Inhibitory Effects of Glutamate-stimulated Astrocytes on Action Potentials Evoked by Bradykinin in Cultured Dorsal Root Ganglion Neurons

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Patch-clamp and Ca^{2+}-imaging techniques have revealed that astrocytes have dynamic properties including ion channel activity and release of neurotransmitters, such as adenosine triphosphate (ATP) and glutamate. Here, we used the patch-clamp technique to determine whether ATP and glutamate is able to modulate the bradykinin (BK) response in neurons cultured with astrocytes in the mouse dorsal root ganglia (DRG) in order to clarify the role of astrocytes in nociceptive signal transmission. Astrocytes were identified using a fluorescent anti-GFAP antibody. The membrane potential of astrocytes was about −39 mV. The application of glutamic acid (GA) to the bath evoked the opening of two types of Cl^- channel in the astrocyte cell membrane with a unit conductance of about 380 pS and 35 pS in the cell-attached mode, respectively. ATP application evoked the opening of two types of astrocyte K^+ channel with a unit conductance of about 60 pS and 29 pS, respectively. Application of BK to the neuron evoked an action potential (spike). Concomitant BK application with ATP increased the frequency of BK-evoked neuron spikes when neurons coexisted with astrocytes. Stimulation of BK with GA inhibited the BK-evoked spike under similar conditions. The application of furosemide, a potent cotransporter (Na^+-K^+-$2Cl^-$) inhibitor, prior to stimulation of BK with GA blocked inhibition of the spike. It is thought that inhibition of the spike is related to Cl^- movement from astrocytes.

Key words: astrocyte, glutamate, chloride channel, allodynia, dorsal root ganglion

INTRODUCTION

Glial cells (glia), including astrocytes and oligodendrocytes, have traditionally been considered the supporting cells of the nervous system. However, recently developed patch-clamp techniques and Ca^{2+}-imaging technology have revealed that astrocytes have dynamic properties, including ion channel activity, release of neurotransmitters such as ATP and glutamate, as well as signal transmission in the neuronal network of the central nervous system [1, 2]. Free nerve endings of pain sensory cells are activated or sensitized by pain-related stimuli and also by compounds that are released locally in damaged tissue [3, 4]. Of these compounds, bradykinin (BK), prostaglandins (PG) and amines activate the pain sensory endings, and are thought to play a role in pain associated with injury and inflammation [3, 4]. It has been suggested that glial cells contribute to enhanced pain. Activated glial cells release pain-enhancing substances, such as nitric oxide (NO), ATP, PG, excitatory amino acids (EAA), interleukins (IL1, IL6) and tumor necrosis factor (TNF) [5].

On the other hand, it has been reported that glutamate microinjection into the lateral reticular nucleus [6] and the nucleus raphe magnus [7] in the medulla, and the locus coeruleus/subcoeruleus in the pons [7, 8], reduced the neuronal response to skin heating. Glutamate microinjection into the locus coeruleus in the dorsolateral pons reduced the nociceptive tail-flick reflex [9]. Our previous study demonstrated that free nerve endings of cultured neurons from dorsal root ganglia (DRG) are activated by BK [10]. In the present study, the electrophysiological properties of cultured astrocytes were investigated using the patch-clamp technique. Specifically, we studied the inhibitory effects of glutamate-stimulated astrocytes on the action potentials of cultured neurons stimulated by BK in the cell body when neurons coexisted with astrocytes.

MATERIALS AND METHODS

Cells

The following procedures were performed in accordance with the Guidelines for the Care and Use of Animals for Science Purposes at Tokai University. Mice (Jcl: ICR; age, 7–8 weeks; male) were obtained from CLEA Japan (Shizuoka, Japan). Animals had free access to water and food until the day of the experiment. Mice were killed by section of the neck above the spinal column under anesthesia with diethyl ether. About 40–45 DRG were aseptically removed from each mouse. DRG were dissociated in 2 ml of Ham's F12 medium with 0.2% collagenase at 37°C for 90 min in a shaking bath. After a further 15 min of incubation in Ca^{2+} - and Mg^{2+} -free Hank's balanced salt solution (HBSS) containing 0.25% trypsin, tissue was triturated in HBSS containing a trypsin inhibitor (0.05 mg/ml) and was added to a 30% Percoll solution. It was then subjected to density gradient centrifugation at 200 x *g* for 4 min, resulting in a yield of more than 5 x 10^4 neurons per mouse with high purity. The dispersed neurons were washed three times with F12 containing 10% fetal bovine serum (FBS), and were seeded onto

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35-mm culture dishes coated with poly-L-lysine. Cell density was adjusted to 3 x 10^3 cells/cm^2. DRG neurons were kept in medium and were allowed to adhere to the substrate for 4 h in a humidified atmosphere containing 5% CO_2 at 37ºC. Culture solution containing cellular debris was removed and replaced with fresh medium containing 10% FBS and 100 ng/ml 7S nerve growth factor (NGF). After 24 h, culture solution was removed and replaced with serum-free Ham's F12 culture medium containing 5 μg/ml insulin, 5 μg/ml transferrin, 2 nM progesterone, 0.1 mM putrescine, 3 mM selenium and 100 ng/ml NGF. Medium was changed every other day. In DRG culture, cells smaller than 25 μm in diameter were used in all experiments. Cell size (smaller than 25 μm), resting potential characteristics (around −45 mV), and long spike duration (10–15 ms), indicated that the cells used in the experiments were unmyelinated C-fibers [10].

In order to extract astrocytes, the layer including astrocytes and tissue debris was separated from above the Percoll layer in the centrifuge tube. It was then subjected to density gradient centrifugation, as described above, to remove tissue debris. Dispersed astrocytes were seeded onto 35-mm culture dishes coated with poly-L-lysine. Same medium without NGF was used as a culture solution. To culture neurons with astrocytes, neurons were seeded onto astrocytes that had been cultured for 5–7 days in culture dishes. Neurons accompanied by astrocytes were cultured for 3–4 days in the same culture medium with NGF, as described for the neuron-culture methods, and the patch-clamp experiment was performed.

Glial cells were identified by fluorescence microscopy (TMD-EFQ; Nikon, Tokyo, Japan) and a rabbit polyclonal antibody against glial fibrillary acidic protein (GFAP) (G9269; Sigma, St. Louis, MO). Binding was detected with fluorescein-conjugated goat anti-rabbit secondary antibody (anti-rabbit IgG FITC conjugate, F1262; Sigma) using UV irradiation. Cells were fixed using 4% paraformaldehyde in phosphate buffered saline for 15 min, and then using acid alcohol (methanol) at −20ºC for 10 min prior to incubation with primary antibody (60 min) and secondary antibody (30 min).

Patch-clamp recordings

Membrane potentials (intracellular potential) and single-channel currents were recorded using the patch-clamp technique. Voltage recordings were obtained by the whole-cell current-clamp method. Single-channel current recordings were obtained using the voltage-clamp method. Experiments were performed on a heated microscope stage (30ºC).

Two types of physiological saline solution were used: Na^+-rich and K^+-rich. The Na^+-rich solution contained (mM) 140 NaCl, 4.7 KCl, 1.13 $MgCl_2$, 10 glucose, and 10 HEPES (pH 7.4 with NaOH). The K^+-rich solution contained (mM) 145 KCl, 10 NaCl, 1.13 $MgCl_2$, 10 glucose, and 10 HEPES (pH 7.4 with KOH). The dish solution for recording contained Na^+-rich solution with 2.5 mM $CaCl_2$. The patch pipette solution contained K^+-rich solution with 0.5 mM EGTA.

Single-channel currents were recorded with a patch-clamp amplifier (CEZ2300; Nihon Kohden, Tokyo, Japan) filtered at 1 kHz low pass, and stored on tape (KS609; Sony, Tokyo, Japan) for subsequent replay and analysis. Current amplitude was obtained by measuring the amplitude of the current on the recorder chart with Vernier calipers.

Chemicals

Percoll and mouse nerve growth factor (7S mNGF) were purchased from Amersham Biosciences (Piscataway, NJ) and Alomone Labs (Jerusalem, Israel), respectively. Pyridoxal phosphate-6-azo-tetrasodium salt, DL-2-amino-3-phosphonopropionic acid, and NS102 were obtained from Sigma.

RESULTS

1. Electrophysiological properties of astrocytes

Chloride channels

Glial astrocytes were identified using an antibody against GFAP and were visualized with a fluorescent secondary antibody. A polygonal shaped cell was used for patch-clamp recording. Membrane potential (intracellular potential) was recorded using current-clamp mode in the whole-cell configuration of the patch-clamp technique. The membrane potential was −39.5 ± 1.7mV (mean ± SEM, n = 33). DRG astrocytes were mostly silent with respect to ion channel activity. In the central nervous system, glutamate is known to act as a neurotransmitter, and in astrocytes, it induces a response resulting in Ca^{2+}-changes [1, 11]. Glutamate's ability to evoke the opening of the Cl^- channels in DRG astrocytes was assessed. When glutamic acid (GA) (20 μM, final concentration) was added to the bath solution, single-channel currents were observed in the cell-attached configuration using a pipette containing Na^+-rich solution, which contained 2.5 mM Ca^{2+} (Fig. 1A). The unitary conductance of the single-channel was about 380.6 ± 21.8 pS (mean ± SEM, n = 8). The Cl^- equilibrium potential was about −30 mV under the quasi-physiological ion concentration, given that the internal Cl^- concentration of glial cells is about 40 mM [12]. The membrane potential of astrocytes was about −39 mV, and the reversal (null) potential was between 0 mV and −10 mV of the pipette potential (VP) (Fig. 1B). This channel must be Cl^- selective, as in this experimental situation, the only ion with a reversal potential of around 0 mV of VP is Cl^-.

Ca^{2+}-sensitivity of the Cl^- channel was assessed under conditions when the Cl^- channel was certain to be observed using a pipette containing Na^+-rich solution (145 mM Cl^-); the patch membrane was excised to create an inside-out configuration. The bath solution was replaced by a K^+-rich solution without Ca^{2+} and 5 mM EGTA was added to the bath. Under low Ca^{2+} concentration (less than 10^{-9} M), no decrease in the frequency of Cl^- channel opening was seen (Fig. 1C).

When GA was added to the bath solution, low-conductance Cl^- channel currents were also observed in the cell-attached configuration using a pipette containing Na^+-rich solution (Fig. 2). Unitary conductance was about 35.5 ± 1.7 pS (mean ± SEM, n = 7).

GA addition to the bath solution evoked Cl^- channel opening under the patch membrane, which is isolated from the bath solution. As it is thought that GA response is involved in a second messenger cascade, cyclic AMP was assessed as to whether it evokes

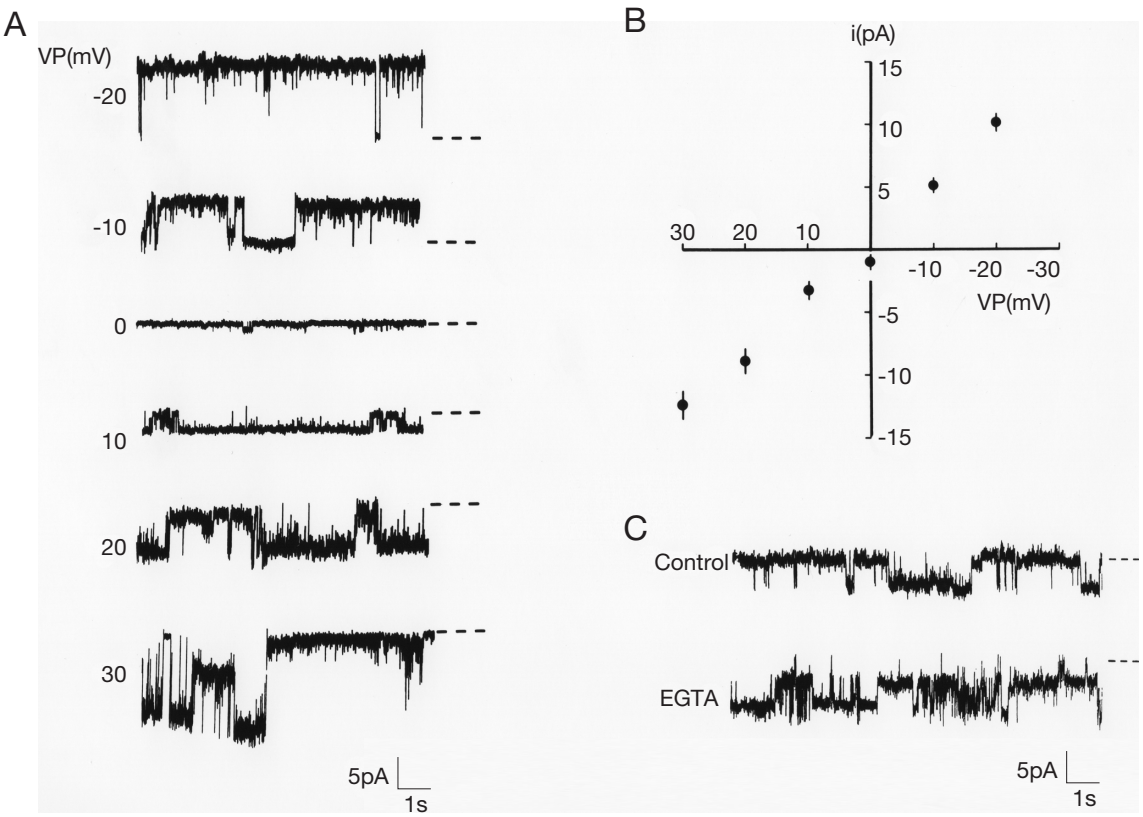

Fig. 1 Glutamic acid (GA)-evoked opening of high-conductance Cl^- channel.
A: Tracings were obtained from the cell-attached configuration using a pipette containing Na^+-rich solution with 2.5 mM Ca^{2+}. All tracings were obtained from the same patch at 1 min after application of GA (20 μM) to the bath solution. The dashed line indicates the current level when all channels are closed. Upward deflection represents outward current. B: Relationship between amplitude of single-channel current and pipette potential (VP). Ordinate shows the mean ± SEM (n = 6) of single-channel current amplitude. C: Ca^{2+}-sensitivity of high-conductance Cl^- channel. Tracings of single-channel current were obtained from excised inside-out configuration using a pipette containing Na^+-rich solution with 2.5 mM Ca^{2+}, and bath was filled with K^+-rich solution without Ca^{2+}. Unitary currents were obtained 1 min after addition of 5 mM EGTA to the bath (bottom). All tracings with respect to membrane potential (MP) of 20 mV were obtained from the same patch.

Cl^- channel opening. Addition of dibutyryl cyclic AMP (dbcAMP) (0.5 mM), a membrane permeable cyclic AMP analog, to the bath solution evoked an opening of the high-conductance Cl^- channel currents in the cell-attached configuration (Fig. 3A). In six patches, high-conductance Cl^- channel currents were also observed by application of dbcAMP.

In order to test whether a non-NMDA(N-Methyl-D-aspartic acid)-receptor blocker is able to inhibit the opening of Cl^- channels due to GA application, experiments with the cell-attached configuration using a pipette containing Na^+-rich solution with 2.5 mM Ca^{2+} was performed. When GA was added to the bath solution concomitantly with NS102 (10 μM), a non-NMDA-receptor antagonist, opening of the high- (Fig. 4A) and low- (Fig. 4B) conductance Cl^- channels was not observed.

Potassium channels

Spontaneous opening of K^+ channels was observed in only 10 of 367 patches in which the experiment was attempted. In the central nervous system, ATP is known as a gliotransmitter, an excitatory substance that increases the excitability of neurons [13–15]. ATP's ability to evoke the opening of K^+ channels in DRG astrocytes was assessed. When ATP (0.25 mM) was added to the bath solution, single-channel currents were observed in the cell-attached configuration using a pipette containing Na^+-rich solution (Fig. 5A). The unitary conductance of the single-channel was about 60.2 ± 3.3 pS (mean ± SEM, n = 5). Outward currents were seen at the pipette potential (VP) in the range of −50 to 40 mV (Fig. 5B). In situ (cell-attached configuration), the reversal potential (null potential) of the K^+ channel current was around 55 mV of VP, as the resting potential was around −35 mV. Given that under these experimental conditions, the only ion with a reversal potential above 40 mV of VP is K^+, this channel must be K^+ selective. To test the Ca^{2+}-sensitivity of the K^+ channel, after opening the K^+ channel, the patch membrane was excised to create an inside-out configuration in Na^+-rich bath solution, and the bath solution was then replaced with a K^+-rich solution containing 5 mM EGTA without Ca^{2+}. K^+ channel opening was seen under a low Ca^{2+} concentration (Fig. 5C).

When ATP (0.25 mM) was added to the bath solution, low-conductance K^+ channel currents were also observed in an outside-out configuration using a K^+-rich solution. The unitary conductance was about 29.3 ± 1.3 pS (mean ± SEM, n=4) (Fig. 6). To test the Ca^{2+}-

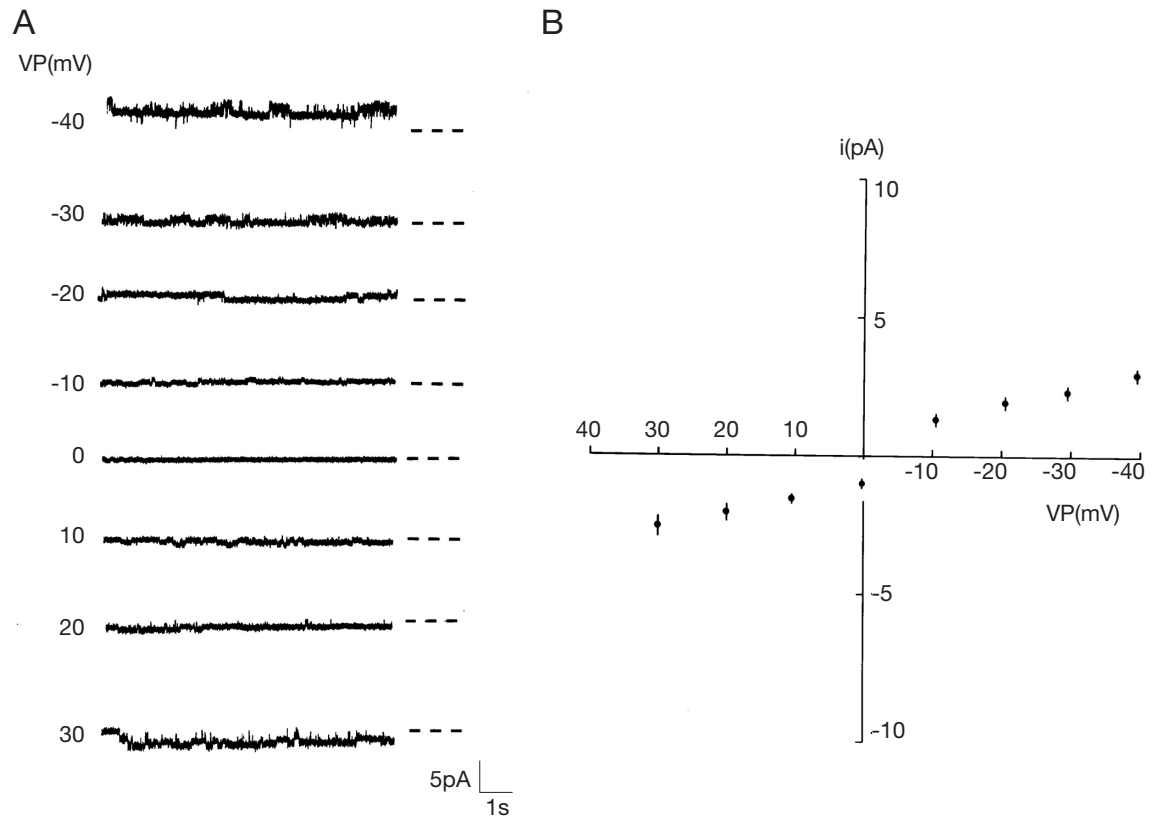

Fig. 2 GA evoked-opening of low-conductance Cl^- channels.
A: Tracings were obtained under the same experimental conditions as described in Fig. 1. B: Relationship between single-channel current amplitude and pipette potential (VP). Ordinate shows the mean ± SEM (n = 5) of single-channel current amplitude.

sensitivity of the low-conductance K^+ channel, after the opening of the channel in a cell-attached configuration using Na^+-rich solution, the patch membrane was excised to an inside-out configuration, and the bath solution was replaced with K^+-rich solution containing 5 mM EGTA without Ca^{2+}. Membrane potential was held at 0 mV. K^+ channel opening was seen under low Ca^{2+} concentrations (Fig. 6C).

Responses of membrane potential to neurotransmitter, and effects of receptor blocker

In order to delineate the glutamate receptor, the membrane potential response was assessed in the whole-cell current-clamp mode using a pipette containing K^+-rich solution including 145 mM Cl^- with 0.5 mM EGTA. Under these experimental conditions, as the equilibrium potential of Cl^- is 0 mV and the resting membrane potential (intracellular potential) is about −35 mV, a larger change in the membrane potential related to Cl^- movement is expected. The use of GA (20 μM) evoked a depolarization (Fig. 7). The average membrane potential evoked by GA was −10.2 ± 2.4 mV (mean ± SEM, n = 6). When GA was given in the presence of NS102 (10 μM), a non-NMDA-receptor antagonist, no depolarization response was observed (Fig. 7, bottom trace).

We then determined whether ATP is able to induce a membrane potential response under the same experimental conditions. Application of ATP (0.25 mM) to the bath solution induced hyperpolarization of the cell (Fig. 8). The average membrane potential evoked by ATP was −76.1 ± 2.3 (mean ± SEM, n = 5). When ATP was given concomitantly with pyridoxal phosphate-6-azo-tetrasodium salt (4 μM), a P_2 purinoceptor antagonist, no hyperpolarization was observed, as shown in the bottom trace of Fig. 8.

2. Effects of astrocytes on neuron activity when the neuron is cultured with astrocytes

As GA opened the Cl^- channel in astrocytes, we tested whether GA-stimulated astrocytes are able to influence neuron activity by culturing a neuron with astrocytes. To test BK-evoked action potentials (spikes), the whole-cell current-clamp mode was used with a pipette containing K^+-rich solution and 0.5 mM EGTA. A small neuron was used (less than 25 μm diameter). After application of BK (6 μM) evoked a spike (Fig. 9A), GA was added to the bath solution, and BK was added to the bath again. The BK-evoked spike was not observed (Fig. 9B) the second time. Third application of BK at high concentration (30 μM) resulted in a small depolarization. The experiment was repeated ten times and similar results were obtained each time. When the density of cultured astrocytes was low and the recorded neuron was not in contact with the astrocytes, no inhibition was observed (not shown). BK application, concomitantly with ATP, increased the frequency of the BK-evoked spike in neurons cultured with astrocytes (Fig. 9C).

In order to clarify the inhibitory mechanisms of GA on the BK-evoked spike, furosemide (0.5 mM), a diuretic and potent blocker of Na^+-K^+-Cl^- cotrans-

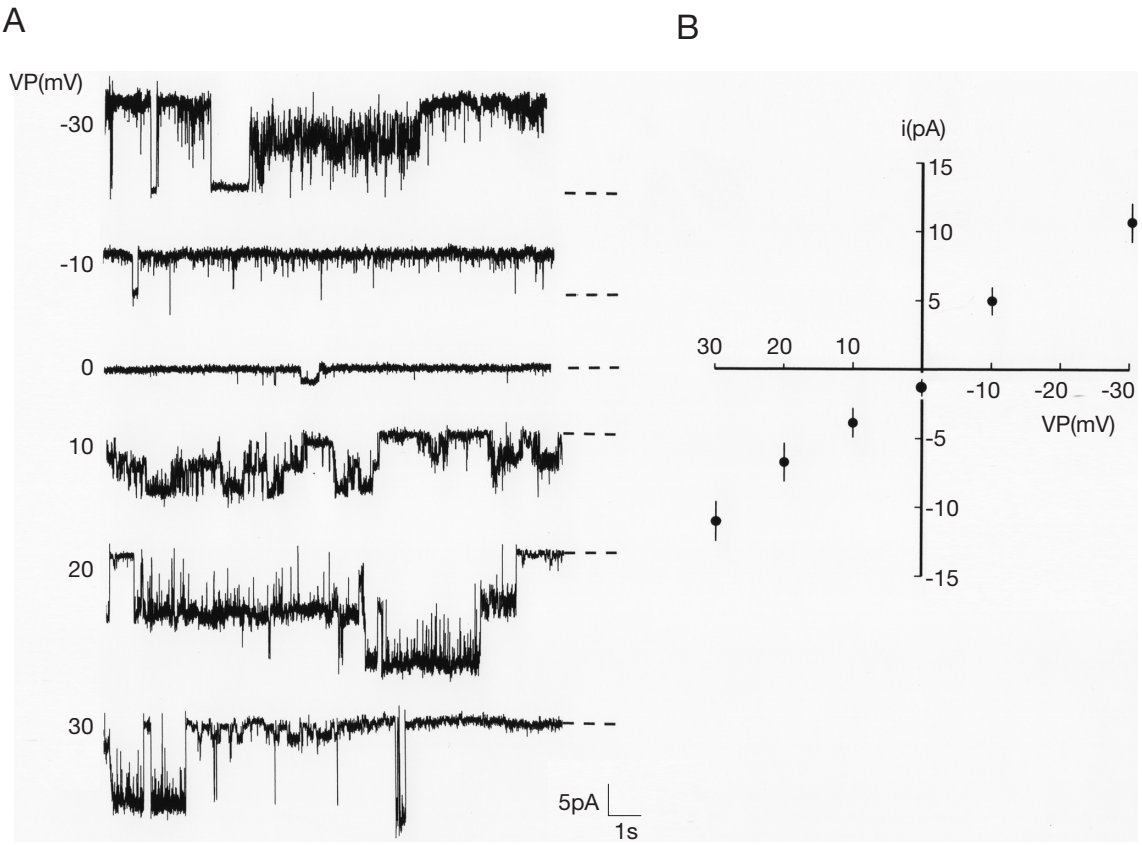

Fig. 3 Dibutyril cyclic AMP (dbcAMP) evoked-opening of high-conductance Cl^- channels.
A: Tracings were obtained under the same experimental conditions as described in Fig. 1. All tracings were obtained from the same patch at 1 min after addition of dbcAMP (0.5 mM) to the bath solution.
B: Relationship between single-channel current and pipette potential (VP). Ordinate shows the mean ± SEM (n = 6) of single-channel current amplitude.

porter was added to the bath solution prior to GA application. Next, addition of BK induced the spike (Fig. 10). To test the direct effects of furosemide on the single-Cl^- channel current, the patch membrane was excised into an outside-out configuration using a pipette containing K^+-rich solution and a bath that contained Na^+-rich solution. Furosemide application to the bath solution did not inhibit the opening of Cl^- channels (data not shown).

Experiments were performed using a neuron cultured onto astrocytes obtained from different animals. When the experiment was performed under conditions in which few neurons coexisted with astrocytes from a single animal due to incomplete centrifugal separation, a similar inhibitory result with GA was also observed (data not shown).

DISCUSSION

It has been reported that satellite- and pancake-shaped astrocytes are present in the nervous systems of various animals [16, 17]. However, these types of astrocyte are rarely observed in the mouse DRG. Although polygonal-shaped cells cultured from the DRG resemble fibroblasts, GFAP-antibody binding demonstrates that they are polygonal-shaped astrocytes.

The average membrane potential of satellite astrocytes cultured in rat DRG using the microelectrode technique is −56.5 mV [18]. The membrane potential of satellite astrocytes is more negative than that of polygonal astrocytes, and it is important to understand the reasons for these different results.

Voltage-gated Cl^- channels [19, 20] and Ca^{2+}-activated Cl^- channels [21] have been observed in the central nervous system. The present study identified high- and low-conductance Cl^- channels in the DRG. However, voltage-gated Cl^- channels and Ca^{2+}-activated Cl^- channels were not observed in the DRG.

The Cl^- channels in the DRG are likely to be transmitter operated, as these Cl^- channels were activated by glutamate. Our results suggest that glutamate induces the opening of Cl^- channels through a non-NMDA receptor, and that the Cl^- channels are activated by involvement of the cyclic AMP pathway. It has been reported that glutamate acts via a non-NMDA receptor, and that GABA opens the Cl^- channel [22]; however, there have been no reports on how the cyclic AMP pathway influences Cl^- channel opening.

GA induced depolarization of astrocyte membrane potential. The average membrane potential was about −10 mV. Cl^- equilibrium potential is 0 mV under these experimental conditions. The difference in potential may be due to the motion of other ions and imperfections in Cl^- channel opening.

ATP evoked activation of K^+ channels in the cell-attached configuration. The channel current was recorded from the patch-membrane, which was isolated from the bath solution. Ca^{2+}-activated K^+ channels have been found in cultured astrocytes from

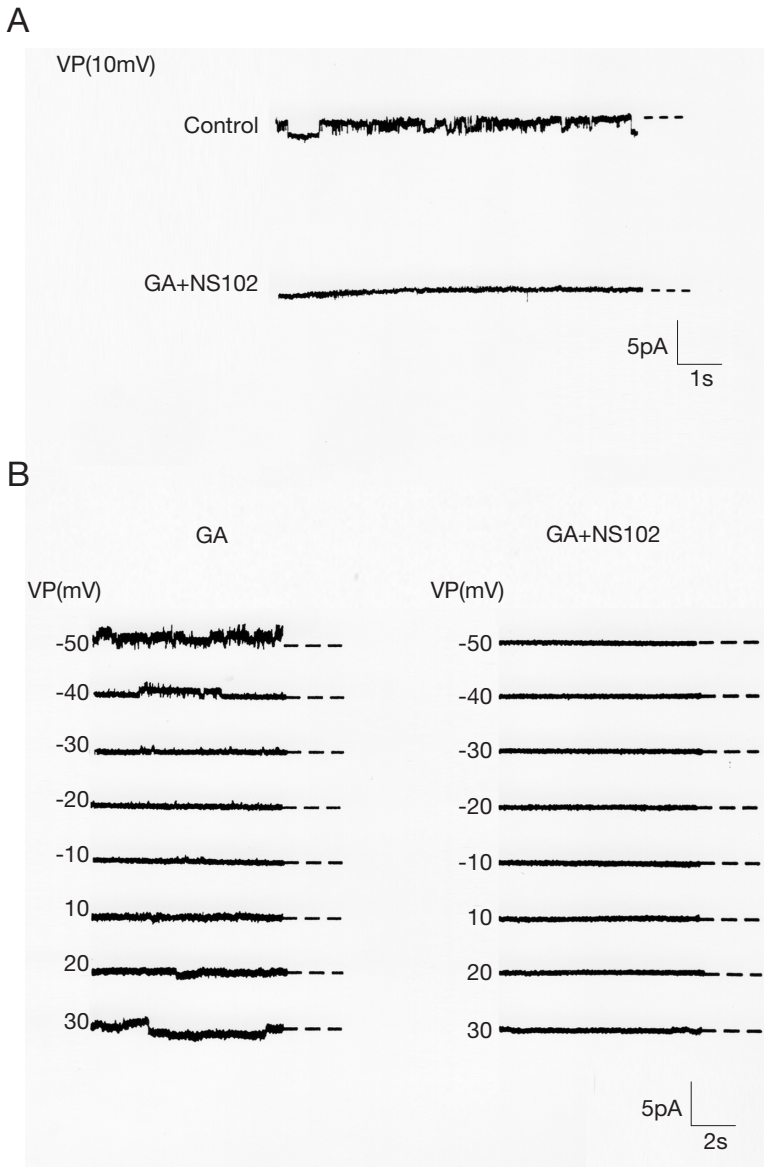

Fig. 4 Effects of non-NMDA-receptor blocker on opening of Cl^- channels.
A: Effects of non-NMDA-receptor blocker on opening of high-conductance Cl^- channels. Tracings were obtained from the cell-attached configuration under the same experimental conditions as described in Fig. 1. Unitary currents were obtained at 2 min after addition of GA to the bath (control), and addition of GA to the bath concomitantly with 10 μM NS102, a non-NMDA-receptor antagonist (GA + NS102), with respect to a pipette potential (VP) of 10 mV. B: Effects of non-NMDA-receptor blocker on opening of low-conductance Cl^- channels. Tracings were obtained from the cell-attached configuration under the same experimental conditions as described in Fig. 1. Unitary currents were obtained at 2 min after addition of GA to the bath (GA), and addition of GA concomitantly with 10 μM NS102 (GA + NS102).

neonatal rat brain [23] and cultured rat hippocampal astrocytes [24]. At present, although it is not known which messenger is directly related to the opening of the K^+ channels, those in the astrocytes in the DRG are unlikely Ca^{2+}-activated K^+ channels.

ATP-induced hyperpolarization was inhibited by application of a P_2 purinoceptor antagonist (Fig. 8). The present results suggest that astrocytes in the DRG possess a P_2 purinoceptor. Rat cortical astrocytes [25], retinal astrocytes and Müller cells [26] possess P_2 purinoceptors.

ATP induced hyperpolarization of astrocyte membrane potential. The average membrane potential was about −76 mV. K^+ equilibrium potential under these experimental conditions was −90 mV. The difference in potential may have been due to the motion of other ions.

In DRG astrocytes, ATP evokes the opening of K^+ channels, and induces K^+ efflux into the extracellular space surrounding the neuron. This could then induce an increase in excitation. The cell-attached configuration at a pipette potential (VP) of 0 mV, the astrocyte near resting potential, resulted in an inward Cl^- channel current when GA was given (Fig. 1). In these astrocytes, the transmitter induced Cl^- efflux (inward current) and Cl^- efflux, thereby further inducing inhibitory effects on neuronal excitation. It has been reported that glial cells possess a Na^+-K^+-$2Cl^-$ cotransporter [27].

Concentration of Cl^- efflux from astrocytes could not be calculated in our experiments. Our previous results showed that a 3 mV membrane potential change reduced spike frequency in neurons [28]. If the membrane potential change is 5 mV, membrane capacitance is about 1 μF/cm² and Cl^- movement is twice that of K^+ movement by the Na^+-K^+-$2Cl^-$ cotransporter, Cl^- movement is presumed to be 0.1 pM/cm². Electric charge (coulombs are converted into moles), potential and capacitance were used as described by DeVoe and Maloney [29].

The present results demonstrated that application of furosemide, a potent blocker of the cotransporter, did not induce the inhibitory effects of GA on the BK-spike. Given that Cl^- efflux stimulates the Na^+-K^+-$2Cl^-$ cotransporter and then K^+ influx via a cotransporter, together they may induce an inhibitory effect on the

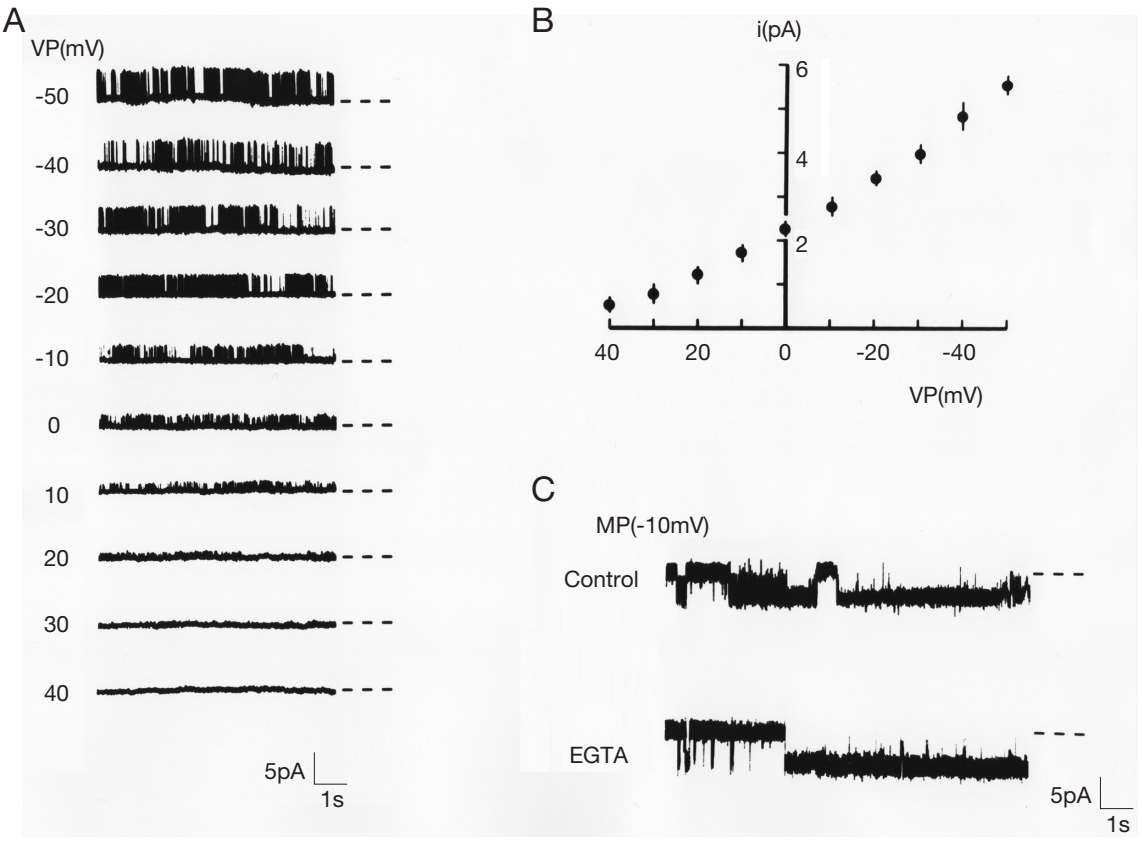

Fig. 5 ATP-evoked opening of high-conductance K^+ channels.
A: Tracings were obtained under the same experimental conditions as described in Fig. 1. All tracings were obtained from the same patch at 2 min after addition of ATP (0.25 mM) to the bath solution. B: Relationship between single-channel current and pipette potential (VP). Ordinate shows mean ± SEM (n = 6) of single-channel current amplitude. C: Tracings of single-channel current were obtained from the excised inside-out configuration using a pipette containing Na^+-rich solution with 2.5 mM Ca^{2+}, and the bath was filled with K^+-rich solution containing 5 mM EGTA without Ca^{2+}. All tracings with respect to a membrane potential (MP) of −10 mV were obtained from the same patch at 1 min after the replacement by K^+-rich solution with EGTA.

neuron.

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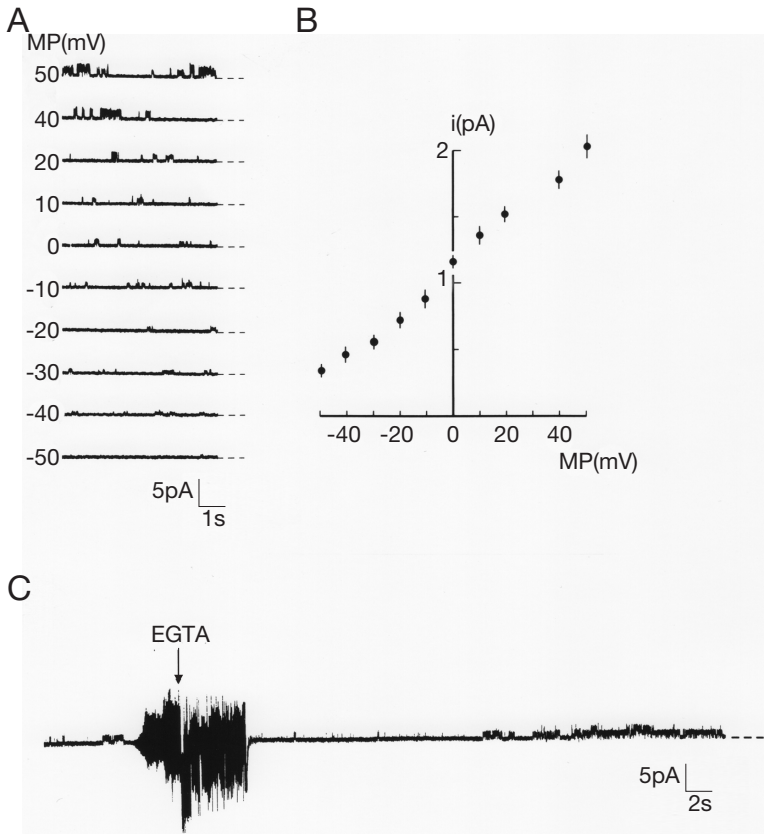

Fig. 6 ATP evoked-opening of low-conductance K^+ channels.
A: Tracings were obtained from the outside-out configuration using a pipette containing K^+-rich solution, and the bath was filled with Na^+-rich solution with 2.5 mM Ca^{2+}. Unitary currents with respect to membrane potential (MP) were obtained from the same patch. B: Relationship between single-channel current amplitude and membrane potential (MP). Ordinate shows the mean ± SEM (n = 5) of single-channel current amplitude. C: Tracing of single-channel current was obtained from the excised inside-out configuration using a pipette containing Na^+-rich solution with 2.5 mM Ca^{2+}, and the bath was filled with K^+-rich solution containing 5 mM EGTA without Ca^{2+}. Membrane potential was held at 0 mV.

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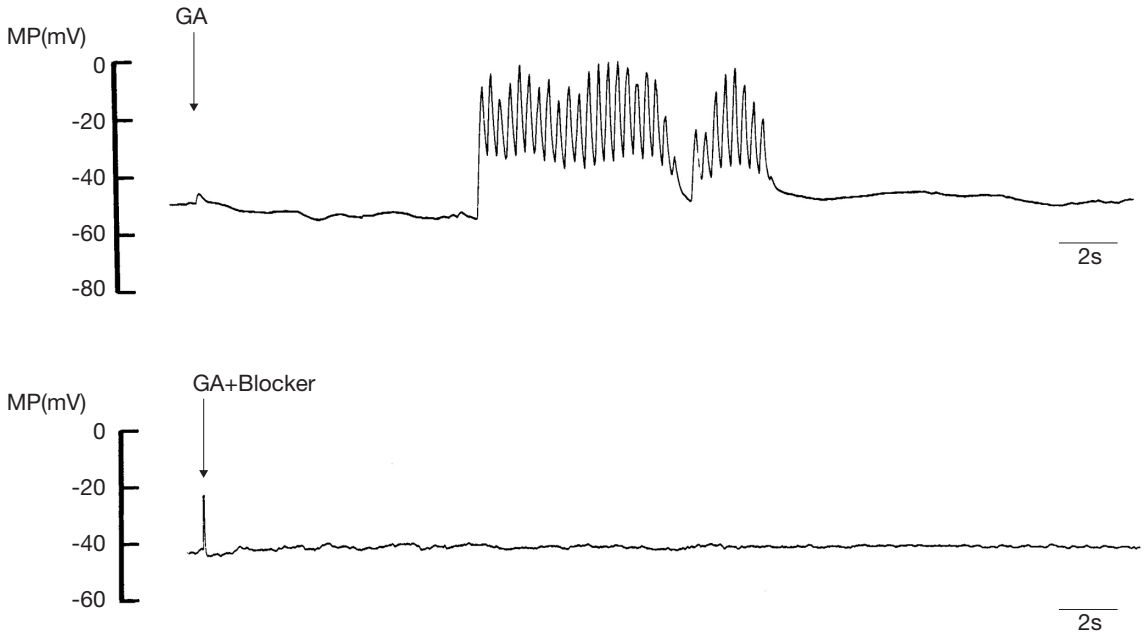

Fig. 7 Response of membrane potential to addition of GA.
Whole-cell configuration using K^+-rich solution (145 mM Cl^-) that included 0.5 mM EGTA; current-clamp voltage recordings were obtained. The membrane potential pattern is shown after addition of GA and after addition of GA concomitantly with NS102 (10 μM), a non-NMDA receptor antagonist (GA + Blocker).

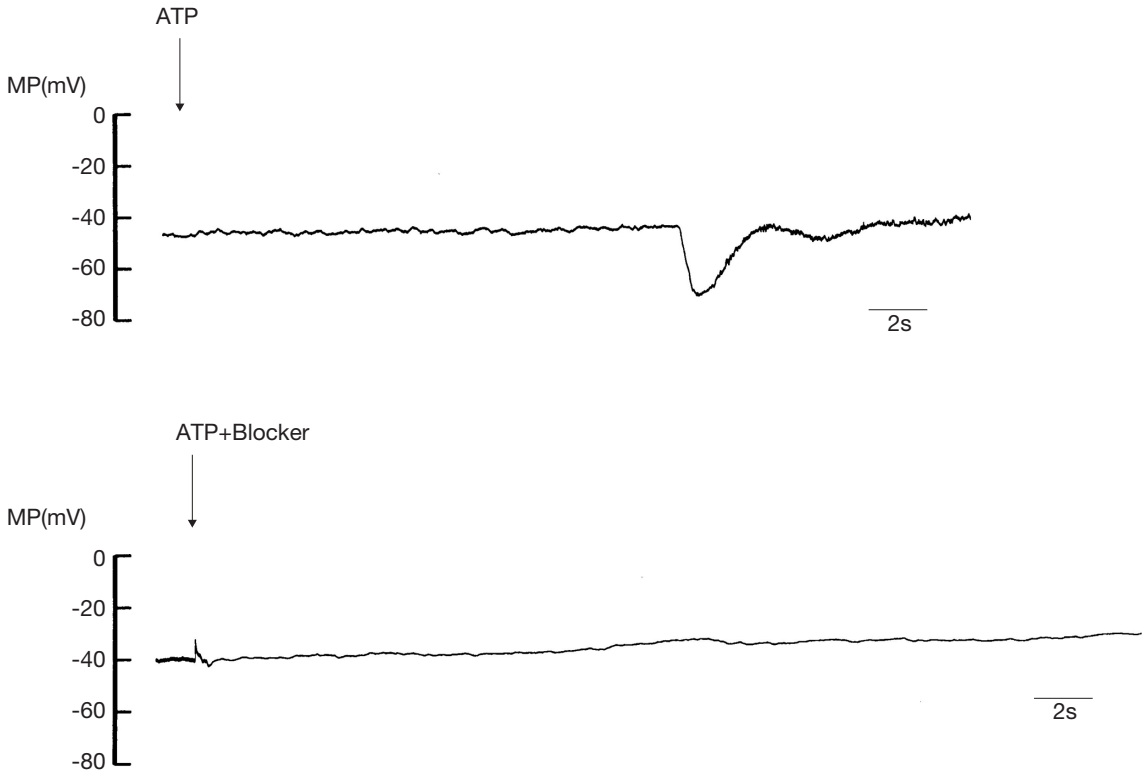

Fig. 8 Response of membrane potential to addition of ATP.
Recording methods were the same as described in Fig. 7. Membrane potential pattern is shown after addition of ATP and after addition of ATP concomitantly with pyridoxal phosphate-6-azo-tetrasodium salt (4 μM), a P_2 purinoceptor antagonist (ATP + Blocker).

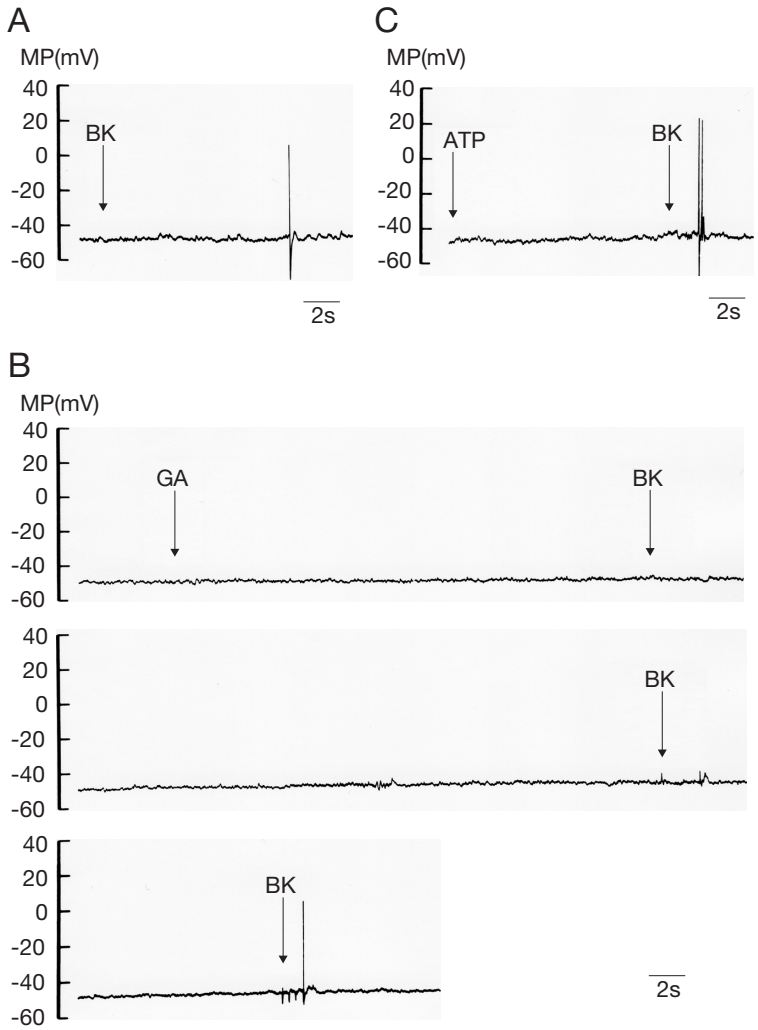

Fig. 9 Response of neuron spike to stimulation by bradykinin (BK).
Whole-cell configuration using a K^+-rich solution that included 0.5 mM EGTA; current-clamp voltage recordings were obtained. A: Tracing was obtained after BK (6 μM) application to bath solution. B: All tracings were obtained from the same cell and a continuous recording. When GA was added to the bath solution prior to BK, and BK was again added to the bath, no BK spike was observed. Second application of BK also did not evoke a spike. Third application of a high concentration of BK (30 μM) evoked a small depolarization. C: Effects of ATP application on BK-evoked spike. Prior to application of BK, 0.25 mM ATP was added to the bath solution.

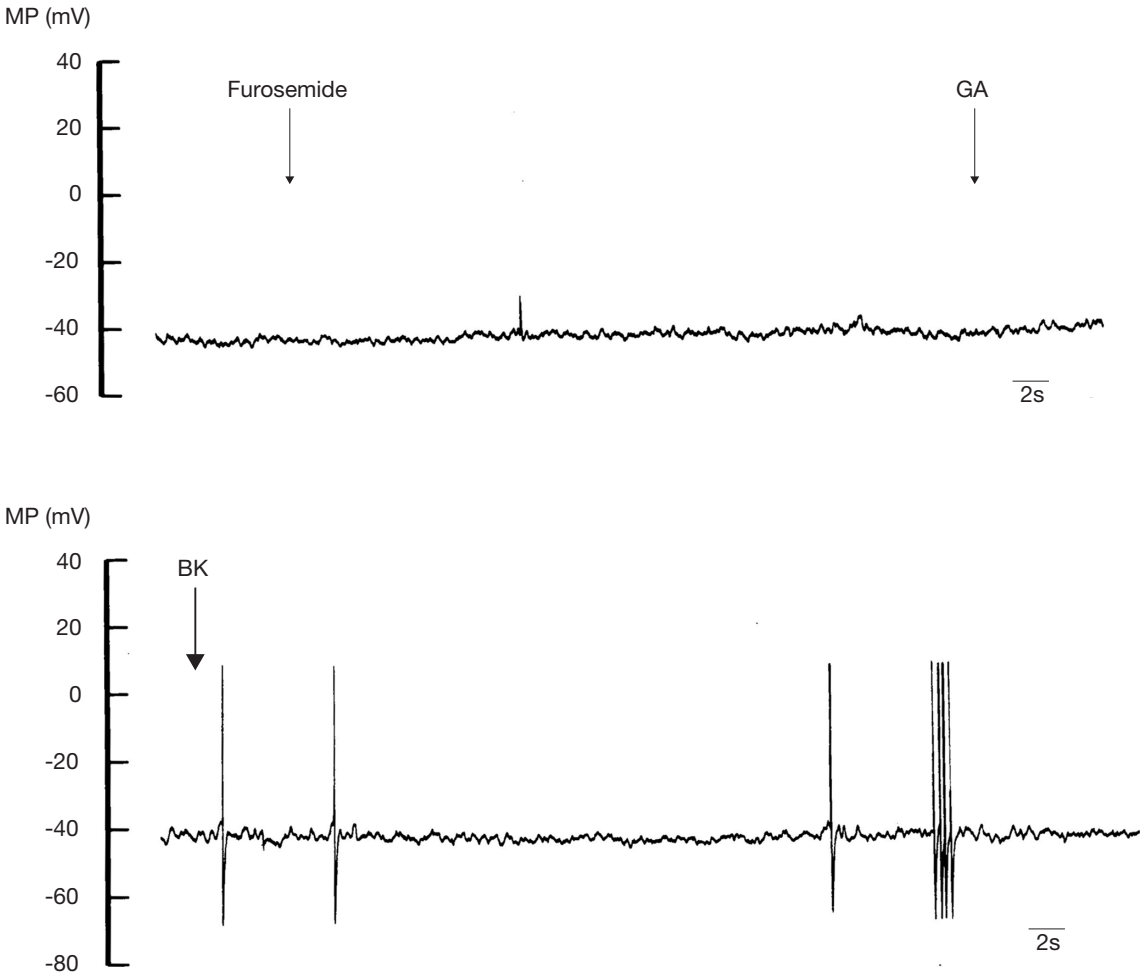

Fig. 10 Effects of furosemide on GA induced-inhibition of BK spike.
Recording methods were the same as described in Fig. 9. All tracings were obtained from the same cell and a continuous recording. When furosemide (0.5 mM), a diuretic and a potent blocker of the Na^+-K^+-Cl^- cotransporter, was added to the bath solution prior to GA application, stimulation with BK evoked a spike.