-CM + 100 mM glycine, pH 9.0), rinsed, and then treated for 30 min at 4°C with lysis buffer (1% Triton X-100, 10 mM Tris, 150 mM NaCl, 5 mM EDTA, 10 μg/ml pepstatin, 10 μg/ml leupeptin, and 0.5 mM PMSF). The lysed cells were centrifuged, and the solubilized, biotinylated proteins in the supernatant were incubated overnight at 4°C with ImmunoPure immobilized streptavidin beads (Pierce). The beads were washed sequentially with lysis buffer, high-salt buffer (0.1% Triton X-100, 10 mM Trisazma base, and 500 mM NaCl, pH 7.5), and no-salt buffer (50 mM Trizma base, pH 7.5) and then heated to 85°C for 10 min in SDS sample buffer [50 mM Tris-Cl, pH 7.0, 1% (vol/vol) glycerol, 4% (wt/vol) SDS, 2% (vol/vol) 2-mercaptoethanol, and 0.13 mg/ml Coomassie blue R-250]. The beads were removed by centrifugation, and the eluted samples and MagicMark Western standards (Invitrogen, Carlsbad, CA) were separated by SDS-PAGE on Tricine gels containing 7.5% polyacrylamide (40). For Western blot analysis, the proteins were transferred to nitrocellulose membranes. The membranes were blocked overnight at 4°C with PBS-CM containing 0.05% Tween 20 and 0.5% nonfat dry milk (PBS-TM) and were then incubated for 2 h at room temperature with rabbit anti-P2X7 polyclonal antibody (4 μg/ml) in PBS-TM. After being washed, the blot was exposed for 1 h at room temperature to horseradish peroxidase-linked donkey anti-rabbit Ig (Amersham, Piscataway, NJ) that was diluted 1:5,000 in PBS-TM. Antibody binding was detected by enhanced chemiluminescence with SuperSignal West Dura extended duration substrate (Pierce).

The primary antibody used for Western blot analysis was affinity-purified rabbit anti-P2X7 receptor that was raised against a synthetic peptide corresponding to extracellular residues 136–152 of mouse P2X7 (Alomone Labs, Jerusalem, Israel). As a control, the blot was also probed with the same concentration of primary antibody that was preabsorbed by incubation for 1 h at 37°C with antigenic peptide (1 μg peptide/μg antibody).

Statistics. Results are reported as means ± SE; absence of error bars in the figures indicates an error that is too small for representation. Statistical significance (P < 0.05) was determined using Student’s t-test when analyzing pairs or Kruskal-Wallis one-way
ANOVA on ranks and Dunn’s multiple comparison test (SigmaStat 2.0 software; SPSS, Chicago, IL).

Chemicals. Hydrocortisone, bovine apotransferrin, bovine insulin, somatostatin, glycy1-1, histidyl-1, lysine acetate, calf serum from donor herd, ATP sodium salt, guanosine 5’-O-(2-thiodiphosphate) (GDPβS) trilithium salt, pyridoxal phosphate 6-azophenyl-2’,4’-disulfonic acid (PPADS) tetrasodium salt, ADF sodium salt, α,β-methyleneadenosine 5’-triphosphate (αβ-MeATP) lithium salt, 2’,3’-O-(4-benzoylbenzoyl)-adenosine 5’-triphosphate (BzATP) trimethylammonium salt, brilliant blue G sodium salt (BBG), and oxoATP sodium salt were obtained from Sigma (St. Louis, MO). 2-Methylthioadenosine 5’-triphosphate (2-MeSATP) tetrasodium salt and UTP sodium salt were obtained from Calbiochem (La Jolla, CA), YO-PRO-1 diiodide and FM1-43 dibromide were obtained from Molecular Probes (Eugene, OR), and TSH was provided by Dr. A. F. Parlow (National Hormone and Peptide Program, Torrance, CA).

RESULTS

Extracellular ATP-activated current. A 5-min application of ATP to an FRTL cell bathed in standard saline activated an inward current at the −80-mV holding potential. In response to 500 μM ATP, there was an initial rapid desensitization, but the majority of current was long lasting and reversed quickly upon washout of ATP (Fig. 1). The responses to additional applications of the same dose were larger than the first response and slowly grew in amplitude. A higher dose (2 mM), applied to a different cell, produced a larger initial current that slowly grew during the first application and then began to slowly desensitize after ~90 s (Fig. 1). The response to the second application decayed with time and failed to completely deactivate upon washout of ATP. The dose-dependent time courses of the illustrated currents were highly reproducible in other cells. Concentrations of ATP of 200 μM or less had no effect. A current-voltage (I-V) plot of the ATP-activated current, measured during the second application of ATP, showed slight inward rectification and a mean reversal potential of +8.6 ± 2.2 mV (n = 6) in standard bath saline (Fig. 2). When external Na+ was replaced with NMDG+, the reversal potential shifted to −34 ± 3 mV (n = 3). Replacement of external Cl− in the standard saline with gluconate− changed the Cl− equilibrium potential from −36 mV to +62 mV but had no effect on the reversal potential (+9.0 ± 3.8 mV, n = 4). These results indicate that ATP activates a channel permeable to small cations (Cs+ and Na+) but relatively impermeable to NMDG+ and Cl−.

Pharmacology of the ATP-activated current. The current evoked by 500 μM ATP is mediated by a P2 purinergic receptor, because PPADS (100 μM), a nonspecific antagonist at most P2X and P2Y receptors, abolished the response to 500 μM ATP (Fig. 3A). UTP (500 μM), a potent P2Y-selective agonist, had no effect. The response to ATP was the same when 2 mM GDPβS, which interferes with G protein activation, was included in the pipette solution. These results, and the cation selectivity of the channel, indicate that ATP is activating a P2X receptor-gated channel rather than a G protein-coupled P2Y receptor. The ATP analog BzATP (200 μM) activated the current (Fig. 1) with a time course that was similar to that seen with a higher dose of ATP (2 mM). Cu2+ (50 μM), an inhibitor of P2X1 and P2X7 receptors, abolished the response. Pretreatment of cells for 2 h with 200 μM oxoATP, followed by extensive rinsing to remove the drug, rendered the cells unresponsive to ATP, indicating irreversible inhibition. OxoATP is an irreversible inhibitor of P2X7 receptors but a reversible inhibitor of P2X1 and P2X2 receptors (39). The effect of Zn2+, which has differential actions (potentiation and/or inhibition) on P2X receptors depending on P2X subtype, is shown in Fig. 3B. In this example, the slow time course of ATP activation of current partially reflects slow equilibration of concentration as the ATP (500 μM) was applied by bath perfusion. In the presence of bath ATP, subsequent brief application of 100 μM Zn2+ plus ATP from a large-bore pipette resulted in rapid and reversible inhibition of current. Data in the bar graph summarize the effects of ATP when applied from a large-bore pipette in the presence and absence of bath solution containing Zn2+. Under these unpaired conditions, Zn2+ inhibition of 500 μM ATP failed to reach significance as a result of large cell-to-cell variability in current amplitude but was significant when tested on the larger amplitude currents evoked by 1 mM ATP. The overall pharmacological profile is most characteristic of the homomeric rat P2X7 receptor subtype (38).
were continuously superfused with 5 μM FM1-43 in standard bath saline and were then switched to saline that additionally contained 500 μM ATP. ATP caused a slow, delayed increase in the relative fluorescence intensity per cell, indicating exocytotic exposure of internal membranes to the cell surface. Upon washout of FM1-43 (and ATP) from the bath, the cell fluorescence decreased to a new level as the dye partitioned out of the plasma membrane. The remaining cell-associated fluorescence represents dye located on internalized membranes. Lowering the temperature from 37°C to 23°C attenuated the extent of ATP-induced exocytosis and internalization. In contrast, application of 500 μM UTP had no discernible effect, and the results looked identical to the basal rates of constitutive exocytosis and membrane internalization (not shown). Additional studies of the temperature dependence of membrane internalization are shown in Fig. 6A. For these studies, cells were incubated at the indicated temperature for 10 min in 5 μM FM1-43 in the presence or absence of 500 μM ATP and were then extensively rinsed before measurement of cell-associated fluorescence. The results show that ATP induces a 27-fold stimulation of internalization over basal levels at 37°C; 3.5-fold stimulation at 23°C, and complete block of internalization at 4°C. Thus the phenomenon shows the temperature dependence expected for exocytic and endocytic events.

The electrophysiological studies (Fig. 1) show that the cells recovered rapidly from a 5- to 10-min exposure to 500 μM ATP. A similar recovery was seen for ATP stimulation of membrane trafficking. ATP stimulated FM1-43 internalization to a similar extent with or without prior exposure to ATP (Fig. 6B). Moreover, prior exposure to ATP did not cause subsequent internalization when ATP was not present during incubation with FM1-43.

**Pharmacology of ATP-induced membrane internalization.** Pharmacological studies were performed under conditions that permit measurement of only the internalized fluorescence. P2X and/or P2Y receptor agonists such as 500 μM UTP, 2-MeSATP, ADP, and αβ-MeATP were relatively ineffective compared with ATP (Fig. 7A). BzATP (500 μM), however, stimulated internalization to a greater extent than 500 μM ATP. Antagonists that inhibited the ATP-stimulated internalization were 100 μM PPADS, 50 μM Cu2+, 5 μM BBG, or a 2-h preincubation with 200 μM o xoATP (Fig. 7B). The effects of Zn2+ were also tested (Fig. 7C). At 20 μM, Zn2+ had little effect on ATP-stimulated internalization, but dose-dependent inhibition was observed at 100 and 500 μM. Zn2+ had no effect on basal activity (whether added at the time of FM1-43 incubation or included in the prewash). The effects of ATP on membrane internalization show the same pharmacological specificity as the activation of ionic current and are overall most consistent with activation of a P2X7 receptor.

The effect of ATP on FM1-43 membrane internalization was dose dependent (Fig. 8A) with an EC50 of 440 μM in standard saline, which contained 2 mM each of Ca2+ and Mg2+. In external solution that was nominally Ca2+ free and low in Mg2+ (a small amount was added from the stock ATP solution), ATP stimulation of FM1-43 internalization occurred with a markedly lower EC50 (33 μM). When the same data were plotted as a function of free, uncomplexed ATP (i.e., ATP4+), the EC50 values were 13 μM in standard saline and 28 μM in the low-divalent cation solution (Fig. 8B). The similarities in the EC50 values when based on the free ATP concent-
Fig. 3. Pharmacology of ATP-activated current. A: ATP (500 µM) in standard saline was applied to the cell from a large-bore pipette. Steady-state amplitude of the ATP-activated current at the holding potential of ~80 mV was measured in response to the first application of ATP and then normalized to the cell capacitance (ATP current density). Where indicated, the antagonists pyridoxal phosphate 6-azophenyl-2',4'-disulfonic acid (PPADS; 100 µM) and Cu²⁺ (50 µM) were included in standard bath saline superfusing the cells and also in ATP solution. The antagonist adenosine 5'-triphosphate-2',3'-dialdehyde (oxoATP; 200 µM) was not present during electrophysiological measurements but was present during a 2-h preincubation. Effects of guanosine 5'-O-(2-thiodiphosphate) (GDPβS; 2 mM) were tested by including it in the patch-pipette solution. B: current is shown for a cell that was held at ~80 mV. During time indicated by top horizontal bar, 500 µM ATP was applied for 6 min by bath perfusion; during times indicated by bottom horizontal bars, 500 µM ATP + 100 µM Zn²⁺ was applied to the cell from a large-bore pipette. With this protocol, similar reversible inhibition of ATP-activated current by 100 µM Zn²⁺ was observed in 4 additional cells. C: ATP current density measured using 2 different concentrations (0.5 and 1.0 mM) of ATP under conditions identical to those reported in A but in the presence or absence of 100 µM Zn²⁺ in the bath solution and in the large-bore pipette. Numbers in parentheses represent the number of cells examined. *Significantly different from the response to ATP under control (Cont) conditions.

Fig. 10 shows that addition of 1 mM ATP to cells bathed in saline that is Mg²⁺ free and low in extracellular Ca²⁺ (0.1 mM), a condition that favors pore development (53), the basal rate of increase in YO-PRO-1 fluorescence was usually greater than in standard saline, but addition of ATP had no additional effect. Thus the P2X receptor in FRTL does not undergo pore formation. Because YO-PRO-1 has a slightly smaller molecular mass (375 daltons for the free base) than FM1-43 (451 daltons for the free base) and a similar charge density (2+), it is unlikely that the ATP-induced labeling of internal membranes with FM1-43 was secondary to FM1-43 entry into cells via a large-cation-permeable pore.

**P2X7 receptor protein expression in FRTL.** The pharmacological analyses indicate that ATP stimulation of membrane current and membrane internalization are mediated by the P2X7 receptor subtype. To test for P2X7 receptor expression, we performed Western blot analysis on FRTL plasma membrane proteins. For comparison, we also tested a human Jurkat leukemic cell line that is known to express P2X7 receptors (7). The P2X7 receptor subtype. To test for P2X7 receptor expression, we performed Western blot analysis on FRTL plasma membrane proteins. For comparison, we also tested a human Jurkat leukemic cell line that is known to express P2X7 receptors (7). Cell surface proteins were biotinylated with a membrane-impermeant reagent, isolated with streptavidin-agarose beads, separated by SDS-PAGE, and then transferred to nitrocellulose. As shown in the immunoblot (Fig. 11), FRTL and Jurkat

**Role of external ions in ATP-induced membrane internalization.** The ability of ATP to stimulate internalization in low-divalent cation solutions suggests that the transduction mechanism does not involve Ca²⁺ or Mg²⁺ influx. Further assurance of the lack of effect of Ca²⁺ influx is the finding that ATP stimulated internalization even when 2 mM EGTA was included in the Ca²⁺-free external solution (Fig. 9). ATP also stimulated FM1-43 internalization in the absence of external Na⁺ and K⁺ (Fig. 9). These results suggest that cation (Na⁺, Mg²⁺, or Ca²⁺) influx is not required for ATP stimulation of internalization.

**Permeability to YO-PRO-1.** In addition to a channel that is permeable to small cations, activation of some P2X receptor subtypes (mainly P2X₇, but also P2X₂, P2X₄, and P2X₂,₃) leads to slow development of a pore that is permeable to large organic cations (25, 53). YO-PRO-1, a large organic cation that permeates the pore and fluoresces upon binding to intracellular nucleic acids, is typically used to monitor pore activation. Figure 10 shows that addition of 1 mM ATP to cells bathed in YO-PRO-1 did not increase cell fluorescence above basal levels. In saline that is Mg²⁺ free and low in extracellular Ca²⁺ (0.1 mM), a condition that favors pore development (53), the basal rate of increase in YO-PRO-1 fluorescence was usually greater than in standard saline, but addition of ATP had no additional effect. Thus the P2X receptor in FRTL does not undergo pore formation. Because YO-PRO-1 has a slightly smaller molecular mass (375 daltons for the free base) than FM1-43 (451 daltons for the free base) and a similar charge density (2+), it is unlikely that the ATP-induced labeling of internal membranes with FM1-43 was secondary to FM1-43 entry into cells via a large-cation-permeable pore.

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exhibited P2X7 receptor immunoreactivity that was inhibited by preabsorption of the antibody with the antigenic peptide. The estimated size was 70 kDa for both Jurkat and FRTL, which is appropriate for the molecular mass of the P2X7 subtype (68 kDa in nonglycosylated form).

DISCUSSION

In this study, we describe the effects of purinergic P2 receptor stimulation on membrane permeability and plasma membrane trafficking in the FRTL cell line. Using RT-PCR and subtype-specific primers for G protein-coupled rat P2Y receptors, Ekokoski et al. (17) found mRNA for purinergic P2Y2, P2Y4, and P2Y6 and an absence of P2Y1 receptors in FRTL-5 cells, a subclone of FRTL. No tests were performed for the presence of P2Y11 and P2Y12 receptors, which have thus far been cloned only from humans and dogs. Transcripts were also found for the ATP-gated P2X receptor channel, an oligomeric structure composed of three or more homo- or heteromeric subunits (38). Using specific primers for each of the seven known mammalian P2X receptor subunits, Ekokoski et al. (17) found rat P2X3, P2X4, and P2X5 mRNA in FRTL-5 cells. ATP is an agonist of all P2X and P2Y receptors except P2Y6 and P2Y12. UTP activates P2Y2 and P2Y4 but is a poor agonist of P2X receptors (44, 46). In FRTL-5 and cultured thyroid epithelial cells (17), low concentrations of ATP (EC50 ≤ 0.5 μM) increase PLC, PLA2, and PLD activity, mobilize Ca2+, and influence a number of pathways that contribute to thyroid growth and function. These actions are primarily mediated by
P2Y receptors, because the effects can be mimicked by UTP. The present study represents the first report of a functional ionotropic P2X receptor in the FRTL line.

**ATP activation of a P2X receptor-gated cation channel.** In whole cell, ruptured-patch recording of FRTL cells, prolonged (5 min) application of ATP activated a long-lasting, but rapidly reversible, cation-selective current. PPADS, an antagonist of most rat P2X (P2X1, P2X2, P2X3, P2X5, and P2X7) and P2Y (P2Y1, P2Y2, and P2Y6) receptors (44) inhibited the current. However, P2Y receptors were not involved, because UTP did not activate the ionic current and the ATP-activated current persisted when G protein activation was inhibited by inclusion of GDPβS in the pipette solution. Current activation required high concentrations of ATP, >200 μM in our standard saline containing 2 mM Ca2+ and 2 mM Mg2+. Low affinity for ATP (EC_{50} = 0.1–1 mM) is a characteristic feature of the P2X7 receptor subtype, whereas a higher affinity (EC_{50} < 20 μM) is seen for P2Y and the other P2X subtypes (26, 46). BzATP was more effective than ATP in activating the current in FRTL. BzATP is an agonist of most P2X receptors (22, 39, 55) and of a few P2Y (P2Y2 and hP2Y11) receptors (13, 56). A relative potency of BzATP > ATP, however, is most characteristic of P2X7 receptors (38, 39). Both Cu^{2+} (50 μM) and Zn^{2+} (100 μM) inhibited the ATP-activated current in FRTL cells. Cu^{2+} inhibits ATP binding to P2X7 and P2X4 and potentiates current at P2X7 receptor subtypes (12, 53). Zn^{2+} (2–100 μM) potentiates homomeric P2X2, P2X3, P2X4, and P2X5 and heteromeric P2X_{2/6} and P2X_{4/6} receptors, with an additional inhibitory effect occurring at higher concentrations of Zn^{2+} (10, 12, 29, 53, 55). In contrast, only the inhibitory effect is seen with P2X1 (55) and P2X2 receptors (53). Finally, preincubation with 200 μM oxoATP irreversibly inhibited the current. OxoATP is an irreversible inhibitor of P2X7 but a reversible inhibitor of P2X1 and P2X2 receptors (39). Overall, the long-lasting nature of the current and the pharmacological profile are most consistent with the involvement of a P2X7 receptor. The P2X7 subtype is unique in its inability to form heteromers with other P2X subunits (38).

Pore activation is a common (25, 38, 53), but not obligatory (20, 43, 45), property of heterologously expressed and endogenous P2X7 receptors. In FRTL cells, the ATP-activated receptor remained relatively impermeant to NMDG+ and YO-PRO-1 during a 5- to 10-min exposure to ATP at 37°C (in the presence or absence of divalent cations). Thus, as in bovine endothelial cells (45), human retina glial cells (41), and parotid acinar and duct cells (33), ATP activates a P2X7-like receptor channel in FRTL without large pore formation. This could be due to absence of an accessory protein (47) or a variation of the COOH terminus of the P2X7 receptor that is essential for pore activation (51).

The ATP-activated current increases in amplitude and/or grows with successive applications of 500 μM ATP or increases upon the first application of 2 mM ATP. The growth is not due to slow activation of pores because the channel remains impermeant to NMDG+. Furthermore, ion selectivity does not change during the rising phase of the current (Kochukov MY and Ritchie AK, unpublished observations). Such P2X7 receptor kinetics have been observed by others and have been proposed to be due to a slow increase in potency with submaximal concentrations of ATP (23) and/or a slow assembly step involving the cytoskeleton that is required for channel gating (33).

**A P2X7-like receptor mediates ATP stimulation of plasma membrane trafficking.** Confocal and epifluorescence measurements of the cell surface marker FM1-43 showed that ATP stimulated a very large increase in cell-associated fluorescence with dramatic labeling of internal membranes. Although this labeling pattern could occur secondary to permeation of FM1-43 through an ATP-activated pore, this seems unlikely because ATP did not increase permeability to YO-PRO-1. FM1-43 influx may also enter cells via nonselective cation channels, as occurs in sensory hair cells (36). FM1-43 influx through such channels, however, is characterized by rapid kinetics (in seconds) and influx at 4°C. In contrast, the ATP-induced fluorescence changes in FRTL began after a delay of several minutes, gradually increased over the next 11 min, was markedly attenuated at 23°C, and was completely absent at 4°C. The slow kinetics and temperature sensitivity are consistent with ATP stimulation of membrane trafficking in which the increase in fluorescence is due to exocytotic fusion of internal membranes with the plasma membrane and the pronounced labeling of internal membranes is due to increased internalization of dye-labeled plasma membrane.
BzATP was a more potent stimulator of membrane internalization than ATP, whereas agonists such as 2-MeSATP (9, 57), 2-MeATP, and ADP (39), which are ineffective or less effective than ATP (39) at P2X7 receptors, had relatively little effect on membrane internalization. The response to ATP was inhibited by PPADS, oxoATP, Zn2⁺, Cu²⁺, and 5 μM BBG (38). At 5 μM, BBG inhibits P2X7 and P2X2, but much higher concentrations are needed to inhibit P2X1, P2X2, P2X3, P2X2/3, P2X4, and P2X1/5 receptors. Two distinguishing features of P2X7 receptors, low affinity for ATP and potentiation upon removal of extracellular Ca²⁺ and Mg²⁺, were also present. The EC₅₀ value for ATP stimulation of FM1-43 internalization was ~440 μM in standard saline and 14 times lower (EC₅₀ ~ 33 μM) in nominally Ca²⁺-free, low-Mg²⁺ saline. This is consistent with other reports that the active ligand for the P2X7 receptor is the fully ionic ATP⁴⁻ rather than ATP that is complexed with a divalent cation (30, 38). Finally, replacement of Na⁺ with NMDG⁺, which enhances the potency of ATP (37) for P2X7 receptors, also greatly enhanced the effect of ATP on membrane internalization in FRTL. The pharmacological profile indicates that ATP stimu-
A P2X<sub>7</sub> RECEPTOR STIMULATES MEMBRANE TRAFFICKING

Fig. 9. External ion dependence of ATP-stimulated FM1-43 internalization. Following the same protocol as in Fig. 8, cells were incubated with FM1-43 in the presence and absence of 500 μM ATP in different external saline solutions. In the first set of cells, Ca<sup>2+</sup> was removed from external saline and 2 mM EGTA was added (0 Ca<sup>2+</sup>, 2 EGTA). In the second set of cells, Na<sup>+</sup> and K<sup>+</sup> were replaced with NMDG<sup>+</sup>. The internalized fluorescence was normalized to the amount of internalization observed in the same experiment with 500 μM ATP in standard saline. Each bar represents the average of >170 cells, and data were taken from 3 coverslips per condition. *Significantly different from basal; **significantly different from ATP in standard saline.

Fig. 10. Effect of ATP on YO-PRO-1 uptake. Fluorescence of cells bathed in 5 μM YO-PRO-1 at 37°C was monitored by confocal microscopy. The bathing solution was standard saline (circles) or saline that was low in divalent cations (0 Mg<sup>2+</sup>, 0.1 mM Ca<sup>2+</sup>; triangles). ATP at a final bath concentration of 1 mM (open symbols) or vehicle (filled symbols) was added to the chamber at time indicated by the arrow. The RFI was measured from all cells in the image field for each condition (4 and 8 cells for control and ATP, respectively, in standard saline; 9 and 7 cells for control and ATP, respectively, in low-divalent cation saline). The occasional damaged cells were highly fluorescent, exceeding an endocytosis in FRTL is unknown. Because the stimulation of trafficking persists in Ca<sup>2+</sup>-free (with 2 mM EGTA), low-Mg<sup>2+</sup> (~20 μM), or Na<sup>+</sup>-free solutions, these actions of ATP occur independently of the influx of ions through the receptor-gated channel. A Ca<sup>2+</sup>-independent effect of extracellular ATP on plasma membrane trafficking has been previously observed in rat brown adipocytes (31); however, the receptor in adipocytes does not appear to involve P2X<sub>7</sub> receptors, because it is activated by low concentrations of ATP, ADP, and 2-MeSATP (42). Some previously reported Ca<sup>2+</sup>-independent actions of P2X<sub>7</sub> receptors in other cell types include stimulation of MAP kinases (3) and the Ca<sup>2+</sup>-insensitive forms of PLA<sub>2</sub> (11) and PLD (24). MAP kinases (27), PLA<sub>2</sub> (6), and PLD (11) are known to affect plasma membrane trafficking. The P2X<sub>7</sub> receptor forms a complex with a number of membrane, extracellular matrix, and intracellular proteins that could be involved in signaling from the receptor to the cytoskeleton or other scaffolding proteins involved in trafficking (28, 58).

Function. The P2X<sub>7</sub> subtype is highly expressed in many epithelial and hematopoietic cells, where it activates a number of signaling cascades. It appears to be a regulator of inflammation, because some of the activities evoked by the receptor include T-cell activation and maturation and killing of invading microorganisms via apoptotic death of macrophages (38). In monocytes, activation of P2X<sub>7</sub> receptors mediates rapid secretion of IL-1β via plasma membrane microvesicle shedding, a process opposite to the pronounced stimulation of plasma membrane internalization that we observed in FRTL cells (34). The function of the P2X<sub>7</sub> receptor in stimulation of membrane trafficking in FRTL is unknown. Because the stimulation requires very high concentrations of ATP, it is unlikely to play a physiological role in the exocytosis and internalization of thyroglobulin. We speculate that activation by high concentrations of ATP may reflect a response to ATP released from damaged cells that could influence the uptake of nutrients, or changes in the composition of membrane proteins such as channels or transporters, of nearby healthy cells.

In summary, ATP activates a nonselective cation conductance and plasma membrane trafficking via a P2X<sub>7</sub> receptor that does not undergo pore expansion. The stimulation of internalization does not involve Na<sup>+</sup> or Ca<sup>2+</sup> influx; hence, P2X<sub>7</sub> receptor activation may couple to other proteins that initiate the membrane trafficking independently of channel activity. This is the first evidence of functional P2X<sub>7</sub> receptors in cells of thyrocyte origin.

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REFERENCES


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