ATP-sensitive K+ channels and cellular actions of morphine in periaqueductal gray slices of neonatal and adult rats

Lih-Chu Chiou and Cheng-Hung How

Department of Pharmacology, College of Medicine, National Taiwan University, Taipei, Taiwan

Send reprint requests to:
Dr. Lih-Chu Chiou,
Department of Pharmacology, College of Medicine, National Taiwan University, No. 1, Jen-Ai Rd., Section 1, Taipei 100, Taiwan.
FAX: 886-22-341-4788
E-mail: lcchiou@ha.mc.ntu.edu.tw

Running title: K\textsubscript{ATP} channels and morphine action in the periaqueductal gray

Number or text pages: 34
Number of tables: 1
Number of figures: 9
Number of references: 40
Number of words in the Abstract: 241
Number of words in the Introduction: 422
Number of words in the Discussion: 1074

Abbreviation:
ACSF, artificial cerebral spinal fluid; EPSCs, excitatory postsynaptic currents; i.c.v., intracerebroventricular; I\textsubscript{hold}, holding current; IPSCs, inhibitory postsynaptic currents; K\textsubscript{ATP} channels, ATP-sensitive potassium channels; PAG, periaqueductal gray; QX-314, lidocaine N-ethyl bromide.

Abstract
ATP-sensitive K+ (K\textsubscript{ATP}) channels were reported to be involved in morphine analgesia \textit{in vivo}. This study, using patch clamp technique in brain slices of neonatal (P12-P16) and adult rats, investigated cellular actions of K\textsubscript{ATP} channel ligands and their interactions with morphine in the ventrolateral periaqueductal gray (PAG), a crucial site for morphine analgesia. In neonatal PAG neurons, morphine depressed evoked inhibitory postsynaptic currents (IPSCs) by in almost all neurons and elicited an inwardly rectifying K+ current in one third of recorded neurons. Glibenclamide (1-10 µM), a K\textsubscript{ATP} channel blocker, did not affect the membrane current or synaptic current \textit{per se} but also failed to affect the effects of morphine. No outward current was elicited upon using microelectrodes containing ATP-free internal solution. In adult PAG neurons, morphine, up to 300 µM, failed to activate K+ current in all 25 neurons recorded but depressed IPSCs to a comparable extent as that in neonatal neurons. Glibenclamide also did not affect the latter action of morphine. The openers of K\textsubscript{ATP} channels, lemakalim (10-30 µM) and diazoxide (10-500 µM), unlike morphine, did not increase membrane current of both neonatal and adult PAG neurons. However, in hippocampal CA1 neurons, diazoxide induced a glibenclamide-sensitive outward current. It is concluded that K\textsubscript{ATP} channels display little functional role \textit{per se} and might not be involved in morphine actions in the ventrolateral PAG. The correlation between the age-dependent susceptibility in K+ channel activation and the ontogeny of morphine analgesia was discussed.

Introduction
It has been reported since 1956 (Davis et al.) that insulin affected morphine-induced antinociceptive response. Singh et al. (1983) further substantiated that blood glucose level affected morphine analgesia and
the effect was due to lower level of intracellular ATP. After ATP-sensitive K⁺ (K$_{ATP}$) channels were explored in the brain (Bernardi et al., 1988), several in vivo studies suggested that K$_{ATP}$ channels are involved in morphine-induced supraspinal analgesia. Glibenclamide, a K$_{ATP}$ channel blocker, when administered intracerebroventricularly (i.c.v.), dose-dependently decreased the antinociceptive response of morphine administered either intravenously (Ocana et al., 1990; Roane and Boyd, 1993) or i.c.v. (Narita et al., 1992). This reversal of morphine analgesia was substantiated by using other K$_{ATP}$ channel blockers but not nonselective K⁺ channel blockers, tetraethylammonium (TEA) or 4-aminopyridine (Ocana et al., 1993; Ocana et al., 1995). The potencies of K$_{ATP}$ channel blockers in reversing morphine analgesia were correlated to their blocking activities of K$_{ATP}$ channels (Ocana et al., 1993). On the other hand, K$_{ATP}$ channel openers were found to potentiate the analgesic effect of morphine (Vergoni et al., 1992; Narita et al., 1993; Lohmann and Welch, 1999). In addition, glibenclamide (i.c.v.) also decreased the analgesic effect induced by other opiates including levorphanol, methadone and buprenorphine (Ocana et al., 1995; Raffa and Martinez, 1995).

Nevertheless, no in vitro study substantiated the possible correlation of K$_{ATP}$ channels and morphine actions at cellular level of pain-related brain regions. Given that the ventrolateral periaqueductal gray (PAG) is a crucial site of morphine-induced supraspinal analgesia (Yaksh et al., 1976), it is interesting to investigate the interactions of K$_{ATP}$ channel blockers or openers with morphine in the ventrolateral PAG. The cellular mechanism for morphine-induced supraspinal analgesia is attributed mostly to an inhibition of inhibitory synaptic transmission and partly to membrane hyperpolarization resulting from an activation of inwardly rectifying K⁺ channels (Osborne et al., 1996; Vaughan and Christie, 1997; Chiu and Huang, 1999). Therefore, effects of morphine in the ventrolateral PAG were assessed by these two actions. The possible functional roles of K$_{ATP}$ channels in the ventrolateral PAG were also explored by investigating the effects of K$_{ATP}$ ligands alone and their interactions with glibenclamide.

It has been reported that the density of K$_{ATP}$ channels is higher in adult rats than that in neonates (Mourre et al., 1990; Xia and Haddad, 1991). We therefore examined interactions of K$_{ATP}$ channel ligands with morphine in ventrolateral PAG neurons from both neonatal and adult rats. Given that there is ontogeny in morphine-induced antinociception, we also explored the possible ontogeny of cellular effects of morphine in the ventrolateral PAG.

Materials and Methods

Brain slice preparations

Coronal brain slices (400 µm) containing the PAG were dissected as previous report (Chiou and Chou, 2000) from Wistar rats at the age of 12-16 days (neonates) or 8-12 weeks (adults). In some experiments, transverse hippocampal slices of 400 µm from adult rats were used. After equilibrium, slices were transferred to a submerged chamber and perfused with artificial cerebral spinal fluid (ACSF) at 2-3 ml min⁻¹. ACSF contained (mM) NaCl 117, KCl 4.5, CaCl$_2$ 2.5, MgCl$_2$ 1.2, NaH$_2$PO$_4$ 1.2, NaHCO$_3$ 25 and dextrose 11.4 and was oxygenated with 95% O$_2$ plus 5% CO$_2$.

Electrophysiological recordings

Blind patch clamp whole cell recordings were conducted in ventrolateral neurons of the PAG or in CA1 pyramidal neurons of the hippocampus at 30 ± 1°C. The resistance of patch microelectrodes was 4-8 MΩ. The input resistance was monitored with the
MEMBRANE TEST function of Clampex 7.0 (Axon Instrument, Foster City, CA) by applying small hyperpolarization pulses (-3 mV).

Membrane currents were recorded with an Axopatch 200A amplifier (Axon Instrument, Foster City, CA) and acquired with a Pentium III computer installed with pClamp 7 software (Axon instrument) through a DigiData 1200A A/D converter (Axon instrument). The signal was filtered at 2-5 kHz and sampled at 10 kHz. When investigating the inwardly rectifying K+ current, the hyperpolarization ramp protocol was used as reported previously (Chiou, 2001). Briefly, neurons were held at −70 mV and hyperpolarization ramp commands from −60 mV to −140 mV at the rate of 0.2 mV/ms were applied every 30 s. Membrane currents elicited by voltage ramps were recorded simultaneously with a chart recorder (Gould 3200, Gould Electronics, Valley View, OH) at a very low frequency response (10K) to monitor the time course of drug effects.

Synaptic currents were evoked and recorded as previous report (Chiou and Chou, 2000). Square pulses of 0.05 – 0.1 ms were applied at 0.03 Hz from a Grass S88 stimulator (Quincy, MA) with a bipolar concentric stimulating electrode (50 µm in diameter, FHC, Brunswick, ME). The stimulation electrode was placed 125 ~ 375 µm away from the recording electrode to focally stimulate the afferent fibers. Excitatory postsynaptic currents (EPSCs) were recorded at −72 mV, which is close to the reversal potential of GABA_A receptor-mediated inhibitory postsynaptic currents (IPSCs), the major IPSCs in the ventrolateral PAG (Chiou and Chou, 2000). IPSCs were recorded in the presence of 1 mM kynurenic acid, an ionotropic glutamate receptor antagonist.

The normal internal solution contained (in mM): K+-gluconate 125, KCl 5, CaCl_2 0.5, BAPTA 5, HEPES 10, MgATP 5 and GTPtris 0.33. In certain experiments, microelectrodes were filled with ATP-free internal solution in which MgATP was omitted. To improve the space clamp efficiency when synaptic currents were recorded, the internal solution was changed to (in mM): Cs+-gluconate 110, TEA chloride 5, lidocaine N-ethyl bromide (QX-314) 5, CaCl_2 0.5, BAPTA 5, Hepes 10, MgATP 5 and GTPtris 0.33. The liquid junction potentials of -11, -15 and -7 mV, respectively, were corrected for normal, Cs+-gluconate and ATP-free internal solutions.

**Data analysis and Chemicals**

The effect of drugs was measured at steady state as previous report (Chiou, 2001). Drug-induced shifting of holding current (I_hold) and percent increase of membrane current at -140 mV (I_-140) were calculated. The increment of membrane current was normalized to the control current in each individual neuron. Data were expressed as mean ± S.E.M. The n number is the number of neurons recorded, each of which was taken from one slice from different rats. Usually, 2-3 slices of caudal PAG were used from one rat. For a comparison, sequential treatments were applied in the same neuron, if possible. The Student’s t-test was used for statistical analysis.

Bicuculline methiodide, kynurenic acid, QX-314, glibenclamide and diazoxide were purchased from Sigma (St. Louis, USA). Morphine chloride was purchased from National Bureau of Controlled Drugs (Taipei, R.O.C.). Lemakalim is a generous gift from Dr. C. Y. Cheng (School of Pharmacy, National Taiwan University). Glibenclamide, lemakalim and diazoxide were dissolved in dimethylsulfoxide and other drugs were in deionized water as stock solutions. Kynurenic acid was dissolved in ACSF directly before use.

All experiments conformed to the guidelines of the IACUC of National Taiwan University, College of
Results

Part A  PAG neurons from neonatal rats

First, we performed experiments in PAG slices isolated from neonatal rats, which are better preparations for a successful whole cell recording than those from adult rats (Plant et al., 1995).

Glibenclamide alone had no effect on evoked synaptic currents of neonatal neurons

Glibenclamide, a K<sub>ATP</sub> channel blocker, at concentrations of 1-10 µM, affected neither EPSCs nor IPSCs (Fig. 1). The amplitude of EPSCs in the presence of 10 µM glibenclamide was 97 ± 4 % of the control (n=8) and that of IPSCs was 99 ± 3 % of the control (n=7). The holding currents (dotted lines in Fig. 1) recorded with microelectrodes containing ATP-free internal solution were also not affected by glibenclamide. They were –60.2 ± 6.6 pA vs. –55.9 ± 5.6 pA (n=8) and –2.7 ± 1.9 vs. –1.9 ± 1.4 pA (n=7), respectively, when EPSCs and IPSCs were recorded.

Glibenclamide failed to affect morphine-induced inhibition of IPSCs

Morphine concentration-dependently decreased the amplitude of IPSCs, which were recorded in the presence of 1 mM kynurenic acid (Fig. 2), an ionotropic glutamate receptor blocker. The magnitude of inhibition by 50 µM morphine was 44 ± 6 % (n=5). Further addition of 10 µM glibenclamide did not affect the inhibition induced by morphine (Fig. 2). Increasing the concentration of glibenclamide or decreasing that of morphine also did not display any antagonism (Fig. 2).

Glibenclamide failed to affect morphine-induced activation of inwardly rectifying K<sup>+</sup> channels

In 7 out of 27 neurons, morphine increased the membrane current elicited by a hyperpolarization ramp from –60 mV to –140 mV and shifted the holding current outwardly (Fig. 3). The current increased by morphine was characterized with inward rectification and reversed at –86 mV which is close to the equilibrium potential of K<sup>+</sup> ions, being –91 mV, according to Nernst equation. Therefore, the current induced by morphine is an inwardly rectifying K<sup>+</sup> current. The current at –140 mV was increased by 50 µM morphine to 135 ± 12 % of control (n=7) (Fig. 4). In morphine-sensitive neurons, further treatment with 10 µM glibenclamide did not show any further change of the membrane current (Fig. 3, 4). However, naloxone (1 µM) effectively antagonized the effect of morphine (Fig. 3).

K<sub>ATP</sub> channel openers had no effect on neuronal membrane current

The negative result of glibenclamide on membrane current might be due to that the K<sub>ATP</sub> channels, if any, in our recording condition have been blocked by intracellular ATP. Therefore, we further investigated the action of K<sub>ATP</sub> channel openers. The results show that neither lemakalim (10-30 µM) nor diazoxide (100-500 µM) affected the membrane current elicited by hyperpolarization ramp or the holding current (Fig. 4, open bars). Further addition of glibenclamide, 10 µM, also failed to produce any effect (Fig. 4, filled bars).

Effect of ATP-free internal solution on the holding current

Further experiments were conducted with microelectrodes containing ATP-free internal solution to see if K<sub>ATP</sub> channels could be disclosed by washing
out the intracellular ATP. Figure 5 shows that, after whole cell configuration was formed, the holding current recorded with ATP-free internal solution was shifted inwardly but not outwardly.

Part B. PAG neurons from adult rats

It has been reported from immunohistochemical studies that the density of K\textsubscript{ATP} channels increases age-dependently (Mourre et al., 1990; Xia and Haddad, 1991). To examine if the ineffectiveness of K\textsubscript{ATP} ligands is attributed to low density of K\textsubscript{ATP} channels in neonatal PAG neurons, further experiments were performed in neurons from adult rats of 8-12 weeks.

Neither K\textsubscript{ATP} channel ligands nor morphine affect the membrane current of adult PAG neurons

In adult ventrolateral PAG neurons, diazoxide at concentrations up to 500 µM, alone or in combination with glibenclamide, affected neither the membrane current elicited by hyperpolarization ramp nor the holding current (Fig. 6). Interestingly, morphine, at concentration of 50 µM which caused one third of neonatal PAG neurons hyperpolarization, failed to affect membrane currents in all neurons recorded (n=9). When the concentration of morphine was increased to 100 µM in 7 neurons or to 300 µM in 9 neurons, membrane currents were still not affected (Fig. 6). No effect was observed by further addition of glibenclamide. However, in those morphine-resistant neurons, baclofen, a GABA\textsubscript{B} receptor agonist, increased the membrane current reversibly (Fig. 6,7). The current increased by baclofen reversed at potential near the equilibrium potential of K\textsuperscript{+} ions and was characterized with inward rectification (Fig. 7).

Glibenclamide failed to affect morphine-induced inhibition of IPSCs in adult rats

To see if morphine sensitivity is generally lower in adult neurons, the effect of morphine on IPSCs of adult PAG neurons was examined. In all of five neurons recorded, IPSCs recorded in the presence of 1 mM kynurenic acid were depressed by 50 µM morphine (Fig. 8). The magnitude of inhibition is 41 ± 5 % (n=5), which is comparable to the magnitude, 44 ± 6 % (n=5), obtained in neonatal neurons (Fig. 2). Further addition of 10 µM glibenclamide did not make further change in IPSCs (Fig. 8)

Part C. Hippocampal neurons from adult rats

K\textsubscript{ATP} channel ligands did affect the membrane current of hippocampal CA1 neurons

To see if the K\textsubscript{ATP} channel ligands used in our recording system are valid, we examined their effects on hippocampal CA1 neurons where K\textsubscript{ATP} channel openers were reported to induce membrane hyperpolarization (Fujimura et al., 1997). In contrast to the negative finding in PAG neurons, diazoxide (500 µM) induced an outward current, which was reversed by glibenclamide (10 µM) in hippocampal CA1 neurons (Fig. 9).

Discussion

In the present study, we demonstrated that: 1) In neonatal ventrolateral PAG neurons, morphine depressed the inhibitory synaptic transmission and activated inwardly rectifying K\textsuperscript{+} channels; 2) In adult PAG neurons, morphine failed to activate K\textsuperscript{+} channels in all recorded neurons but produced a comparable inhibition of IPSC as that in neonatal neurons; 3) Glibenclamide, a K\textsubscript{ATP} channel blocker, did not affect the effects of morphine in both neonatal and adult neurons; 4) Neither openers nor blockers of K\textsubscript{ATP} channels affected the synaptic transmission or
membrane current of ventrolateral PAG neurons from both neonatal and adult rats.

**Little functional role of K\textsubscript{ATP} channels in the ventrolateral PAG**

\( K_{\text{ATP}} \) channels are inhibited by intracellular ATP at the concentration as low as 100 \( \mu \text{M} \) (Ashcroft and Kakei, 1989) which is far lower than the intracellular concentration of ATP in our recording condition. This might explain the failure in obtaining any effect of glibenclamide alone on the membrane current. However, the openers of \( K_{\text{ATP}} \) channels, either lemakalim or diazoxide, also did not have any effect on the membrane current. Although lemakalim was reported to be less effective in neuronal \( K_{\text{ATP}} \) channels (Stanford and Lacey, 1996; Schwanstecher and Bassen, 1997), diazoxide was effective in several central neurons (Ben-Ari et al., 1990; Hausser et al., 1991; Fujimura et al., 1997). It caused membrane hyperpolarization in both substantial nigra (Hausser et al., 1991) and hippocampal (Fujimura et al., 1997) neurons. In pancreatic β cells, the opening of \( K_{\text{ATP}} \) channels by diazoxide was hindered if intracellular concentration of ATP was higher than 3 mM (Trube et al., 1986) and intracellular MgADP is essential for the activation of \( K_{\text{ATP}} \) channels (Larsson et al., 1992). It is not clear if these requirements are also applied to neuronal \( K_{\text{ATP}} \) channels. Nevertheless, using the same recording condition, we demonstrated that diazoxide induced a glibenclamide-sensitive outward current in hippocampal CA1 neurons, as reported by Fujimura et al. (1997). Therefore, the negative result of diazoxide in the PAG is not due to a possible limitation from whole cell recording technique, a dialysis of intracellular MgADP, neither due to a deterioration of \( K_{\text{ATP}} \) channel ligands used.

In the substantia nigra, a glibenclamide-sensitive outward current was gradually elicited when whole cell recording was performed with microelectrodes containing ATP-free internal solution (Hausser et al., 1991; Stanford and Lacey, 1996). However, we failed to obtain an outward shifting of holding current with ATP-free internal solution in PAG neurons. Instead, the holding current was shifted inwardly, which might be resulted from a decrease of \( \text{Na}^+\text{-K}^+\text{-ATP} \) pump activity due to a deprivation of intracellular ATP. Therefore, the negative finding of \( K_{\text{ATP}} \) channel ligands in ventrolateral PAG neurons is unlikely resulted from a pharmacological resistance in this area. It is, therefore, suggested that the \( K_{\text{ATP}} \) channels play no significant role in the regulation of resting membrane potential of ventrolateral PAG neurons.

It was reported that the density of \( K_{\text{ATP}} \) channels increases age-dependently (Mourre et al., 1990; Xia and Haddad, 1991). Nevertheless, the failure in obtaining any positive result with \( K_{\text{ATP}} \) channel ligands in either neonatal or adult neurons suggests that the density of \( K_{\text{ATP}} \) channels in the ventrolateral PAG is too low to have any functional role.

***K\textsubscript{ATP} channels are not involved in the effects of morphine in the ventrolateral PAG***

Several *in vivo* studies demonstrated that i.c.v. injection of glibenclamide decreased morphine-induced analgesia (Narita et al., 1992; 1993; Ocana et al., 1990; 1993; 1995; Roane and Boyd, 1993; Raffa and Martinez, 1995). Nevertheless, no antagonism by glibenclamide of morphine-induced effects was observed *in vivo* in the ventrolateral PAG from either neonatal or adult rats, even though morphine exerted different actions on them. Therefore, the site of action for glibenclamide antagonism of morphine analgesia might be at the areas other than the ventrolateral PAG where the density of \( K_{\text{ATP}} \) channel is moderate.
(Treheren and Ashford, 1991; Dunn-Meynell et al., 1998). Alternatively, the findings of K_{ATP} channel ligands in nociceptive studies in vivo might be due to mechanism(s) distinct from K_{ATP} channel activation (Lohmann and Welch, 1999).

**Different proportions of neurons hyperpolarized by morphine or µ-opioids**

The findings that morphine inhibits the inhibitory synaptic transmission and activates the inwardly rectifying K^+ channels in the ventrolateral PAG is in agreement with previous experiments using µ-opioid peptides (Chieng and Christie, 1994a; Chieng and Christie, 1994b; Vaughan and Christie, 1997; Chiou and Huang, 1999; Han et al., 1999; Chiu, 2001). However, the proportion of neurons which were hyperpolarized by morphine or µ-opioid peptides is different (Tab. 1). It seems that less neurons sensitive to morphine than to µ-opioid peptides in either neonatal or adult neurons (Tab. 1). It is not known if this lower incidence of morphine response is due to the partial agonist property of morphine (Osborne et al., 2000). Differences have been reported in the phosphorylation, internalization or desensitization of µ-opioid receptors activated by µ-opioid peptides or alkaloids (Law et al., 2000). It remains to be elucidated if these differences between morphine and DAMGO can explain the different susceptibility found in the ventrolateral PAG.

**Ontogeny of morphine actions in the ventrolateral PAG**

The finding that none of adult neurons is hyperpolarized by morphine is interesting. In morphine-resistant adult neurons, baclofen did activate the inward rectifying K^+ channels, which shares the same G protein-coupled cascade with µ-opioids (Christie and North, 1988). Therefore, the insensitivity to morphine is not attributed to a defect in the signaling(s) downstream to receptor activation in adult neurons. Interestingly, for µ-opioid peptides, the population of sensitive neurons is also higher in the neonates (Tab. 1). The reason for this discrepancy among ages is not clear. Since morphine induced a comparable inhibition of synaptic transmission in neonatal and adult PAG neurons, it is unlikely that there is a global difference between adult and neonatal neurons in opioid receptor density or receptor coupling efficacy. The possibility remains to be elucidated that a developmental shift occurs in the pre- or post-synaptic site in the distribution of µ-opioid receptors, G-protein activation efficacy or G-protein-coupled receptor kinase activity.

Morphine analgesia was found to be more profound in neonatal rats than the adults (Auguy-Valette et al., 1978; Windh and Kuhn, 1995). It was suggested to be not solely due to the poor blood-brain barrier in the neonates (Windh and Kuhn, 1995). The present finding that morphine is effective in both pre- and post-synaptic sites of neonatal PAG neurons but only in the presynaptic site of adult neurons might be one of the contributors to the ontogeny of morphine analgesia.

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Acknowledgement
This work was supported by the grant NSC 89-2320-B-002-273 from National Science Council, ROC (L.C.C.).

Fig 1. Effects of glibenclamide on evoked synaptic currents in neonatal ventrolateral PAG neurons
IPSCs (A) and EPSCs (B) were evoked at 0.03 Hz by focal stimulation in the absence (left) or presence of 10 \(\mu M\) glibenclamide (right).

IPSCs were recorded at –63 mV in the presence of 1 mM kynurenic acid. EPSCs were recorded at –73 mV. Note that the holding current (dotted line) was not changed by glibenclamide. ATP-free internal solution was used.

Fig. 2 Effect of glibenclamide on morphine-induced inhibition of IPSCs in neonatal neurons
IPSCs were recorded at~15 mV in the presence of 1 mM kynurenic acid. (A): Traces of IPSCs were recorded from a neuron in control, 50 \(\mu M\) morphine and 50 \(\mu M\) morphine plus 10 \(\mu M\) glibenclamide. (B): Summary of the amplitude of IPSCs, normalized to the control, in the presence of morphine (open bars) and morphine plus glibenclamide (filled and striped bars). Figures in the parentheses indicate the number of neurons, each of which was treated with morphine and further with 10 or 30 \(\mu M\) glibenclamide. \(\text{Cs}^+\)-gluconate internal solution was used. * \(p < 0.05\) vs. control.

Fig.3 Effect of glibenclamide on the activation of inward rectifier by morphine in a neonatal neuron
Membrane currents were elicited by hyperpolarization ramps (B, inset) from –60 mV to –140 mV for 400 ms every 30 s. Holding potential was –70 mV. (A): The chart recording of membrane currents elicited by voltage ramps in a neuron treated with 50 \(\mu M\) morphine, followed by further treatment with 10 \(\mu M\) glibenclamide and further with 1 \(\mu M\) naloxone. (B, left): I-V curves of membrane currents elicited by voltage ramps in control, 50 \(\mu M\) morphine and 50 \(\mu M\) morphine plus 10 \(\mu M\) glibenclamide. (B, right): I-V curves of morphine-induced currents, that were obtained
by subtracting the current in the presence of morphine from that of the control, in the absence or presence of glibenclamide. Note that the current-induced by morphine reversed at –86 mV which is close to the equilibrium potential of K⁺ ion and characterizes with inward rectification. Normal K⁺-gluconate internal solution.

Fig. 4 Statistical analysis of the interaction of glibenclamide with morphine, lemakalim and diazoxide on voltage ramp-elicited membrane currents in neonatal neurons
Membrane currents were elicited by the same protocol as those in figure 3. Shown are the effects of morphine (50 µM), lemakalim (10–30 µM) and diazoxide (100 ~ 500 µM) in the absence (open bars) or presence of 10 µM glibenclamide (filled bars). (A): Changes of holding currents (I_{hold}) were denoted as positive for outward shifting and as negative for inward shifting. B: Changes in membrane currents at –140 mV (I_{-140}) were expressed as the percentage of control current at –140mV. Insets: (Left): I-V curves of membrane currents in control, 30 µM lemakalim and 30 µM lemakalim plus 10 µM glibenclamide. (Right): I-V curves of membrane currents in control, 500 µM diazoxide and 500 µM diazoxide plus 10 µM glibenclamide. Figures in parentheses are the number of neurons. For each group of bars, sequential treatments were applied in the same neuron, if possible. * p < 0.05 vs. control.

Fig. 5 Changes of holding currents after neurons were dialyzed with normal or ATP-free internal solution in neonatal neurons
The changes of holding current (I_{hold}) after whole-cell configuration was formed were recorded with microelectrodes filled with K⁺-gluconate internal solution with 5 mM ATP (filled circles, n=5) or without ATP (open circles, n=5). The abscissa is the time after whole cell configuration was obtained.

Fig. 6 Statistical analysis of the effects of morphine, diazoxide and baclofen on membrane currents and their interactions with glibenclamide in adult neurons
Membrane currents were elicited by the same protocol as those in figure 3. Doublet bars: Neurons were treated with morphine (50 ~ 300 µM) and 500 µM diazoxide in the absence (open bars) or presence of glibenclamide (filled bars). Triplet bars: Neurons were treated with 50 µM morphine (vertical strips), 3 µM baclofen (oblique strips) and washout (horizontal strips). Changes of I_{hold} (A) or I_{-140} (B) were calculated and expressed as those in figure 4. * p < 0.05 vs. control.

Fig. 7 Inwardly rectifying K⁺ current induced by baclofen but not morphine in an adult neuron
Membrane currents were elicited by the same protocol as those in figure 3. (A): The chart recording from a neuron which was insensitive to 300 µM morphine but responsive to 3 µM baclofen. (B): I-V curves of membrane currents (left) elicited by voltage ramp in control, 300 µM morphine and 3 µM baclofen. Baclofen-induced current (right) was obtained by subtracting the current in the presence of baclofen from that of the control.

Fig. 8 Effect of glibenclamide on morphine-induced inhibition of IPSCs in adult neurons
IPSCs were recorded at –50 mV in the presence of 1 mM kynurenic acid. (A): Traces of IPSCs from a neuron in control, 50 µM morphine and 50 µM morphine plus 10 µM
glibenclamide. (B): Summary of the amplitude of IPSCs, normalized to the control, in the presence of morphine (open bar) and morphine plus glibenclamide (filled bar). Normal K+-gluconate internal solution was used. * p < 0.05 vs. control.

Fig. 9 Effects of diazoxide and glibenclamide on the membrane current of a hippocampal CA1 neuron

The holding current was recorded at –70 mV in a hippocampal neuron treated with 500 µM diazoxide followed by 10 µM glibenclamide. Note that diazoxide elicited an outward current which was antagonized by glibenclamide.

Index terms:
morphine, periaqueductal gray, K_{ATP} channels,
pain, ontogeny, patch clamp