Potassium Conductance Causing Hyperpolarization of CA1 Hippocampal Neurons During Hypoxia

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Erdemli, G., Y. Z. Xu, and K. Krnjević. Potassium conductance causing hyperpolarization of CA1 hippocampal neurons during hypoxia. J. Neurophysiol. 80: 2378–2390, 1998. In experiments on slices (from 100- to 150-g Sprague-Dawley rats) kept at 33°C, we studied the effects of brief hypoxia (2–3 min) on CA1 neurons. In whole cell recordings from submerged slices, with electrodes containing only KMeSO₄ and N-2-hydroxyethylpipеразине-N'-2-ethanesulfonic acid, and in the presence of kynurenate and bicuculline (to minimize transmitter actions), hypoxia produced the following changes: under current clamp, 36 cells were hyperpolarized by 2.7 ± 0.5 (SE) mV and their input resistance (Rᵢ) fell by 23 ± 2.7% in 30 cells under voltage clamp, membrane current increased by 114 ± 22.3 pA and input conductance (Gᵢ) by 4.9 ± 0.9 nS. These effects are much greater than those seen previously with K gluconate whole cell electrodes, but only half those seen with ‘‘sharp’’ electrodes. The hypoxic hyperpolarizations (or outward currents) were not reduced by intracellular ATP (1–5 mM) or bath-applied glyburide (10 μM); therefore they are unlikely to be mediated by conventional ATP-sensitive K channels. On the other hand, their depression by internally applied ethylene glycol-bis-(β-aminoethyl ether) (1.1 and 11 mM) and especially 1,2-bis-(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid (11–33 mM) indicated a significant involvement of Ca-dependent Kₐ channels. The β-adrenergic agonist isoprenaline (10 μM) reduced hypoxic hyperpolarizations and decreases in Rᵢ (n = 4) (and in another 11 cells corresponding changes in Gᵢ); and comparable but more variable effects were produced by internally applied 3',5'-adenosine cyclic monophosphate (CAMP, 1 mM, n = 6) and bath-applied 8-bromo-cAMP (n = 8). Thus afterhyperpolarization-type Kₐ channels probably take part in the hypoxic response. A major involvement of G proteins is indicated by the near total suppression of the hypoxic response by guanosine 5'-O-(3-thiotriphosphate) (0.1–0.3 mM, n = 23) and especially guanosine 5'-O-(2-thiodiphosphate) (0.3 mM, n = 26), both applied internally. The adenosine antagonist 8-(p-sulfophenyl) theophylline (10–50 μM) significantly reduced hypoxic hyperpolarizations and outward currents in whole cell recordings (with KMeSO₄ electrodes) but not in intracellular recordings (with KCl electrodes) from slices kept at gas/saline interface. In further intracellular recordings, antagonists of γ-aminobutyric acid-B or serotonin receptors also had no clear effect. In conclusion, these G-protein-dependent hyperpolarizing changes produced in CA1 neurons by hypoxia are probably initiated by Ca²⁺ release from internal stores stimulated by enhanced glycolysis and a variable synergistic action of adenosine.

INTRODUCTION

Brief periods of hypoxia (2–3 min) result in a total, but reversible, loss of CA1 neuronal function. After a transient initial depolarization, the characteristic changes are cessation of firing, moderate hyperpolarization, and marked decrease in input resistance (Rᵢ), probably caused by enhanced potassium conductance (Gₖ) (Belousov et al. 1995; Croning et al. 1995; Fujimura et al. 1997; Fujiwara et al. 1987; Haddad and Jiang 1993; Hansen et al. 1982; Hyllienmark and Brismar 1996; Krnjević 1993; Krnjević and Leblond 1989; Yamamoto et al. 1997). Under voltage clamp, the predominant effect is a corresponding outward current and increased input conductance (Gᵢ) (Haddad and Jiang 1993; Krnjević 1993; Krnjević and Leblond 1989).

The initial depolarization reflects an inward current that probably persists throughout the hypoxic period, as indicated by the sustained inward current seen in many cells during hypoxia when outward current are suppressed by K channel blockers such as Ba and carbachol (Krnjević and Xu 1990). According to our unpublished observations, the associated conductance changes (<< 0.5 nS) are only some 3% of the mean input conductance of our cells and <10% of the observed hypoxic changes in conductance (Table 3). Although not negligible (they explain why the hypoxic outward current often has a more positive reversal potential than would be expected of a pure K current), they are not a major component of the overall response and therefore are not further analyzed in the present paper.

The nature of the Gₖ responsible for the prevailing hyperpolarization and how it is triggered by hypoxia has not been fully clarified. Obvious possibilities include a Ca-sensitive Gₖ (Gₖ(Ca)) activated by cytosolic Ca²⁺ ([Ca²⁺]ₜ) (Krnjević 1993; Krnjević and Lisiewicz 1972); a ATP-sensitive Gₖ (Gₖ(ATP)) opened by ATP depletion (Ashcroft 1988); and a G-protein-dependent Gₖ activated by adenosine, which is known to be released during hypoxia (Berne et al. 1974), or some other endogenous ligand, such as GABA or serotonin (Nicoll 1988).

The hypoxic response of CA1 neurons is suppressed by dantrolene, heparin, thapsigargin, procaine and, less consistently, by ryanodine (Belousov et al. 1995; Krnjević and Xu 1989; Yamamoto et al. 1997)—agents that prevent Ca²⁺ release from internal stores (Henzi and MacDermott 1992; Hill et al. 1987; Kobayashi et al. 1988; Mody and MacDonald 1995; Thastrup et al. 1990). In the hippocampus, as in the locus coeruleus (Murai et al. 1997), internal Ca²⁺ release thus plays an important role in the cellular response to hypoxia. But why should hypoxia elicit an early release of Ca²⁺? A likely answer is suggested by recent evidence that reduced nicotinamide adenine dinucleotide (NADH; formed by glycolysis during hypoxia, the Pasteur effect) (Clarke et al. 1989) enhances Ca²⁺ release from InsP₃-sensitive stores (Kaplin et al. 1996).
In experiments with “sharp” electrodes, Ca chelators do not suppress hypoxic hyperpolarizations very consistently (Leblond and Krnjević 1989; Yamamoto et al. 1997), either because of inadequate injection of chelators or because other types of G\(_K\), such as G\(_K\)\(_{ATP}\), also are involved. There is convincing evidence from single channel studies that K\(_{ATP}\) channels are present in some central neurons (Ashford et al. 1990; Jiang and Haddad 1997; Jiang et al. 1994). But for hippocampal neurons, the evidence has been indirect, relying mainly on tests of sulfonyleurea blockers of G\(_K\)\(_{ATP}\) (Fujimura et al. 1997; Grigg and Anderson 1989; Riepe et al. 1992), although sulfonureas (especially tolbutamide) can block other K channels (Crépel et al. 1993; Erdemli and Krnjević 1996; Godfraind and Krnjević 1993; Yamada et al. 1997); moreover, tolbutamide does not block some hypothalamic K\(_{ATP}\) channels (Ashford et al. 1990).

Whole cell recording might be expected to resolve these issues by allowing more efficient internal applications of agents such as ATP and Ca chelators. However, in recordings with gluconate- or Cl-containing electrodes (Zhang and Krnjević 1993), hypoxia had much weaker effects than in recordings with sharp electrodes. Subsequently, Zhang et al. (1994) and Velumian et al. (1997) found that hippocampal K and Ca currents soon run down when electrodes contain K gluconate or KCl but not KMeSO\(_4\).

In the present experiments with KMeSO\(_4\) electrodes, we observed substantial hypoxic changes in membrane properties, under both current and voltage clamp. The underlying mechanisms were investigated by whole cell recording with electrodes containing various combinations of relevant agents such as Ca chelators [ethylene glycol-bis-(β-aminomethyl ether)-N,N',N'',N''-tetraacetic acid (EGTA) or 1,2-bis (2-aminophenoxy) ethane-N,N',N'',N''-tetraacetic acid (BAPTA)], ATP, and cyclic AMP, and an activator or blocker of G proteins. In further attempts to identify the main G\(_K\) involved in the hypoxic responses, we also recorded (with whole cell or sharp electrodes) the effects produced by isoprenaline and antagonists of adenosine, GABA\(_A\), and serotonin receptors. Preliminary reports of some of these results have appeared as abstracts (Erdemli and Krnjević 1994a, 1997).

**Methods**

The brain was removed from young Sprague-Dawley rats (100–150 g) fully anesthetized with halothane. The hippocampus was dissected out in ice-cold oxygenated saline, and 400-μm transverse slices were cut with a Vibroslice (Campden Instruments, Loughborough, UK). They were kept for ≈1 h at room temperature. The standard artificial cerebrospinal fluid (ACSF) contained (in mM) 124 NaCl, 3.0 KCl, 2.0 CaCl\(_2\), 2.0 MgCl\(_2\), 1.25 NaH\(_2\)PO\(_4\), 26 NaHCO\(_3\), and 10 glucose. It was aerated continually with carbogen (95% O\(_2\),5% CO\(_2\)) at 15°–37°C. The pH was adjusted to 7.2 with 10 mM NaOH.

After filling with 3 M KOH, they had a resistance of 60–90 MΩ. The 2.5- to 3-μm-tip patch electrodes were made from 1.5 mm OD borosilicate glass. The “simple” internal solution (sIS) contained only 150 mM potassium methylsulfate (KMeSO\(_4\)) buffered to pH 7.2 with 10 mM N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid (HEPES). The following were added to some electrodes (alone or in various combinations, Table 1): adenosine 3′,5′-cyclic monophosphate (cAMP, 1 mM); ATP di-K salt (ATP, 1–5 mM); MgCl\(_2\) (1–5 mM); ethylene glycol-bis(β-aminomethyl ether)-N,N',N'',N''-tetraacetic acid (EGTA, 1.1 and 11 mM); 1,2-bis(2-aminophenoxy)ethane-N,N',N'',N''-tetraacetic acid tetra-K salt (BAPTA, 11 and 33 mM); Na\(_2\)BAPTA (11 mM, Fluka Chemicals, Ronkonkoma, NY); CaCl\(_2\), (0.1, 1.1, or 3.3 mM); GTTP tris salt (GTP, 0.2 mM); guanosine 5′-O-(3-thiotriphosphate) tetralithium salt (GTPyS, 0.1–0.3 mM); guanosine 5′-diphosphate Na salt (GDP, 1 mM); and guanosine 5′-O-(2-thiodiphosphate) trilithium salt (GDPβS, 0.1 mM). The pH was always adjusted to 7.2 with KOH. The osmolality (measured with a micro-osmometer, Precision Systems, Natick, MA) was kept within 280–310 mosm/kg by changing [KMeSO\(_4\)] as needed. The patch electrodes initially had a resistance of 2–3 MΩ. All recordings were done “blind” in the stratum pyramidale. The electrode series resistance was monitored by applying brief current pulses; in useful whole cell recordings, it remained <15 MΩ.

Because all the internal solutions of patch electrodes contained the same concentration of the predominant electrolyte (150 mM KMeSO\(_4\)), it was assumed that whatever junctional potential was formed at the tip would not vary systematically between different cells. According to our own measurements, 0.15 M KMeSO\(_4\), and 0.15 M NaCl differ by 7% in specific conductivity and 1.5% in osmotic coefficients. Using the Henderson equation, one can calculate a junctional potential of 5–7 mV between the KMeSO\(_4\) electrodes and ACSF. On the other hand, unknown, but possibly comparable, Donnan potentials may be generated during whole cell recording, owing to the presence of “immobile” anions in the cell. Therefore no attempt was made to correct the recorded values of membrane potential.

The signals were amplified by an Axoclamp 2 (Axon Instruments, Burlingame, CA) in current (“bridge”)- or voltage-clamp mode. The discontinuous voltage-clamp operated at a frequency of 3 kHz, a gain of 25 nA/mV, and an upper bandwidth limit of 300 Hz; the usual precautions were taken to optimize the efficacy of the clamp.

In bridge mode, the cell input resistance (\(R_i\)) was assessed by measuring the peak voltage produced by hyperpolarizing current pulses (0.1–0.2 nA, lasting 200 ms). To generate afterhyperpolarizations (AHPs), spike trains were evoked by 200-ms depolarizing pulses. Medium AHPs (Storm 1990) were measured at the early peak, slow AHPs 1 s after the start of the AHP. Under voltage clamp, the baseline current (\(I_b\)) was minimized by initially holding the membrane potential (\(V_m\)) near its resting level (about equal to −55 mV). The input conductance (\(G_i\)) was calculated by fitting a first-order regression to the linear portion of “instantaneous” current-voltage (I-V) plots, in the region negative to the holding potential (\(V_h\)). Voltage-dependent currents were elicited with voltage pulses lasting 500 ms.

Slices were made hypoxic with 95% N\(_2\)-5% CO\(_2\), and the superfuse was bubbled vigorously with this gas. Indirect synaptic effects were minimized by adding kynurenic acid (1–2 mM), bicuculline (10 μM) and, in some experiments, tetrodotoxin (1 μM). The following agents also were tested: by balanced iontophoresis from three-barreled micropipettes, AMP (from a 0.2 M electrode solution at pH 7) and serotonin [5-hydroxytryptamine (5-HT)], from a 40 mM 5-HT creatine sulfate solution at pH 3.2, the third barrel containing 1 mM NaCl; by bath-application, carbacol (20 μM), isoprenaline (5–10 μM), diluted from Isuprel; given by Sterling Winthrop, Markram, Ontario, Canada; and, in some experiments, by infusion. Solutions were prepared in standard artifical cerebrospinal fluid (ACSF) and the carbogen were warmed to 33–37°C. Culture media were transferred to a Haas-type chamber (Medical Systems, Greenvale, NY) where their upper surface was either exposed to humidified 95% N\(_2\)-5% CO\(_2\) or submerged under 0.1–0.5% O\(_2\)-99.5% N\(_2\) (95% O\(_2\)-5% CO\(_2\)) , which kept its pH 7.2 ± 0.3. All recordings were done “blind” using a Axoclamp 2 (Axon Instruments, Burlingame, CA) in current (“bridge”) or voltage-clamp mode. The discontinuous voltage-clamp operated at a frequency of 3 kHz, a gain of 25 nA/mV, and an upper bandwidth limit of 300 Hz; the usual precautions were taken to optimize the efficacy of the clamp.
tréal, Québec) from a stock solution in dimethyl sulfoxide (DMSO; ICN Biomedicals, Aurora, OH), 8-(p-sulfophenyl)theophylline (8-SPT, 10 μM, Research Biochemicals), 8-bromo-cyclic AMP Na salt (8-Br-cAMP, 1 mM), the GABA<sub>B</sub> antagonist CGP-35348 [Ciba-Geigy, Basel, Switzerland; given by Dr. R. Ticku and Ciba-Geigy Canada, Calgary (Dr. J. A. Zidichouski)], and the serotonin antagonist spiperone. To eliminate possible artifacts, all control and test media contained the same amounts of DMSO (0.1%) or NaOH. Chemicals were purchased from Sigma, except where indicated. Means ± SE are given throughout. The significance of differences was assessed by Student’s t-test (for paired results) or the d-statistic and its Fisher-Behrens distribution (Campbell 1989, p. 420) for unpaired differences with unequal population variances.

**RESULTS**

In agreement with previous reports (Belousov et al. 1995; Zhang and Krnjević 1993), when compared with conventional intracellular recordings, the whole cell recordings with simple solutions (sIS, Table 1) were characterized by large action potentials (∼100 mV), a more positive <i>V<sub>m</sub></i> (∼58 ± 0.80 mV) and high <i>R<sub>m</sub></i> (76 ± 3.7 MΩ; Table 2).

**Effects of brief hypoxia under current clamp**

As mentioned in the Introduction, after a brief depolarization, which can be seen in several figures (e.g., Figs. 1, A and B, 4A, 6C, and 9A), there is a sustained, predominantly hyperpolarizing change in membrane potential (<i>V<sub>m</sub></i>). In 36 CA1 neurons recorded with sIS electrodes, 2–3 min of hypoxia evoked a mean hyperpolarization of 2.7 ± 0.50 mV, accompanied by a 23 ± 2.7% fall in <i>R<sub>m</sub></i> (Fig. 1A; see also Table 2).

**Tests of possible activation of K<sub>ATP</sub> channels**

**HIGH INTERNAL ATP.** To counteract any depletion of cellular ATP during hypoxia, 1–5 mM ATP was added to sIS. This reduced hypoxia but did not abolish the effects of hypoxia (Fig. 1B). The mean hypoxic fall in <i>R<sub>m</sub></i> (∆<i>R<sub>m</sub></i>) diminished by half—the unpaired difference from the control mean of ∆23 ± 2.7% was ∆11 ± 4.8% (for <i>n</i> = 7, <i>P</i> < 0.05); but ∆<i>V<sub>m</sub></i> was slightly increased (Table 2).

In these cells, both <i>V<sub>m</sub></i> and <i>R<sub>m</sub></i> were significantly higher than in cells recorded with sIS electrodes. Thus ATP/Mg may suppress a G<sub>q</sub> that is at least partly open in the presence of sIS. This would be consistent with block of K<sub>ATP</sub> channels, but also with phosphorylation- and/or G-protein-dependent modulation of some other K channels (Brown 1990; Egan et al. 1993; Sadoshima et al. 1988). A further possibility is significant chelation by ATP (Sellers et al. 1992) of the substantial residual Ca<sup>2+</sup> present in such nominally Ca-free solutions as sIS (could be ∼40 μM) (Zhang et al. 1995). The fact that adding EGTA/Ca (which should hold [Ca<sup>2+</sup>]), well <100 nM) restored the lower <i>R<sub>m</sub></i> suggests that these channels are indeed Ca sensitive.

**SULFONYLUREAS.** The K<sub>ATP</sub> channel blocker glyburide (10 μM) was applied to 12 cells. As reported by Godfraind and Krnjević (1993), glyburide tended to reinforce the hyperpolarizing responses of some cells. Thus the inconspicuous potential change elicited initially by hypoxia in the cell shown in Fig. 1C became a clear hyperpolarization after the application of glyburide (Fig. 1D). But overall, the hypoxic ∆<i>V<sub>m</sub></i> and ∆<i>R<sub>m</sub></i> were not significantly altered by glyburide: the respective paired differences from control values were 0.4 ± 1.8 mV and 0.3 ± 3.5%. Glyburide was equally ineffective as blocker of the outward current induced by hypoxia in cells under voltage clamp (cf. Fig. 2, A and B).

Tolbutamide, another K<sub>ATP</sub> channel blocker (Ashford et al. 1990; Henquin and Meissner 1982), suppresses both hypoxic outward currents (Godfraind and Krnjević 1993) and slow AHPs in hippocampal slices (Erdemli and Krnjević 1993).

**TABLE 1. Composition of filling solutions for patch electrodes**

<table>
<thead>
<tr>
<th>Internal Solution</th>
<th>K-EGTA</th>
<th>BAPTA</th>
<th>CaCl&lt;sub&gt;2&lt;/sub&gt;</th>
<th>MgCl&lt;sub&gt;2&lt;/sub&gt;</th>
<th>K-ATP</th>
<th>GTP</th>
</tr>
</thead>
<tbody>
<tr>
<td>sIS(EGTA)</td>
<td>1.1</td>
<td>0.1</td>
<td>1</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>sIS(EGTA, GTP)</td>
<td>1.1</td>
<td>0.1</td>
<td>1–5</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>sIS(EGTA, GTP, EGTA)</td>
<td>1.1</td>
<td>0.1</td>
<td>1</td>
<td>0.5</td>
<td>1</td>
<td>0.2</td>
</tr>
<tr>
<td>sIS(EGTA, BAPTA)</td>
<td>11–33</td>
<td>1.1–3.3</td>
<td>1</td>
<td>1</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Simple internal solution (sIS) contained only 150 mM KMeSO<sub>4</sub> and 10 mM N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid (HEPES; pH 7.2); others had the above additions (in mM). EGTA, ethylene glycol-bis(β-aminoethyl ether)-N,N’,N’’-tetraacetic acid; BAPTA, 1,2-bis(2-aminophenoxyethyl)amine-N,N’,N’’-tetraacetic acid.

**TABLE 2. Resting membrane properties and hypoxic responses of CA1 neurons recorded under current clamp with different internal electrode solutions**

<table>
<thead>
<tr>
<th>Internal Solution</th>
<th>n</th>
<th>V&lt;sub&gt;m&lt;/sub&gt; mV</th>
<th>R&lt;sub&gt;m&lt;/sub&gt; mΩ</th>
<th>∆V&lt;sub&gt;m&lt;/sub&gt; mV</th>
<th>∆R&lt;sub&gt;m&lt;/sub&gt; %</th>
</tr>
</thead>
<tbody>
<tr>
<td>sIS</td>
<td>36</td>
<td>−58 ± 0.80</td>
<td>76 ± 3.7</td>
<td>−2.7 ± 0.50*</td>
<td>−23 ± 2.7*</td>
</tr>
<tr>
<td>sIS (ATP)</td>
<td>7</td>
<td>−43 ± 3.7†</td>
<td>102 ± 5.6†</td>
<td>−3.1 ± 1.1†</td>
<td>−12 ± 4.0††</td>
</tr>
<tr>
<td>sIS (EGTA)</td>
<td>10</td>
<td>−57 ± 1.6</td>
<td>87 ± 5.4</td>
<td>−2.3 ± 0.80†‡</td>
<td>−12 ± 4.5†‡</td>
</tr>
<tr>
<td>sIS (ATP, EGTA)</td>
<td>18</td>
<td>−56 ± 1.1</td>
<td>85 ± 7.4</td>
<td>−2.1 ± 0.70$</td>
<td>−26 ± 2.6*</td>
</tr>
<tr>
<td>sIS (ATP, GTP, EGTA)</td>
<td>10</td>
<td>−55 ± 1.6</td>
<td>102 ± 6.1†</td>
<td>−3.0 ± 0.56*</td>
<td>−23 ± 4.3*</td>
</tr>
<tr>
<td>sIS (ATP, BAPTA)</td>
<td>18</td>
<td>−65 ± 1.3†</td>
<td>51 ± 3.6†</td>
<td>−0.60 ± 0.40‡</td>
<td>−19 ± 2.5*</td>
</tr>
</tbody>
</table>

Values are means ± SE; <i>n</i> is number of cells. <i>V<sub>m</sub></i>; resting membrane potential; <i>R<sub>m</sub></i>, resting input resistance; ∆<i>V<sub>m</sub></i> and ∆<i>R<sub>m</sub></i> peak changes during 2–3 min of hypoxia. Compositions of internal solutions are listed in Table 1. * <i>P</i> ≤ 0.001. † <i>V<sub>m</sub></i> or <i>R<sub>m</sub></i> significantly different from sIS value. ‡ <i>P</i> ≤ 0.05. $ <i>P</i> ≤ 0.01.

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All recordings were in presence of kynurenic acid (KYN) and bicuculline shown on 100-fold accelerated traces before, during, and after hypoxic tests. These pulses and the corresponding potential changes are illustrated. Initial membrane potentials are indicated by numbers.

Input resistance was measured with current pulses lasting 200 ms and intensity 0.1 nA for A and 0.2 nA for B and C and D; only those for A and C actually are illustrated. These pulses and the corresponding potential changes are shown on 100-fold accelerated traces before, during, and after hypoxic tests. All recordings were in presence of kynurenic acid (KYN) and bicuculline (BIC), with 0.1% dimethyl sulfoxide (DMSO) added for both C and D. Initial membrane potentials are indicated by numbers.

Internal Ca chelators: tests of possible activation of \(K_{Ca}\) channels

HYPERPOLARIZING ACTION OF BAPTA. During the first 5–15 min after the break through the membrane, cells recorded with BAPTA-containing electrodes typically showed a gradual hyperpolarization and substantial fall in \(R_{in}\) (Fig. 3, A and B). Similar changes were seen with electrodes containing 11 mM K4-BAPTA (\(n = 13\)), 33 mM K4-BAPTA (\(n = 15\)), or 11 mM Na4-BAPTA (\(n = 4\)) (Table 1). According to the pooled data from 32 cells, \(V_{m}\) (initially \(-60 \pm 0.85\) mV) changed by \(-7.7 \pm 1.1\) mV (\(P < 0.001\)) and \(R_{in}\) (initially \(91 \pm 5.8\) MΩ) dropped by \(40 \pm 3.4\%\) (\(P < 0.001\)). Most of the \(R_{in}\) change was not secondary to hyperpolarization because it persisted when the initial \(V_{m}\) was restored by current injection (Fig. 3 B). Similar changes in \(V_{m}\) and \(R_{in}\) were recorded in 0.2 mM tetrathylammonium (TEA; \(n = 2\)). Low concentrations of TEA block a K current that appears to be suppressed by Ca2+ (Constanti et al. 1993) and therefore could mediate the slow hyperpolarizing shift, but they were abolished by 20 μM carbachol (\(n = 6\); Fig. 3 C). In its presence, \(\Delta V_{in}\) was \(-0.25 \pm 0.57\) mV (from \(-44 \pm 3.4\) mV) and \(\Delta R_{in}\) 12 ± 6.7% (from 124 ± 19.4 MΩ). No comparable hyperpolarizing trend was seen with EGTA-containing electrodes (Fig. 3 D).

Under voltage clamp, resting values of \(V_{m}\) and baseline current (\(I_{b}\)) did not differ significantly when recorded with SiS or SiS (EGTA) electrodes (Table 3). With BAPTA-containing electrodes, \(I_{b}\) and \(G_{m}\) rose gradually after the onset of recording, resulting in a higher steady \(I_{b}\) and \(G_{m}\) than was seen with any other type of electrode (Table 3).

EFFECTS OF BRIEF HYPOXIA. Under current clamp. Simply adding EGTA (1.1 mM with 0.1 mM CaCl2) to SiS reduced \(\Delta R_{in}\) by nearly half (\(-11 \pm 5.2\%\); unpaired differ-

FIG. 1. Hypoxic changes in \(V_{m}\) and \(R_{in}\) are not abolished by either internal ATP or external glyburide. Current-clamp traces show the responses to 2 min of hypoxia \((N_{2})\) in 3 cells recorded with patch electrodes containing: simple internal solution \([\text{siS} (\text{KMeSO}_{4} \text{ and HEPES}) \text{ A}]; \text{siS} (\text{ATP} 5 \text{ mM}) \text{ B}, \text{and siS} \text{C} \text{for this cell, effect of hypoxia was recorded before (C) and during bath application of } 10 \text{ μM glyburide (D): note inconspicuous hypoxic hyperpolarization in C was enhanced by glyburide in D. Input resistance was measured with current pulses lasting 200 ms and intensity 0.1 nA for A and B and 0.2 nA for C and D; only those for A and C actually are illustrated. These pulses and the corresponding potential changes are shown on 100-fold accelerated traces before, during, and after hypoxic tests. All recordings were in presence of kynurenic acid (KYN) and bicuculline (BIC), with 0.1% dimethyl sulfoxide (DMSO) added for both C and D. Initial membrane potentials are indicated by numbers.\n
 FIG. 2. Hypoxic outward current recorded under voltage-clamp shows little effect of glyburide but it is blocked by Ba and carbachol. In presence of tetrodotoxin (TTX), KYN, and BIC, with potential held at \(-40\) mV, outward currents were evoked by 20-nV depolarizing pulses or by 2 min of hypoxia (between vertical arrows); before, during, and after hypoxic tests, 20-nV hyperpolarizing pulses monitored input conductance [at bottom the voltage steps, which lasted 500 ms]; 0 current levels are indicated by horizontal arrows (left). A: control traces. B: after 12 min of glyburide (10 μM) application. C: 5 min after start of Ba (1 mM) and carbachol (20 μM) application, depolarizing pulse evokes only inward current and hypoxia minimal outward current and conductance increase.
FIG. 3. Internally applied 1,2-bis (2-aminophenoxy) ethane-N,N,N′,N′-tetraacetic acid (BAPTA) causes hyperpolarization and decrease in input resistance. Whole cell recording from CA1 neurons in presence of kynurenic acid (KYN, 1 mM) and bicuculline (BIC, 10 µM) with electrodes containing sIS (ATP, NaBAPTA 11 mM) (A); sIS (ATP, K/BAPTA 11 mM) (B); sIS (ATP, K/BAPTA 33 mM) (C); and sIS (ATP, EGTA 11 mM) (D). Bottom of each trace, membrane potential (initial value is indicated) traces just after breakthrough as well as later. Cells in A and B show characteristic hyperpolarization (after transient depolarization) and marked drop in Rm—which was not abolished by restoring initial Vm with 330 pA depolarizing current injection (B). C: in similar recording from another cell, carbachol (20 µM) prevented such BAPTA-induced hyperpolarization and decrease in Rm. D, in recording with EGTA-containing electrode, there was no comparable hyperpolarization and decrease in Rm after the initial membrane breakthrough.

ence, P = 0.05), although it made no difference to the hypoxic ∆Vm (Table 2). Thus either ATP or EGTA alone depressed ∆Rm. However, when both were added (with or without GTP), the hypoxic changes were virtually identical to those recorded with sIS alone (Table 2).

When electrodes contained BAPTA (11 or 33 mM), the hypoxic hyperpolarization was abolished, but ∆Rm was not significantly depressed (Table 2). Figure 4 illustrates depolarizing responses to hypoxia recorded with 11 and 33 mM BAPTA electrodes (B and C) and, for comparison, a small but clear hyperpolarization seen with an 11 mM EGTA electrode (A).

Under voltage clamp. The substantial increases in Gm and Ih observed with sIS electrodes (Fig. 2A) were two-thirds smaller when EGTA was added to the electrodes (Table 3)—the unpaired reductions (−3.2 ± 1.07 nS for ∆Gm and −80 ± 26.4 pA for ∆Ih) were both significant at P < 0.05. Further addition of ATP to the electrodes restored the larger hypoxic changes obtained with sIS electrodes (Table 3): ∆Gm was increased by 3.2 ± 0.83 nS (P < 0.01) and ∆Ih by 58 ± 23 pA (P < 0.05). Although ∆Gm was not diminished, the hypoxic outward currents seen with BAPTA electrodes were significantly smaller than those recorded with sIS electrodes (by 70 ± 34.9 pA, P = 0.05) (Table 3). The voltage- and current-clamp observations are thus in good agreement.

TABLE 3. Resting membrane properties and hypoxic responses of CA1 neurons recorded under voltage clamp with different internal electrode solutions

<table>
<thead>
<tr>
<th>Internal Solution</th>
<th>n</th>
<th>Vm, mV</th>
<th>Gm, nS</th>
<th>Ih, pA</th>
<th>∆Gm, nS</th>
<th>∆Ih, pA</th>
</tr>
</thead>
<tbody>
<tr>
<td>sIS (EGTA)</td>
<td>30</td>
<td>−55 ± 0.8</td>
<td>14 ± 2.6</td>
<td>22 ± 7.4</td>
<td>4.9 ± 0.90*</td>
<td>114 ± 22.3*</td>
</tr>
<tr>
<td>sIS (ATP)</td>
<td>9</td>
<td>−53 ± 2.1</td>
<td>7.7 ± 2.1</td>
<td>−7.8 ± 15.8</td>
<td>1.7 ± 0.58† ‡</td>
<td>34 ± 14.1† ‡</td>
</tr>
<tr>
<td>sIS (ATP, GTP)</td>
<td>12</td>
<td>−49 ± 1.9</td>
<td>12 ± 0.83</td>
<td>−63 ± 24.6†</td>
<td>4.9 ± 0.60*</td>
<td>92 ± 17.8*</td>
</tr>
<tr>
<td>sIS (ATP, GDP)</td>
<td>8</td>
<td>−53 ± 3.1</td>
<td>18 ± 1.4</td>
<td>−10 ± 23</td>
<td>5.3 ± 1.6†</td>
<td>−18 ± 38‡</td>
</tr>
<tr>
<td>sIS (ATP, BAPTA)</td>
<td>13</td>
<td>−56 ± 1.9</td>
<td>24 ± 2.6‡</td>
<td>373 ± 84.1‡</td>
<td>5.3 ± 0.90*</td>
<td>44 ± 26.8‡</td>
</tr>
</tbody>
</table>

Values are means ± SE; n is number of cells. Vm, holding potential; Gm, resting input conductance; Ih, baseline current; ∆Gm and ∆Ih, peak changes during 2–3 min of hypoxia. Compositions of internal solutions are listed in Table 1; in sIS (EGTA, GDP) 1 mM GDP was substituted for ATP. * P ≤ 0.001. † P ≤ 0.05. ‡, Significantly different from sIS value. § P ≤ 0.01.
Tests of G-protein involvement in hypoxic response

EFFECTS OF GDPβS AND GTPγS. G-protein-mediated activations of \( G_\kappa \) are well known (Brown 1990; Jan and Jan 1997; Nicoll 1988). A relevant example is the pertussis toxin-sensitive K current elicited by adenosine (Trussell and Jackson 1987). If such a \( G_\kappa \) contributes significantly to the hypoxic response, it should be sensitive to agents that stimulate or inhibit G proteins, especially GTPγS and GDPβS, the actions of which are irreversible (Eckstein et al. 1979).

Indeed the effects of hypoxia were either undetectable or greatly diminished in both current- and voltage-clamp recordings with electrodes containing GTPγS or GDPβS.

Under current clamp. Cells recorded with GDPβS- or GTPγS-containing electrodes showed no hypoxic hyperpolarization and a greatly reduced \( \Delta R_m \), especially with GDPβS (Fig. 5C and Table 4): mean \( \Delta R_m \) diminished by 71\% (unpaired difference was \(-18 \pm 3.1\%\), \( P < 0.01 \)). Although sometimes equally striking (Fig. 5B), the reductions seen with GTPγS were less consistent (means down by 31\%; unpaired differences \(-8.0 \pm 4.7\%\), \( P < 0.1 \)).

Under voltage clamp. Both agents abolished the hypoxic outward current; GDPβS also fully suppressed the increase in \( R_m \), whereas GTPγS reduced it by 69\% (the unpaired difference, \(-3.4 \pm 0.78\) nS, was very significant, \( P < 0.01; \) Table 5). The reversible ligands GTP and GDP were less effective (Table 3), the only notable change being the suppression of the outward current by GDP (down by \( 110 \pm 42\) pA, \( P < 0.05 \)).

The impressive actions of GDPβS and GTPγS, which confirm earlier tests of GTPγS (Krnjević and Xu 1990), are strong evidence that G-protein activation is an early consequence of hypoxia. Several ligands that open \( G_\kappa \) via a G protein could be released during hypoxia. They include GABA and serotonin (Andrade et al. 1986) and particularly adenosine (Trussell and Jackson 1987).

Adenosine. In previous intracellular recordings from slices in an interface-type chamber (Leblond and Krnjević 1989), adenosine antagonists such as 8-SPT did not suppress the hypoxic changes in \( V_m \) and \( R_m \); and 1 mM adenosine, which had a pronounced hyperpolarizing effect, did not occlude the hypoxic conductance increase. Further tests under the same conditions have confirmed these results. A marked hyperpolarization, associated with a sharp conductance increase, could be evoked reproducibly by iontophoretic applications of AMP (AMP; Figs. 6 and 7).

The traces in Fig. 6 illustrate well the contrast between the purely hyperpolarizing action of adenosine (or serotonin) and the mixed effects of hypoxia: initial depolarization, smaller hyperpolarization, and clear posthypoxic hyperpolarization (not seen after AMP or 5-HT applications). The hypoxic hyperpolarization is relatively small because of an underlying continued depolarizing effect (inward current), which is revealed when, as in Fig. 10B, the hyperpolarizing action (or outward current) is suppressed (Krnjević and Xu 1990).

The adenosine antagonist 8-SPT markedly enhanced both ongoing firing and synchronically evoked responses and virtually abolished the effect of AMP but not the small hyperpolarization and large conductance increase induced by hypoxia (Fig. 7B). Comparable tests of 10–50 \( \mu \)M 8-SPT on another 12 cells under current- or voltage-clamp consistently showed clear preservation of hypoxic hyperpolarizations (or outward currents) and especially the conductance changes.

By contrast, in a recent study on submerged slices, including whole cell recording, 8-SPT partly reduced the hypoxic outward current and conductance (Zhu and Krnjević 1997); and

### Table 4. Hypoxia-induced changes in membrane properties (under current clamp) recorded with patch-electrodes containing GDPβS, GTPγS, or cAMP

<table>
<thead>
<tr>
<th>Internal Solution</th>
<th>( n )</th>
<th>( V_m ), mV</th>
<th>( R_m ), M( \Omega )</th>
<th>( \Delta V_m ), mV</th>
<th>( \Delta R_m ), %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control [sIS (EGTA, ATP)]</td>
<td>18</td>
<td>(-56 \pm 1.1)</td>
<td>85 ± 7.4</td>
<td>(-2.1 \pm 0.70^*)</td>
<td>(-26 \pm 2.6^\dagger)</td>
</tr>
<tr>
<td>GDPβS (0.3 mM)</td>
<td>11</td>
<td>(-58 \pm 1.3)</td>
<td>52 ± 6.7*</td>
<td>(-2.0 \pm 1.2)</td>
<td>(-7.6 \pm 1.7^\ddagger)</td>
</tr>
<tr>
<td>GTPγS (0.1–0.3 mM)</td>
<td>14</td>
<td>(-53 \pm 1.2)</td>
<td>90 ± 6.8</td>
<td>(-0.90 \pm 0.80)</td>
<td>(-18 \pm 3.9^\ddagger)</td>
</tr>
<tr>
<td>Control (sIS)</td>
<td>12</td>
<td>(-51 \pm 1.9)</td>
<td>73 ± 5.6</td>
<td>(-2.7 \pm 1.2)</td>
<td>(-30 \pm 3.8^\ddagger)</td>
</tr>
<tr>
<td>cAMP (1 mM)</td>
<td>6</td>
<td>(-54 \pm 4.0)</td>
<td>71 ± 10.4</td>
<td>(-3.0 \pm 0.68^*)</td>
<td>(-18 \pm 5.1^\ddagger)</td>
</tr>
</tbody>
</table>

Values are means ± SE; \( n \) is number of cells tested. Agents were added to control internal solution [sIS (EGTA, ATP), see Table 1]; except adenosine 3',5'-cyclic monophosphate (cAMP), which was added to simple sIS used for control recordings in the same slices. \( V_m \), resting potential; \( R_m \), input resistance; \( \Delta V_m \) and \( \Delta R_m \), hypoxia-induced changes. GDPβS, guanosine 3',5'-O-(3-thiotriphosphate) tetralithium salt; GTPγS, guanosine 5'-O-(3-thiotriphosphate) tetralithium salt. *\( P < 0.01 \). †\( P < 0.001 \). ‡Significantly different from control value. §\( P < 0.05 \).
in the current experiments on submerged slices, 8-SPT eliminated the hypoxic changes in recordings under both current and voltage clamp (\(n = 5\) and 4, respectively). In 10 \(\mu\)M 8-SPT, hypoxia elicited depolarizations and no drop in \(R_n\), as illustrated in Fig. 8. Under voltage clamp, the hypoxic outward current and increase in \(G_m\) were similarly abolished. These data are summarized in Table 6. A relevant point is that 8-SPT also depressed (reversibly) both the medium and the slow AHP evoked by bursts of spikes (Fig. 8D).

**Serotonin (5-HT).** In recordings with 3 M KCl-containing sharp electrodes, the 5-HT antagonist spiperone (6–50 \(\mu\)M) selectively suppressed hyperpolarizations evoked by microiontophoretic applications of 5-HT (Figs. 6 and 7A); but the hypoxic responses were little changed (Fig. 7A). Comparable observations were made on four other cells tested under similar conditions.

**GABA.** The GABA\(_B\) receptor antagonist CGP-35348 was tested on five cells, two recorded under current clamp, three under voltage clamp, mainly during intracellular (sharp electrode) recordings. When bath-applied at 0.5 mM, this agent did not depress the hypoxic hyperpolarization and \(R_n\) fall (Fig. 9, A and B) or the corresponding outward current and \(G_m\) rise (not shown). Overall, for the five cells, the hypoxic \(\Delta G_m\) changed by \(-0.4 \pm 9.3\%\), \(\Delta V_m\) by \(-0.35 \pm 0.35\) mV and \(\Delta I_h\) by \(-13 \pm 14.9\) pA; none of these was significant. In three other cells, there was no sign of occlusion between marked increases in \(G_m\) produced by hypoxia and by the GABA\(_B\) agonist baclofen (not shown).

**Role of AHP-type \(K_{Ca}\) channels in hypoxic response**

Several findings suggested the possibility that a \(K_{Ca}\) (AHP) is activated by hypoxia, more specifically that responsible for the slow component of AHPs: notably the suppression of

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**TABLE 5.** Hypoxia-induced changes in membrane properties (under voltage clamp) recorded with patch-electrodes containing GDP\(_S\), GTP\(_{\gamma}\)S, or cAMP

<table>
<thead>
<tr>
<th>Internal Solution</th>
<th>(n)</th>
<th>(V_m), mV</th>
<th>(G_m), nS</th>
<th>(I_h), pA</th>
<th>(\Delta G_m), nS</th>
<th>(\Delta I_h), pA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control [sIS (EGTA, ATP)]</td>
<td>27</td>
<td>(-50 \pm 1.5)</td>
<td>13 (\pm 0.90)</td>
<td>(-63 \pm 24.6)</td>
<td>4.9 (\pm 0.60*)</td>
<td>92 (\pm 17.8*)</td>
</tr>
<tr>
<td>GDP(_S) (0.3 mM)</td>
<td>15</td>
<td>(-57 \pm 1.3)</td>
<td>14 (\pm 1.2)</td>
<td>(-4.0 \pm 12.5†)</td>
<td>(-0.1 \pm 0.47†)</td>
<td>(-21 \pm 8.01†)</td>
</tr>
<tr>
<td>GTP(_{\gamma})S (0.1–0.3 mM)</td>
<td>9</td>
<td>(-58 \pm 1.2)</td>
<td>9.9 (\pm 1.1†)</td>
<td>(-23 \pm 14.6)</td>
<td>1.5 (\pm 0.50‡)</td>
<td>(-7.8 \pm 12.4†)</td>
</tr>
<tr>
<td>Control (sIS)</td>
<td>9</td>
<td>(-46 \pm 1.6)</td>
<td>17 (\pm 1.7)</td>
<td>34 (\pm 31.7)</td>
<td>7.3 (\pm 12*)</td>
<td>201 (\pm 38*)</td>
</tr>
<tr>
<td>cAMP (1 mM)</td>
<td>6</td>
<td>(-46 \pm 3.5)</td>
<td>11 (\pm 1.6†)</td>
<td>14 (\pm 33.8)</td>
<td>3.4 (\pm 1.1‡)</td>
<td>59 (\pm 21‡)</td>
</tr>
</tbody>
</table>

Values are means \(\pm SE;\) \(n\) is number of cells tested. Agents were added to control internal solution [sIS (EGTA, ATP), see Table 1]; except cAMP, which was added to simple sIS solution used for control recordings in the same slices. \(V_m\), holding potential; \(G_m\), input conductance; \(I_h\), baseline current; \(\Delta s\) are corresponding changes at peak of hypoxia. *\(P \leq 0.001\). †Significantly different from control value. ‡\(P \leq 0.05\).

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[FIG. 6.](#) Effects of 5-hydroxytryptamine (5-HT), adenosine 5’-monophosphophate (AMP) and hypoxia recorded with sharp microelectrode from slice in interface-type chamber. Note marked hyperpolarization and conductance increase produced by iontophoretic applications of 5-HT (40 nA for 60 s, A) or AMP (80 nA for 30 s, B); and initial depolarization then small hyperpolarization during 2 min of hypoxia (C). Identical hyperpolarizing current pulses (0.2 nA, 0.2 s; illustrated top) were injected at regular 5-s intervals to monitor input resistance; a constant stimulus, at same frequency, was applied to stratum radiatum to evoke synaptic response.

[FIG. 7.](#) In same CA1 neuron (cf. Fig. 6), selective antagonists greatly diminish the responses to 5-HT and adenosine but not the effects of hypoxia. Similar iontophoretic tests of 5-HT and AMP show that spiperone (20 \(\mu\)M, bath-applied) largely blocked effect of 5-HT but not that of AMP or hypoxia (A); whereas 8-(p-sulfophenyl)theophylline (8-SPT; 10 \(\mu\)M, also bath-applied) largely blocked effect of AMP, but small hyperpolarization and marked drop in resistance were still elicited by 2 min of hypoxia (B). As in other current-clamp recordings, input resistance was measured throughout with hyperpolarizing current pulses: these 0.2-nA pulses, which lasted 200 ms, are monitored on top trace (only for A); they are shown on 100-fold accelerated traces shortly before and during hypoxic tests in both A and B.
FIG. 8. In whole cell recording from submerged slices, 8-SPT depresses both medium AHP and hypoxic hyperpolarization. All traces were obtained from same CA1 neuron recorded with sIS (ATP, EGTA) electrode, in presence of KYN and BIC; 0.2-s, 0.1-nA pulses applied to monitor $R_{in}$ are shown top: A: hyperpolarization and $R_{in}$ drop during 2 min of hypoxia in control conditions. B: in 10 μM 8-SPT (10 min), hypoxia elicited depolarization and $R_{in}$ rise; these were largely reversed by 35-min wash (C). D: examples of afterhyperpolarizations (AHPs) induced by depolarizing pulses that evoked 9 spikes, recorded before, during 8-SPT (10 μM) application, and after wash; all were recorded at initial $V_{m}$. Note greater depression of mAHP than sAHP.

### TABLE 6. Effects of 8-SPT (10 μM, bath-applied) on resting membrane properties and hypoxia-induced changes

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>8-SPT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Current Clamp</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$V_{m}$ mV</td>
<td>$-45 \pm 7.3$</td>
<td>$-45 \pm 7.3$</td>
</tr>
<tr>
<td>$R_{in}$ MΩ</td>
<td>$80 \pm 10$</td>
<td>$80 \pm 10$</td>
</tr>
<tr>
<td>$\Delta V_{m}$ mV</td>
<td>$2.8 \pm 1.1^*$</td>
<td>$2.8 \pm 1.1^*$</td>
</tr>
<tr>
<td>$\Delta R_{in}$ %</td>
<td>$14 \pm 10.2^*$</td>
<td>$14 \pm 10.2^*$</td>
</tr>
<tr>
<td>Voltage Clamp</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$I_{b}$ pA</td>
<td>$-20 \pm 25$</td>
<td>$-20 \pm 25$</td>
</tr>
<tr>
<td>$G_{in}$ nS</td>
<td>$38 \pm 7.6$</td>
<td>$38 \pm 7.6$</td>
</tr>
<tr>
<td>$\Delta I_{b}$ pA</td>
<td>$25 \pm 35^*$</td>
<td>$25 \pm 35^*$</td>
</tr>
<tr>
<td>$\Delta G_{in}$ nS</td>
<td>$1.5 \pm 0.60$</td>
<td>$1.5 \pm 0.60$</td>
</tr>
</tbody>
</table>

Values are means ± SE. Data were obtained by whole cell recording under current and voltage clamp [with sIS (EGTA, ATP, GTP) electrodes; $V_{m} = -30$ mV]. Number of cells tested was 5 for current clamp and 4 for voltage clamp. $V_{m}$, resting potential; $R_{in}$, input resistance; $V_{h}$, holding potential; $I_{b}$, baseline current; $G_{in}$, input conductance; $\Delta V_{m}$, $\Delta R_{in}$, $\Delta I_{b}$, and $\Delta G_{in}$ are hypoxia-induced changes. *Significantly different from control values (paired). 8-SPT, 8-(β-sulfophenyl)theophylline; † $P < 0.05$.

which acts via β receptors and cyclic AMP (Madison and Nicoll 1986; Pedarzani and Storm 1993), might therefore also be expected to depress the hypoxic changes.

### ISORENALINE

When it was applied to 15 cells under various conditions of recording, mostly in the whole cell mode, isoprenaline (5–10 μM) either abolished ($n = 3$) or reduced by about half ($n = 11$) hypoxic hyperpolarizations and outward currents (Fig. 9, C and D, and Table 7). Most cells also showed a corresponding reduction in hypoxic $\Delta G_{in}$. In agreement with previous reports, isoprenaline markedly depressed sAHPs (by $61 \pm 13.9^\%$, $P < 0.01$). In three cells, slowly decaying tail currents after depolarizing pulses to approximately equal to $-10$ mV (probably largely consisting of $I_{AHP}$) (Lancaster and Adams 1986) were reduced by $52 \pm 14.7^\%$ ($P < 0.05$).

### CYCLIC AMP

We also tested the effects of cAMP, the internal mediator of isoprenaline’s action on AHPs (Madison and Nicoll 1986; Pedarzani and Storm 1993). A series of current-clamp recordings were made with electrodes containing either plain sIS or sIS with 1 mM cAMP added.
TABLE 7. Effects of isoprenaline (Isoprel, 10 μM, bath applied) on resting membrane properties and hypoxia-induced changes

<table>
<thead>
<tr>
<th>Current Clamp</th>
<th>Control</th>
<th>Isoprel</th>
</tr>
</thead>
<tbody>
<tr>
<td>$V_m$, mV</td>
<td>$-53 \pm 1.6$</td>
<td>$-56 \pm 1.8$</td>
</tr>
<tr>
<td>$R_m$, MΩ</td>
<td>$119 \pm 19$</td>
<td>$132 \pm 17$</td>
</tr>
<tr>
<td>$\Delta V_m$, mV</td>
<td>$-4.8 \pm 1.1^*$</td>
<td>$1.3 \pm 1.1^+$</td>
</tr>
<tr>
<td>$\Delta R_m$, %</td>
<td>$-23 \pm 7.2^*$</td>
<td>$-2.6 \pm 2.5$</td>
</tr>
</tbody>
</table>

Voltage Clamp

<table>
<thead>
<tr>
<th>Whole Cell</th>
<th>Intracellular</th>
</tr>
</thead>
<tbody>
<tr>
<td>$I_h$, pA</td>
<td>$139 \pm 67$</td>
</tr>
<tr>
<td>$G_{in}$, nS</td>
<td>$18 \pm 3.7$</td>
</tr>
<tr>
<td>$\Delta I_h$, pA</td>
<td>$219 \pm 72^*$</td>
</tr>
<tr>
<td>$\Delta G_{in}$, nS</td>
<td>$5.8 \pm 1.5^{*+}$</td>
</tr>
</tbody>
</table>

| $I_h$, pA  | $413 \pm 54$ | $500 \pm 135$ |
| $G_{in}$, nS | $28 \pm 13.4$ | $25 \pm 12.5$ |
| $\Delta I_h$, pA | $500 \pm 104^{**}$ | $250 \pm 132^{*+}$ |
| $\Delta G_{in}$, nS | $11.4 \pm 1.8^{*+}$ | $2.5 \pm 1.5$ |

Values are means ± SE. Data were obtained by whole cell recording under current and voltage clamp (with sIS electrodes; $V_m = -41 \pm 3.1$ mV) and by intracellular recording under voltage clamp (with 3M KCl electrodes; $V_m = -57 \pm 3.3$ mV). Number of cells tested was 4 for whole cell current clamp and 8 and 3 for whole cell and intracellular voltage clamps, respectively. $V_m$, resting potential; $R_m$, input resistance; $I_h$, holding potential; $I_h$, baseline current; $G_{in}$, input conductance; $\Delta V_m$, $\Delta R_m$, $\Delta I_h$, and $\Delta G_{in}$ are hypoxia-induced changes. $^* P \leq 0.05$. $^{*+}$ Significantly different from control values (paired). $\dagger P \leq 0.01$.

When compared with 12 control cells (Table 4), six cells recorded in the same group of slices with cAMP-containing electrodes showed no significant change in resting membrane properties and no reduction of hypoxic hyperpolarization, but the associated drop in $R_m$ was diminished by 40%. Under voltage clamp, the hypoxic changes were significantly smaller (by 50–70%) when recorded with cAMP-containing electrodes ($n = 6$) than in nine control cells (Fig. 10, C and D, and Table 5).

As a further test, eight cells were treated with 1 mM 8-Br-cAMP, the membrane permeable derivative of cAMP, applied in the bath. In its presence, the hypoxic response was either abolished or reversed (as in Fig. 10, A and B): overall the potential changes were no longer significant ($0.14 \pm 2.2$ mV), in contrast to the clear hyperpolarizations seen in the same neurons during preceding control hypoxic tests ($-3.7 \pm 0.87$ mV, $P < 0.005$). The hypoxic resistance fall also was depressed by 8-Br-cAMP, from a mean of $-29 \pm 5.3$ ($P < 0.005$) to a less consistent $-22 \pm 9.9%$ (from $R_m = 76 \pm 12$ MΩ).

**Discussion**

By providing better access to the neuronal interior, whole cell recording should clarify the mechanism(s) underlying the G$_K$-mediated hypoxic hyperpolarization of CA1 neurons. However, as reported by previous authors (Kurachi et al. 1992; Lenz et al. 1997; Morita et al. 1980; Velumian et al. 1997; Zhang et al. 1994), K currents, especially those that are activated via a G protein, are very sensitive to the anion content of recording electrodes. The hypoxic K current is no exception. By substituting MeSO₄ for gluconate as the main anion (Velumian et al. 1997; Zhang et al. 1994), more substantial hypoxic effects were obtained, confirming the usefulness of KMeSO₄, at least for such experiments (cf. Belousov et al. 1995; Zhang and Krnjević 1993). Nevertheless, even the largest changes in $R_m$ ($-26%$; Table 2) were only half the near-50% reductions in $R_m$ typically seen with intracellular electrodes (Croning et al. 1995; Fujiwara et al. 1987; Hansen et al. 1982; Leblond and Krnjević 1989; Yamamoto et al. 1997).

**Ca-dependent G$_K$ and effects of chelators**

**ON RESTING-MEMBRANE PROPERTIES.** The carbamol-sensitive hyperpolarization (or outward current) that developed gradually after the start of whole cell recording with BAPTA-containing electrodes is a curious phenomenon. Similar changes in dorsal vagal and hippocampal neurons...

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**FIG. 10.** In some neurons, 3′,5′-adenosine cyclic monophosphate (cAMP) suppresses hypoxic hyperpolarization or outward current as well as AHP or outward after-current. A and B: sharp electrode recordings from a CA1 cell show AHP (after 8-spike burst) and response to 2 min of hypoxia obtained initially (A) and 19 min after start of 8-Br-cAMP (1 mM) bath application (B). All traces were recorded at same resting potential ($-52$ mV). In B, 8-spike burst, which was evoked by smaller current pulse (top), was followed by much smaller AHP, and hypoxia had a depolarizing effect, not associated with fall in resistance. C and D: in another slice, 2 CA1 cells were recorded with whole cell electrodes containing sIS, either with no cAMP (C) or with cAMP (D, 1 mM) added. For both cells, holding potential was $-40$ mV and horizontal arrows mark 0 current. Left: are the outward currents evoked by 30-nm depolarizing pulses lasting 500 ms (shown below); note prominent slow outward tail in C and its absence in D. Changes in input conductance during hypoxia were monitored by applying 20-nm hyperpolarizing pulses seen on bottom traces. Note in D, hypoxia induced small inward current and no conductance increase; it was followed by typical postanoxic outward current.
were ascribed respectively to activation of $G_{K(\text{ATP})}$ (Trapp et al. 1994) or $G_{K(\text{Ca})}$ (Zhang et al. 1995) — the latter because the outward current was blocked by carbachol and Ba. Zhang et al. (1995) suggested that $G_{K(\text{Ca})}$ somehow might be activated by redistribution of internal Ca$^{2+}$ by the diffusible BAPTA (see also Schwindt et al. 1992, who also observed lower values of $V_m$ and $R_m$ in neocortical recordings with “high-BAPTA” sharp electrodes).

Another possibility is activation of a $G_K$ that is normally suppressed by cytoplasmic Ca$^{2+}$, such as r-eag-type $K$ channels (Stansfeld et al. 1996), which also are blocked by muscarinic agents. Why EGTA, an equally potent and diffusible buffer, does not have a similar effect is not clear: its slower kinetics should not affect such a slow process.

ON HYPOXIC HYPERPOLARIZATION. Considering the strong circumstantial evidence in favor of Ca$^{2+}$-release as an important factor (Belousov et al. 1995; Krnjević 1993; Yamamoto et al. 1997), it is surprising that Ca chelators do not have more conclusive effects; Duchen (1990) also observed only variable effects of BAPTA on hypoxic responses of sensory neurons. This may be explained if some other messenger also plays an important role in the activation of $K_{Ca}$ channels (Lasser-Ross et al. 1997).

Alternatively, like $K_{ATP}$ channels, $K_{Ca}$ channels may be regulated in a highly localized “microdomain.” In both cases, the critical signals are probably generated by glycolysis: ATP for $K_{ATP}$ channels (Weiss and Lamp 1989), and NADH for InsP$_3$ receptors (Kaplin et al. 1996). If the ATP-forming enzyme phosphoglycerate kinase is situated close to $K_{ATP}$ channels (Schackow and Ten Eick 1994; Weiss and Lamp 1989), its neighbor in the glycolytic pathway, the glyceraldehyde-3-phosphate dehydrogenase that converts NAD to NADH, may release Ca$^{2+}$ from very superficial InsP$_3$-sensitive stores (Berridge 1993, 1997; Henkart 1980; Liévremon et al. 1996; Mody and MacDonald 1995; Takei et al. 1992), in a microdomain that is not fully accessible to exogenous chelators (Adachi-Akahane et al. 1996; Matthews 1997; Narahgi and Neher 1997).

Chelators may not obviously suppress the hypoxic $G_K$ rise for another reason: by lowering cytosolic [Ca$^{2+}$], they would diminish any ongoing suppression of a r-eag type of $G_K$ (Stansfeld et al. 1996). A combination of Ca$^{2+}$-induced decrease of a r-eag $G_K$ and increase in $G_{ATP}$ could generate the bidirectional changes in $G_m$ and membrane current elicited by hypoxia (Krnjević and Xu 1990), although other explanations (Krnjević 1993; Nieber et al. 1995; Tanaka et al. 1997) have not been excluded.

Another intriguing possibility is that hypoxia releases an agent like $\beta$-amyloid precursor protein, which activates $G_{K(\text{Ca})}$, although it does not raise [Ca$^{2+}$], and is insensitive to chelators (Furukawa et al. 1996).

$K_{ATP}$ channels

The weak effects of internally applied 1–5 mM ATP are in agreement with some previous reports (Duchen 1990; Hyllienmark and Brismar 1996). Together with the absence of any depression by glyburide, they strongly argue against a major involvement of conventional $K_{ATP}$ channels in our experiments, in keeping with the minimal hyperpolarizing actions that we have observed with $K_{ATP}$ channel openers (Erdemli and Krnjević 1994b, 1995). The partial disagreement with other authors, who found some effects of glyburide, although much less consistently than with tolbutamide (Fujimura et al. 1997; Grigg and Anderson 1989), may be explained by technical differences; for example, any changes produced by hypoxic glutamate and GABA release largely were eliminated in our experiments. On the other hand, in spite of the use of KMeSO$_4$ electrodes for our whole cell recordings (Lenz et al. 1997; Zhang et al. 1994), there may have been some selective loss of a $K_{ATP}$ component of the hypoxic response.

A possible objection might be that unconventional $K_{ATP}$ channels are present in CA1 neurons (as in hypothalamic glucocceptive cells) (Ashford et al. 1990) that have a low sensitivity to ATP and glyburide. In the hypothalamic cells, however, glyburide prevents the blocking action of tolbutamide, which was clearly not the case in our CA1 neurons.

Could these K channels be both Ca and ATP sensitive (Hunter and Giebisch 1988; Jiang et al. 1994; Sellers et al. 1992)? But in our recordings with electrodes containing both ATP and a chelator, the hypoxic changes were not diminished. Moreover, according to Jiang et al. (1994), currents of this type in nigral neurons are blocked by glyburide.

Is also unlikely that 2–3 min of hypoxia would cause a major drop of ATP level (cf. Lipton and Whittingham 1984; Takata and Okada 1995) to well <1 mM, where $K_{ATP}$ channels open (Jiang et al. 1994). As Hochachka (1996) has emphasized, perhaps the most remarkable feature of energy depletion is the quick suppression of ATP utilization. The tight coupling between ATP production and utilization prevents a rapid fall in cellular ATP, in spite of a large reduction in ATP turnover. Hence changes in ATP level do not accurately reflect the almost instantaneous cellular adaptation to hypoxia.

Only a minor role of $K_{ATP}$ channels in the early hypoxic response of CA1 neurons is not surprising in view of the very low incidence of $K_{ATP}$ channels in neocortical and hippocampal neurons (Ashford et al. 1988, 1990; Lee et al. 1996): nearly all the $K_{ATP}$ channels observed by Lee et al. (1996) were on nerve terminals and not on cell bodies (although cf. Jiang and Haddad 1997). Such a presynaptic localization would be fully consistent with previous evidence that $K_{ATP}$ channel blockers reduce hypoxic hyperpolarizations and enhance glutamate release-mediated depolarizations (Ben-Ari 1990; Krnjević 1990; Mourre et al. 1989).

Suppressant action of GDP$\beta$S and GDP$\gamma$S

The powerful effect of GTP$\gamma$S, and especially that of GDP$\beta$S, are strong evidence that the hypoxic response involves a G protein (Eckstein et al. 1979). This suggests that hypoxia releases a ligand which, acting via a G protein, either directly opens $G_K$ or enhances the release of internal Ca$^{2+}$. Of the numerous ligand-triggered activations of $G_K$ that are G-protein-mediated (Brown 1990; Jan and Jan 1997; Nicoll 1988), those that do not involve a second messenger typically are sensitive to pertussis toxin. In Spuler and Grafe’s (1989) sharp electrode study of submerged hippocampal slices, pertussis toxin did not prevent the effects of hypoxia, though hyperpolarizations via adenosine or GABA$_A$ receptors were abolished. Their conclusion that the anoxic
effects could not be mediated by adenosine is in good agreement with the lack of effect of 8-SPT in our intracellular recordings (see also Croning et al. 1995; Leblond and Krnjević 1989) but in contradiction with the clear effects of 8-SPT on submerged slices (seen also by Zhu and Krnjević 1997). This may be explained if 8-SPT reaches its target more quickly in submerged slices or if adenosine accumulates faster because the slices are underoxygenated (Fredholm et al. 1984).

An alternative possibility is that adenosine, acting on A1 receptors but via a nonpertussis-sensitive G protein, initiates the ligand-triggered production of InsP3 (Burnatowska-Hledin and Spielman 1991; Kohl et al. 1990; Ogata et al. 1994; Rugolo et al. 1993; Yaku et al. 1992), the Ca2+-releasing action of which (Berridge 1993) would be reinforced greatly during hypoxia by increased glycolysis and NADH production (Kaplin et al. 1996). Without the synergistic effect of adenosine-triggered InsP3 formation, NADH may not be able to induce a significant release of Ca2+. This scheme is more plausible, being consistent with the evidence that hypoxic hyperpolarizations are suppressed by blockers of InsP3-mediated Ca2+ release (Belousov et al. 1995; Murai et al. 1997; Yamamoto et al. 1997) and that they are variably sensitive to adenosine antagonists. As already mentioned, it does not exclude the participation of some other G-protein ligand(s) that activate(s) phospholipase C.

**Nature of the G_{K(Ca)} postulated to be activated by hypoxia**

Several features favor an AHP-type conductance: the greater hypoxic changes recorded with MeSO4 than with gluconate-containing electrodes, the strong block by carbamol, the depression of both AHPs and hypoxic hyperpolarizations by 8-SPT, and the substantial block by the β-adrenergic agonist isoprenaline. The suppression of slow AHPs by β agents being mediated mainly by cAMP (Blitzer et al. 1994; Madison and Nicoll 1986; Pedarzani and Storm 1993), internally applied cAMP, and its membrane-permeable analogue 8-bromo-cAMP were expected to be consistently effective.

There are several possible explanations for the partial dissociation between the effects of isoprenaline and those of cAMP. Isoprenaline partly may affect AHP-type channels by a more direct action, only G-protein mediated (Welling et al. 1992). At the relatively high concentration of 1 mM, cAMP may have some other action that counteracts the suppression of I_{AHP}. For example, cAMP strongly potentiates maxi-K_{Ca} channel activity of olfactory bulb neurons (Egan et al. 1993). Another relevant target for cAMP is the enzyme phosphofructokinase, the principal regulator of glycolysis (Siesjö 1978, p. 204–206). By reinforcing glycolysis, stimulation of phosphofructokinase would enhance the hypoxic production of NADH enhancement and thus potentiate any rise in cytoplasmic [Ca2+]i. Alternatively, isoprenaline may be more effective because it produces a high local concentration of cAMP at the critical site (Blitzer et al. 1994).

Near resting potential, hypoxia would activate mainly K_{AHP} channels (SK type), which are sensitive to relatively small increases in [Ca2+] (Gurney et al. 1987; Lancaster and Zucker 1994; Prakriya et al. 1996); but large, voltage-dependent BK channels (large conductance K_{Ca} channels) (Brown and Griffith 1983; Egan et al. 1993; Lee et al. 1995) are also likely to be involved in view of the marked voltage dependence of the hypoxic outward current and its depression by TEA (Krnjević and Leblond 1989).

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