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Adenosine activates the K^+ channel and enhances cytosolic Ca^{2+} release via a P_{2Y} purinoceptor in hippocampal neurons

Youji Ikeuchi, Tomoyuki Nishizaki *, Masahiro Mori, Yasuhiro Okada

Department of Physiology, Kobe University School of Medicine, 7–5-1 Kusunoki-cho, Chuo-ku, Kobe 650, Japan

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Abstract

The effects of adenosine on hippocampal neurons were examined by patch-clamp recording and Ca^{2+} imaging using fura-2 fluorescence. In the whole-cell patch-clamp configuration, adenosine evoked outwardly rectifying K^+ currents in a dose-dependent manner. These currents were not inhibited by a nonselective P_1 purinoceptor antagonist or selective adenosine A_1, A_{2A} receptor antagonists and moreover, selective adenosine A_1, A_{2A} receptor agonists evoked no current. In contrast, P_2 purinoceptor agonists produced similar outward currents with the order of potency: ADP ≥ 2-methylthio ATP > ATP > adenosine ≫ AMP. No response was obtained to UTP, α,β-methylene ATP or β,γ-methylene ATP. The intracellular perfusion of a broad G-protein inactivator, guanosine-5′-*O*-(2-thiodiphosphate) (GDPβS), abolished adenosine-evoked currents, whereas a G_i/G_o-protein inhibitor, pertussis toxin, had no effect. Furthermore, the currents were blocked by a phospholipase C inhibitor, neomycin, or specific protein kinase C inhibitors, GF109203X (bisindolyl maleimide, $C_{25}H_{24}N_4O_2$) and protein kinase C inhibitor peptide. In the cell-attached patch-clamp configuration, adenosine elicited single-channel currents with two major kinds of slope conductances. Likewise, application of adenosine outside the patch electrode again produced single-channel currents with same conductances. A potent protein kinase C activator, 12-*O*-tetradecanoylphorbol-13-acetate (TPA), induced single-channel currents in a fashion that mimics the effect of adenosine. The evoked currents were blocked by GF109203X. In addition, adenosine enhanced intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$). This $[Ca^{2+}]_i$ increase was inhibited by GDPβS or neomycin, but was not affected by pertussis toxin. These results, thus, suggest that adenosine activates the K^+ channel and enhances cytosolic Ca^{2+} release via a P_{2Y} purinoceptor linked to a pertussis toxin-insensitive G-protein, which is involved in a phospholipase C-mediated phospholipid-signaling pathway.

Keywords: P_{2Y} purinoceptor; G-protein; Protein kinase C; K^+ channel; Ca^{2+} release, cytosolic; Hippocampal neuron

1. Introduction

Purinoceptors are classified as P_1 and P_2 (Ollson and Pearson, 1990); the P_3 purinoceptor (Shinozuka et al., 1988) has been also identified on the basis of preference for nucleosides or nucleotides. The adenosine-sensitive P_1 sites are the adenosine receptors, which have now been further subdivided into A_1, A_{2A}, A_{2B}, A_3, and A_4 subtypes on the basis of their differential selectivity for adenosine analogs (Stiles, 1992; Tucker and Linden, 1993; Linden, 1994). Adenosine is known to serve as a neuromodulator in both peripheral and central nervous systems (Stiles, 1986). Its main role in neurotransmission has been considered to be inhibitory, but there are some reports that adenosine exerts excitatory actions as well (Nishimura et al., 1990; Okada et al., 1990; Garaschuk et al., 1992; Sebastião and Ribeiro, 1992). Concerning the presynaptic effects, adenosine has been reported to reduce noradrenaline release in rat kidney, hippocampus, and brain cortex (Hedqvist and Fredholm, 1976; Allgaier et al., 1987; Von Kügelgen et al., 1992). In addition, the adenosine A_1 receptor is involved in the inhibition of electrically evoked dopamine and/or acetylcholine release in the rat striatum (Jin et al., 1993; Kirkpatrick and Richardson, 1993) and rat phrenic nerve endings (Correia-de-Sá et al., 1991), or K^+-evoked glutamate release in goldfish cerebellum (Lucchi et al., 1994). In contrast, adenosine A_{2A}-selective receptor agonists enhance electrically evoked or K^+-evoked acetylcholine release in the rat striatum (Kirkpatrick and Richardson, 1993; Kirk and Richardson, 1994), and excitatory amino acid release in ischemic cerebral cortex of the rat (Simpson et al., 1992). The inhibitory effect of adeno-

* Corresponding author. Tel.: (81) (78) 341-7451; fax: (81) (78) 341-5732.

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sine at the presynaptic site, thus, appears to be mediated by the adenosine A_1 receptor, and the excitatory effects by the adenosine A_{2A} receptor (Correia-de-Sá et al., 1991; Fuder et al., 1992; Von Kügelgen, 1994). Otherwise, adenosine acts as an inhibitory modulator of electrical activity at postsynaptic site and this effect has been attributed to enhancement of K^+ conductance (Segal, 1982). There is, however, less information regarding the inhibitory mechanisms of adenosine at the postsynaptic site.

We show here that adenosine exerts an inhibitory effect at the postsynaptic site by activating the outwardly rectifying K^+ channel via a P_{2Y} purinoceptor linked to a G-protein, which is involved in the phospholipase C-mediated protein kinase C activation pathway and which furthermore, enhances intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) in rat-cultured hippocampal neurons.

2. Materials and methods

2.1. Tissue culture

Hippocampal neurons from neonatal rat on day 1 were cultured as described by Banker and Cowan (1977). The hippocampus 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 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% CO_2. To suppress the growth of glial cells, cytosine-β-D-arabinofuranoside (AraC) (final concentration, 10 μM) was added to the culture medium 1–3 days after plating. Cultured neurons were used 1–2 weeks after plating.

2.2. Whole-cell patch-clamp recording

The cells were bathed at room temperature (20–22°C) in a standard extracellular solution (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, 5 EGTA, and 10 HEPES, pH 7.2. Membrane currents from the whole-cell voltage clamp were recorded using an Axopatch-200A amplifier (Axon Instrument, USA) according to established methods (Hamill et al., 1981). After formation of whole-cell patches, series resistance (Rs) compensation was made up to ~ 95%. The drugs were applied to cells for 3 s by an air pressure microinjector (PV 830 Pneumatic Picopump, World Precision Instruments, USA). The currents were filtered at 5 kHz, stored on a magneto-optical disk (MK128D, Mitsubishi-Kasei, Japan), and analysed with a laboratory computer using pClamp software (Axon Instrument; version 6). To normalize current amplitudes induced by purinoceptor agonists, cell capacitance was measured and current density (peak current amplitude/cell capacitance) was calculated. To get current/voltage relations, 20-ms voltage pulses were applied to cells from − 120 mV to + 120 mV in 20-mV steps.

2.3. Single-channel recording

Single-channel currents were recorded in the cell-attached patch clamp configuration using an Axopatch-200A according to previously described methods (Hamill et al., 1981). The currents were filtered at 2 kHz, digitized at 1 kHz, and analysed using pClamp software. The patch electrode was filled with the standard extracellular solution in the presence and absence of adenosine or 12-*O*-tetradecanoylphorbol-13-acetate (TPA). In patches using the patch electrode-filling solution without adenosine or TPA, adenosine or TPA was bath-applied to cells outside the patch electrode after gigaseal formation.

2.4. Assay of $[Ca^{2+}]_i$

The cells were incubated at 37°C for 1 h with 4 μM fura-2/AM. Fura-2 loaded cells were placed in a recording chamber onto the stage of a Nikon DIAPHOT 300 microscope and were bathed in the standard Ca^{2+}-containing extracellular solution as described above and Ca^{2+}-free solution (in mM): 145 NaCl, 5 KCl, 1.8 glucose, 10 HEPES, 5 EGTA and 0.3×10^{-3} tetrodotoxin, pH 7.4. The cells were viewed with a fluorescence 20 × dry objective lens and with a 20 × dry phase-contrast objective. The neurons were selected in the portion without glial cells as background and the average fura-2 signal throughout the neuronal soma was calculated. Adenosine was applied to cells for 3 s with an air pressure microinjector. To confirm whether adenosine-evoked currents were obtained from the selected cells, whole-cell patches were made to the same cells after or before assay of Ca^{2+}. Fura-2 was excited at 340 and 380 nm switched alternatively every 500 ms. The fluorescence signal was filtered through a bandpass filter transmitting 500-511nm and detected by an intensified charge-coupled device camera (ARGUS-50/CA, Hamamatsu Photonics, Japan). Ratio images were calculated in real time, stored on hard disk, and analysed using ARGUS-50/CA software (version 3.0). The $[Ca^{2+}]_i$ was calculated from the fluorescence ratio/Ca^{2+} concentration calibration curve made before the experiments. In some experiments, co-assay of $[Ca^{2+}]_i$ and whole-cell current was carried out. Whole-cell patches was made to the fura 2-loaded cells using the patch electrode-filling solution containing 0.4 μM fura 2.

2.5. Drugs

N^6-Cyclohexyladenosine (CHA), 2-*p*-(2-carboxyethyl)phenetylamino-5′-*N*-ethylcarboxamidoadenosine hy-

drochloride (CGS21680), 2-*p*-(2-carboxyethyl) phenetylamino-5′-*N*-ethylcarboxamidoadenosine hydrochloride (8-CPT), 3,7-dimethyl-1-propargylxanthine (DMPX), 2-methylthio ATP, α,β-methylene ATP, β,γ-methylene ATP, and UTP were purchased from RBI (USA), pertussis toxin and AraC from Sigma (USA). GDPβS from Boehringer Mannheim (Germany), fura-2/AM from Molecular Probes (USA), GF109203X (bisindolyl maleimide, $C_{25}H_{24}N_4O_2$), TPA, neomycin, and 1,2-bis(2-aminophenoxy)ethane-*N,N,N′,N′*-tetraaceticacid, tetrapotassium salt, hydrate (BAPTA) from Wako Pure Chemical Industries (Japan), and all other chemicals from Nacalai Tesque (Japan).

3. Results

3.1. Adenosine-evoked whole-cell membrane currents

Adenosine (10 μM) produced an outward current with a latency of 2 ± 0.6 s at a holding potential of +60 mV (Fig. 1A, left). This current was fully blocked by the replacement of KCl with CsCl in the patch electrode-filling solution (Fig. 1A, right). Additionally, the currents were inhibited by a nonselective K^+ channel blocker, tetraethylammonium (1–10 mM) (Fig. 1A, right). The adenosine-evoked current/voltage relations obtained from 20-ms voltage steps (-120 mV ~ +120 mV) showed an outward rectifier with a reversal potential of -80 ± 5 mV ($n = 5$) (Fig. 1B), well consistent with the Nernst equilibrium potential for K^+ (−86 mV) (Lewis and Stevens, 1979) under the ionic conditions used in this experiment. These results indicate that adenosine activates the K^+ channel responsible for outwardly rectifying currents. Furthermore, the currents were not affected by reduction of the extracellular Ca^{2+} concentration, increasing the intracellular EGTA concentration (10 mM), or replacement of intracellular EGTA with BAPTA (10 mM) (data not shown), suggesting that the adenosine-sensitive K^+ channel is not dependent on Ca^{2+}. Application of adenosine at concentrations varying from 0.1 to 100 μM increased the current amplitude in a dose-dependent manner, reaching a maximum at 10 μM (Fig. 3).

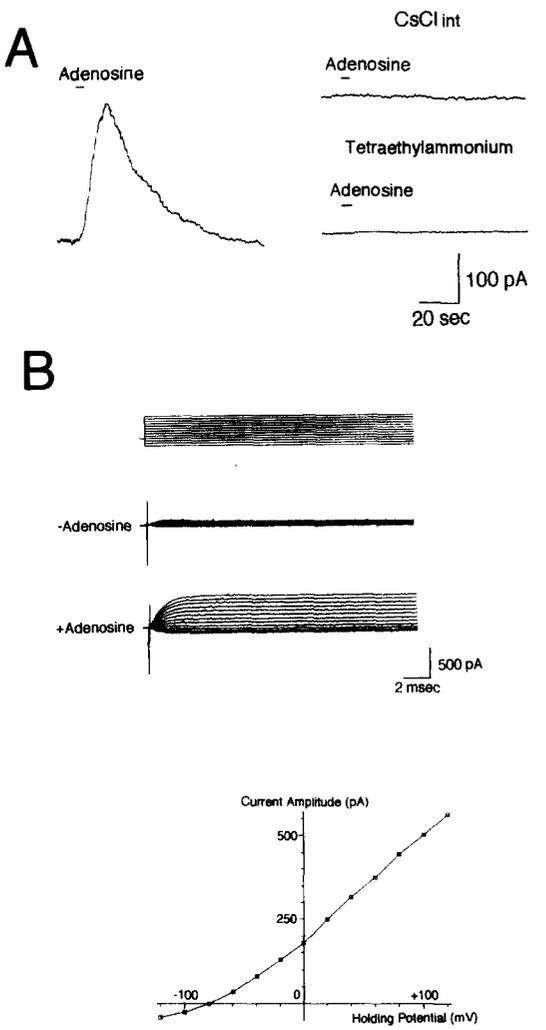

Fig. 1. Adenosine-evoked whole-cell membrane currents. (A) Adenosine (10 μM) was applied to cells by air pressure microinjector for 3 s as indicated by the bars. Holding potential was +60 mV. To examine the ion species concerned with adenosine-evoked currents, 150 mM KCl in the patch electrode-filling solution was replaced by 150 mM CsCl ($n = 5$) or adenosine was applied to cells in the presence of tetraethylammonium (10 mM) ($n = 5$). In this and all further figures, outward currents correspond to upward deflections. (B) 20-ms voltage pulses (-120 mV to +120 mV in 20-mV increments from a holding potential of −60 mV) were applied to patched cells without (−Adenosine) or with adenosine (10 μM) (+Adenosine). The voltage steps after application of adenosine were started when an adenosine-induced current reached a peak as monitored with an oscilloscope. The current–voltage relation resulting from adenosine is shown at the bottom.

3.2. An adenosine-bound purinoceptor

In an attempt to define whether adenosine-evoked currents are mediated by an adenosine receptor (P_1 purinoceptor), adenosine receptor agonists and antagonists were applied to the cells. A selective adenosine A_1 receptor agonist, CHA (0.1–10 μM), or a selective adenosine A_{2A} receptor agonist, CGS21680 (0.01–1 μM), never produced current in 10 cells (Fig. 2A). On the other hand, adenosine-induced currents were not inhibited by a nonselective adenosine receptor antagonist, theophylline (100 μM) (Fig. 2A). Additionally, a selective adenosine A_1 receptor antagonist, 8-CPT (10 μM), or a selective adenosine A_{2A} receptor antagonist, DMPX (0.1 μM), had no effect on the currents (Fig. 2A). These observations provide an indication that adenosine receptors are not concerned with activating the K^+ channel.

To further examine what purinoceptor mediates activation of the adenosine-sensitive K^+ channel, P_2 or P_3 purinoceptor agonists were applied to the cells. P_2 purinoceptor agonists, such as 2-methylthio ATP, ATP, ADP, and AMP at a concentration of 10 μM produced similar outward currents at a holding potential of +60 mV

(Fig. 2B). These agonists dose-dependently elicited currents with the order of their potency being: ADP ≥ 2-methylthio ATP > ATP ≥ adenosine ≫ AMP (Fig. 3). In contrast, a P_{2X}, P_{2U}, or P_3 purinoceptor agonist, α,β-methylene ATP, UTP, or β,γ-methylene ATP, respectively, elicited no response (Fig. 2B). These results, thus, indicate that adenosine activates the K^+ channel via a P_2 purinoceptor.

3.3. A P_2 purinoceptor-mediated signaling pathway

It was investigated whether the adenosine-bound P_2 purinoceptor is linked to a G-protein and what signaling pathway is involved in activation of the K^+ channel. The intracellular perfusion of a broad G-protein inhibitor, GDPβS (1 mM) (Eckstein et al., 1979), abolished adenosine-elicited currents, whereas a G_i/G_o-protein inhibitor, pertussis toxin (1 μg/ml), had no effect (Fig. 4A). In addition, the currents were completely blocked by a phospholipase C inhibitor, neomycin (500 μM) (Fig. 4B), and selective protein kinase C inhibitors, GF109203X (500 nM) (Oiu and Leslie, 1994), or protein kinase C inhibitor peptide (200 μg/ml) (Fig. 4C). These results suggest that the adenosine-bound P_2 purinoceptor is linked to a pertussis toxin-insensitive G-protein and that the K^+ channel is regulated by phospholipase C-mediated protein kinase C activation.

3.4. Adenosine-elicited single-channel currents

In the cell-attached patch-clamp configuration, adenosine (10 μM) inside the patch electrode elicited single-

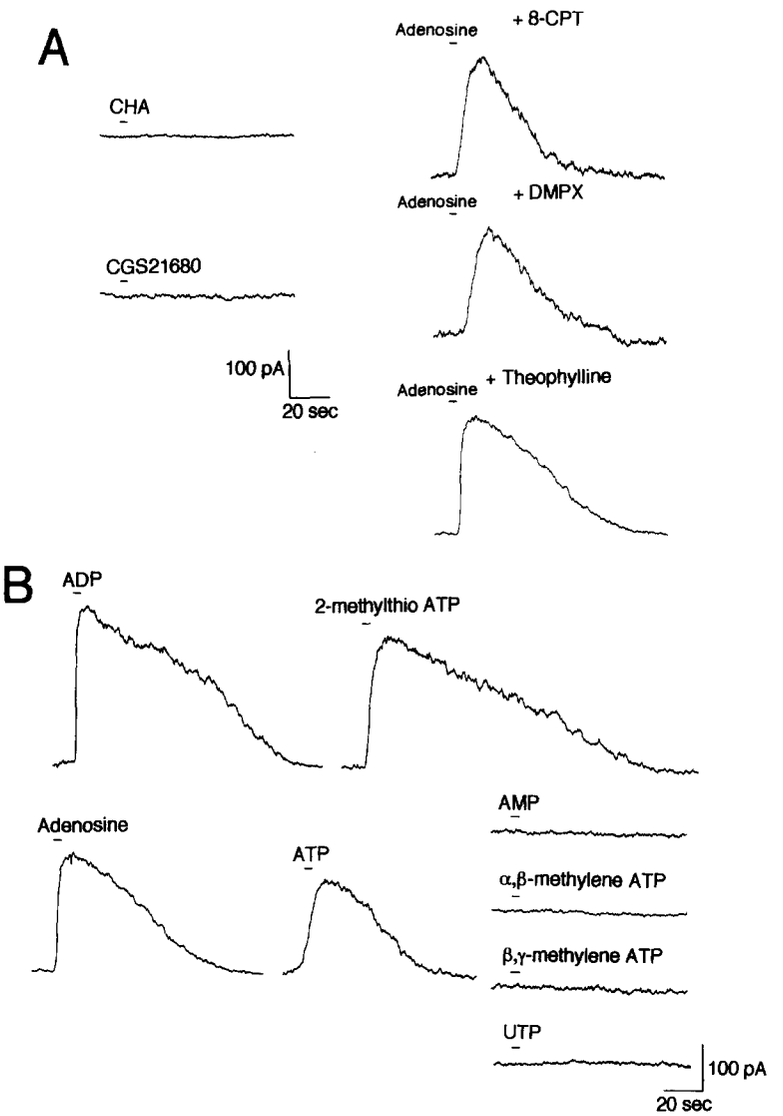

Fig. 2. The currents induced by purinoceptor agonists. (A) The responses to application of CHA (10 μM) or CGS21680 (1 μM) are illustrated in the left column. Each experiment was carried out in 10 cells. The effects of 8-CPT (10 μM), DMPX (0.1 μM), and theophylline (100 μM) on adenosine-evoked currents are shown in the right column. Cells were treated with these antagonists for more than 1 min and adenosine (10 μM) was applied to cells in the presence of these antagonists. Each effect was examined in 8–10 cells. (B) P_2 and P_3 purinoceptor agonist-produced currents are illustrated. 2-methylthio ATP, ATP, ADP, AMP, α,β-methylene ATP, β,γ-methylene ATP, and UTP at a concentration of 10 μM were applied to cells ($n = 7–10$). Drugs were applied for 3 s as indicated by the bars. Holding potential was +60 mV.

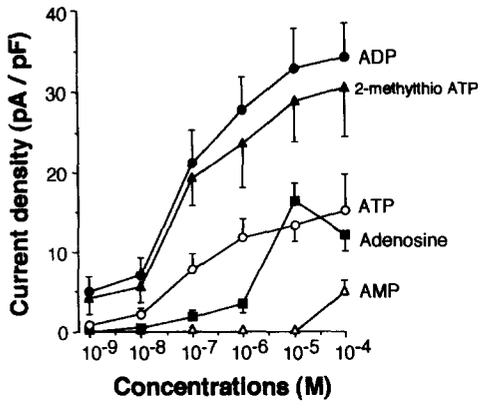

Fig. 3. Dose–response curves of P_2 purinoceptor agonists. ADP, 2-methylthio ATP, ATP, adenosine, and AMP at concentrations varying from 10^{-9} to 10^{-4} M were applied to cells at a holding potential of +60 mV. Each point represents the average from 5–8 cells and S.D. is shown by the bar.

channel currents with two major classes of slope conductances (28 ± 5 pS and 89 ± 7 pS, $n = 5$) (Fig. 5A,B). In patches using the patch electrode-filling solution without adenosine, bath-applied adenosine (10 μM) outside the patch electrode again evoked single-channel currents with slope conductances of 30 ± 3 pS and 90 ± 7 pS ($n = 5$) (Fig. 5A,B) and the latency to the start was within 2 s, suggesting that adenosine evokes K^+ currents via an intracellular signaling pathway. The currents induced by bath applications of adenosine were markedly inhibited by GF109203X (500 nM) without flickering (Fig. 5A). This indicates that GF109203X does not serve as a direct channel blocker but as an inhibitor of protein kinase C. Likewise, application of TPA (50 nM) inside or outside the patch electrode produced single-channel currents with slope conductances 30 ± 4 pS/90 ± 7 pS ($n = 5$) or 33 ± 6 pS/92 ± 5 pS ($n = 5$), respectively, with 60–90 s latency (Fig. 5A,B). These currents were also blocked by GF109203X (Fig. 5A). These data, thus, support the concept that the adenosine-sensitive K^+ channel is regulated by protein kinase C activated via a G-protein-mediated intracellular signaling pathway.

3.5. Adenosine-enhanced $[Ca^{2+}]_i$

So far several kinds of protein kinase C activation pathways, such as via phospholipase C, phospholipase A_2, and phospholipase D, have been proposed (Nishizuka, 1992). It is widely known that phospholipase C hydrolyzes phosphatidylinositol 4,5-biphosphate to produce diacylglycerol and inositol 1,4,5-triphosphate (IP_3). The latter stimulates Ca^{2+} release from intracellular calcium stores through IP_3 receptors. To further determine whether the adenosine-bound P_2 purinoceptor is involved in phospholipase C stimulation, $[Ca^{2+}]_i$ was assayed using fura-2 fluorescence. Adenosine (10 μM) enhanced $[Ca^{2+}]_i$ with an initiation time of 31 ± 5 s in the presence of extracellular Ca^{2+} (Fig. 6A). The fluorescence ratio F340/F380 was 0.80 ± 0.21 and the calibrated Ca^{2+} concentration was 2381 ± 536 nM ($n = 5$). Similarly, adenosine increased the ratio to 0.71 ± 0.13 and the calibrated concentration to 1970 ± 562 nM ($n = 4$) with an initiation time of 29 ± 8 s in Ca^{2+}-free extracellular solution also (Fig. 6B). These results indicate that adenosine stimulates Ca^{2+} release from intracellular calcium stores. Adenosine-induced $[Ca^{2+}]_i$ enhancement was completely blocked by GDPβS (1 mM), but was not affected by pertussis toxin (1 μg/ml) (Fig. 6C). Furthermore, this increase was inhibited by neomycin (500 μM) (Fig. 6C), suggesting that adenosine enhances $[Ca^{2+}]_i$ by IP_3 production associated with phosphatidylinositol hydrolysis catalyzed by phospholipase C. These results give additional evidence that the P_2

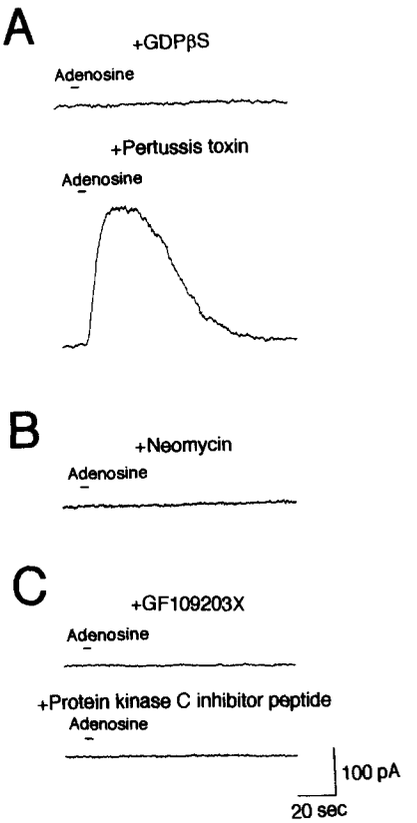

Fig. 4. The effects of G-protein inhibitors, a phospholipase C inhibitor, and a protein kinase C inhibitor on adenosine-evoked currents. (A) To examine the effects of GDPβS and pertussis toxin on the currents, GDPβS (1 mM) or pertussis toxin (1 μg/ml) was added to the patch electrode-filling solution and adenosine (10 μM) was applied to cells 5 min after whole-cell patch formation ($n = 8$). (B) To examine the effect of neomycin on the current, cells were treated with neomycin (500 μM) for 15 min and adenosine (10 μM) was applied to the patched cells in the presence of neomycin ($n = 5$). (C) The effect of GF109203X or protein kinase C inhibitor peptide on the current. Cells were treated with GF109203X (500 nM) for 3 min or protein kinase C inhibitor peptide (200 μg/ml, final concentration) was added to the patch electrode-filling solution ($n = 5$). Holding potential was +60 mV.

purinoceptor for adenosine is linked to a pertussis toxin-insensitive G-protein, which regulates phospholipase C-mediated phospholipid intracellular signaling.

The co-assay of $[Ca^{2+}]_i$ and whole-cell current showed no adenosine-induced $[Ca^{2+}]_i$ increase when EGTA inside the patch pipette was increased, although a current was

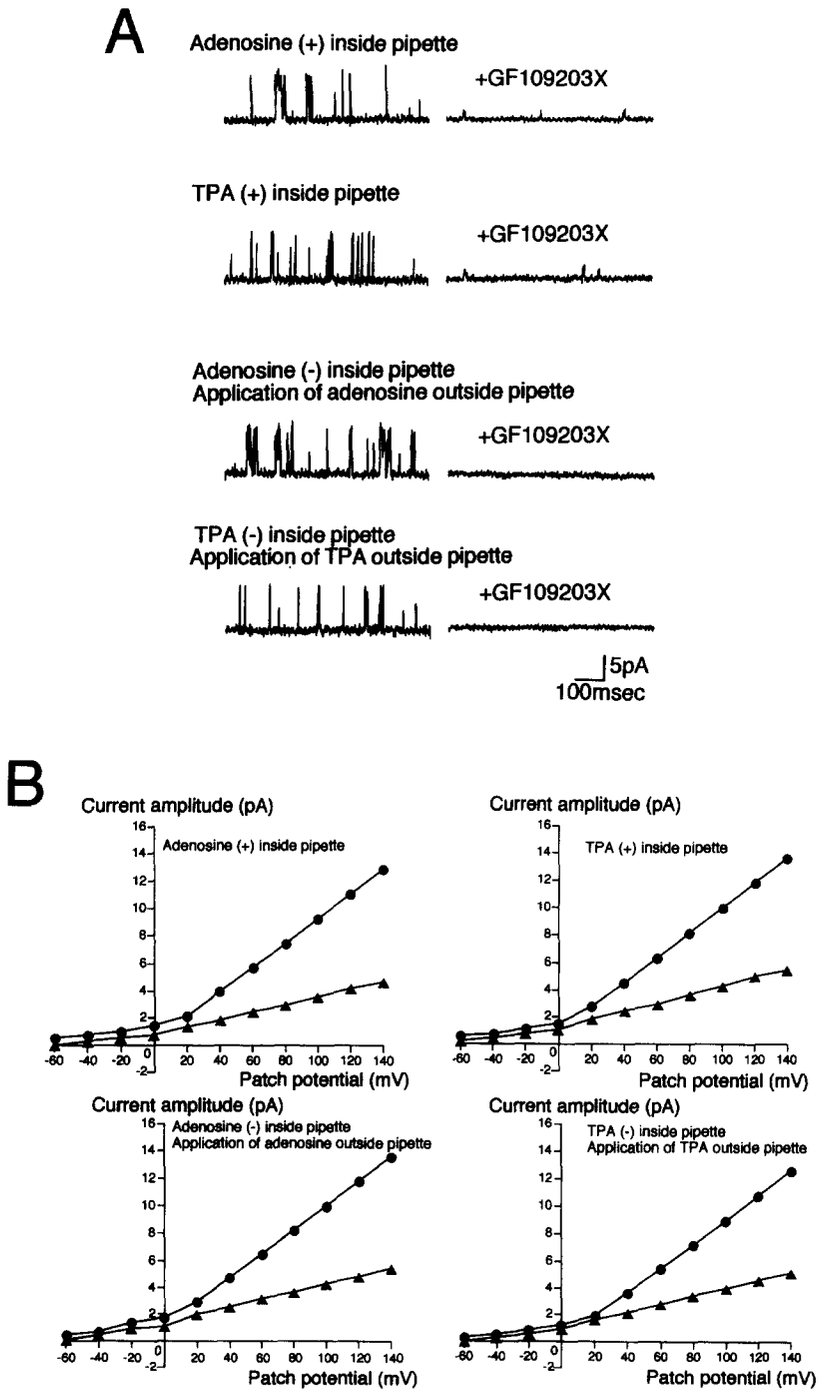

Fig. 5. Adenosine- and TPA-elicited single-channel currents. Cell-attached patches were made using the patch electrode filled with an extracellular solution in the presence and absence of adenosine (10 μM) or TPA (50 nM). In patches using the patch electrode-filling solution without adenosine or TPA, adenosine (10 μM) or TPA (50 nM) was bath-applied to cells outside the patch electrode during recordings in the presence and absence of GF109203X (500 nM). Typical single-channel currents are illustrated in A. Each experiment was carried out in 5 cells. Patch potential, which indicates the voltage loaded on the inside membrane, was +100 mV. Outward currents correspond to upward deflections. The adenosine- or TPA-activated single-channel current–voltage (I–V) relations are shown in B. Each slope conductance (closed triangle: lower conductance events, closed circle: higher conductance events) was measured by linear regression fitted to single-channel I–V relations ($n = 5$).

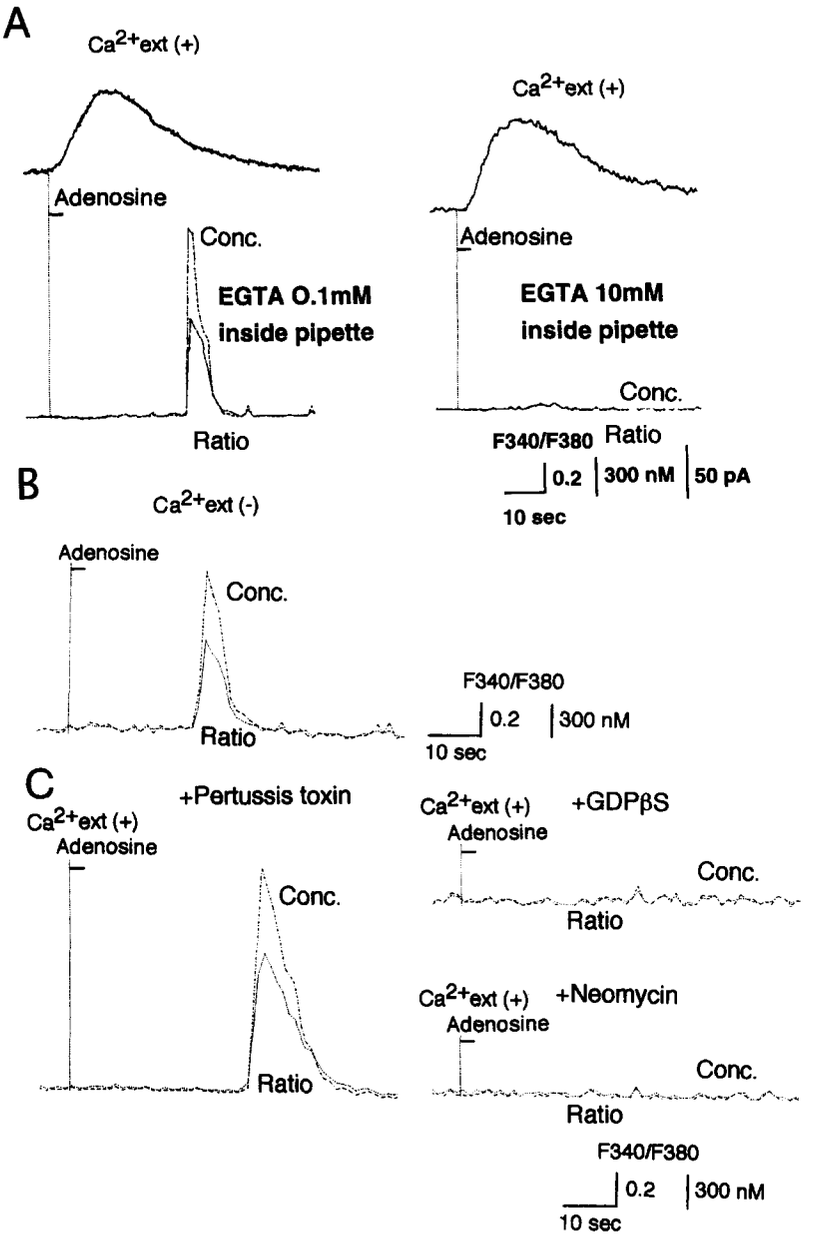

Fig. 6. Adenosine-induced $[Ca^{2+}]_i$ enhancement. (A) Co-assay of $[Ca^{2+}]_i$ and whole-cell current was carried out using the patch electorode-filling solution containing 0.1 or 10 mM EGTA. Holding potential was +20 mV. Adenosine (10 μM) was applied to cells for 3 s as indicated by the bars. (B) The adenosine-induced $[Ca^{2+}]_i$ increase was assayed in the Ca^{2+}-free extracellular solution (Ca^{2+} ext(−)). (C) To examine the effects of G-protein on the $[Ca^{2+}]_i$, whole-cell patches were made to fura-2-loaded cells using the patch electrode-filling solution containing GDPβS (1 mM) or pertussis toxin (1 μg/ml), and the $[Ca^{2+}]_i$ assay was carried out 5 min after patch formation. To examine the phospholipase C effect on the $[Ca^{2+}]_i$, cells were treated with neomycin (500 μM) after fura-2 loading. Ratio: the fluorescence ratio F340/F380, Conc.: the calibrated intracellular free Ca^{2+} concentration. Each effect was investigated in 4–7 cells.

evoked (Fig. 6A). This indicates that adenosine activates the K^+ channel by a mechanism independent of intracellular Ca^{2+}.

4. Discussion

Adenosine is widely known to exert an inhibitory effect as a neuromodulator on synaptic transmission (Stiles, 1986). The inhibitory mechanisms of adenosine at the postsynaptic site, however, are not fully understood. The present data clearly demonstrate that adenosine activates the K^+ channel responsible for outwardly rectifying currents in hippocampal neurons and thereby exerts an inhibitory effect. Recently, we found that P_2 purinoceptor agonists produced outwardly rectifying K^+ currents in a variety of regions of the central nervous systems with marked differences in the order of their potencies: ATP $\gg$ 2-methylthio ATP $\geq$ ADP > ATP > adenosine > AMP in

striatal neurons (Ikeuchi and Nishizaki, 1995a), 2-methylthio ATP > ADP > ATP > AMP > α,β-methylene ATP $\gg$ UTP in inferior colliculus neurons (Ikeuchi and Nishizaki, 1995b), 2-methylthio ATP > ADP > ATP $\geq$ α,β-methylene ATP $\geq$ AMP > UTP in medullar neurons (Ikeuchi et al., 1995a), 2-methylthio ATP > ADP > adenosine > ATP $\gg$ AMP in superior colliculus neurons (Ikeuchi et al., 1995b), 2-methylthio ATP > ADP > ATP > adenosine > α,β-methylene ATP > AMP > UTP in cerebellar neurons (Ikeuchi and Nishizaki, 1996), 2-methylthio ATP > ADP > ATP > adenosine > 2-methylthio ATP $\geq$ ATP $\geq$ ADP $\gg$ adenosine $\ggg$ AMP = α,β-methylene ATP in spinal neurons (unpubl. data). In striatal, spinal, and superior colliculus neurons, the ATP- and adenosine-sensitive K^+ channels are regulated by protein kinase C as is the channel in hippocampal neurons. Interestingly, the ADP-sensitive K^+ channel in inferior colliculus and medullar neurons and the 2-methylthio ATP-sensitive K^+ channel in cerebellar neurons appear to be regulated by direct coupling of the β,γ subunits of a G-protein. Thus, these data suggest that the different activation pathways of the K^+ channel may be crucial for fine tuning of inhibitory transmission in neurons. In contrast, there is a report of an adenosine-sensitive inwardly rectifying K^+ channel in cultured hippocampal neurons (Trussell and Jackson, 1987). The K^+ channel is activated via an adenosine receptor linked to a pertussis toxin-sensitive G-protein and this is entirely distinct from the channel described here. The inward rectification is supposed to be due to a quick voltage-dependent block of Mg^{2+}, plugging the channel from the intracellular side during outward current flow. We used a Mg^{2+}-free intracellular and extracellular solution for patches, and this may be the reason that the adenosine-evoked inwardly rectifying currents were not observed in our experiments. It remains unknown what roles the inwardly rectifying K^+ channel plays and whether the outward and inward rectifier interact with each other in hippocampal neurons. We are currently carrying out further experiments to address these questions.

Early on, we demonstrated that adenosine has dose-dependent excitatory and inhibitory biphasic actions on postsynaptic potentials elicited from hippocampal slices (Nishimura et al., 1990). In the present studies, the effect of adenosine on the postsynaptic site shows only inhibition in a dose-dependent manner and the maximal effect is observed at more than 10 μM. This is consistent with the concentrations (10 μM–1 mM) showing an inhibitory effect on the postsynaptic field potentials. Otherwise, lower concentrations of adenosine (10 nM–1 μM) exhibit excitation (Nishimura et al., 1990), presumably due to enhancement of glutamate release from the presynaptic site, and the inhibition at the postsynaptic site may be masked by this effect.

The pharmacological study using adenosine receptor agonists and antagonists demonstrates that the adenosine activation of the K^+ channel is not mediated by an adenosine receptor. In contrast, P_2 purinoceptor agonists produce outwardly rectifying currents. A large response is obtained with a P_{2Y} purinoceptor agonist, 2-methylthio ATP, but surprisingly, ADP is more potent than 2-methylthio ATP. ADP is known to be a specific agonist of the P_{2T} purinoceptor expressed in platelets, and ATP acts as an antagonist (Gordon, 1986). The P_{2X} purinoceptor is activated by α,β-methylene ATP (Burnstock and Kennedy, 1985) and the P_{2Z} purinoceptor that is found in mast cells is characterized by responding to ATP^{4-} (Gordon, 1986). Furthermore, the P_{2U} purinoceptor is activated by a variety of nucleotides including UTP, but does not respond to adenosine (Lin et al., 1993). The P_3 purinoceptor is potentiated by β,γ-methylene ATP and antagonized by α,β-methylene ATP (Shinozuka et al., 1988; Todorov et al., 1994). The receptor for adenosine described here, thus, appears to be a P_{2Y} purinoceptor, although the order of potency is not consistent with that established for the P_{2Y} purinoceptor (2-methylthioATP $\gg$ ATP = ADP $\gg$ α,β-methylene ATP) (O'Connor et al., 1991).

The P_{2Y} and P_{2U} purinoceptors are reported to be linked to a G-protein (Lin et al., 1993; Barnard et al., 1994). The P_{2Y} purinoceptor is involved in a phospholipase C signal transduction pathway (O'Connor et al., 1991), forming diacylglycerol, to activate protein kinase C and IP_3, to enhance intracellular Ca^{2+} release by hydrolysis of membrane phospholipid. In the present study, a potent protein kinase C activator, TPA, produced currents in a fashion that mimics the effect of adenosine and both TPA- and adenosine-evoked currents are inhibited by a selective protein kinase C inhibitor. These results indicate that an adenosine-bound receptor is relevant to protein kinase C activation and that the channel is regulated by activated protein kinase C. In addition, the finding that the currents and $[Ca^{2+}]_i$ enhancement induced by adenosine are blocked by a phospholipase C inhibitor suggest that the receptor stimulates a phosphatidylinositol-signaling pathway. Together, these data could lead to the conclusion that adenosine binds to a subtype of the P_{2Y} purinoceptor.

Among G-proteins, G_q- and G_i/G_o-proteins are known to activate protein kinase C via phospholipase Cβ stimulation (Wu et al., 1993) and phospholipase A_2 stimulation (Leurs et al., 1994), respectively. The finding that the adenosine-evoked currents are inhibited by GDPβS, but not by a G_i/G_o-protein inhibitor, pertussis toxin, provides an indication that a P_{2Y} purinoceptor for adenosine is linked to G_q-protein and, furthermore, the results of the $[Ca^{2+}]_i$ assay support this concept.

In conclusion, the results presented here clearly demonstrate that adenosine activates the K^+ channel responsible for outwardly rectifying currents and enhances cytosolic Ca^{2+} release via a P_{2Y} purinoceptor linked to G_q-protein, which is involved in a phospholipase C-mediated phospholipid-signaling pathway, in hippocampal neurons.

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