Contribution of ATP-Sensitive Potassium Channels to Hypoxic Hyperpolarization in Rat Hippocampal CA1 Neurons In Vitro

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Fujimura, N., E. Tanaka, S. Yamamoto, M. Shigemori, and H. Higashi. Contribution of ATP-sensitive potassium channels to hypoxic hyperpolarization in rat hippocampal CA1 neurons in vitro. J. Neurophysiol. 77: 378–385, 1997. To investigate the mechanism of generation of the hypoxia-induced hyperpolarization (hypoxic hyperpolarization) in hippocampal CA1 neurons in rat tissue slices, recordings were made in current-clamp mode and single-electrode voltage-clamp mode. Superfusion with hypoxic medium produced a hyperpolarization and corresponding outward current, which were associated with an increase in membrane conductance. Reoxygenation produced a further hyperpolarization, with corresponding outward current, followed by a recovery to the preexposure level. The amplitude of the posthypoxic hyperpolarization was always greater than that of the hypoxic hyperpolarization. In single-electrode voltage-clamp mode, it was difficult to record reproducible outward currents in response to repeated hypoxic exposure with the use of electrodes with a high tip resistance. The current-clamp technique was therefore chosen to study the pharmacological characteristics of the hypoxic hyperpolarization. In 60–80% of hippocampal CA1 neurons, glibenclamide or tolbutamide (3–100 μM) reduced the amplitude of the hypoxic hyperpolarization in a concentration-dependent manner by up to ~70%. The glibenclamide or tolbutamide concentrations producing half-maximal inhibition of the hypoxic hyperpolarization were 6 and 12 μM, respectively. The chord conductance of the membrane potential between ~80 and ~90 mV in the absence of glibenclamide (30 μM) or tolbutamide (100 μM) was 2–3 times greater than that in the presence of glibenclamide or tolbutamide. In contrast, the reversal potential of the hypoxic hyperpolarization was approximately ~83 mV in both the absence and presence of tolbutamide or glibenclamide. In ~40% of CA1 neurons, diazoxide (100 μM) or nicorandil (1 mM) mimicked the hypoxic hyperpolarization and pretreatment of these drugs occluded the hypoxic hyperpolarization. When ATP was injected into the impaled neuron, hypoxic exposure could not produce a hyperpolarization. The intracellular injection of the nonhydrolyzable ATP analogue 5′-adenylylimidodiphosphate lithium salt reduced the amplitude of the hypoxic hyperpolarization. Furthermore, application of dinitrophenol (10 μM) mimicked the hypoxic hyperpolarization, and the dinitrophenol-induced hyperpolarization was inhibited by either pretreatment of tolbutamide or intracellular injection of ATP, indicating that the hypoxic hyperpolarization is highly dependent on intracellular ATP. It is therefore concluded that in the majority of hippocampal CA1 neurons, exposure to hypoxic conditions resulting in a reduction in the intracellular level of ATP leads to activation of ATP-sensitive potassium channels with concomitant hyperpolarization.

INTRODUCTION

In the CNS, hippocampal CA1 neurons are known to be extremely vulnerable to anoxia and ischemia (Siesjö 1988). In tissue slices, exposure of hippocampal CA1 neurons to hypoxia for a short period (2–4 min) induces a hyperpolarization with decreases in input resistance and in synaptic noise (Fujiwara et al. 1987; Krnjević and Leblond 1989; Leblond and Krnjević 1989). The hypoxia-induced hyperpolarization (hypoxic hyperpolarization) is most likely to be mediated by an increase in K+ conductance (Fujiwara et al. 1987; Hansen et al. 1982; Leblond and Krnjević 1989), but the subtype(s) of K+ channels involved in the hyperpolarization is still unclear. It has been suggested that the hypoxic hyperpolarization is due to activation of a Ca2+-dependent K+ conductance, because hypoxia induces an early increase in the intracellular Ca2+ concentration as a result of Ca2+ mobilization from intracellular stores (Belousov et al. 1995; Katchman and Hershkovitz 1993; Krnjević and Xu 1989). Direct evidence supporting this is, however, either lacking (Fujiwara et al. 1987) or inconclusive (Leblond and Krnjević 1989). The involvement of ATP-sensitive potassium (KATP) channels in the hyperpolarization has been suggested by an inverse correlation between the ATP content of patch electrodes and conductance changes induced by hypoxia (Zhang and Krnjević 1993). The highest binding densities of glibenclamide, a KATP channel blocker, have been reported in the cortex, hippocampus, cerebellum, and substantia nigra in the CNS (Jiang et al. 1992; Mourre et al. 1989; Xia and Haddad 1991). Moreover, it has been reported that in substantia nigra neurons and dorsal vagal neurons, anoxia induces a hyperpolarization that is mediated by an activation of KATP channels (Jiang et al. 1994; Murphy and Greenfield 1992; Trapp and Ballanyi 1995). Nevertheless, the contribution of the KATP channel to the hypoxic hyperpolarization is still controversial in hippocampal CA1 neurons. KATP channel blockers, such as tolbutamide and glibenclamide, have yielded equivocal results: tolbutamide depressed the hypoxic hyperpolarization (Godfraind and Krnjević 1993; Grigg and Anderson 1989), but neither tolbutamide nor glibenclamide had comparable effects (Godfraind and Krnjević 1993; Leblond and Krnjević 1989).

The aim of this study is, therefore, to investigate the involvement of activation of KATP channels in the hypoxic hyperpolarization. By observing the effects of selective KATP channel antagonists, agonists and intracellular injection of ATP, attempts were made to examine whether the hypoxic hyperpolarization is specifically mediated by KATP channels. In addition, attempts were made to mimic the hyperpolarization with the use of a metabolic inhibitor. The accompanying paper (Yamamoto et al. 1997) describes the contribution of the Ca2+-dependent K+ conductance to the hypoxic hyperpolarization.
Hypoxia-induced hyperpolarization

Methods

The forebrains of adult Wistar rats (male, weight 200–250 g) were quickly removed under ether anesthesia and placed in chilled (4–6°C) Krebs solution that was aerated with 95% O₂-5% CO₂. The composition of the solution was (in mM) 117 NaCl, 3.6 KCl, 2.5 CaCl₂, 1.2 MgCl₂, 1.2 NaH₂PO₄, 25 NaHCO₃, and 11 glucose. The hippocampus was dissected and then sliced with a Vibratome (Oxford) to a thickness of ~400 μm. A slice was placed on a nylon net in a recording chamber (volume 500 μl) and immobilized with a titanium grid placed on the upper surface of the section. The preparation was completely submerged in the superfusing solution. The temperature in the recording chamber was continuously monitored and maintained at 36–37°C, and the solution was perfused at a constant rate of 6–8 ml/min. Intracellular recordings from CA1 pyramidal cells were made with glass micropipettes filled with potassium acetate (2 M) or KCl (2 M). The electrode resistance was 20–90 MΩ. The membrane potential was determined by the baseline level recorded from an X-Y recorder with a low-pass filter. In another series of experiments, voltage clamping was performed with a single-electrode voltage-clamp amplifier (Axon Instruments, Axoclamp-2A), employing a switching frequency of 4–5 kHz and a 30% duty cycle. The headstage voltage was continuously monitored to ensure complete settling of the voltage at the end of each switching cycle. Particular efforts were made to select electrodes with optimal current-passing characteristics: tip resistance of 20–90 MΩ and minimal rectification. In some neurons, recording electrodes containing adenosine 5'-triphosphate dipotassium salt (K₆-ATP, 20 mM), adenosine 5'-triphosphate magnesium salt (Mg-ATP, 10 mM), or 5'-adenylylimidodiphosphate lithium salt (AMP-PNP, 20 mM)/potassium acetate (2 M) were used for intracellular ATP or AMP-PNP injection. ATP or AMP-PNP was injected by passing hyperpolarizing current pulses (0.4–0.9 nA in intensity, 200 ms in duration, and 3.3 Hz in frequency) for 10–15 min.

Slice preparations were made "hypoxic" by superfusing medium equilibrated with 95% N₂-5% CO₂ (hypoxic medium). The dead space of the superfusing system resulted in a delay of 15–20 s before the new medium reached the chamber.

Drugs used were tolbutamid and glibenclamide (Research Biochemicals International); diazoxide (Sigma); nicorandil (gift from Chugai Pharmaceutical); 2,4-dinitrophenol (Tokyo Kasei Organic Chemical); and Mg-ATP, K₆-ATP, and AMP-PNP (Sigma). All quantitative results are expressed as means ± SD. The number of neurons examined is given in parentheses. The unpaired and paired Student’s t-test was used to compare data, with P < 0.05 considered significant.

Results

This study was based on recordings from ~100 CA1 pyramidal neurons of adult rats with stable membrane potentials more negative than −60 mV. The resting membrane potential and the apparent input resistance were −71.4 ± 4.9 mV and 33.3 ± 8.8 MΩ (n = 67), respectively, recorded with 2 M potassium acetate electrodes and −70.1 ± 2.7 mV and 40.4 ± 4.2 MΩ (n = 38), respectively, recorded with 2 M KCl electrodes. In either current-clamp or voltage-clamp recording, unless specified otherwise, the membrane potential was held at −60 mV by current injection from the recording electrode before hypoxic exposure.

Hypoxia-induced hyperpolarization

Figure 1 illustrates typical changes in membrane potential or membrane current in response to oxygen deprivation, with the use of 2 M KCl-filled electrodes with a high tip resistance (80 MΩ). Superfusion with hypoxic medium produced a hyperpolarization that was associated with a reduction in apparent input resistance. When oxygen was readmitted, the membrane potential was transiently shifted to a more negative level (posthypoxic hyperpolarization) before it recovered to the preexposure level. In the same neuron under the voltage-clamp mode, hypoxia now evoked a corresponding outward current that was accompanied by a large increase in conductance. Reoxygenation prolonged the decay of the outward current by a transient outward shift, which corresponded to the posthypoxic hyperpolarization. Our previous study indicates that the hypoxic hyperpolarization is mainly due to an increase in K⁺ conductance and the posthypoxic hyperpolarization is caused by reactivation of the Na⁺-K⁺ pump (Fujiwara et al. 1987).

The peak amplitudes of the hypoxic responses, the posthypoxic hyperpolarization, and the corresponding currents, with the use of KCl or potassium acetate electrodes with high and low tip resistances, are summarized in Table 1. As the tip resistance of KCl or potassium acetate electrodes decreased, the peak amplitudes of the hypoxic hyperpolarization and its corresponding outward current were reduced, whereas the posthypoxic hyperpolarization and its corresponding outward current were not altered. When the neurons were impaled by KCl or potassium acetate electrodes with extremely low tip resistances (20- to 30-MΩ KCl electrodes and 35- to 45-MΩ potassium acetate electrodes), hypoxia produced a depolarization and its corresponding current. Both responses were associated with an increase in conductance. The peak amplitudes of the hypoxic depolarization, the hypoxic hyperpolarization, and their corresponding currents were not significantly different between potassium acetate and KCl electrodes.
TABLE 1. Peak amplitudes of hypoxic depolarization or hyperpolarization, posthypoxic hyperpolarization, and corresponding currents using KCl and potassium acetate electrodes

<table>
<thead>
<tr>
<th>KCl electrode</th>
<th>Hypoxic Depolarization, mV</th>
<th>Hypoxic Inward Current, pA</th>
<th>Hypoxic Hyperpolarization, mV</th>
<th>Hypoxic Outward Current, pA</th>
<th>Posthypoxic Hyperpolarization, mV</th>
<th>Posthypoxic Outward Current, pA</th>
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<tr>
<td>20–30 MΩ</td>
<td>7.0 ± 1.0 (5)</td>
<td>228 ± 74 (5)</td>
<td>None</td>
<td>None</td>
<td>11.9 ± 1.7 (5)</td>
<td>282 ± 111 (5)</td>
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<td>30–50 MΩ</td>
<td>None</td>
<td>None</td>
<td>5.7 ± 2.5 (8)</td>
<td>161 ± 64 (5)</td>
<td>11.2 ± 5.2 (8)</td>
<td>259 ± 73 (5)</td>
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<td>&gt;50 MΩ</td>
<td>None</td>
<td>None</td>
<td>9.8 ± 2.6 (30)*</td>
<td>242 ± 78 (5)</td>
<td>13.4 ± 4.8 (30)</td>
<td>278 ± 78 (5)</td>
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<td>Potassium acetate</td>
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<td>35–45 MΩ</td>
<td>6.1 ± 1.6 (5)</td>
<td>192 ± 70 (5)</td>
<td>None</td>
<td>None</td>
<td>11.4 ± 2.0 (5)</td>
<td>276 ± 102 (5)</td>
</tr>
<tr>
<td>45–70 MΩ</td>
<td>None</td>
<td>None</td>
<td>6.3 ± 2.3 (10)</td>
<td>168 ± 61 (5)</td>
<td>10.8 ± 5.5 (10)</td>
<td>253 ± 70 (5)</td>
</tr>
<tr>
<td>&gt;70 MΩ</td>
<td>None</td>
<td>None</td>
<td>9.2 ± 4.0 (57)*</td>
<td>234 ± 84 (5)</td>
<td>13.6 ± 4.4 (57)</td>
<td>283 ± 68 (5)</td>
</tr>
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</table>

Values are means ± SD, with number of neurons in parentheses. P < 0.01, unpaired t-test.

Moreover, when the neuron was impaled by electrodes with relatively high tip resistances (60–80 MΩ), repeated exposure to hypoxia at the interval of every 10 min gradually attenuated both the hypoxic and posthypoxic outward currents, but not both the hypoxic and posthypoxic hyperpolarizations. This result suggests that the neuron in the voltage-clamp mode would be deteriorated more easily than that in the current-clamp mode. In other words, the hypoxic hyperpolarization would prevent the accumulation of intracellular Ca\textsuperscript{2+} concentration that triggers the cell deterioration (cf. Siesjö 1988). Thus we used the current-clamp technique to study the pharmacological characteristics of the hypoxic hyperpolarization in the following section.

**Effects of K\textsubscript{ATP} channel blockers**

The hippocampal slice preparation was pretreated with the K\textsubscript{ATP} channel blockers tolbutamide (3–100 μM) or glibenclamide (3–100 μM) for 10 min before hypoxic exposure. Each drug caused a small, sustained depolarization that was accompanied by an increase in apparent input resistance. The amplitude of the depolarization was 3.6 ± 1.5 mV (n = 9) in the presence of 100 μM tolbutamide and 3.4 ± 1.5 mV (n = 7) in the presence of 100 μM glibenclamide. In the majority of neurons tested, both tolbutamide and glibenclamide reduced the amplitude of the hypoxic hyperpolarization and/or prolonged the onset time of the hyperpolarization in a concentration-dependent manner (Fig. 2, A and B). In 8 of 10 hippocampal CA1 neurons tested, the minimal effective concentration and the maximal inhibitory concentration of tolbutamide were 3 and 100 μM, respectively. Tolbutamide (100 μM) significantly reduced the amplitude of the hypoxic hyperpolarization by 71 ± 16% (n = 8, P < 0.01) of the control. The half-maximum inhibition was achieved at a concentration of 12 μM (Fig. 2B). In the remaining two neurons, tolbutamide had no effect. Glibenclamide had similar effects; in 13 of 22 CA1 neurons, the minimal effective concentration, half-maximum inhibition and maximal inhibitory concentration were 3, 6, and 100 μM, respectively (Fig. 2C). Glibenclamide (100 μM) significantly reduced the amplitude of the hypoxic hyperpolarization by 67 ± 15% (n = 13, P < 0.01) of the control. In two other neurons, glibenclamide prolonged the onset time without affecting the amplitude. In the remaining five neurons, glibenclamide had no effect. Moreover, tolbutamide or glibenclamide at the high concentration (100 μM) did not significantly affect the amplitude of the posthypoxic hyperpolarization (Fig. 2A for tolbutamide). Thus, in 60–80% of the neurons, tolbutamide and glibenclamide depressed the hypoxic hyperpolarization in a concentration-dependent manner, but were unable to block the posthypoxic hyperpolarization.

To further study the inhibitory action of glibenclamide or tolbutamide, steady-state current-voltage plots before and during the hypoxic hyperpolarization were obtained by passing hyperpolarizing and subsequent depolarizing ramp currents through the recording electrode. The conductance change during hypoxia was much greater than that in normoxic media (Fig. 3, A and C), indicating that it is not likely to result from the reduction of a tonic inward current. The net outward current induced by hypoxia was markedly depressed by tolbutamide (100 μM) or glibenclamide (30 μM) (compare Fig. 3, A and C, insets, with Fig. 3, B and D, insets, respectively). The chord conductance of the membrane potential between −80 and −90 mV in the absence of tolbutamide was significantly greater than that in the presence of tolbutamide (100 μM), being 12.3 ± 6.1 nS (n = 8) in the absence and 3.7 ± 1.5 nS (n = 6) in the presence of tolbutamide. Similar results were obtained in the absence and presence of glibenclamide (30 μM), being 14.3 ± 7.5 nS (n = 4) in the absence and 6.3 ± 3.3 nS (n = 4) in the presence of glibenclamide. In contrast, the reversal potential of the hypoxic hyperpolarization was not different at −83.7 ± 3.5 mV (n = 8) and −83.3 ± 2.6 mV (n = 6) in the absence and presence of tolbutamide (100 μM), respectively. The reversal potential of the hypoxic hyperpolarization was −82.6 ± 1.9 mV (n = 4) and −83.4 ± 1.7 mV (n = 4) in the absence and presence of glibenclamide (30 μM), respectively.

**Effects of activators of K\textsubscript{ATP} channels**

Diazoxide and nicorandil are well known to be activators of K\textsubscript{ATP} channels in pancreatic β-cells (Ashcroft and Ashcroft 1990; Dunne 1990; Trube et al. 1986). To confirm the contribution of K\textsubscript{ATP} channels to the hypoxic hyperpolarization, these activators were applied to hippocampal CA1 neurons. In 10 of 36 neurons, application of diazoxide (100
μM) or nicorandil (1 mM) produced a hyperpolarization that was accompanied by a fall in apparent input resistance. The hyperpolarization reversed rapidly when the application was discontinued. The peak amplitudes of the hyperpolarization induced by diazoxide (100 μM) and nicorandil (1 mM) were 6.0 ± 2.6 mV (n = 4) and 2.8 ± 1.2 mV (n = 6),

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**FIG. 2.** Effects of the ATP-sensitive potassium (K<sub>ATP</sub>) channel blockers on the hypoxic hyperpolarization. A: hypoxic hyperpolarization before blockers (top trace), after pretreatment with tolbutamide at a concentration of 10 μM (2nd trace), 30 μM (3rd trace), and 100 μM (4th trace) for 10 min, and after washing out of the drug for 30 min (bottom trace). B and C: concentration dependence of inhibition by tolbutamide (B) and glibenclamide (C). The peak amplitudes of the hypoxic hyperpolarizations at various concentrations of tolbutamide or glibenclamide were normalized with those of the respective controls. Error bars: SD. The curves fitting the points were drawn by the Hill’s equation. The Hill coefficient was 1.8 for tolbutamide and 1.0 for glibenclamide. Downward deflections in voltage recordings are hyperpolarizing electrotonic potentials elicited by anodal current pulses (0.4 nA, applied for 200 ms every 3 s).

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**FIG. 3.** Effects of the K<sub>ATP</sub> channel blockers on the membrane conductance during the hypoxic hyperpolarization. Slow hyperpolarizing and depolarizing DC ramp currents (1–2 mV/s) were passed through the recording electrode to obtain steady-state current-voltage relationships before and during hypoxic exposure (not shown). A and B: steady-state current-voltage curves before (Control) and during hypoxic exposure (Hypoxia) in the absence (A) and presence (B) of tolbutamide (100 μM). Insets: net outward currents produced by hypoxia, which were obtained by subtraction of the steady-state current-voltage relation at preexposure level from that during hypoxic exposure. For subtraction, steady-state current-voltage relations, which were continuously recorded from the most hyperpolarizing level (0 mV) to −60 mV, were used. Note that the net outward current was markedly depressed by tolbutamide. C and D: steady-state current-voltage curves before (Control) and during hypoxic exposure (Hypoxia) in the control condition and in the presence of glibenclamide (30 μM), respectively. The outward current in the control condition was markedly depressed by glibenclamide. Note that the reversal potential of the net outward current was not affected by either tolbutamide or glibenclamide.
respectively. The neuronal input resistance measured at the peak of the hyperpolarization was reduced to 79 ± 11% (n = 4) of the control before application of diazoxide and to 85 ± 11% (n = 6) of the control before application of nicorandil. Next, the hypoxic hyperpolarization was compared in the absence and presence of the K$_{ATP}$ channel activator. The peak amplitude of the hypoxic hyperpolarization was significantly reduced to 82 ± 4% (n = 4) of the control in the presence of 100 μM diazoxide and to 63 ± 12% (n = 6) of the control in the presence of 1 mM nicorandil. In the remaining 26 neurons, application of diazoxide or nicorandil did not significantly affect the membrane potential and apparent input resistance.

**Effects of a metabolic inhibitor**

Metabolic inhibitors, such as 2,4-dinitrophenol and cyanide, have been used to produce the anoxic/ischemic insult by blocking oxidative respiration and thereby lowering intracellular ATP levels ([ATP]$_i$) (Murphy and Greenfield 1991; Reiner et al. 1990). A short period (15–60 s) of superfusion with dinitrophenol (10 μM) caused a hyperpolarization that was associated with a reduction of the neuronal input resistance. As the application period increased, the duration of the hyperpolarization was prolonged (Fig. 4A). The amplitude of the dinitrophenol-induced hyperpolarization was 4 ± 2 mV (n = 4), 7 ± 3 mV (n = 4), and 9 ± 4 mV (n = 9) with 15, 30, and 60 s of superfusion with medium containing dinitrophenol (10 μM). The duration of the dinitrophenol-induced hyperpolarization was 1.4 ± 0.3 min (n = 4), 4 ± 0.5 min (n = 4), and 5.2 ± 0.7 min (n = 9) with 15, 30, and 60 s of superfusion with medium containing dinitrophenol (10 μM). On superfusion with dinitrophenol (10 μM) for 60 s, the neuronal input resistance measured at the peak of the dinitrophenol-induced hyperpolarization was decreased to 60 ± 13% (n = 9) of the control.

Because the posthypoxic hyperpolarization is generated by reactivation of the Na$^+$–K$^+$ pump (Fujisawa et al. 1987), it is possible that a hyperpolarization generated by reactivation of the Na$^+$–K$^+$ pump may follow some periods after superfusion with metabolic inhibitors. It was, however, difficult to detect two components in dinitrophenol-induced hyperpolarization. It may be possible to detect reactivation of the Na$^+$–K$^+$ pump in the presence of a high concentration of tolbutamide (100 μM), because the hypoxic hyperpolarization was inhibited by ~70% whereas the posthypoxic hyperpolarization was not affected (see Fig. 2A). We therefore compared the peak amplitude and neuronal input resistance of the dinitrophenol-induced hyperpolarization in the absence and presence of tolbutamide (100 μM) by comparison of two arbitrary points of the time when the dinitrophenol-induced hyperpolarization reached a peak in the absence (t$_1$) and presence (t$_2$) of tolbutamide, as shown in Fig. 4B. The amplitudes of the dinitrophenol-induced hyperpolarization at t$_1$ and t$_2$ in the absence of tolbutamide were 12.7 ± 1.1 mV (n = 4) and 11.9 ± 3.9 mV (n = 4), respectively. In the absence of tolbutamide, the neuronal input resistances measured at t$_1$ and t$_2$ were reduced to 51 ± 8% (n = 4) and 58 ± 8% (n = 4) of the controls, respectively. The amplitudes of the dinitrophenol-induced hyperpolarization at t$_1$ and t$_2$ in the presence of tolbutamide were 1.0 ± 1.7 mV (n = 4) and 6.3 ± 2.8 mV (n = 4), respectively. In the presence of tolbutamide, the neuronal input resistances measured at t$_1$ and t$_2$ were reduced to 88 ± 6% (n = 4) and 84 ± 18% (n = 4) of the controls, respectively (Fig. 4B).

Thus tolbutamide significantly depressed the peak amplitude of the dinitrophenol-induced hyperpolarization at t$_1$ (P < 0.01), but not the peak amplitude at t$_2$. Tolbutamide reversed the reduction in both the neuronal input resistances during the dinitrophenol-induced hyperpolarizations at t$_1$ (P <
Effects of intracellular injection of ATP on the hyperpolarization induced by hypoxia (A) or dinitrophenol (bars). ATP was injected after recording of the 1st response to hypoxic medium or dinitrophenol-containing medium by passing hyperpolarizing current pulses (0.3–0.9 nA for 200 ms every 3 s) through the recording electrode for 10 min. A: hypoxic hyperpolarization recorded 5 min after impalement with an electrode containing 20 mM adenosine 5’-triphosphate dipotassium salt (K₂-ATP)/2 M KCl (top trace) was virtually abolished after intracellular ATP injection (bottom trace). B: dinitrophenol-induced hyperpolarization 5 min after impalement with an electrode containing 20 mM adenosine 5’-triphosphate magnesium salt (Mg-ATP)/2 M KCl (top trace) was abolished after intracellular ATP injection (bottom trace).

Antagonists and agonists of K\textsubscript{ATP} channels

The highest binding densities of radioactively labeled glibenclamide, a sulfonylurea, which is a specific K\textsubscript{ATP} channel blocker (Bernardi et al. 1988; Geisen et al. 1985), have been reported in the cortex, hippocampus, cerebellum, and substantia nigra in the CNS (Jiang et al. 1992; Mouri et al. 1989; Xia and Haddad 1991). The binding densities of glibenclamide in rat brain appear to be dense after birth; most of the binding sites are well developed within 3 wk after birth (Xia et al. 1993). The present study shows that in ~80% of hippocampal CA1 neurons tested, the sulfonylureas tolbutamide and glibenclamide depressed both the hypoxic hyperpolarization and the corresponding net outward current. The half-maximum inhibition of tolbutamide for the K\textsubscript{ATP} channel activity is 3–17 \mu M in pancreatic \beta-cells (Belles et al. 1987; Gillis et al. 1989; Trube et al. 1986; Zünkler et al. 1988), 60 \mu M in skeletal muscle (Woll et al. 1989), and 380 \mu M in cardiac muscle (Strugess et al. 1988). On the other hand, the half-maximum inhibition of glibenclamide is 4–27 nM in pancreatic \beta-cells (Belles et al. 1987; Zünkler et al. 1988) and 20 \mu M in smooth muscle cells (Standen et al. 1989; also cf. Ashcroft and Ashcroft 1990). In the present study, the values for the half-maximum inhibition of tolbutamide and glibenclamide inhibition of the hypoxic hyperpolarization were 12 and 6 \mu M, respectively. Thus these values are comparable with those in \beta-cells and smooth muscle, respectively.

Neither tolbutamide nor glibenclamide at high concentrations (100 \mu M) affected a slow spike afterhyperpolarization, which is induced by increased Ca\textsuperscript{2+}-dependent K\textsuperscript{+} conductance (Lancaster and Nicoll 1987), and a posthypoxic hyperpolarization, which is produced by reactivation of the electrogenic Na\textsuperscript{+}-K\textsuperscript{+} pump (Fujiwara et al. 1987). Moreover, the reversal potential of the hypoxic hyperpolarization was

DISCUSSION

Our previous study demonstrates that the hypoxic hyperpolarization in hippocampal CA1 neurons is unaffected by Co\textsuperscript{2+} (2 mM) or tetrodotoxin (0.3 \mu M) medium, which completely blocks spontaneous and evoked synaptic potentials (Fujiwara et al. 1987). This finding indicates that the response is brought about by a direct action of hypoxia on the impaled neuron. The hyperpolarization is markedly enhanced in K\textsuperscript{+}-free media and is depressed in high-K\textsuperscript{+} (10 mM) solutions, whereas the hyperpolarization is not significantly affected by low-Cl\textsuperscript{−} or low-Na\textsuperscript{+} medium. The hyperpolarization reverses in polarity at −83 mV (Fujiwara et al. 1987), which is comparable with the value in the present study. These results suggest that the hypoxic hyperpolarization is mainly due to activation of K\textsuperscript{+} channels. The contribution of the activation of K\textsubscript{ATP} channels in the hypoxic hyperpolarization will be discussed in the following section.
not affected by these sulfonylurea compounds. The results suggest that tolbutamide and glibenclamide (3–100 μM) act on sulfonylurea receptors in CA1 neurons, with concomitant inactivation of the coupled K<sub>ATP</sub> channels. The K<sub>ATP</sub> channels characterized in heart, skeletal and smooth muscles, and pancreatic β-cells are only activated when the [ATP], declines by 30–50% of normal levels (Noma and Shibasaki 1985).

In guinea pig hippocampal slice preparations, [ATP], is decreased by ~15% within 2 min of exposure to hypoxia (Lipton and Whittingham 1982). Thus a brief application of hypoxia for 2–4 min would be expected to activate K<sub>ATP</sub> channels in ~80% of hippocampal CA1 neurons. Even in the presence of 100 μM tolbutamide or glibenclamide, however, the hypoxic hyperpolarization was not completely blocked; 30–36% of the amplitude of the hypoxic hyperpolarization was resistant to K<sub>ATP</sub> channel blockers. Furthermore, in the remaining 20% of CA1 neurons, up to 100 μM concentrations of tolbutamide or glibenclamide did not affect the hypoxic hyperpolarization. The sensitivity of K<sub>ATP</sub> channels for tolbutamide or glibenclamide is different in various tissues, described above. In epithelial cells, K<sub>ATP</sub> channels are not affected by either tolbutamide or glibenclamide (cf. Ashcroft and Ashcroft 1990). It is therefore possible that the K<sub>ATP</sub> channels in hippocampus are heterogeneous and some of them are insensitive to tolbutamide and glibenclamide.

On the other hand, there is another possibility that the sulfonylurea-insensitive hypoxic hyperpolarization could be mediated by activation of another species of K<sup>+</sup> channels, such as Ca<sup>2+</sup>-dependent K<sup>+</sup> channels (Yamamoto et al. 1997).

In ~40% of CA1 neurons tested in the present study, activators of K<sub>ATP</sub> channels, such as diazoxide and nicorandil (Trube et al. 1986; also cf. Ashcroft and Ashcroft 1990), mimicked the hypoxic hyperpolarization, and pretreatment with these drugs occluded the hypoxic hyperpolarization. These results indicate that in these neurons the activation of K<sub>ATP</sub> channels may contribute to the hypoxic hyperpolarization. Nevertheless, diazoxide or nicorandil did not produce any hyperpolarization in the remaining 60% of neurons, whereas tolbutamide or glibenclamide produced a depolarization and depressed the hypoxic hyperpolarization in ~80% of the neurons tested. This discrepancy may be due to the fact that in the experiments in which diazoxide and nicorandil were used the slices were prepared from relatively young (just after 3 wk postnatal) rats, which may only have a small number of K<sub>ATP</sub> channels. The activation of K<sub>ATP</sub> channels by diazoxide depends on the [ATP]; diazoxide induces openings of the channel at <1 mM [ATP], but has little effect at >1 mM (Trube et al. 1986). The cortical concentration of [ATP], is ~3 μM per g wet wt (Hansen 1985). Thus an alternative explanation is that the infrequent, small responses to activators of K<sub>ATP</sub> channels are due to high concentrations (>1 mM) of [ATP], in hippocampal CA1 neurons (Lipton and Whittingham 1982).

**ATP dependency of the hypoxic hyperpolarization**

When ATP was injected into the recording neuron, hypoxia failed to induce a hyperpolarization, suggesting that the hypoxic hyperpolarization is highly dependent on [ATP]. Furthermore, application of the metabolic inhibitor dinitrophenol mimicked the hypoxic hyperpolarization, and the dinitrophenol-induced hyperpolarization was reduced by pretreatment with tolbutamide and inhibited by intracellular injection of ATP. Dinitrophenol reduces the transmembrane proton concentration gradient across an inner mitochondrial membrane, which results in a decline of ATP synthesis from mitochondria (Darnell et al. 1990). Taken together, these results support the hypothesis that deprivation of ATP induces the hypoxic hyperpolarization.

Intracellular ATP injection by the use of electrodes containing Mg-ATP or K<sub>2</sub>-ATP similarly inhibited the generation of the hypoxic hyperpolarization. It is therefore unlikely that some leakage of Mg<sup>2+</sup> or K<sup>+</sup> through the electrode is involved in the depression of hypoxic hyperpolarization after ATP injection. Like ATP, the nonhydrolyzable ATP analogues AMP-PNP and adenylyl (β,γ-methylene)-diphosphate have been reported to block K<sub>ATP</sub> channels in heart, pancreatic β-cells, and skeletal muscle (Ashcroft and Kakei 1989; Cook and Hales 1984; Kakei et al. 1985; Spruce et al. 1987; Trube and Hescheler 1984). Similar results were observed in the present study; intracellular injection of AMP-PNP markedly reduced the hypoxic hyperpolarization. This result suggests that the blocking action of ATP on the generation of the hypoxic hyperpolarization is not due to energy supply by hydrolyzation of ATP.

In conclusion, the present study suggests that the hypoxic hyperpolarization is mainly due to the activation of K<sub>ATP</sub> channels caused by the reduction of [ATP], following hypoxic exposure.

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**REFERENCES**


