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P_2 Purinoceptor-Operated Potassium Channel in Rat Cerebellar Neurons

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P_2 purinoceptor agonists produced whole-cell potassium currents in cerebellar neurons with the order of potency 2-methylthio ATP (2-MeSATP) > ADP > ATP > adenosine > α,β-methylene ATP > AMP > UTP. In the outside-out patch clamp configuration, 2-MeSATP evoked single channel currents with two major classes of slope conductances without latency. The currents were blocked by a G-protein inhibitor, GDPβS, although they were not affected by a phospholipase C inhibitor, a selective protein kinase C or A inhibitor. In contrast, a potent G-protein activator, GTPγS, produced single channel currents with same conductances as those of the currents induced by 2-MeSATP. These provide an indication that the P_2 purinoceptor-operated potassium channel is regulated by the $\beta\gamma$ subunits of a G-protein.

In recent studies, we found that a P_2 purinoceptor is involved in the activation of the potassium channel in a variety of regions of the brain. The regulatory mechanisms, however, are different between the regions and the receptors for individual P_2 purinoceptor agonist. The potassium channel activated by ATP in striatal neurons (1) and by adenosine in superior colliculus and hippocampal neurons (submitting elsewhere) is regulated by protein kinase C. The ADP-sensitive potassium channel in inferior colliculus (2) and medullar neurons (3) appears to be activated by a direct action of the $\beta\gamma$ subunits of a G-protein. We were prompted to further ensure the regulation of the potassium channel in neurons by the G-protein $\beta\gamma$ subunits using outside-out patch clamp technique. The results presented here provide an additional evidence that the P_2 purinoceptor-operated potassium channel in cerebellar neurons is regulated by the $\beta\gamma$ subunits of a G-protein.

METHODS

Cell culture. Cerebellar neurons from neonatal rat on day 1 were cultured, as described before (1–3). Cerebellum was removed from the brain under ether anesthesia. The tissues were incubated in 0.25% trypsin in Ca^{2+}-, Mg^{2+}-free saline for a few min at room temperature and then mechanically dissociated by triturating with a Pasteur pipette. The dissociated cells were plated on collagen-coated cover-slips 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% CO_2. To suppress the growth of glial cells, AraC (final conc., 10 μM) was supplemented to the culture medium 1–3 days after plating. Cultured neurons were used 1–2 weeks after plating.

Whole-cell patch clamp recording. Cells were bathed at room temperature (20–22°C) in a standard extracellular solution containing (in mM) 145 NaCl, 5 KCl, 2.4 $CaCl_2$, 1.8 glucose, 10 HEPES, and 0.3×10^{-3} tetrodotoxin, pH 7.4. The basic patch electrode-filling solution was 150 KCl, 10 EGTA, and 10 HEPES, pH 7.2. Membrane currents from whole-cell voltage clamp were recorded using an Axopatch-200A amplifier (Axon, Instrument, Inc., USA). After formation of whole-cell patches, series resistance (Rs) compensation was made up to about 95%. P_2 purinoceptor agonists were applied to cells for 5 sec by an air pressure microinjector (PV 830 Pneumatic Picopump, World Precision Instruments, Inc., USA). The currents were filtered at 5 KHz, stored on magneto optical disk (MK 128D, Mitsubishi-Kasei, Inc., Japan). To normalize current amplitudes, a cell capacitance was measured and a current density (peak current amplitude/cell capacitance) was calculated.

Single channel recording. Single channel currents were recorded with an Axopatch-200A amplifier in the outside-out patch clamp configuration. The patch electrode was filled with a same intracellular solution as used in the whole-cell patches and 2-MeSATP was bath-applied during recording. The currents were filtered at 2 KHz and digitized at 500 Hz, and analyzed on a laboratory computer using pClamp software (Axon Instrument, Inc.; version 6).

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RESULTS

P_2 purinoceptor agonists-evoked whole cell potassium currents. P_2 purinoceptor agonists produced currents at a holding potential of +60 mV with the order of potency: 2-methylthio ATP (2-MeSATP) > ADP > ATP > adenosine (AD) > α,β-methylene ATP (α,β-MeATP) > AMP > UTP, but no current was induced by β,γ-methylene ATP (β,γ-MeATP) (Fig. 1A). A most potent agonist, 2-MeSATP had a response with a latency of <100 msec (Fig. 1A). It is notable that the latency to the current onset differs between agonists (2-MeSATP: 52 ± 16 msec, ADP: 63 ± 22 msec, ATP: 1.3 ± 0.6 sec, AD: 2.2 ± 0.3 sec, α,β-MeATP: 2.4 ± 0.7 sec, AMP: 4.0 ± 1.1 sec, UTP: 13.4 ± 2.2 sec, n = 5–7). The 2-MeSATP-evoked current/voltage relation obtained from 20 msec voltage pulses had an outwardly rectifier with a reversal potential of −82 ± 5 mV (Fig. 1B). Furthermore, the currents were blocked by intracellular Cs^+ (Fig. 1C). These indicate that 2-MeSATP activates the potassium channel responsible for outwardly rectifying currents in cerebellar neurons. A broad G-protein inhibitor, GDPβS inhibited 2-MeSATP-evoked currents, but a G_i/G_o-protein inhibitor, pertussis toxin (PTX) had no effect (Fig. 1C). In addition, the currents were not affected by a phospholipase C inhibitor, neomycin, a selective protein kinase C inhibitor, GF109203X, or a selective cAMP-dependent protein kinase inhibitor, H-89 (Fig. 1C).

2-MeSATP-induced single channel currents. 2-MeSATP elicited single channel currents without latency with slope conductances of 50 ± 7 pS and 95 ± 6 pS at a holding potential of +60 mV, although no current was induced before application (Fig. 2A,B). The intracellular perfusion of GDPβS completely abolished the currents (all of 5 trials) (Fig. 2A), indicating that the channel is activated by a G-protein-mediated signaling. The slope conductances of the currents were not affected by neomycin, GF109203X, or H-89 (51 ± 9 pS and 102 ± 23 pS, 48 ± 5 pS and 98 ± 9 pS, 46 ± 8 pS and 97 ± 11 pS, respectively)(Fig. 2A,B). These suggest that the currents are not regulated by protein kinase C or cAMP-dependent protein kinase C.

Single channel currents induced by a potent G-protein activator. Outside-out patches were made to cells using the patch electrode-filling solution containing a potent G-protein activator, GTPγS. The intracellular perfusion of GTPγS for 3 min induced single channel currents (all of 8 patches) (Fig. 3A). The slope conductances were 47 ± 9 pS and 104 ± 12 pS (Fig. 3B) and these are well consistent with those of the currents evoked by 2-MeSATP. This observation provides an evidence that the potassium channel is regulated directly by activation of a G-protein linking to the receptor for 2-MeSATP.

DISCUSSION

The present data demonstrate that P_2 purinoceptor agonists produce whole-cell currents in cerebellar neurons with the order of potency: 2-MeSATP > ADP > ATP > AD > α,β-MeATP > AMP > UTP. This sequence is similar to that for the P_{2Y} purinoceptor (2-MeSATP > ATP ≥ ADP > α,β-MeATP>>>UTP)(4). Differences in the latency to the current onset between P_2 purinoceptor agonists, however, may imply that these agonists evoke the currents not through a single receptor but through mixed ones and therefore, the purinoceptor can not be classified according to the order of potency as established previously.

2-MeSATP activated the potassium channel responsible for outwardly rectifying currents with a latency of <100 msec in whole-cell patches. Furthermore, 2-MeSATP induced single channel currents without latency even in outside-out patches, suggesting that the channel is activated only by plasma membrane factors without intracellular components. The channel is not regulated by a phospholipase C-mediated phosphatidylinositol signaling, protein kinase C, or cAMP-dependent protein kinase. Additionally, the channel is insensitive to voltage, since no current was observed by voltage steps, and is independent of intracellular Ca^{2+}, since the patch electrode-filling solution contains 10 mM EGTA to fully chelate calcium. A possible mechanism for activation of the potassium channel could be that the channel is regulated directly by the $\beta\gamma$ subunits of a G-protein.

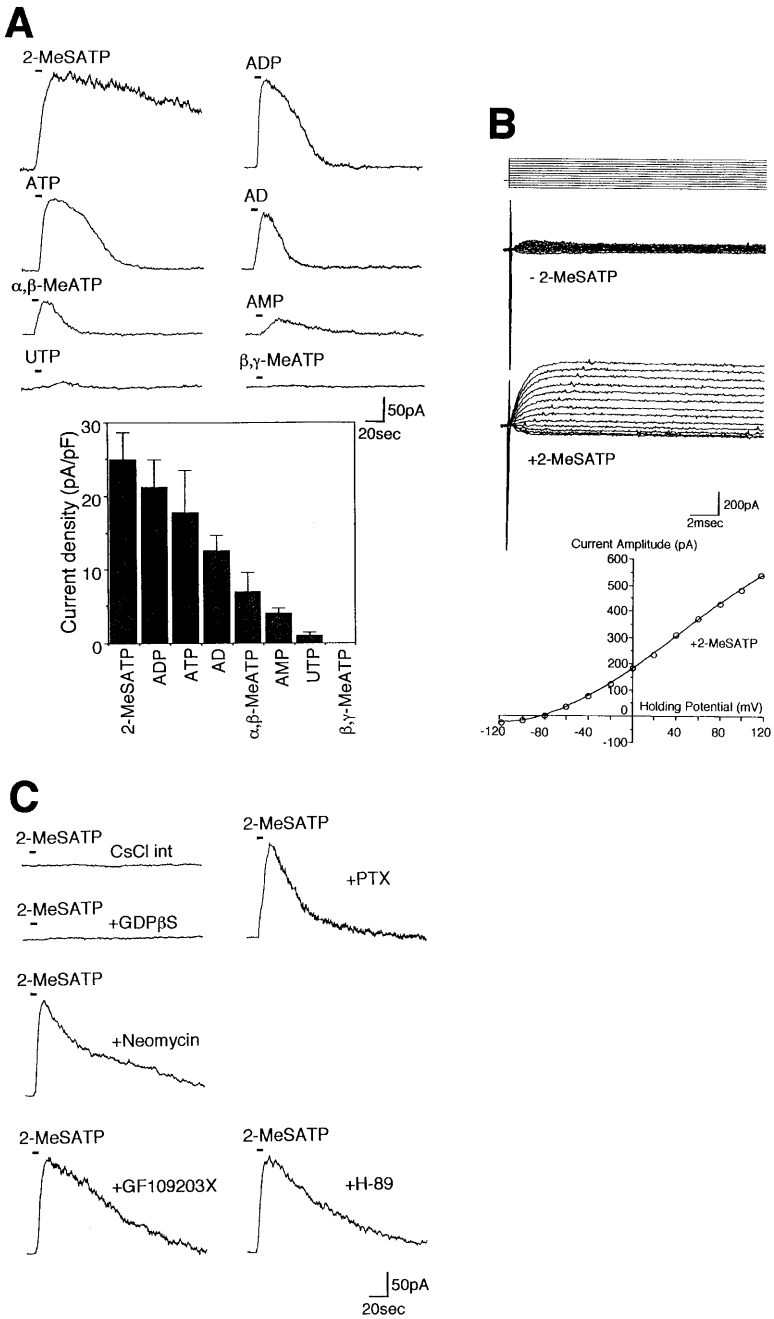

FIG. 1. 2-MeSATP-evoked whole-cell currents. (A) P_2 purinoceptor agonists, such as 2-MeSATP, ADP, ATP, AD, α,β-MeATP, AMP, UTP, and β,γ-MeATP, at a concentration of 10 μM were applied to cells clamped at +60 mV for 5 sec indicated bars. Each typical current is illustrated in upper column. Outward currents correspond to upward deflections. The relative potency for the current amplitudes is shown in lower column. Each value represents the average from 5–7 cells and SD is indicated by the bars. (B) Voltage pulses for 20 msec from −120 mV to +120 mV were applied to cells before (−2-MeSATP) and after application of 2-MeSATP (+2-MeSATP) (10 μM). This experiment was carried out 5 times. The current/voltage relation is shown in lower column. (C) Effects of several kinds of inhibitors on 2-MeSATP-activated whole-cell currents were investigated. To examine the effect of intracellular Cs^+, 150 mM KCl in the patch electrode-filling solution was replaced by 150 mM CsCl. GDPβS (1 mM) was added to the patch electrode-filling solution. Cells were treated with neomycin (500 μM), GF109203X (500 nM), or H-89 (1 μM) for 15 min, 3 min, or 3 min, respectively, prior to application of 2-MeSATP (10 μM). Each effect was examined in 5 cells.

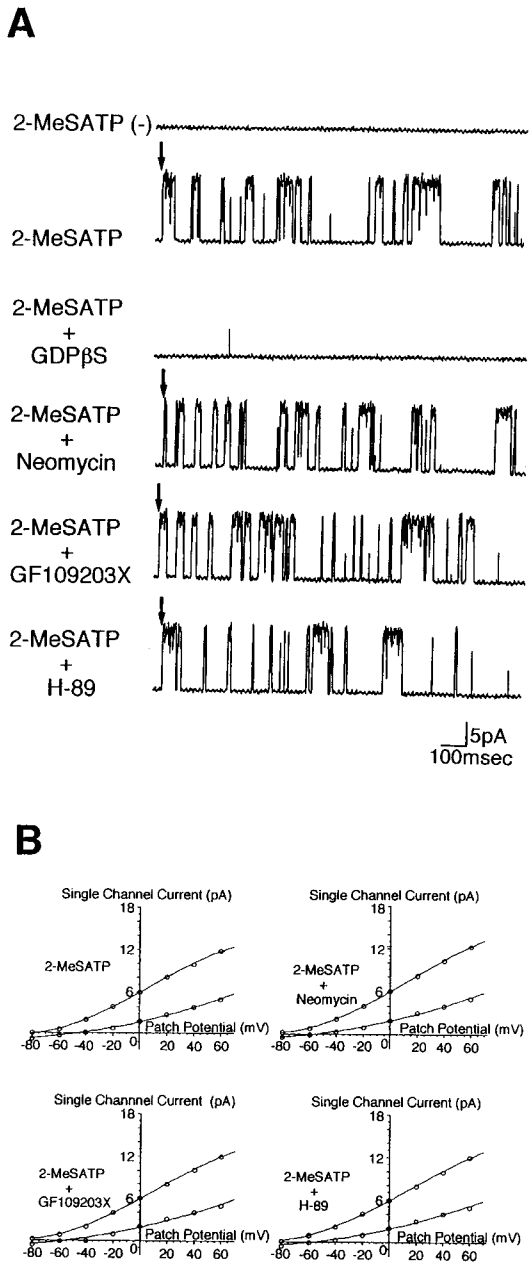

FIG. 2. 2-MeSATP-elicited single channel currents. (A) Outside-out patches were made using the patch electrode filling solution with or without GDPβS. 2-MeSATP (10 μM) was applied to cells clamped at +60 mV during recording in the presence and absence of neomycin (500 μM), GF109203X (500 nM), or H-89 (1 μM). The beginning of application of 2-MeSATP is indicated by the arrows. Each experiment was carried out in 5–7 cells. (B) The slope conductances were measured by linear regression fitted to the single channel current/voltage relations shown.

The results that 2-MeSATP-evoked single channel currents were activated without latency by a plasma membrane factor alone, blocked by GDPβS, and GTPγS induced currents with same slope conductances as those obtained by 2-MeSATP give a strong evidence for supporting this concept. However, further experiments are required in order to define what the relevant G-protein is and

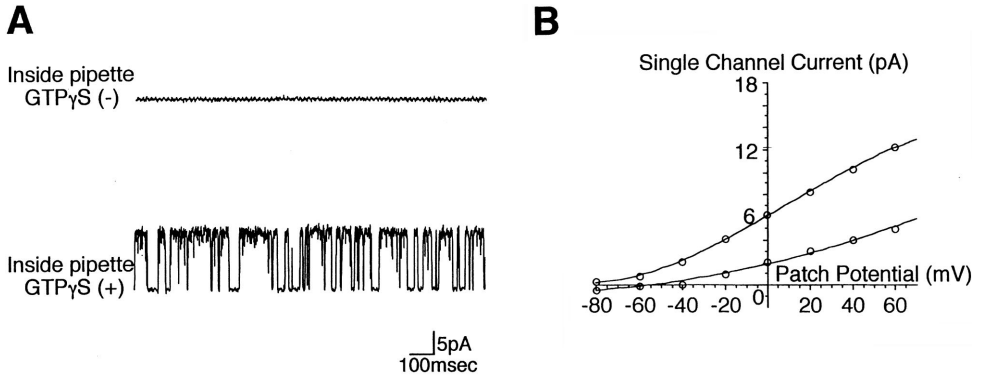

FIG. 3. GTPγS-induced single channel currents. (A) GTPγS (500 μM), which is added to the patch electrode-filling solution, was perfused into the cells. Holding potential was +60 mV. The effect of GTPγS was examined in 8 cells. (B) The slope conductance was measured from the GTPγS-induced single channel current/voltage relation shown.

whether the $\beta\gamma$ subunits of the G-protein activates the potassium channel in the inside-out patch clamp configuration.

The K_{ACh} channel (GIRK1)(5–10), K_{ATP} channel (ROMK1)(11,12), L-type Ca^{2+} channel (13), Na^+ channel (14) and I_f channel (15) in heart are known to be regulated by the $\beta\gamma$ subunits of a G-protein. The K_{ACh} and K_{ATP} channels are activated by the $\beta\gamma$ subunits of a PTX-sensitive G-protein, producing inwardly rectifying currents. Their channel properties and the coupled G-protein are different from those observed here. The potassium channel in cerebellar neurons is regulated by a PTX-insensitive G-protein and has an outwardly rectifier. Besides in cerebellar neurons, we demonstrate that the ADP-sensitive potassium channel in inferior colliculus neurons (2) and medullar neurons (3) is likely regulated by a same mechanism. The physiological significance for regulation by the $\beta\gamma$ subunits of a G-protein in central neurons is unclear. The channel activation by this mechanism, however, may have an integral function in rapid signal transduction.

In conclusion, the results presented here demonstrate that the purinoceptor for 2-MeSATP is involved in activation of the potassium channel responsible for outwardly rectifying currents by a PTX-insensitive G-protein-mediated signaling, most likely by a direct action of the $\beta\gamma$ subunits, in cerebellar neurons.

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