ABSTRACT

ATP-sensitive K⁺ (KATP) channels were reported to be involved in morphine analgesia in vivo. The present study, using patch-clamp technique in brain slices of neonatal (P12-P16) and adult rats, investigated cellular actions of KATP 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) in almost all tested neurons and elicited an inwardly rectifying K⁺ current in one-third of tested neurons. Glibenclamide (1–10 μM), a KATP channel blocker, did not affect the membrane current or synaptic current 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 neurons, morphine, at the concentration up to 300 μM, failed to activate K⁺ current in all 25 neurons tested but depressed IPSCs to a comparable extent as that in neonatal neurons. Glibenclamide also failed to alter the effect of morphine in adult neurons. The openers of KATP channels, lemakalim (10–30 μM) and diazoxide (10–500 μM), unlike morphine, did not increase membrane currents in both neonatal and adult neurons. However, diazoxide induced a glibenclamide-sensitive outward current in hippocampal CA1 neurons. It is concluded that KATP channels display little functional role per se and might not be involved in effects of morphine in the ventrolateral PAG. The correlation between the insensitivity in K⁺ channel activation and the less antinociceptive response in morphine in adults was discussed.

Since 1956, it has been reported that insulin pretreatment enhanced morphine-induced antinociceptive response (Davis et al., 1956). Singh et al. (1983) further suggested that this enhancement obtained in hypoglycemic animals is due to a lower level of intracellular ATP. After ATP-sensitive K⁺ (KATP) channels were explored in the brain (Bernardi et al., 1988), several in vivo studies suggested that KATP channels are involved in morphine-induced supraspinal analgesia: glibenclamide, a KATP channel blocker, when administered i.c.v., dose dependently decreased the antinociceptive response of morphine administered either intravenously (Ocaña et al., 1990; Roane and Boyd, 1993) or i.c.v. (Narita et al., 1992). Other KATP channel blockers but not the nonselective K⁺ channel blocker tetraethylammonium or 4-aminopyridine (Ocaña et al., 1993, 1995) also decreased the antinociceptive response of morphine. The potencies of KATP channel blockers in reversing morphine analgesia were correlated with their blocking activities of KATP channels (Ocaña et al., 1993). On the other hand, KATP channel openers potentiated the antinociceptive response of morphine (Vergoni et al., 1992; Narita et al., 1993; Lohmann and Welch, 1999). In addition, glibenclamide (i.c.v.) also decreased antinociceptive responses induced by other opiates, including levorphanol, methadone, and buprenorphine (Ocaña et al., 1995; Raffa and Martinez, 1995).

Nevertheless, the correlation between activation of KATP channels and morphine cellular actions in pain-related brain regions has not been studied in vitro. Given that the ventrolateral periaqueductal gray (PAG) is a crucial site for morphine-induced supraspinal analgesia (Yaksh et al., 1976), the present study was designed to test the hypothesis that effects of morphine in the ventrolateral PAG are mediated by activation of KATP channels. The cellular mechanism in the ventrolateral PAG for morphine-induced supraspinal analgesia is attributed mostly to inhibition of inhibitory synaptic transmission and partly to membrane hyperpolarization resulting from activation of inwardly rectifying K⁺ channels (Osborne et al., 1996; Vaughan et al., 1997; Chiu and Huang, 1999). Therefore, effects of morphine in the ventrolateral PAG were assessed by these two actions. The possible functional role(s) of KATP channels in the ventrolateral PAG were also explored by investigating effects of KATP ligands on synaptic transmissions and membrane currents.

It has been shown that the density of KATP channels is

ABBREVIATIONS: KATP, ATP-sensitive K⁺; PAG, periaqueductal gray; ACSF, artificial cerebral spinal fluid; EPSC, excitatory postsynaptic current; IPSC, inhibitory postsynaptic current; Ihold, holding current.
higher in adult rats compared with neonates (Mourre et al., 1990; Xia and Haddad, 1991). In addition to neonatal slices that are better preparations for blind patch-clamp recordings (Plant et al., 1995), experiments were also conducted in adult slices to see whether greater effects on morphine actions are observed when K_{ATP} channels are blocked. Given that the antinociceptive response induced by morphine is age-dependent (Auguy-Valette et al., 1978; Windh and Kuhn, 1995), the present study also examined whether the cellular actions of morphine in the ventrolateral PAG are dependent on age.

**Materials and Methods**

**Brain Slice Preparations.** Coronal brain slices (400 μm) containing the PAG were dissected as described previously (Chiou and Chou, 2000) from Wistar rats at the age of 12 to 16 days (neonates) or 8 to 12 weeks (adults). In some experiments, transverse hippocampal slices of 400 μm from adult rats were used. After equilibrated in the artificial cerebral spinal fluid (ACSF) for at least 1 h, slices were transferred to a submerged chamber and perfused with the ACSF at 2 to 3 ml min⁻¹. The ACSF contained 117 mM NaCl, 4.5 mM KCl, 2.5 mM CaCl₂, 1.2 mM MgCl₂, 1.2 mM NaH₂PO₄, 25 mM NaHCO₃, and 11.4 mM dextrose and was oxygenated with 95% O₂ plus 5% CO₂ (pH 7.4).

All experiments conformed to the guidelines of the Institutional Animal Care and Use Committee of National Taiwan University, College of Medicine. All efforts were made to minimize both the suffering and the number of animals used.

**Electrophysiological Recordings.** Blind patch-clamp recording was conducted in ventrolateral neurons of PAG slices or in CA1 pyramidal neurons of hippocampal slices at 30°C. The membrane input resistance was monitored with the MEMBRANE TEST function of Clampex 7.0 (Axon Instruments, Foster City, CA) by applying small hyperpolarization pulses (−3 mV). Membrane currents were recorded with an Axopatch 200B amplifier (Axon Instruments), digitized, and acquired with an A/D converter (DigiData 1200A; Axon Instruments) in a Pentium III PC computer running pClamp 7 (Axon Instruments).

When investigating the inwardly rectifying K⁺ current, membrane currents were elicited by a voltage ramp protocol and recorded as reported previously (Chiou, 2001). Briefly, hyperpolarization ramp commands from −60 to −140 mV at 0.2 mV/ms were applied every 30 s. The holding potential was −70 mV. Membrane currents elicited by voltage ramps were recorded simultaneously with a chart recorder (Gould 3000; Gould Electronics, Valley View, OH) to monitor the time course of drug effects.

Synaptic currents were evoked and recorded as previous report (Chiou and Chou, 2001). Excitatory postsynaptic currents (EPSCs) were recorded at −72 mV, which is close to the reversal potential of γ-aminobutyric acid (GABA) receptor-mediated inhibitory postsynaptic currents (IPSCs), the major IPSCs in the ventrolateral PAG. IPSCs were recorded in the presence of 1 mM kynurenic acid, an ionotropic glutamate receptor antagonist.

The normal internal solution contained 125 mM K⁺-gluconate, 5 mM KCl, 0.5 mM CaCl₂, 5 mM 1,2-bis(2-aminoophenoxy)ethane-N,N,N',N'-tetraacetic acid, 10 mM HEPES, 5 mM MgATP, and 0.33 mM GTPtris. 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 110 mM Cs⁺-gluconate, 5 mM tetraethylammonium chloride, 5 mM lidocaine-N-ethyl bromide, 0.5 mM CaCl₂, 5 mM 1,2-bis(2-aminoophenoxy)ethane-N,N,N',N'-tetraacetic acid, 10 mM HEPES, 5 mM MgATP, and 0.33 mM GTPtris. The liquid junction potentials of −11, −15, and −7 mV, respectively, were corrected for normal, Cs⁺-gluconate and ATP-free internal solutions. The resistance of microelectrodes was 4 to 8 MΩ.

**Data Analysis and Chemicals.** The effect of drugs was measured at the steady state of treatments as reported previously (Chiou, 2001). The shifting of holding current (I_{hold}) was expressed as positive for an outward shifting and negative for an inward one. The membrane current at −140 mV (I_{−140}) was normalized by reference to the control current at −140 mV for each neuron. Data were expressed as means ± S.E.M. with n indicating the number of neurons recorded, each of which was taken from one slice from different rats. From each rat, usually two to three slices at caudal segment of the PAG were used. For a comparison, sequential treatments were applied in the same neuron, if possible. Student’s t test was used for statistical analysis.

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

**Results**

**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 Had No Effect on Evoked Synaptic Currents in Neonatal Neurons.** Glibenclamide, a K_{ATP} channel blocker, at concentrations of 1 to 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

![Fig. 1. Lack of effect 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 μM glibenclamide (right). IPSCs were recorded at −63 mV in the presence of 1 mM kynurenic acid. EPSCs were recorded at −73 mV. Dotted line indicates the holding current of the control. ATP-free internal solution was used.](https://doi.org/10.1093/ajpaj/33.4.140)
with microelectrodes containing ATP-free internal solution were also not affected by glibenclamide. They were \(-60.2 \pm 6.6\) versus \(-55.9 \pm 5.6\) pA (n = 8) and \(-2.7 \pm 1.9\) versus \(-1.9 \pm 1.4\) pA (n = 7), respectively, when EPSCs and IPSCs were recorded.

**Glibenclamide Did Not Affect Morphine-Induced Inhibition of IPSCs.** Morphine concentration dependently decreased the amplitude of IPSCs that were recorded in the presence of 1 mM kynurenic acid (Fig. 2), an ionotropic glutamate receptor blocker. The magnitude of inhibition by 50 \(\mu\)M morphine was \(44 \pm 6\%\) (n = 5). Further addition of 10 \(\mu\)M 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 Did Not Affect Morphine Activation of Inwardly Rectifying K\(^+\) Channels.** In 7 of 27 neurons tested, morphine increased the membrane current elicited by a hyperpolarization ramp from \(-60\) to \(-140\) mV and shifted the holding current outwardly (Fig. 3). The current increased by morphine showed inward rectification and had a reversal potential at \(-86\) mV, which is close to the equilibrium potential of K\(^+\) ions, being \(-91\) mV, according to the Nernst equation. Therefore, the current induced by morphine is an inwardly rectifying K\(^+\) current. The current at \(-140\) mV was increased by 50 \(\mu\)M morphine to \(135 \pm 12\%\) of the control (n = 7) (Fig. 4). In morphine-sensitive neurons, further treatment with 10 \(\mu\)M glibenclamide did not show any further change on the membrane current (Figs. 3 and 4). However, naloxone (1 \(\mu\)M) significantly antagonized the effect of morphine (Fig. 3).

**\(K_{ATP}\) Channel Openers Had No Effect on Membrane Current.** The negative result of glibenclamide on the membrane current might be due to that the \(K_{ATP}\) channels, if any, have been blocked by intracellular ATP in our recording condition. The effects of \(K_{ATP}\) channel openers were, therefore, further investigated. Neither lemakulim (10–30 \(\mu\)M) nor diazoxide (100–500 \(\mu\)M) affected the membrane current elicited by a hyperpolarization ramp or the holding current (Fig. 4, open columns). Further addition of glibenclamide, 10 \(\mu\)M, also did not produce any effect (Fig. 4, filled columns).

**Effect of ATP-Free Internal Solution on Holding Current.** Further experiments were conducted with microelectrodes containing ATP-free internal solution to see whether \(K_{ATP}\) 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.

**PAG Neurons from Adult Rats**

Immunohistochemical studies have shown that the density of \(K_{ATP}\) channels in neonates is lower compared with adults (Mourre et al., 1990; Xia and Haddad, 1991). To examine whether the ineffectiveness of \(K_{ATP}\) ligands is attributed to the low density of \(K_{ATP}\) channels in neonatal PAG neurons, further experiments were performed in slices isolated from adult rats.

**Neither \(K_{ATP}\) Channel Ligands nor Morphine Affected Membrane Currents of Adult PAG Neurons.** In adult ventrolateral PAG neurons, diazoxide at the concentration up to 500 \(\mu\)M, alone or in combination with glibenclamide, affected neither the membrane current elicited by a hyperpolarization ramp nor the holding current (Fig. 6). Interestingly, morphine, at the concentration of 50 \(\mu\)M, which induced membrane hyperpolarization in one-third of neonatal PAG neurons tested, failed to affect membrane currents in all recorded neurons (n = 9). When the concentration of morphine was increased to 100 \(\mu\)M in seven neurons or to 300 \(\mu\)M in nine neurons, membrane currents were still not affected (Fig. 6). No effect was observed by further addition of glibenclamide. However, in these morphine-resistant adult neurons, baclofen, a \(\gamma\)-aminobutyric acid\(B\) receptor agonist, increased the membrane current reversibly (Figs. 6 and 7). The current increased by baclofen reversed at the potential close to the equilibrium potential of K\(^+\) ions and was characterized with inward rectification (Fig. 7).

**Glibenclamide Did Not Affect Morphine Inhibition of IPSCs in Adult Neurons.** To see whether the sensitivity to morphine is generally lower in adult PAG neurons, the effect of morphine on IPSCs of adult PAG neurons was examined. In all of the five neurons tested, IPSCs recorded in the presence of 1 mM kynurenic acid were depressed by 50 \(\mu\)M morphine (Fig. 8). The magnitude of depression is \(41 \pm 5\%\) (n = 5), which is comparable with the magnitude of 44 ±
6% (n = 5) obtained in neonatal slices (Fig. 2). Further addition of 10 μM glibenclamide did not gain further change in IPSCs (Fig. 8).

**Hippocampal Neurons from Adult Rats**

**K<sub>ATP</sub> Channel Ligands Did Affect Membrane Current of Hippocampal CA1 Neurons.** To see whether the K<sub>ATP</sub> channel ligands used in our recording system are valid, we examined their effects on hippocampal CA1 neurons where K<sub>ATP</sub> channel openers can 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 ventrolateral PAG neurons, we demonstrated the following: 1) In neonates, morphine depressed the inhibitory synaptic transmission and activated inwardly rectifying K<sup>+</sup> channels. 2) In adults, morphine failed to activate K<sup>+</sup> channels in all recorded neurons but produced an inhibition of IPSC comparable with that in neonates. 3) Glibenclamide, a K<sub>ATP</sub> channel blocker, did not alter morphine effects in both neonates and adults. 4) Neither openers nor blockers of K<sub>ATP</sub> channels affected the synaptic transmission or membrane current in both neonates and adults.

**Little Functional Role of K<sub>ATP</sub> Channels in Ventrolateral PAG.** K<sub>ATP</sub> channels are inhibited by intracellular ATP at the concentration as low as 100 μM (Ashcroft and Kakei, 1989), which is far lower than the intracellular concentration of ATP in our recording condition. While this might explain why glibenclamide did not affect the membrane current per se, the openers of K<sub>ATP</sub> channels, either lemakalim or diazoxide, also have no effect on the membrane current of ventrolateral PAG neurons. Although lemakalim was less effective in neuronal K<sub>ATP</sub> channels (Stanford and Lacey, 1996; Schwanstecher and Bassen, 1997), diazoxide caused membrane hyperpolarization in both substantial nigra (Häsuser et al., 1991) and hippocampal (Ben-Ari et al., 1990; Fujimura et al., 1997) neurons. In pancreatic β cells, the opening of K<sub>ATP</sub> channels by diazoxide was hindered if the concentration of intracellular ATP was higher than 3 mM (Trube et al., 1986) and the intracellular MgADP is essential for the activation of K<sub>ATP</sub> channels (Larsson et al., 1992). It is not clear whether these requirements are also applied to neuronal K<sub>ATP</sub> 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). There-
fore, the negative result of K<sub>ATP</sub> channel ligands in the PAG is not due to a limitation from whole cell recording technique such as a dialysis of intracellular MgADP, or a deterioration of K<sub>ATP</sub> channel ligands used. The latter inference is further supported by two assays (B.-H. Liang and C. Y. Cheng, personal communication). First, the structure of used glibenclamide has been confirmed by NMR analysis. Second, glibenclamide, from the same lot used in the present study, antagonized lemakalim-induced inhibition of spontaneous contraction of rat portal veins.

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 (Häusser et al., 1991; Stanford and Lacey, 1996). However, in PAG neurons, no outward shifting of holding current was obtained with electrodes containing ATP-free internal solution. Instead, the holding current was shifted inwardly, which might be resulted from a decrease of Na<sup>+</sup>-K<sup>+</sup>-ATP pump activity due to a deprivation of intracellular ATP. Therefore, the negative finding with K<sub>ATP</sub> channel ligands in ventrolateral PAG neurons is unlikely to have resulted from a pharmacological resistance in this area. It is, therefore, suggested that the K<sub>ATP</sub> channels do not play a significant role in the regulation of resting membrane potential of ventrolateral PAG neurons.
It was reported that the density of KATP channels is higher in adult rats (Mourre et al., 1990; Xia and Haddad, 1991). Nevertheless, the failure in obtaining any positive result with KATP channel ligands in either neonatal or adult neurons suggests that the density of KATP channels in the ventrolateral PAG is too low to have any functional role.

**K<sub>ATP</sub> Channels Are Not Involved in Effects of Morphine in Ventrolateral PAG.** Glibenclamide, of which the stability has been confirmed by NMR assay or bioassays in other preparations, did not affect morphine actions, either the presynaptic inhibition of IPSCs or postsynaptic activation of K<sup>+</sup> channels, in the ventrolateral PAG. These findings suggest that K<sub>ATP</sub> channels are not involved in morphine actions in the ventrolateral PAG. The mechanism of opioid inhibition of IPSCs in the PAG was proposed to be activation of a 4-aminopyridine- and dendrotoxin-sensitive K<sup>+</sup> conductance through 12-lipoxygenase products and was suggested to be the mechanism of the supraspinal analgesic action of opioids (Vaughan et al., 1997). Nevertheless, it disagrees with the finding that 4-aminopyridine (i.c.v.) fails to affect the antinociceptive response of morphine (Ocaña et al., 1995).

Several in vivo studies demonstrated that i.c.v. injection of glibenclamide decreased morphine-induced analgesia (Ocaña et al., 1990, 1993, 1995; Narita et al., 1992, 1993; Roane and Boyd, 1993; Raffa and Martinez, 1995). However, glibenclamide failed to affect the actions of morphine in the PAG. The neurons recorded in the present study were confined to the ventrolateral area of the caudal PAG, the most active site in the brain responsive to morphine-induced antinociception (Yaksh et al., 1976). 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<sub>ATP</sub> channel is moderate (Treheren and Ashford, 1991; Dunn-Meynell et al., 1998). However, the possibility that K<sub>ATP</sub> channel ligands affect morphine analgesia in vivo by a mechanism(s) distinct from a direct activation of K<sub>ATP</sub> channels (Lohmann and Welch, 1999) cannot be excluded.

**Different Proportions of Neurons Hyperpolarized by Morphine and μ-Opioid Peptides.** The findings that morphine inhibits the inhibitory synaptic transmission and acti-
K<sub>ATP</sub> Channels and Morphine Action in Periaqueductal Gray


References


TABLE 1 Percentage of neurons that were hyperpolarized by morphine or μ-opioid peptides in the ventralateral PAG

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<th>Neonates</th>
<th>Adults</th>
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<tr>
<td>Morphine</td>
<td>26&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>DAMGO(Met)-Enkephalin</td>
<td>65&lt;sup&gt;c&lt;/sup&gt;</td>
<td>25–30&lt;sup&gt;d&lt;/sup&gt;</td>
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<sup>a</sup> Conducted by whole cell recording.
<sup>b</sup> Chiou (2001).
<sup>c</sup> Conducted by conventional intracellular recording.
<sup>d</sup> Behbehani et al. (1990).
<sup>e</sup> Chieng and Christie (1994a).


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