Diverse Signal Transduction Pathways Mediated by Endogenous P2 Receptors in Cultured Rat Cerebral Cortical Neurons

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In earlier studies using cultured rat neurons from different regions of the CNS, we demonstrated that the P2 receptor agonists activate potassium channels via a pertussis toxin (PTX)–insensitive G-protein–regulated receptor, possibly involving a P2Y purinoceptor (Ikeuchi and Nishizaki 1995a,b, 1996a,b; Ikeuchi et al. 1995, 1996). ATP receptors evoke potassium currents by interacting with a protein kinase C (PKC) pathway in striatal neurons and spinal neurons (Ikeuchi and Nishizaki 1995a,b). In contrast, 2-methylthio ATP (2-MeSATP) and ADP generated currents by a mechanism independent of phospholipase C stimulation and IP3 production. ATP-evoked currents were partially inhibited by either neomycin, or GF109203X, or G-protein (Song and Chueh 1996; Webb et al. 1996a). These findings suggest that the effects of the P2 receptor agonist on neurons cannot be explained by a single P2Y purinoceptor and that coupled potassium channels are activated by different mechanisms. This prompted the present study to assess the intracellular signal transduction pathways responsible for ATP, 2-MeSATP, and uridine triphosphate (UTP) by monitoring patch-clamp currents and cytosolic calcium mobilization in cultured rat cerebral cortical neurons. The results of the present study culminated in the finding that either 2-MeSATP or UTP activates potassium channels and regulates intracellular calcium mobilization in cultured rat cerebral cortical neurons. The results of the present study culminated in the finding that either 2-MeSATP or UTP activates potassium channels and regulates intracellular calcium mobilization in cultured rat cerebral cortical neurons. The results of the present study culminated in the finding that either 2-MeSATP or UTP activates potassium channels and regulates intracellular calcium mobilization in cultured rat cerebral cortical neurons. The results of the present study culminated in the finding that either 2-MeSATP or UTP activates potassium channels and regulates intracellular calcium mobilization in cultured rat cerebral cortical neurons.

INTRODUCTION

Purinoceptors play a significant role in the regulation of a variety of physiologically important processes in excitable and nonexcitable cells. These receptors were originally classified as P1 (adenosine > AMP ≥ ADP ≥ ATP) and P2 purinoceptors (AMP ≥ ADP ≥ AMP ≥ adenosine) depending on their preference for adenosine or adenyne nucleotides (Burnstock and Kennedy 1985). The P2 purinoceptors can be further subdivided into P2X, P2Y, P2Z, P2T, and P2I subtypes (Gordon 1986; Lin et al. 1993; O’Connor et al. 1991), although this nomenclature has been superceded by subdivision into two families (Abbracchio and Burnstock 1994). The P2X purinoceptors, a family of the ligand-gated receptors, have been cloned as P2X1, 2, 3, and P2X7 (P2Z) (Bo et al. 1995; Brake et al. 1994; Chen et al. 1995; Collo et al. 1996; Lewis et al. 1995; Soto et al. 1996; Surprenant et al. 1996; Valera et al. 1994), and otherwise, the P2Y purinoceptors, linked to G-proteins (Barnard et al. 1994), have been cloned as P2Y1, P2Y2, P2Y6, and P2Y4 (Chang et al. 1995; Communi et al. 1995; Janssens et al. 1996; Nguyen et al. 1995; Tokuyama et al. 1995; Webb et al. 1993, 1994, 1996a). The P2Y purinoceptors mainly appear to be coupled to the phospholipase C–mediated signaling pathway (O’Connor et al. 1991), and in addition, to the activation of mitogen-activated protein kinase (MAPK) (King et al. 1996; Patel et al. 1996) and G protein (Song and Chueh 1996; Webb et al. 1996a). Some of the P2Y purinoceptors are expressed in the CNS, suggesting an association with synaptic transmission. Little is known, however, about actual function of P2Y purinoceptors in neurons and the relevant intracellular signaling pathways.

METHODS

Cell culture

Cerebral cortical neurons from neonatal rat on day 1 were cultured as described previously (Ikeuchi and Nishizaki 1995a).
cerebral cortex was removed from the brain under ether-induced anesthesia. The tissues were incubated in 0.25% trypsin in Ca²⁺-, Mg²⁺-free saline for a few minutes at room temperature and then mechanically dissociated by triturating with a Pasteur pipette. The dissociated cells were plated on collagen-coated coverslips and grown in Dulbecco's modified Eagle's medium (DMEM, GIBCO) with 15% fetal bovine serum at 37°C in a humidified atmosphere of 95% air and 5% CO₂. To suppress the growth of glial cells, cytosine β-d-arabinofuranoside (Ara-C; Sigma, St. Louis, MO; final concentration, 10 μM) was supplemented to the culture medium 1–3 days after plating. Cultured neurons were used 1–2 wk after plating.

**Patch-clamp recording**

Whole cell voltage-clamp and single-channel currents were recorded using an Axopatch-200A amplifier (Axon Instruments, Foster, CA). Currents obtained were stored on magneto optical disks (MK128D, Mitsubsihi-Kasei, Tokyo, Japan) and analyzed on a microcomputer using pClamp software (Axon Instruments; Version 6). Cells were bathed at room temperature (20–22°C) in a standard extracellular solution containing (in mM) 145 NaCl, 5 KCl, 2.4 CaCl₂, 1.8 glucose, 10 N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES), and 0.3 × 10⁻³ tetrodotoxin, pH 7.4. The basic patch electrode-filling solution was (in mM) 150 KCl, 10 1,2-bis (2-aminophenoxy) ethane-N,N',N,N'-tetraacetic acid (BAPTA) tetrapotassium salt, hydrate, and 10 HEPES, pH 7.2. In some experiments, the patch electrode-filling solution was (in mM) 150 KCl, 10 HEPES, 0.1 ethylene glycol-bis (β-aminoethyl ether)-N,N',N'-tetraacetic acid (EGTA), and 0.4 μM fura-2/AM, pH 7.2. Cells were viewed using a fluorescence ×20 dry objective lens and a ×20 dry phase-contrast objective. Fura-2 was excited at 340 and 380 nm alternatively switched every 500 ms. The fluorescence signal was measured and current density (peak current amplitude/cell capacitance) was calculated.

For single-channel current recording, cell-attached patches were made using the patch electrode-filling solution with (+) and without 1,2-bis (2-aminophenoxy) ethane-N,N',N'-tetraacetic acid (BAPTA; −). Cells were held at voltages as indicated and afterward, ATP, UTP, or 2-MeSATP was applied. Currents obtained were normalized by current density (peak current amplitude/cell capacitance), and whole cell current-voltage relations were calculated. Each point represents the average from 7–10 independent experiments, and the SD is indicated by the bars.

**Assay of intracellular free Ca²⁺ concentrations ([Ca²⁺]ᵢ)**

Cells were incubated with 4 μM fura-2/AM (Molecular Probes) at 37°C for 1 h in a standard extracellular solution together with 0.02% pluronic F-127; the cell-permeant AM esters serves as a Ca²⁺-sensitive indicator by deesterification in cells. Fura 2-loaded cells were placed into a recording chamber onto the stage of a Nikon DIAPHOT 300 microscope and bathed in the standard Ca²⁺-containing extracellular solution. Whole cell patches were made from fura 2–loaded cells using a patch electrode filled with the following intracellular solution (in mM): 150 KCl, 10 HEPES, 0.1 ethylene glycol-bis (β-aminoethyl ether)-N,N',N'-tetraacetic acid (EGTA), and 0.4 μM fura-2/AM, pH 7.2. Cells were viewed using a fluorescence ×20 dry objective lens and a ×20 dry phase-contrast objective. Fura-2 was excited at 340 and 380 nm alternatively switched every 500 ms. The fluorescence signal was...
filtered through a band-pass filter transmitting 500–511 nm and detected by an intensified charge-coupled device camera (CAM-220, Hamamatsu Photonics, Hamamatsu, Japan). Ratio images were calculated in real time, stored on hard disk, and analyzed using ARGUS-50/CA software (Version 3.0). [Ca\(^{2+}\)] was calculated from the fluorescence ratio/Ca\(^{2+}\) concentration calibration curve made before experiments. Simultaneously, whole cell currents were recorded and analyzed as described above. The Ca\(^{2+}\)-free extracellular solution was (in mM) 145 NaCl, 5 KCl, 2.4 CaCl\(_2\), 1.8 glucose, 10 HEPES, 10 BAPTA, and 0.3 \times 10^{-3}\) tetrodotoxin, pH 7.4.

**RESULTS**

**Whole cell currents evoked by ATP, UTP, and 2-MeSATP**

P\(_2\) purinoceptor agonists (ATP, UTP, and 2-MeSATP) produced outward whole cell currents at a holding potential of +60 mV in cultured cerebral cortical neurons (Fig. 1A). In addition, ADP, AMP, and α,β-MeATP, or the P\(_2\) purinoceptor agonist, adenosine also evoked currents, although no response was induced by β,γ-MeATP (Fig. 1A). The currents were blocked either by replacement of KCl with CsCl in the patch electrode-filling solution (Fig. 2) or by treatment with the nonselective K\(^+\) channel blocker, tetraethylammonium (1 mM; data not shown). The current-voltage relations obtained with voltage steps from −120 to +120 mV exhibited a reversal potential of about −80 mV, consistent with the expected equilibrium potential for K\(^+\) (Fig. 1B). These results indicate that these agonists activated potassium channels.

The currents evoked by ATP, UTP, or 2-MeSATP were inhibited by the broad G-protein inhibitor, GDPβS (100 μM), whereas they were not affected by the G\(_i\)/G\(_o\)-protein inhibitor, PTX (0.1 μg/ml; Fig. 2). This indicates that the receptors for ATP, UTP, and 2-MeSATP were linked to a PTX-insensitive G-protein. The patch electrode-filling solution used here did not contain Mg\(^{2+}\), a necessary factor for activating G-proteins, suggesting that G-proteins could be activated by intrinsic Mg\(^{2+}\) even if it is diluted. ATP-evoked currents were inhibited by 21 ± 6% and 44 ± 8% (mean ± SD) by the phospholipase C inhibitor, neomycin (500 μM), and by the selective PKC inhibitor, GF109203X (500 nM), respectively (Fig. 2). UTP-evoked currents were fully inhibited by neomycin and GF109203X, whereas these inhibitors had no effect on 2-MeSATP-evoked currents (Fig. 2). These results suggest that UTP activated K\(^+\) channels by phospholipase C-mediated PKC activation and that the ATP-sensitive channel was only regulated in part by PKC activation. Otherwise, the 2-MeSATP-sensitive channel was regulated by a mechanism independent of PKC activation.

**Intracellular Ca\(^{2+}\) mobilization induced by ATP, UTP, and 2-MeSATP**

ATP, UTP, and 2-MeSATP increased [Ca\(^{2+}\)] in Ca\(^{2+}\)-containing extracellular solution (Fig. 3C). Increases were also observed in Ca\(^{2+}\)-free extracellular solution (Fig. 3A), suggesting that these agonists stimulated Ca\(^{2+}\) release from intracellular calcium stores. Notably, there was no relation between current amplitude and rise in intracellular Ca\(^{2+}\) induced by these agonists (Fig. 3C). When monitored with the patch electrode-filling solution containing 10 mM BAPTA, neither of these agonists increased [Ca\(^{2+}\)], while currents were evoked (Fig. 3B). These findings, together with the finding that there was no significant difference in the whole cell current-voltage relations between in the presence and absence of BAPTA in the patch pipette (Fig. 1B), indicate that intracellular Ca\(^{2+}\) did not activate K\(^+\) channels.

GDPβS (100 μM) inhibited the rise in intracellular Ca\(^{2+}\)
induced by all the agonists (Fig. 3, B and D), suggesting that intracellular Ca\(^{2+}\) mobilization was regulated by activation of a G-protein. The increase in [Ca\(^{2+}\)]\(_i\) induced by UTP was blocked either by neomycin (500 \(\mu\)M) or by the inositol 1,4,5-trisphosphate (IP\(_3\)) receptor antagonist, heparin (1 mg/ml), indicating that the receptor for UTP was linked to a G-protein involving phospholipase C stimulation; hydrolysis of phosphatidylinositol produces IP\(_3\), to cause cytosolic Ca\(^{2+}\) release, and diacylglycerol, to activate PKC. In contrast, intracellular Ca\(^{2+}\) rise induced by ATP and 2-MeSATP was not affected by neomycin or heparin (Fig. 3, B and D), suggesting that ATP and 2-MeSATP elevated [Ca\(^{2+}\)]\(_i\) by a mechanism independent of phospholipase C stimulation and IP\(_3\) production.

**Single-channel currents elicited by ATP, UTP, and 2-MeSATP**

ATP, UTP, and 2-MeSATP, when added to the patch electrode-filling solution, elicited single-channel K\(^+\) currents in the cell-attached patch-clamp configuration (data not shown). Likewise, bath application of these agonists outside the patch pipette also induced single-channel currents (Fig. 4A). No current was evoked in the presence of tetraethylammonium (1 mM) as observed in whole cell currents (data not shown). UTP-induced currents showed a larger slope conductance (96 ± 9 pS), whereas 2-MeSATP evoked currents with the lower slope conductance (85 ± 7 pS; Fig. 4A). ATP-induced currents had two major classes of the slope conductance (86 ± 8 pS and 95 ± 9 pS; Fig. 4A), each of which was consistent with slope conductances for 2-MeSATP– or UTP-induced currents, respectively.

GF109203X (500 nM) fully inhibited UTP-evoked currents (Fig. 4A), suggesting that UTP regulated the potassium channels as a result of PKC activation. In contrast, 2-MeSATP–evoked currents were not affected by GF109203X (Fig. 4A), suggesting that 2-MeSATP elicited the currents by a mechanism independent of PKC activation. Of ATP-induced currents, only the currents with the higher slope conductance were blocked by GF109203X (Fig. 4A), suggesting that ATP evoked K\(^+\) current in part by PKC activation. The potent PKC activator, TPA (50 nM) induced single-channel currents with the same level of the slope conductance (95 ± 7 pS) as that achieved by UTP, ~1 min after application outside the patch pipette, and the current was blocked by GF109203X (Fig. 4A), further supporting the idea that the UTP receptor regulates potassium channels by interacting with a PKC pathway.

ATP and 2-MeSATP elicited single-channel currents with a slope conductance of 87 ± 6 pS and 88 ± 9 pS, respectively, without latency in the outside-out patch-clamp configuration, although no current was induced by UTP (Fig. 4B). The currents induced by ATP or 2-MeSATP were completely inhibited by GDP/BS (100 \(\mu\)M; Fig. 4B) and otherwise, the G-protein activator, GTP\(\gamma\)S (500 \(\mu\)M), produced single-channel currents with the same level of the slope conductance (88 ± 5 pS) as that achieved by ATP or 2-MeSATP (Fig. 4B). These results suggest that 2-MeSATP activates potassium channels directly by G-proteins and that ATP evokes potassium currents both by the direct action of G-protein subunits and by PKC activation.

**DISCUSSION**

In the present study, the \(P_2\) purinoceptor agonists, ATP, UTP, and 2-MeSATP, evoked potassium currents and increased intracellular calcium concentration in rat cerebral cortical neurons. One assumes that the potassium channels might be activated by calcium. Two kinds of Ca\(^{2+}\)-dependent potassium channels, such as voltage-dependent BK with high single-channel conductance of 100–250 pS and voltage-independent SK channels with low single-channel conductance of 4–14 pS, have been found in neurons (Hille 1992). The findings that the currents by ATP, UTP, and 2-MeSATP were sensitive to tetrodotoxin and that the channels had conductances of 85–95 pS suggest that the relevant channel was a BK type. However, this is unlikely, because these agonists produced potassium currents under conditions of zero intracellular Ca\(^{2+}\); the currents were not affected by intracellular calcium concentrations; and the currents showed no voltage dependency. If depolarizing pulses were applied to cells, then the Ca\(^{2+}\)- and voltage-dependent potassium currents might be induced also here, as observed in cat cortical neurons (Schwindt et al. 1992). Receptors for ATP, UTP, and 2-MeSATP were linked to PTX-insensitive G-proteins; however, the responses could not be explained by activation of a single receptor. UTP did not activate potassium channels in excised patches, indicating that the UTP responses require intracellular second messengers. The finding that UTP-induced currents were blocked by neomycin or GF109203X supports this idea; the UTP receptor is linked to a G-protein involving phospholipase C–mediated PKC activation. 2-MeSATP–induced currents otherwise were not affected by neomycin or GF109203X, suggesting that the 2-MeSATP receptor is not coupled to a phospholipase C pathway. 2-MeSATP elicited single-channel currents in excised patches; the currents were blocked by GDP/BS, whereas GTP\(\gamma\)S produced currents in a fashion that mimics the effect of 2-MeSATP, indicating that 2-MeSATP–operated potassium channels are activated by a G-protein alone. The K\(_{\text{ATP}}\) channel (GIK1) (Breitwieser and Szabo 1985; Clapham and Neer 1993; Ito et al. 1992; Kubo et al. 1993; Paffinger et al. 1985; Takao et al. 1994), K\(_{\text{ATP}}\) channel (ROMK1) (Ho et al. 1993; Kirsch et

**FIG. 3.** Assay of [Ca\(^{2+}\)]\(_i\). Whole cell patches were made in fura 2–loaded cells using the patch electrode-filling solution containing 0.1 mM ethylene glycol-bis(\(\beta\)-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA; A and C) or 10 mM BAPTA (B). The currents (I) and/or intracellular Ca\(^{2+}\) waves ([Ca\(^{2+}\)]; Conc., calibrated [Ca\(^{2+}\)]; Ratio, fluorescence ratio F340/380) were monitored in Ca\(^{2+}\)-free [extCa\(^{2+}\)(−); A] and Ca\(^{2+}\)-containing extracellular solution [extCa\(^{2+}\)(+); B and C]. GDP/BS (100 \(\mu\)M) or heparin (1 mg/ml) was perfused in the patch electrode-filling solution for 5 min before application of ATP, UTP, or 2-MeSATP at a concentration of 10 \(\mu\)M. Cells were treated with neomycin (500 \(\mu\)M) for 15 min before and during application of the drugs. The holding potential here was +30 mV, because fura 2–loaded cells were not stabilized at a holding potential of +60 mV. In the accompanying graph (D), each value (±SD) represents the mean from 7–10 independent experiments. \(*P < 0.01,\) unpaired \(t\)-test.
FIG. 4. Single-channel recordings. Cell-attached patches were made to cells using the patch electrode-filling solution without ATP, UTP, or 2-MeSATP. Each of the agonists (10 μM) or 12-O-tetradecanoyl phorbol-13-acetate (TPA; 50 nM) was applied to a single neuron outside the patch pipette with 5-min intervals, in the presence and absence of GF109203X (500 nM; A). The pipette potential, which represents the voltage loaded on the inside membrane, was +100 mV. a and b, single-channel currents elicited by ATP in the absence; and presence of GF109203X (c); d, single-channel currents elicited by UTP; e, single-channel currents elicited by 2-MeSATP in the absence; and presence of GF109203X (f); g, single-channel currents elicited by TPA. B: ATP, UTP, or 2-MeSATP at a concentration of 10 μM was applied to a single patched cell in the outside-out patch-clamp configuration. GDPβS (100 μM) or GTPγS (500 μM) was perfused in the patch electrode-filling solution. Holding potential was +60 mV. The single-channel current-voltage (I-V) relations were plotted, and the slope conductances were measured by linear regression fitted to the I-V relation (n = 8–10).

al. 1990), L-type Ca^{2+} channel (Yatani and Brown 1989), and I_{f} channel (Yatani et al. 1990) are recognized to be activated directly by the βγ-subunits of G-proteins. Taken together, 2-MeSATP may activate potassium channels by a direct action of G-protein βγ dimer. ATP-evoked currents were partially inhibited by either neomycin or GF109203X. ATP elicited single-channel currents in cell-attached patches with two major classes of the slope conductance, and GF109203X blocked currents with the higher slope conductance, which is in good agreement with that achieved by
UTP. In addition, ATP induced currents with the same slope conductance as 2-MeSATP induced in outside-out patches. These findings suggest that the ATP responses are mediated by both of the UTP and 2-MeSATP receptor.

UTP increased $[\text{Ca}^{2+}]_i$, which originated from intracellular calcium stores, and the increase was blocked by either neomycin or heparin, further supporting the idea that the UTP receptor is linked to a G-protein involving phospholipase C activation. $G_\text{q}^{-}$- and $G_\text{i}/G_\text{o}$-proteins are known to be coupled to phospholipase $C_{\text{b}2}$ (Wu et al. 1993) and phospholipase $A_2$ (Leurs et al. 1994), respectively. The finding that the UTP response was blocked by the broad G-protein inhibitor GDP$\beta$S but not by the $G_\text{i}/G_\text{o}$ protein inhibitor PTX strongly suggests that the corresponding G-protein belongs to the class of $G_\text{q}$ proteins.

The increases in $[\text{Ca}^{2+}]_i$ induced by 2-MeSATP were not affected by either neomycin or heparin, suggesting that 2-MeSATP elevates $[\text{Ca}^{2+}]_i$ by a mechanism independent of phospholipase C activation and IP$_3$ production. In addition to IP$_3$ receptors, the ryanodine receptors are major intracellular Ca$^{2+}$ channels (Berridge 1993). Moreover, recent studies suggest that cyclic ADP-ribose releases cytosolic Ca$^{2+}$ by a mechanism independent of IP$_3$ receptors (Berridge 1993; Galione 1993). It is presently unknown whether the 2-MeSATP-induced intracellular Ca$^{2+}$ rise is due to activation of ryanodine receptors or a third intracellular Ca$^{2+}$ pool related to cADP-ribose. Alternatively, another possible explanation for intracellular calcium mobilization via the 2-MeSATP receptor involves a direct activation by the $\beta\gamma$-subunits of a G-protein. To address this question, we are currently carrying out further examinations.

The rise in intracellular Ca$^{2+}$ induced by ATP was not affected by either neomycin or heparin, whereas the currents were partially inhibited by neomycin or GF109203X. This suggests that, whereas PKC is activated by ATP enough to produce $K^+$ currents, IP$_3$ is not produced enough
to release stored calcium and that, therefore, ATP-induced
cytosolic Ca\textsuperscript{2+} release is mainly mediated by the 2-MeSATP
receptor.

In conclusion, the results presented here demonstrate that
at least two different types of the P\textsubscript{2Y} purinoceptors,
a UTP and 2-MeSATP receptor, linked to a PTX-insensitive
G-protein, are expressed in rat cerebral cortical neurons.
The UTP receptor is involved in stimulation of phospholipase
C, leading to activation of the potassium channels and
increase in [Ca\textsuperscript{2+}]. (Fig. 5). The 2-MeSATP
receptor exerts activation of the potassium channels, possibly
by a direct action by the \( \beta \)-subunits of a G-protein,
whereas enhancement in [Ca\textsuperscript{2+}], occurs by a mechanism
independent of phospholipase C stimulation (Fig. 5). The ATP
responses may be mediated by both receptors for UTP and
2-MeSATP (Fig. 5).

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