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Adenosine evokes potassium currents by protein kinase C activated via a novel signaling pathway in superior colliculus neurons

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Abstract **Adenosine evoked whole-cell potassium currents and enhanced intracellular free Ca^{2+} concentration ($[Ca^{2+}]i$) in superior colliculus neurons through a P_{2Y} purinoceptor linked to a pertussis toxin-insensitive G-protein, possibly Gq-protein, which is involved in a protein kinase C (PKC) activation pathway. The $[Ca^{2+}]_i$ increase was inhibited by a phospholipase C (PLC) inhibitor, whereas the evoked currents were not affected by a PLC inhibitor or a phospholipase A_2 (PLA_2) inhibitor. Adenosine elicited single channel currents via PKC activation in cell-attached patches and furthermore, those currents with conductances of the same slope were induced even in excised patches, suggesting that PKC can be activated only by cell membrane factors without intracellular components. These results thus indicate that the P_{2Y} purinoceptor-coupled potassium channel is regulated via a novel PKC activation pathway independent of PLC or PLA_2.**

Key words: Purinoceptor; Potassium channel; G-protein; Protein kinase C; Patch clamp; Ca^{2+} assay; Superior colliculus neuron

1. Introduction

In earlier studies, we demonstrated that P_2 purinoceptor agonists produce potassium currents in various regions of the brain and the channel is regulated by different mechanisms. The ADP- or 2-methylthio ATP-sensitive potassium channel appears to be activated by a direct action of the G-protein $\beta\gamma$ subunits in inferior colliculus [1], medullar [2], and cerebellar neurons (submitting elsewhere). The ATP-sensitive potassium channel in striatal neurons is regulated by protein kinase C (PKC) activation [3]. Adenosine also produces whole-cell potassium currents via a P_2 purinoceptor but not via an adenosine receptor in cultured rat superior colliculus neurons [4]. Its regulatory mechanism for the potassium channel, however, remains to be investigated.

PKC is known to exert a variety of effects on cellular responses to hormones, neurotransmitters, and some growth factors. Several lines of evidence have pointed to an integral role for PKC in signal transduction. In spite of numerous studies, the activation pathways of this enzyme are not fully understood as of yet and less physiological evidence has been provided. We show here that adenosine activates the P_{2Y}-coupled potassium channel in superior colliculus neurons via a G-protein-regulated PKC activation pathway independent of phospholipase C (PLC) and phospholipase A_2 (PLA_2).

2. Materials and methods

2.1. Tissue culture

Superior colliculus neurons from neonatal rat on day 1 were cultured, as described before [1–4]. Superior colliculus 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 minutes 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 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. Electrophysiology

For whole-cell patch clamp, 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 patch electrode-filling solution (in mM) was 150 KCl, 5 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 (R^s) compensation was made up to about 95%. Adenosine was applied to cells for 4 s by an air pressure microinjector (PV 830 Pneumatic Picopump, World Precision Instruments, Inc., USA). Single channel recordings were carried out in the cell-attached and outside-out patch clamp configurations using a Axopatch-200A. For cell-attached patches, the patch pipette was filled with the standard extracellular solution in the presence and absence of adenosine (0.1–100 μM). Excised patches were made using the same patch electrode-filling solution as used in whole-cell patches. The currents were filtered at 2 kHz, digitized at 500 Hz, and analyzed using pClamp software (Axon Instrument, Inc.; version 6).

2.3. Co-assay of adenosine-induced whole-cell current and intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$)

Cells were incubated at 37°C for 1 h with 4 μM fura-2/AM. Fura-2 loaded cells were placed into 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. Whole-cell patches were made to fura-2 loaded cells using the patch electrode filled with an intracellular solution (in mM): 150 KCl, 10 HEPES, and 0.1 EGTA, pH 7.2. Cells were viewed with a fluorescence 20 × dry objective lens and with a 20 × dry phase-contrast objective. The average fura-2 signal throughout the cell body was calculated. Fura-2 was excited at 340 and 380 nm alternately switched every 500 ms. The fluorescence signal was filtered through a bandpass filter transmitting 500–511 nm and detected by an intensified charge coupled device camera (Argus-50/CA, Hamamatsu Photonics, Inc., Japan). Ratio images were calculated in real time, stored on hard disk, and analyzed using Argus-50/CA software (version 3.0). $[Ca^{2+}]_i$ was calculated from the fluorescence ratio/Ca^{2+} concentration calibration curve made before experiments. Simultaneously, adenosine-evoked currents were recorded and analyzed according to the method described in section 2.1. Adenosine was applied to cells for 4 s by an air pressure microinjector.

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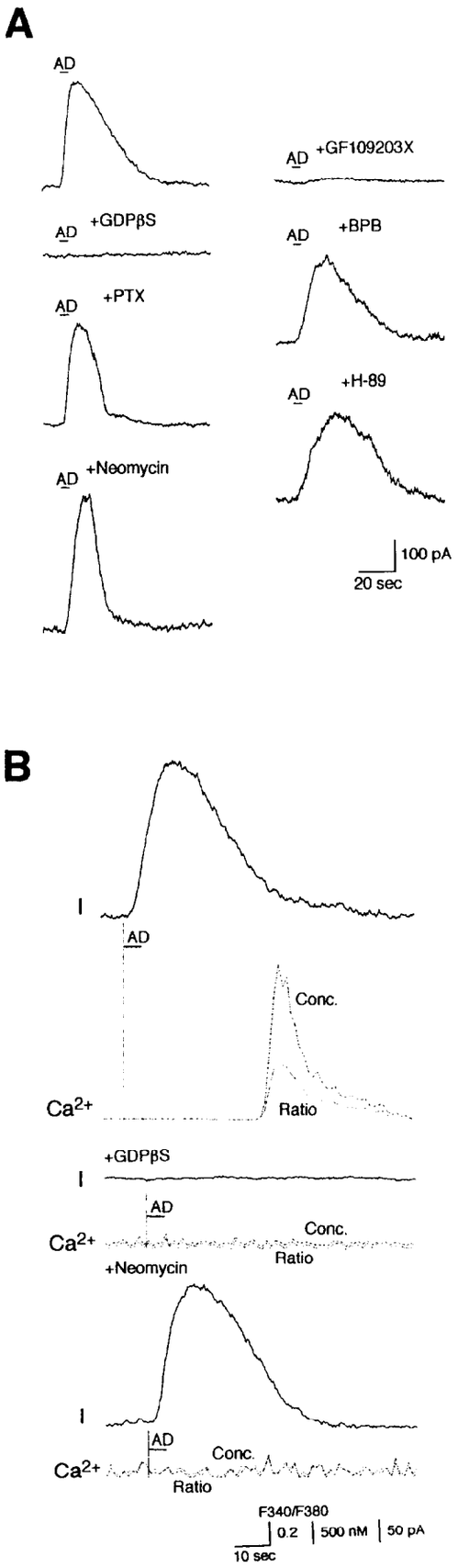

Fig. 1. Adenosine-induced potassium currents and $[Ca^{2+}]_i$ enhancement. (A) Whole-cell membrane currents recorded at a holding potential of +60 mV in the presence and absence of various inhibitors. GDPβS (1 mM) or PTX (0.1 μg/ml) was perfused in the patch electrode-filling solution for 5 min after patch formation and then adenosine (AD) (10 μM) was applied to cells. Cells were treated with neomycin (500 μM) for 15 min, GF109203X (500 nM), BPB (50 μM), and H-89 (1 μM) for 3 min prior to application of adenosine. Outward currents correspond to upward deflections. (B) Co-assay of whole-cell membrane currents and $[Ca^{2+}]_i$. Whole-cell patches were made to fura-2 loaded cells and subsequently adenosine (10 μM) was applied to the cells clamped at +60 mV in the presence and absence of GDPβS or neomycin. The fluorescence ratio *F*340/380 (Ratio) and the calibrated $[Ca^{2+}]_i$ (Conc.) are shown in the same trace.
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3. Results and discussion

3.1. Regulation of adenosine-evoked whole-cell potassium currents

Adenosine produced outward whole-cell membrane currents with a latency of 1.8 ± 0.7 s at a holding potential of +60 mV (Fig. 1A). The currents were completely blocked by a broad G-protein inhibitor, GDPβS, whereas a Gi/Go-protein inhibitor, pertussis toxin (PTX) had no inhibitory effect (Fig. 1A), indicating that the receptor for adenosine is linked to a PTX-insensitive G-protein. A PLC inhibitor, neomycin or a selective PLA_2 inhibitor, 4-bromophenacyl bromide (BPB) [5] had no effect on the currents (Fig. 1A). In contrast, adenosine-evoked currents were fully blocked by a selective PKC inhibitor, GF109203X [6], but not by a selective cAMP-dependent protein kinase (PKA) inhibitor, H-89 (Fig. 1A). These results suggest that the adenosine-sensitive potassium channel is regulated via a PLC- or PLA_2-independent PKC activation pathway.

3.2. Regulation of intracellular Ca^{2+} mobilization by adenosine

Co-assay of the $[Ca^{2+}]_i$ and whole-cell membrane current demonstrated that adenosine enhances $[Ca^{2+}]_i$ with an initiation time of 33.7 ± 7.5 s in the presence (Fig. 1B) and absence (data not shown) of extracellular Ca^{2+} after appearance of the current. GDPβS blocked both an adenosine-induced current and $[Ca^{2+}]_i$ enhancement (Fig. 1B). Neomycin inhibited the $[Ca^{2+}]_i$ enhancement, but failed to inhibit the current (Fig. 1B). These give an indication that the G-protein coupling to a P_2 purinoceptor may stimulate PLC to hydrolyze the lipid precursor phosphatidylinositol 4,5-biphosphate to produce both diacylglycerol (DG) and inositol 1,4,5-triphosphate (IP_3). Adenosine-induced $[Ca^{2+}]_i$ enhancement, therefore, appears to be

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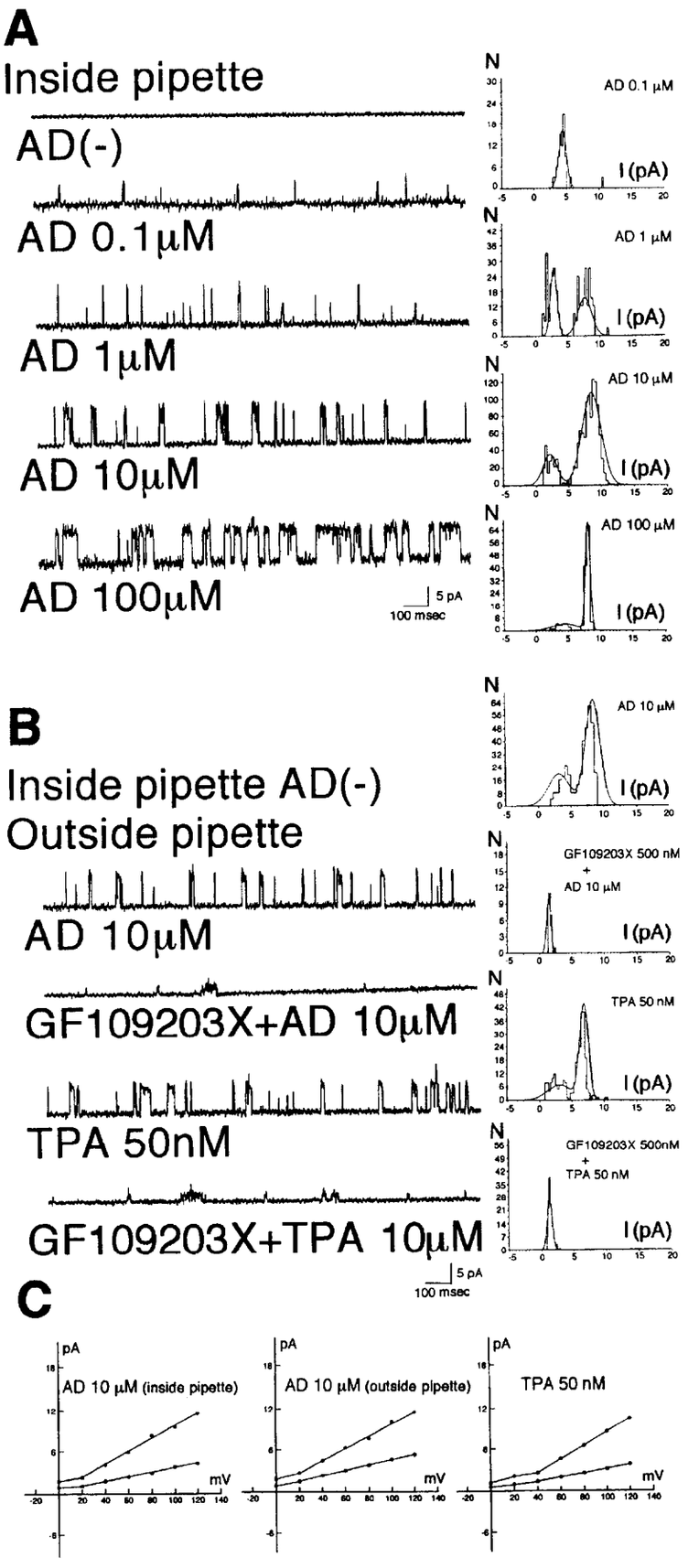
Fig. 2. Single channel recordings in the cell-attached patch clamp configuration. (A) Single channel currents elicited by adenosine (0.1–100 μM). Patch potential, which indicates the voltage loaded on the inside membrane, was +80 mV. In this and all following figures, outward single channel currents correspond to upward deflections. (B) Effects of PKC on the currents. Cell-attached patches were made using the patch electrode-filling solution without adenosine. Adenosine (10 μM) or TPA (50 nM) was bath-applied to cells outside the patch electrode in the presence and absence of GF109203X (500 nM). The currents were evoked within 2 s or 60 s after application of adenosine or TPA. (C) The single channel current/voltage (*I/V*) relations obtained by adenosine and TPA. The slope conductances were measured by linear regression fitted to the *I/V* relations.


A
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AD(-)
AD 0.1μM
AD 1μM
AD 10μM
AD 100μM
5 pA
100 msec
N
AD 0.1 μM
I (pA)
AD 1 μM
AD 10 μM
AD 100 μM
B
Inside pipette AD(-)
Outside pipette
AD 10μM
GF109203X+AD 10μM
TPA 50nM
GF109203X+TPA 10μM
GF109203X 500 nM
+
AD 10 μM
TPA 50 nM
GF109203X 500nM
+
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pA
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AD 10 μM (outside pipette)
TPA 50 nM
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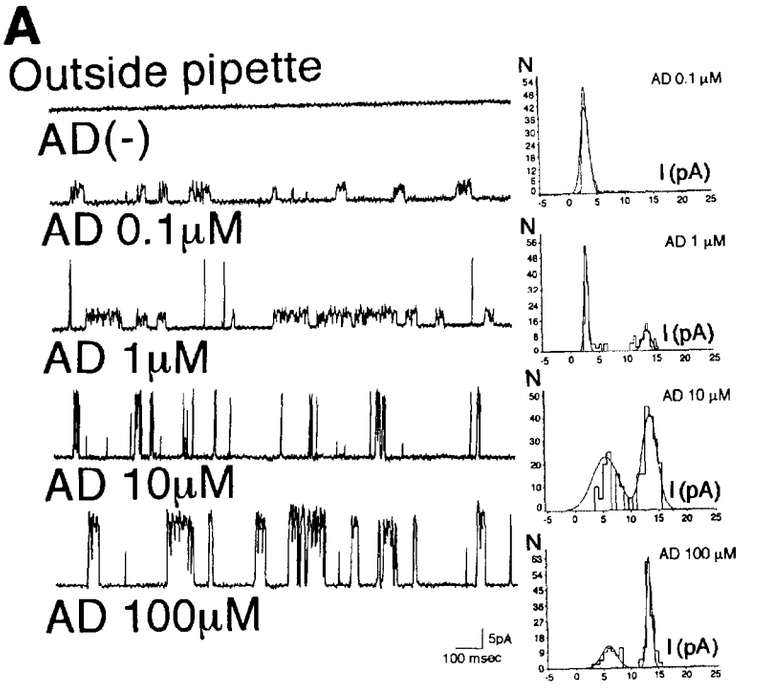
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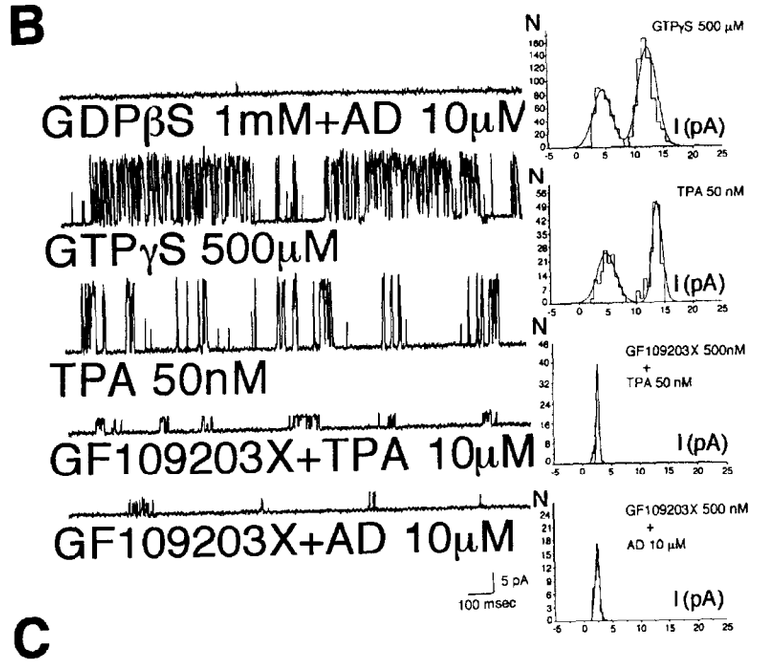
B
GDPβS 1mM+AD 10μM
GTPγS 500μM
TPA 50nM
GF109203X+TPA 10μM
GF109203X+AD 10μM
5 pA
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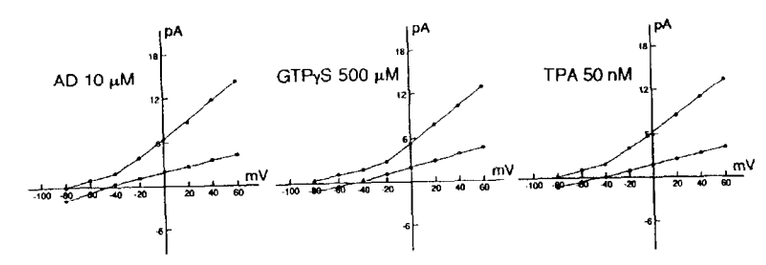
I (pA)
GTPγS 500 μM
TPA 50 nM
GF109203X 500nM
+
TPA 50 nM
GF109203X 500 nM
+
AD 10 μM


C
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AD 10 μM
GTPγS 500 μM
TPA 50 nM

Fig. 3. Single channel currents in the outside-out patch clamp configuration. (A) Single channel currents elicited by bath application of adenosine (0.1–100 μM). The currents were evoked within 2 s after application. Holding potential was +60 mV. (B) Effects of G-protein and PKC on the currents. To examine the effect of G-protein on the currents, GDPβS (1 mM) or GTPγS (500 μM) was added to the patch electrode-filling solution. To examine the effect of PKC, cells were treated with GF109203X (500 nM) for 3 min. The intracellular perfusion of GTPγS or bath-applied TPA (50 nM) induced currents within 5 min or 60 s. (C) The single channel current/voltage (I/V) relations obtained by adenosine, GTPγS, and TPA.
←

due to Ca^{2+} release from intracellular calcium stores through IP_3 receptors, but the channel is not regulated by a mechanism independent of a PLC-mediated phospholipid signaling.

3.3. Cell-attached patch clamp recording

In the cell-attached patch clamp configuration, adenosine elicited single channel currents with two major classes of slope conductances (31 ± 7 and 94 ± 5 pS at 10 μM, $n = 8$) (Fig. 2A,C), although spontaneous currents were not observed (Fig. 2A). Likewise, bath-applied adenosine outside the patch pipette produced single channel currents with conductances of 37 ± 6 and 89 ± 9 pS ($n = 10$) (Fig. 2B,C). Furthermore, a potent PKC activator, 12-*O*-tetradecanoylphorbol 13-acetate (TPA) again induced single channel currents with similar slope conductances (30 ± 4 and 98 ± 7 pS, $n = 10$) (Fig. 2B,C). The currents induced by bath-applied adenosine and TPA were inhibited by GF109203X (Fig. 2B). These data suggest that the channel regulation is mediated by an intracellular signaling associated with PKC activation. Notably the amplitude distribution histograms obtained with adenosine (0.1–100 μM) inside the pipette demonstrated that the lower amplitude events shifted to the larger ones in a dose-dependent manner (Fig. 2A).

3.4. Outside-out patch clamp recording

The G-protein-linked receptor-operated channel has been considered not to be activated in excised patches lacking intracellular second messenger cascades. Adenosine, however, evoked single channel currents with two major kinds of slope conductances (38 ± 4 and 132 ± 8 pS at 10 μM, $n = 10$) in the outside-out patch clamp configuration as well (Fig. 3A,C). The intracellular perfusion of a potent G-protein activator, GTPγS, produced single channel currents with slope conductances of 44 ± 7 and 126 ± 9 pS ($n = 7$) (Fig. 3B,C) and adenosine-evoked currents were completely blocked by GDPβS (Fig. 3B), suggesting that the channel is activated by a G-protein-regulated signaling. Additionally, bath-applied TPA induced single channel currents in a fashion that mimics the effect of adenosine (40 ± 3 and 124 ± 7 pS, $n = 10$) (Fig. 3B,C). Adenosine- and TPA-evoked currents were inhibited by GF109203X (Fig. 3B), suggesting that the regulation of the adenosine-sensitive potassium channel is dependent upon PKC activation. These results thus indicate that the G-protein coupling to the receptor for adenosine is involved in PKC activation, and surprisingly a series of these reactions occur only in cell membrane components, although of course, some intracellular factors including cytosolic PKC are contaminated inside the patch electrode-filling solution in making outside-out patches.

Similarly in the cell-attached patches, the dose-dependent shift from a lower slope conductance to a higher one of the currents elicited by adenosine (0.1–100 μM) was also observed in outside-out patches (Fig. 3A). Furthermore, the slope conductance of adenosine- or TPA-induced currents in the presence of GF109203X (33 ± 6 or 35 ± 4 pS, $n = 8$, respectively) was almost consistent with a lower conductance than those in the absence of GF109203X. The above may imply that PKC is dose-dependently activated by adenosine and that the currents with a higher conductance are regulated by PKC.

3.5. PKC activation pathway for regulation of the adenosine-sensitive potassium channel

So far, several kinds of PKC activation pathways, such as via PLC, PLA_2, and phospholipase D (PLD), have been proposed [7,8]. Among G-proteins, Gq- and Gi/Go-proteins are known to be involved in the activation of PKC via PLCβ stimulation [9–11] and via PLA_2 stimulation [12], respectively. Taken together with the data showing that the P_{2U} and P_{2Y} purinoceptors are linked to a G-protein and that the latter stimulates PLC [13], the receptor for adenosine appears to be a P_{2Y} purinoceptor. The result that adenosine-evoked currents had no response to PTX, which inhibits Gi/Go-protein, may explain that the adenosine-bound P_{2Y} purinoceptor is likely linked to Gq-protein. The observation that the $[Ca^{2+}]_i$ increase induced by adenosine was blocked by a PLC inhibitor, neomycin, supports this concept. However, neomycin had no effect on the currents, suggesting that Gq-protein takes part in a PLC-mediated phospholipid signaling pathway, but the currents are regulated mainly via another PKC activation pathway. A pathway associated with PLA_2 would be ruled out, based upon the data that a selective PLA_2 inhibitor, BPB had no effect on the currents. PLD can activate PKC via a mechanism of phosphatidylcholine hydrolysis into phosphatidic acid (PA) and choline. PA has been reported to be converted to DG [14] or to be a direct activator of PKC [15]. This acidic acid also stimulates PLCγ_1 [16] and phosphatidylinositol-4-phosphate kinase [17]. Furthermore, PLD is activated by GTPγS [7,14], but a cytosolic soluble protein factor, such as the ADP-ribosylation factor and a small G-protein [18,19], is required for its activation. Thus, PLD appears to be a convincing candidate for a PKC activation pathway responsible for regulation of the potassium channel. However, it would be hard to explain the rapid response of the order of 1 s as observed here for a PLD-mediated pathway, since PLD is supposed to activate PKC more slowly and to play a significant role in the maintenance of PKC activity for a duration of several hours [8]. Another possibility includes the possibility that a PKC-binding protein (PKC-BP) in the plasma membrane may be involved in the activation of PKC, as Raf is translocated to the plasma membrane and activated by binding to a specific protein in the cytoskeleton [20]. Indeed, several PKC-binding proteins, which may translocate the activated PKC molecule to the plasma membrane and act as the receptors for the activated form of PKC (RACKs), have been isolated [21–25]. The results presented here indicate that cytosolic inactivated PKC targeted to the plasma membrane may be activated by a PKC-BP in the membrane independent of Ca^{2+}, and that this pathway may be crucial for rapid signal transduction in neuronal cells.

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