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Oxygen Deprivation Activates an ATP-Inhibitable K^+ Channel in Substantia Nigra Neurons

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Depending on its severity and duration, O_2 deprivation activates mechanisms that can lead to profound deleterious changes in neuronal structure and function. Hypoxia also evokes inherent adaptive mechanisms that can possibly delay injury and increase neuronal survival. One of these neuronal adaptive mechanisms is believed to be the activation of K^+ channels, but direct evidence for their activation is lacking. We performed experiments to test the hypothesis that hypoxia induces activation of K^+ channels via changes in cytosolic and membrane factors such as ATP, Ca^{2+}, and membrane potential. The effect of hypoxia on single-channel currents was studied in rat substantia nigra neurons, since these have a high density of glibenclamide binding sites. In cell-attached patches, hypoxia or cyanide reversibly activated an outward current. This hypoxia-activated current in excised inside-out patches was K^+ selective and voltage dependent, and had a high sensitivity to internal ATP, ADP, and AMP-PNP, a nonhydrolyzable ATP analog. Activation of this channel required the presence of free Ca^{2+} on the cytosolic side, but charybdotoxin or apamin did not have any effect on this channel. The effect of ATP on channel activity was not a result of Ca^{2+} chelation because Mg·ATP in high Mg^{2+} background and K_2·ATP in high Ca^{2+} environment inhibited the channel. These results suggest that although this hypoxia-activated K^+ channel shares properties with ATP-sensitive K^+ (K_{ATP}) channels in other tissues, substantia nigra neurons seem to have a different subtype or isoform of K_{ATP} channels. Gating this channel by multiple factors simultaneously would allow this channel to be particularly suitable for activation during metabolic stress.

[Key words: K^+ channel, ATP-sensitive, Ca^{2+}, hypoxia, substantia nigra, patch clamp]

ATP-sensitive K^+ (K_{ATP}) channels have been found in a number of tissues including pancreatic β-cells, vascular smooth and skeletal muscle cells, cardiac myocytes, and central neurons (Noma, 1983; Cook and Hales, 1984; Spruce et al., 1985; Ashford et al., 1988; Standen et al., 1989). The role of these channels in membrane biophysics and cell physiology is not well understood in all tissues, although some progress has been made. For example, we know now that modulation of K_{ATP} channel activity plays an important role in the depolarization of β-cells after glucose entry and increase in cellular ATP levels. This depolarization is crucial for opening voltage-sensitive Ca^{2+} channels and starting a cascade that leads to insulin release (Rorsman et al., 1990). Similarly, the activation of K_{ATP} channels during ischemia leads to hyperpolarization of coronary smooth muscle cells, which in turn causes muscle relaxation and better coronary blood flow (Daut et al., 1990; Nelson et al., 1990).

Autoradiographic, pharmacologic, and electrophysiologic studies have recently provided evidence for the presence of a K_{ATP} channel in the mammalian CNS (Ashford et al., 1988, 1990; Grigg and Anderson, 1989; Mourre et al., 1989, 1990; Amoroso et al., 1990; Röper et al., 1990; Schmid-Antomarchi et al., 1990; Häusser et al., 1991; Jiang and Haddad, 1991; Jiang et al., 1992; Murphy and Greenfield, 1992; Patel et al., 1992; Riepe et al., 1992; Tromba et al., 1992; Xia and Haddad, 1992). This channel appears to be mostly expressed postnatally, reaching adult levels 2–3 weeks after birth in the rat, with the highest binding density expressed in the substantia nigra (SN) (Mourre et al., 1990; Xia and Haddad, 1992).

The function of this channel in the CNS has been implicated in a number of neuronal physiologic phenomena including excitability and seizure activity, neurotransmitter release, and glucose metabolism (Amoroso et al., 1990; Schmid-Antomarchi et al., 1990; Tromba et al., 1992). In addition, previous studies from our laboratory as well as others (Grigg and Anderson, 1989; Mourre et al., 1989; Jiang and Haddad, 1991; Jiang et al., 1992; Murphy and Greenfield, 1992; Patel et al., 1992; Riepe et al., 1992) have strongly suggested that the activation of these channels occurs during hypoxia in the CNS and could play an important role in linking excitability to metabolic and possibly other alterations that occur during O_2 deprivation (Haddad and Jiang, 1993). Using pharmacologic manipulations including sulfonylurea ligands, our previous studies have so far indicated that (1) intracellular K^+ decreases during anoxia, and up to 50% of the K^+ efflux is mediated through these channels; (2) K_{ATP} channels are present on postsynaptic membranes of neurons in regions where K^+ efflux occurs; and (3) blocking these K_{ATP} channels with glibenclamide may be deleterious for neuronal survival during anoxia (Jiang and Haddad, 1991; Jiang et al., 1992).

To understand further the role of K_{ATP} channels in the neuronal response to O_2 deprivation, a number of fundamental questions still need to be addressed. For example, we do not yet have any direct evidence that these channels are actually open during hypoxia in central neurons. Also, if they are acti-

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vated, it is not clear what factors regulate them and how these channels are regulated. Therefore, the aims of these experiments were to (1) provide direct evidence that these K^+ channels alter their open and closed kinetics during hypoxia, (2) characterize these channels in terms of their sensitivity to voltage and changes in nucleotide levels, and (3) study the possible effect of single-channel activation on macroscopic outward currents in the same neurons during hypoxia. In addition, since previous studies have shown that Ca^{2+} increases in central neurons *in vivo* and *in vitro* during anoxia or ischemia (Silver and Erecinska, 1990; Dubinsky and Rothman, 1991; Friedman and Haddad, 1992), we examined the role of Ca^{2+} ions on single-channel activity. We demonstrate in this work for the first time that a K^+ channel that is inhibited by intracellular ATP is also reversibly activated by hypoxia in central neurons. Our results suggest that there are a number of cytosolic factors that modulate the activity of this channel in an important way during hypoxia.

Materials and Methods

Neurons were harvested from the substantia nigra zona compacta (SN) of Sprague–Dawley rats (10–20 d) using the modified methods of Kay and Wong (1988). In brief, rats were deeply anesthetized with methoxyflurane and decapitated. The midbrain was rapidly removed, chilled in 0–1°C Ringer's solution, and prepared as a tissue block. The tissue block was sectioned transversely into 300 μm slices at the level immediately above the pons, and two or three slices were taken. Sections were incubated for 1 hr with oxygenated HEPES buffer containing (in mM) NaCl, 140; KCl, 2.5; $MgCl_2$, 1; $CaCl_2$, 1; D-glucose, 25; HEPES, 10; and trypsin (Sigma type XI), 0.2–0.3%, at 35°C (pH 7.40). Sections were then washed in oxygenated HEPES buffer and maintained for up to 6 hr.

Immediately prior to recording, individual tissue slices were removed and placed in an Na^+-free HEPES buffer. Using a dissecting microscope, the substantia nigra pars compacta on both sides was cut free from the rest of the slice. The tissue of interest was then dissociated by gentle and careful trituration with fire-polished Pasteur pipettes. Cells were plated in 35 mm petri dishes and observed with Hoffman modulation optics. Recordings were obtained only on cells that exhibited the typical morphology of substantia nigra neurons, as has been described previously (Domesick et al., 1983; Grace and Onn, 1989), and did not show visible evidence of injury. Flat or swollen cells or cells with a grainy membrane appearance were not studied (Kay and Wong, 1986; Cummins et al., 1991).

To determine the cell type of the dissociated SN neurons, formaldehyde-glutaraldehyde–induced catecholamine fluorescence (FICF) was examined using a modified method of Furness et al. (1977). After dissociation, the extracellular medium was slowly changed to a solution containing 4% formaldehyde and 0.5% glutaraldehyde in 0.1 mM phosphate buffer saline (pH 7.4). Cells were allowed to stay in this solution overnight. Following two washes, cells were examined and photographed with a fluorescence microscope (filters for excitation, 405 nm; for emission, 430 nm).

Patch-clamp experiments were performed at room temperature (about 24°C). Fire-polished patch pipettes (2–4 MΩ) were made from 1.2 mm borosilicate capillary glass (Sutter P-80/PC puller). Single-channel currents were recorded from cell-attached and inside-out patches. Whole-cell currents were studied in the voltage-clamp mode using the whole-cell configuration (Hamill et al., 1981). The cell was held at −70 mV with a holding current of −0.01 to −0.30 nA (−0.01 to −0.02 nA for most cells). Perforated patches were also performed as described previously (Levitan et al., 1990). The tip of the recording pipette was filled with a solution containing (in mM) KCl, 150; $MgCl_2$, 0.2; D-glucose, 25; and HEPES, 10 (pH 7.4); and the remainder of the pipette was filled with the same solution plus 100 μg/ml nystatin (Sigma; stock solution, 25 mg/ml in dimethylsulfoxide). After formation of gigohm seal, perforation was monitored by measuring series resistance with an Axo-Patch C2 amplifier. Patches were accepted when the series resistance was <50 MΩ in perforated patches and <15 MΩ for whole-cell recordings. Current records were low-pass filtered (0–2,000 Hz, Bessel 4-pole filter, −3 dB), digitized (10 kHz, 12 bit resolution), and stored on computer disk for later analysis (pCLAMP 5.5.1, Axon Instruments). Peak outward currents were quantified. Linear leak subtraction was performed on whole-cell current recordings by applying five hyperpolarizing voltage pulses (5 mV) from a holding potential of −70 mV prior to each test pulse. Since series resistance (after compensation of both fast transient and slow component) in these experiments was relatively low (~10 MΩ for whole-cell recordings) and the sustained currents of interest were ≤1 nA (voltage error should be ≤10 mV), we did not use resistance compensation. Junction potentials between bath and pipette solutions were appropriately nulled before seal formation.

For single-channel recordings, identical solutions were applied to the bath and recording pipettes, and these contained (in mM) KCl, 150; $MgCl_2$, 0.2; D-glucose, 25; and HEPES, 10 (pH 7.4). EGTA (5 mM, pH 7.4) was added to the bath solution when the effect of Ca^{2+} on channel activity was studied. $CaCl_2$ was added to the EGTA-containing solutions to obtain graded concentrations of free Ca^{2+}. Free Ca^{2+} concentration was measured with ion-selective microelectrodes (Ca^{2+} LEX 26, World Precision Instruments) and adjusted against a series of Ca^{2+} standards (World Precision Instruments). For whole-cell recordings, an Na^+-free solution containing (in mM) choline chloride, 150; KCl, 2.5; $MgCl_2$, 1; $CaCl_2$, 1; D-glucose, 25; and HEPES, 10 (pH 7.4) was used in the bath with a pipette solution similar to that for single-channel recordings. A parallel perfusion system was used to administer agents to patches or cells at a rate of ~1 ml/min with no dead space. Hypoxia was instituted by perfusing with a solution bubbled with 100% N_2 (>2 hr) in addition to 2–5 mM $Na_2S_2O_4$, which acts as an O_2 sink (pO_2 < 1 torr, no detectable current passing through the polarographic electrodes) (Jiang et al., 1991). In two of three of these experiments, 5 mM Na^+ was added to the external solution to balance the additional Na^+ in the hypoxic perfusate. We did not see any systematic difference between these experiments and those with Na^+-free external solution. The metabolic inhibitor cyanide (4 mM, K^+ salt) was also used in a few experiments.

For single-channel analysis, data were further filtered (0–1000 Hz) with a Gaussian filter. This filtering causes events shorter than 150 μsec to be ignored. No correction was applied for missed events. The open-state probability (P_{open}) was calculated by first measuring the time, t_j, spent at current levels corresponding to $j = 0, 1, 2, \ldots N$ channels open (Quayle et al., 1988; Standen et al., 1989; Davies et al., 1992). The P_{open} was then obtained as $P_{open} = (\Sigma^N_{j=1}\ t_j)/TN$, where N is the number of channels active in the patch and T is the duration of recordings. P_{open} values were calculated from stretches of data having a total duration of 36–108 sec. Open and closed times were measured from records in which only a single channel was active. The open and closed time distributions were fitted using the method of maximum likelihood (Colquhoun and Sigworth, 1983; Sigworth and Sine, 1987). The current amplitude was described using Gaussian distributions and the difference between two adjacent fitted peaks was taken as unitary current amplitude.

Charybdotoxin was purchased from Research Biochemical International (Natick, MA), 5′-adenylylimidodiphosphate was bought from Boehringer Mannheim (Germany), and BRL 38227 (lemakalim) was a gift of SmithKline Beecham Pharmaceuticals. All other chemicals were obtained from Sigma Chemicals (St. Louis, MO).

Data are presented as means ± SE (n = number of patches), and differences in means were tested with the Student t test and χ^2 test and were accepted as significant if $P \leq 0.05$.

Results

Identification of neurons

After dissociation, SN neurons maintained somatal morphology as seen in Figure 1*A*, with bipolar or triangular soma with proximal dendrites. Cell bodies showed a clear three-dimensional profile with no granular surface. These morphologic characteristics were consistent with those described previously for catecholaminergic neurons in the SN (Domesick et al., 1983; Grace and Onn, 1989; Silva et al., 1990; Yung et al., 1991). In another two experiments, we examined FICF in the dissociated cells. We found that more than 95% of the dissociated neurons showed positive FICF (n > 100), indicating that the majority of cells studied were catecholaminergic (Fig. 1*B*).

Under current clamp, with an extracellular solution containing 140 mM Na^+ and 2.5 mM K^+ and a pipette solution con-

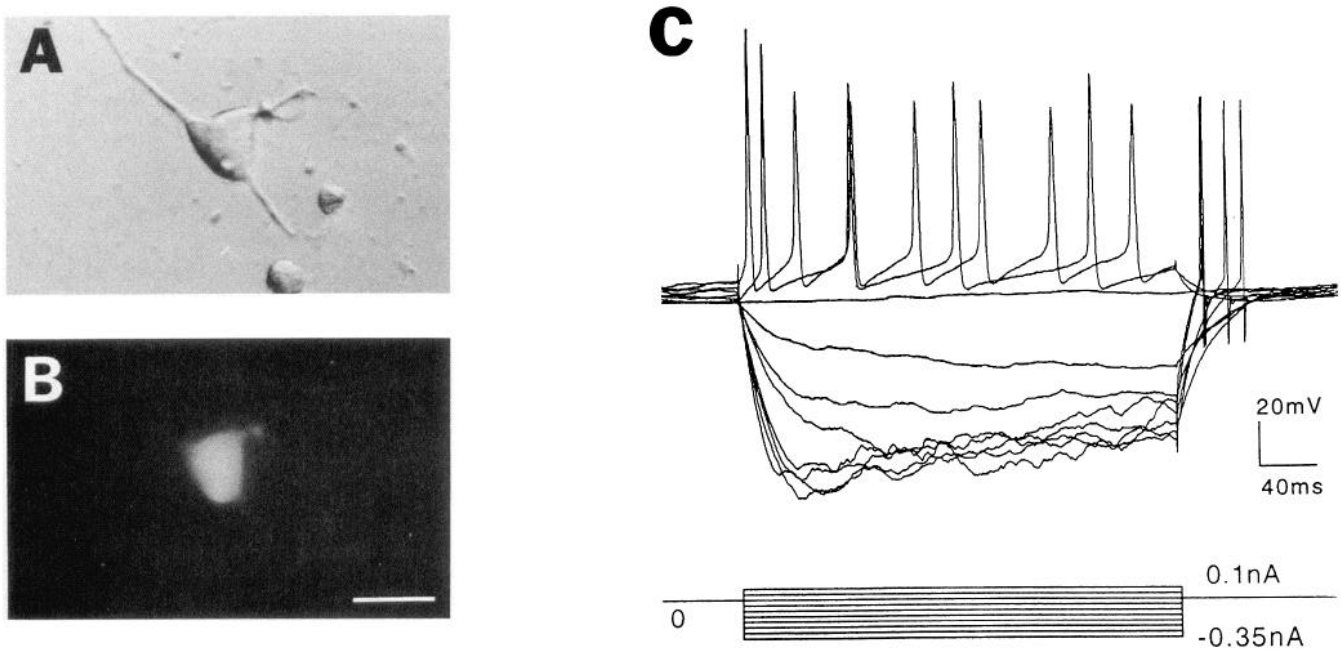

Figure 1. Morphology and cellular properties of dopaminergic neurons in the substantia nigra (SN). *A*, An SN neuron after dissociation. The cell has a triangular soma with proximal dendrites. *B*, The same cell as in *A* after fixation with 4% formaldehyde and 0.5% glutaraldehyde and examined with a fluorescence microscope. This cell shows a positive formaldehyde-glutaraldehyde–induced catecholamine fluorescence. Scale bar, 20 μm. *C*, Current clamp of another dissociated SN neuron (*top traces*). This cell fires action potentials during depolarization and also immediately after hyperpolarization (postinhibitory rebound). Depolarizing and hyperpolarizing currents are shown in the *bottom traces*.

taining 150 mM K^+, the dissociated SN neurons had a membrane potential (V_m) of -51 ± 2 mV (mean ± SE, $n = 6$) and input resistance of 198 ± 27 MΩ ($n = 6$). Action potentials were evoked from baseline potential of about −70 mV (holding current = −0.02 to −0.14 nA, $n = 6$) with depolarizing current pulses in all tested cells. The amplitude (from threshold) of action potentials was 84 ± 3 mV ($n = 6$). In these cells, several cellular properties such as inward rectification and postinhibitory rebound were observed (Fig. 1*C*), but not bursting activity.

A hypoxia-activated outward current

Single-channel activity was studied with a solution containing 150 mM K^+ with 5 mM Na^+ plus 1 μM tetrodotoxin (or with 0 mM Na^+) in the bath and recording pipette. Since V_m was ~0 mV with these solutions (V_m = 0–2 mV, measured in five cells), a holding potential of −40 mV produced a V_m of 40 mV in cell-attached patches. Quantitative changes in channel activity were analyzed using the channel open-state probability (P_{open}) and the average "macroscopic" current (top traces in Fig. 2*A*). P_{open} was obtained from a stretch of single-channel recording (see Materials and Methods). The average current was obtained after an average of at least 16 individual traces of single-channel currents during stepped depolarization (from 0 mV to 40 mV). The amplitude of the average current was measured as the mean level current within the last 20 msec of depolarization.

Figure 2 shows evidence for activation of a large-conductance outward current during hypoxia. Before hypoxia, only a small-conductance (~40 pS) outward current was recorded in cell-attached patches (Fig. 2*A*, 0.0 min). This current had a very low channel activity ($P_{open} = 0.04 \pm 0.01$, $n = 22$). When hypoxia (pO_2 < 1 torr) was instituted, a new large-conductance outward current appeared. This current started to be activated after about 2 min of hypoxia. Its channel activity (measured as the average current amplitude from 48 single traces in Fig. 2*A* and 16 traces in Fig. 2*B*) continuously increased and reached a maximum activation in another 1 min (Fig. 2*A*, 3.0 min; Fig. 2*B*). At maximum current activation, administration of tolbutamide (10 mM, a sulfonylurea K_{ATP} channel blocker) to the hypoxic perfusion medium (pO_2 < 1 torr) strongly and reversibly inhibited this outward current (Fig. 2*A*, 5.5 min; Fig. 2*B*). Washout of these cells with a normoxic external solution for 2–3 min resulted in a return of the channel activity to near the prehypoxia level (Fig. 2*A*, 8.4 min). Excision of membrane patches from the cell led to a strong and rapid reactivation of this large-conductance outward current (Fig. 2*A*, 9.5 min). When this large-conductance current recorded in the cell-attached mode was compared to that in excised patches, this current (1) had an identical conductance (~220 pS) and an identical *I–V* relation with a lack of rectification (from 0 to 60 mV) in symmetric K^+ concentrations across the membrane (Fig. 2*C*), (2) showed a similar sustained activation pattern of the average current trajectory (Fig. 2*A*, 3.0 min and 9.5 min), and (3) was reversibly inhibited by sulfonylurea receptor antagonists such as tolbutamide and glibenclamide, as well as by TEA (Table 1), indicating that it is the same current.

The large-conductance outward current was observed in about one of three patches studied with hypoxia. In the other two of three, only the small-conductance current was seen. At maximum activation, P_{open} of the large-conductance current averaged 0.25 ± 0.04 ($n = 7$) for hypoxia, and 0.71 ± 0.02 ($n = 9$) for cyanide. In seven of eight of the patches, this current was strongly inhibited by tolbutamide ($P < 0.05$, $n = 8$), with the eighth patch not showing any evident change. In addition to tolbutamide, this hypoxia-activated current was also inhibited by glibenclamide (another K_{ATP} channel blocker) and TEA. With 10 μM glibenclamide in the pipette solution, the activation of this

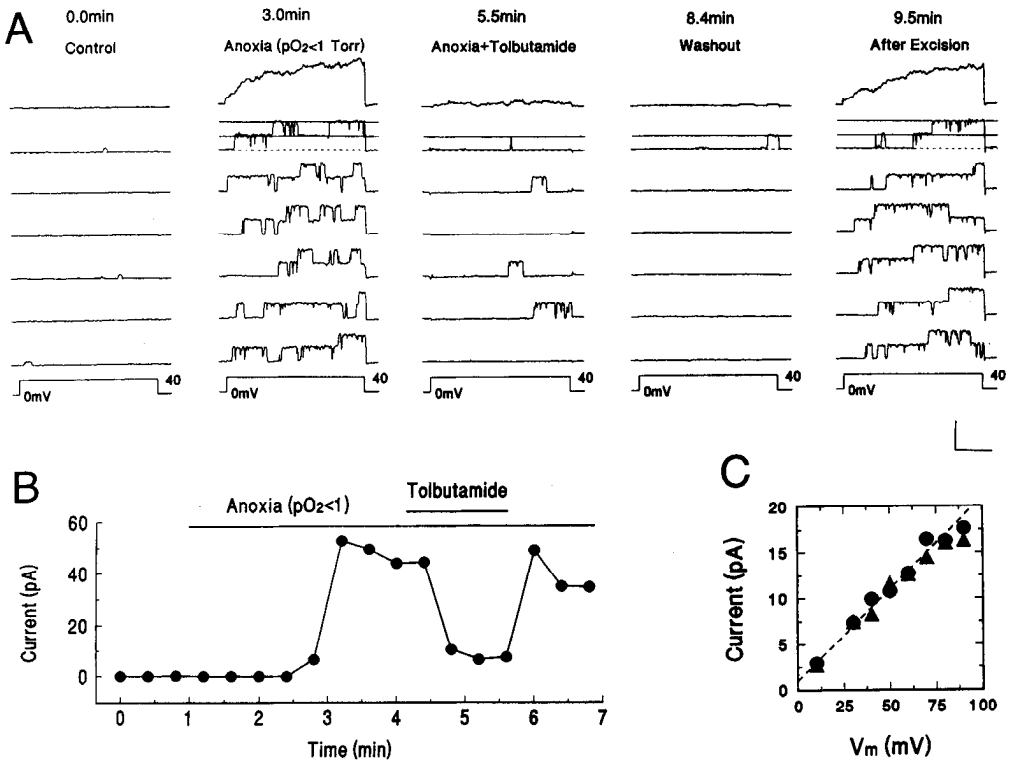

Figure 2. *A*, Activation of an outward current during anoxia. Single-channel currents were recorded from an acutely dissociated SN neuron with the same solution (containing 150 mM K^+) applied to the bath and recording pipette. The membrane potential of the patch was held at 0 mV and stepped to 40 mV every 2 sec (*bottom traces*). Averaged "macroscopic" currents (*upper traces*) were obtained from 48 individual traces in each of the experimental conditions indicated above. In the cell-attached configuration, only a small-conductance current (~40 pS) was observed before anoxia. A large-conductance outward current appeared at about 2 min of anoxic exposure. Addition of tolbutamide (10 mM, an ATP-sensitive K^+ channel blocker) to the anoxic perfusate strongly inhibited these currents. Channel activity returned to almost the baseline level following washout of anoxia and tolbutamide. After excision and recording in inside-out mode, the large-conductance outward currents with the same conductance and a similar sustained activation pattern were seen. Calibration: 20 msec, 8 pA for the average currents (*top traces*) and 16 pA for the rest. The channel closed level is indicated by *dashed lines*, and *solid lines* indicate multiples of the opening level (8.2 pA). *B*, Time profile of the average current amplitude from another SN neuron. Each point represents the average current during the last 20 msec of 16 traces. There was no channel activity during baseline. The outward current started to increase at about 1.5 min into anoxia exposure and reached a maximum at about 2 min. Application of tolbutamide (10 mM) to the anoxic perfusate outside the cell reversibly inhibited this current. *C*, *I–V* relation of the outward current activated during anoxia in the cell-attached patch (●) is identical to the one recorded in excised patch (▲). The *dashed line* represents a slope conductance of 200 pS.

Table 1. Effects of nucleotides and K^+ channel blockers on K_{ATP} channel activity

Agents	mM	$P_{open•CTL}$	$P_{open•blocker}$	i_{CTL} (pA)	$i_{Blocker}$ (pA)	n
K_2·ATP	1.0	0.60 ± 0.01	0.05 ± 0.01	4.9 ± 0.1	4.6 ± 0.1	31
K_2·ATP + Ca^{2+}	1.0 + 5.0	0.71 ± 0.11	0.02 ± 0.03	5.0 ± 0.1	4.4 ± 0.2	4
Mg·ATP + Mg^{2+}	1.0 + 2.5	0.83 ± 0.14	0.24 ± 0.10	4.8 ± 0.2	4.6 ± 0.1	6
ADP	1.0	0.71 ± 0.07	0.40 ± 0.06	4.9 ± 0.1	5.3 ± 0.1	4
AMP-PNP	1.0	0.70 ± 0.13	0.19 ± 0.08	4.7 ± 0.2	4.8 ± 0.2	4
Glibenclamide	0.01	0.62 ± 0.11	0.26 ± 0.12	4.8 ± 0.1	4.6 ± 0.1	5
Tolbutamide	10.0	0.74 ± 0.05	0.36 ± 0.07	3.8 ± 0.2	3.6 ± 0.2	5
External TEA^+	8.0	0.80 ± 0.09	0.01 ± 0.00	4.4 ± 0.4	3.6 ± 0.3	4
Internal TEA^+	40.0	0.99 ± 0.01	0.99 ± 0.00	6.0 ± 0.5	5.0 ± 0.5	3
Cs^+	2.0	0.87 ± 0.07	0.01 ± 0.03	5.1 ± 0.1	1.9 ± 0.1	3
Ba^{2+}	2.0	0.90 ± 0.13	0.01 ± 0.07	4.8 ± 0.3	1.4 ± 0.4	2
Co^{2+}	2.0	0.96 ± 0.11	0.00 ± 0.02	5.0 ± 0.3	1.2 ± 0.4	4
Mg^{2+}	8.0	0.71 ± 0.12	0.56 ± 0.09	4.9 ± 0.2	4.4 ± 0.3	2
4-AP	2.0	0.89 ± 0.03	0.93 ± 0.02	4.8 ± 0.1	5.1 ± 0.2	3

Patches were recorded in inside-out configuration (except external TEA^+) with equal concentrations of K^+ on both internal and external sides of membrane. The measured Ca^{2+} concentration was 1–2 μM and V_m = 20 mV. Agents were applied to the internal solution (except external TEA^+, in which TEA^+ was added to the external solution in the outside-out patch configuration). Data are presented as means ± SE. n, number of patches; $i_{Blocker}$, unitary current amplitude (pA) with blocker; i_{CTL}, unitary current amplitude (pA) during baseline; $P_{open•blocker}$, P_{open} with blocker; $P_{open•CTL}$, baseline P_{open}.

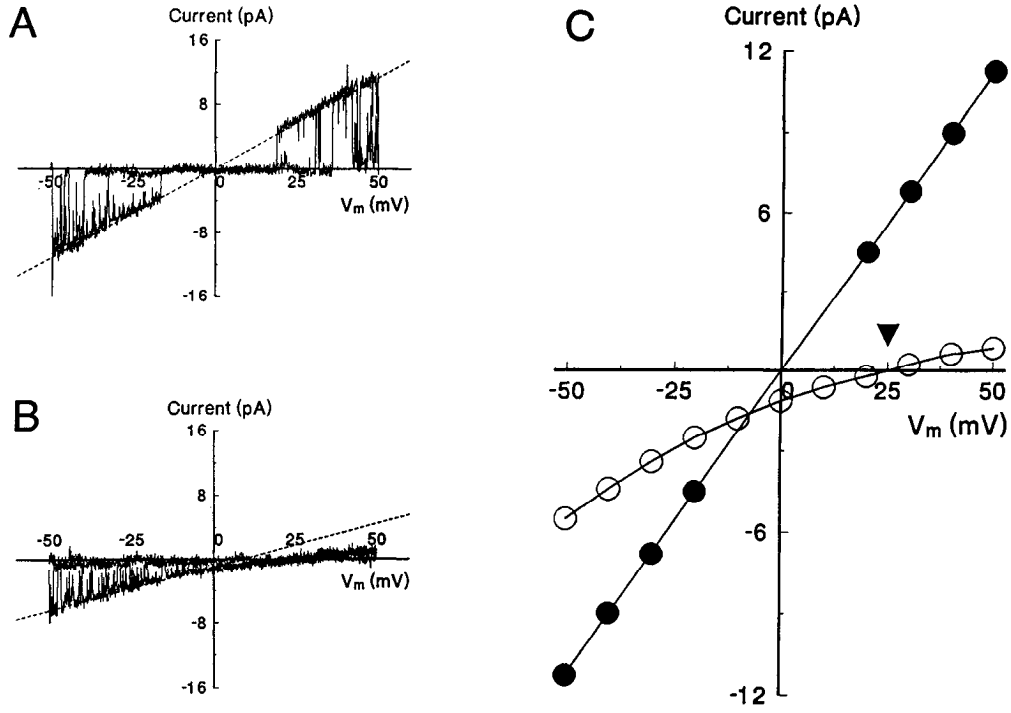

Figure 3. Selective permeability to K^+. A single channel was recorded in an inside-out patch that was held at 0 mV. Repetitive ramp potentials (2 sec) were given from −50 mV to 50 mV. When internal and external solutions containing equal concentrations of K^+ (150 mM) were used (*A*), the channel showed a linear conductance of 226 pS with a reversal potential of 0 mV (*C*, ●). When the internal membrane surface was exposed to a solution containing 54 mM KCl and 96 mM NaCl (*B*), the reversal potential was shifted to +25 mV (*C*, ○, at *arrowhead*) and the unitary conductance (from −50 to −20 mV) was reduced to 109 pS with an inward rectification.

channel was seen in only one patch (out of 10), and not at all in the other nine patches with 5 mM TEA in the pipette medium. The slope conductance in the cell-attached patches was 220 ± 12 pS (n = 6).

The response of small-conductance current to hypoxia, however, was inconsistent and small. In four patches, P_{open} of this current decreased by 5–10% during hypoxia, while it did not change with hypoxia in the other five patches. In addition, since this small-conductance current was not affected by cytosolic nucleotides (e.g., ATP), we focused only on the large-conductance current in the rest of our experiments.

Characterization of the hypoxia-activated current

Since hypoxia can induce major alterations in cytosolic compounds, it is likely that the effect of hypoxia on outward currents is mediated by changes in cytosolic factors during O_2 deprivation. To determine the identity of some of these factors and characterize their role in the hypoxia-activated current, patches were excised from the cell and studied in an inside-out configuration.

The channel is selectively permeable to K^+. With equal concentrations of K^+ applied to both internal and external solutions (150 mM), reversal potential of this current was 0 mV, with a slope conductance of 226 pS (226 ± 30 pS, n = 36) (Fig. 3). When the KCl concentration on the internal surface was reduced to 54 mM (with NaCl added in a concentration of 96 mM), the conductance was reduced to 109 pS. In all five patches studied, the reversal potential was shifted to 24 ± 1 mV, which is very close to the calculated Nernst potential for K^+ (25.7 mV). This indicates that this channel is highly K^+ selective. With equal concentrations of K^+ on both sides of membranes, this current showed a linear conductance (Fig. 3*A*). However, a clear inward rectification was observed when Na^+ (50–100 mM) was applied in the internal solution (Fig. 3*B*).

ATP inhibits channel activity. Although channel activity was very low (P_{open} < 0.01) in the cell-attached patch, P_{open} increased markedly after patches were excised from the cell and exposed to a solution containing no ATP. Since the ATP level drops during O_2 deprivation (Kass and Lipton, 1989), and SN neurons are rich in binding sites of glibenclamide (Mourre et al., 1990; Xia and Haddad, 1991), we hypothesized that this hypoxia-activated outward current in SN neurons is mediated by a reduction of ATP and activation of K_{ATP} channels. We therefore examined the sensitivity of this channel to various concentrations of ATP on the internal side of the membrane. Using inside-out patches, we found that channel activity responded to ATP concentrations of 25–50 μM and was almost totally abolished by 0.5–1.0 mM of ATP (Fig. 4*A*,*B*). The concentration of ATP that induces half-inhibition of channel activity (K_d) was 135 μM (Fig. 4*B*).

As for the K_{ATP} channels in pancreatic β-cell and cardiac myocytes (Ashcroft, 1988; Takano and Noma, 1993), the hypoxia-activated K^+ channel in SN neurons was also sensitive to internal ADP and 5′-adenylylimidodiphosphate (AMP-PNP), a nonhydrolyzable ATP analog. Application of 1 mM ADP (Fig. 4*C*) or 1 mM AMP-PNP (Fig. 4*D*) to the internal surface produced an inhibition of channel activity by 44 ± 4% and 73 ± 5%, respectively.

Channel activity was also significantly inhibited by application of sulfonylurea K_{ATP} channel blockers. Tolbutamide (10 mM) suppressed P_{open} without affecting the unitary conductance (Fig. 5), which was consistently seen in all five tested inside-out patches. The effect of tolbutamide was clearly reversible and reproducible, since three of these patches that were tested with repetitive exposures showed a similar response. Glibenclamide (10 μM), another K_{ATP} channel blocker, also inhibited channel activity. This effect was observed in six (four inside-out and two outside-out) of eight patches. The other two inside-out patches did not show any significant change in channel activity.

The hypoxia-activated channel is also sensitive to Ca^{2+}. Since

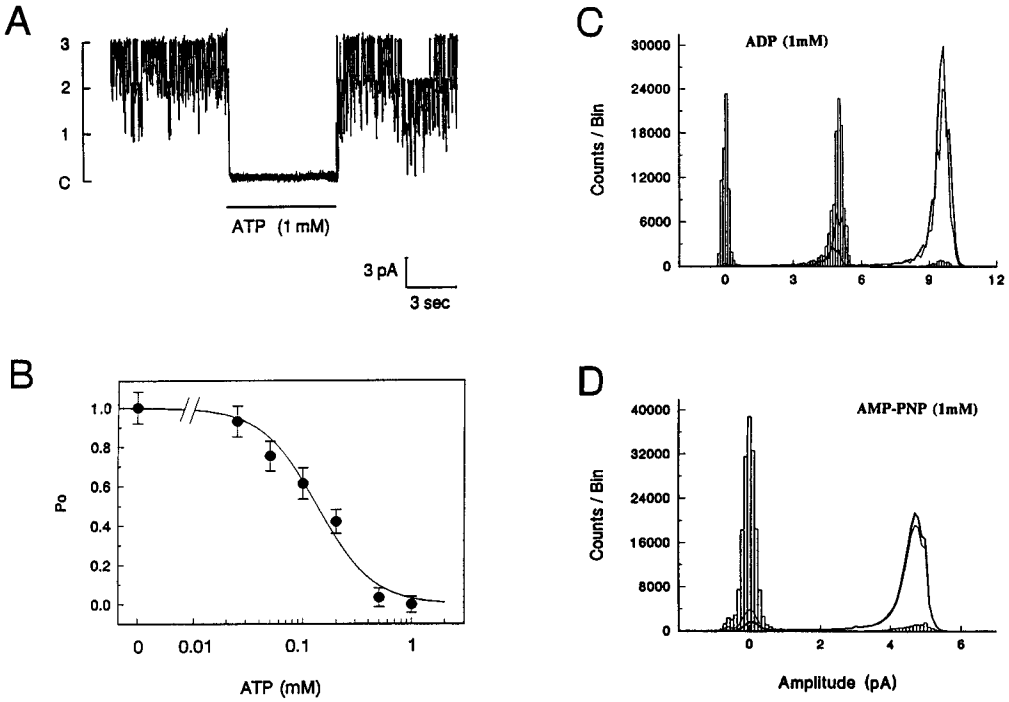

Figure 4. ATP sensitivity of hypoxia-activated K^+ channels. *A,* Single-channel currents recorded from an inside-out patch (V_m = 20 mV) with equal concentrations of K^+ in both internal and external solutions. These solutions are nominally Ca^{2+} free (measured free Ca^{2+} ~ 1.5 μM with no EGTA added). Three active channels were seen during baseline (levels indicated to the *left*; *C,* closing) with similar unitary conductances. ATP (1 mM) rapidly and reversibly suppressed all channels. *B,* Dose-dependent inhibition of the open-state probability by ATP. P_{open} was normalized to baseline level ($P_{open}/P_{open•CTL}$). Data were fitted using the Hill equation $y = 1/\{1 + ([ATP]/K_d)^h\}$, where $y = P_{open}/P_{open•CTL}$ and [ATP] = internal ATP concentration; the half-blocking concentration K_d = 135 μM, and the Hill coefficient h = 1.7. Data are presented as means ± SE (n = 5). ATP at 50 μM has evident inhibitory effect on channel activity and P_{open} reached almost zero when ATP concentration was more than 0.5 mM. *C* and *D,* Effects of other nucleotides on channel activity in the same conditions as *A*. Raw data histograms of current amplitude were obtained from two different inside-out patches (recording length = 18 sec). During baseline (*thick lines*) all patches were active, with two channels active in *C* and only one in *D*. Internal application of ADP (1 mM, *C*) and AMP-PNP (1 mM, *D*) markedly inhibited channel activity (*bars*). This inhibition was reversible and channel activities recovered after washout of these agents (*thin lines*).

the concentration of free Ca^{2+} in the cytosol (Ca^{2+}_i) increases during O_2 deprivation (Silver and Erecinska, 1990; Dubinsky and Rothman, 1991; Friedman and Haddad, 1992), we also tested the hypothesis that these K_{ATP} channels are sensitive to Ca^{2+}. Although no Ca^{2+} was added in the internal solution, the Ca^{2+} concentration measured with ion-selective microelectrodes in this solution was 1–2 μM, a level that may be enough for channel activation. To determine the role of Ca^{2+} on K_{ATP} channels in SN neurons, we exposed inside-out patches to 5 mM EGTA (the calculated free Ca^{2+} concentration was about 20 pM) and found that the hypoxia-activated K_{ATP} channel was completely and reversibly suppressed (Fig. 6*A*). Using various measured concentrations of Ca^{2+}_i, we observed that the Ca^{2+} concentration for half-activation (K_d) was 14 μM.

Although channel activity was dependent on Ca^{2+}, it was not affected by extracellular charybdotoxin (25–50 nM, n = 22) or apamin (5–10 nM, n = 22), two blockers of Ca^{2+}-dependent K^+ channels.

Since ATP and other nucleotides have an affinity for Ca^{2+}, it was possible that the inhibitory effect of ATP on the channel might arise as a consequence of Ca^{2+} chelation (Klöckner and Isenberg, 1992). To test this hypothesis, Mg·ATP was used in addition to a high Mg^{2+} background (2.5 mM). We found that channel activity was inhibited in a similar manner to the inhibition seen with K_2·ATP (Table 1). Furthermore, if the inhibitory effect of ATP were mediated only via Ca^{2+} chelation, a high concentration of Ca^{2+} (enough to saturate the Ca^{2+}-buffering capability of ATP) would totally abolish the ATP effect on channel activity. With a very high concentration of Ca^{2+} (5.0 mM) in the internal solution, however, ATP (1 mM) still markedly lowered channel activity (Fig. 6*B*), while 5 mM Ca^{2+} alone did not inhibit this channel. Therefore, these results suggest that both ATP and Ca^{2+} affect channel activity but act by different mechanisms.

Some conventional K^+ channel blockers inhibit this channel. Table 1 shows the effect of several K^+ channel blockers on channel activity. All of these blockers except TEA were applied to the internal side of patches. External TEA in a concentration of 8 mM completely inhibited the ATP- and Ca^{2+}-sensitive K^+ channel (K_d = 3.1 mM, n = 4), while internal TEA at a concentration of 40 mM only reduced the single-channel conductance by ~20% without affecting P_{open}. Cs^+ (2 mM) significantly and reversibly inhibited P_{open} by >95% and conductance by 60–70%. Divalent cations such as Co^{2+} (2 mM, n = 4), Ba^{2+} (2 mM, n = 2), and Mg^{2+} (8 mM, n = 2) appeared to reduce both P_{open} and single-channel conductance, suggesting that they might have a dual effect on the channel. 4-AP (2 mM) effected neither P_{open} nor current amplitude (n = 3).

The hypoxia-activated K^+ channel showed a slight inward rectification in the presence of 1–2 mM Mg^{2+} in the internal solution. This rectification was larger when Ba^{2+} (0.5 mM) or Na^+ (50–100 mM) was present in the bath solution (Fig. 3*B*).

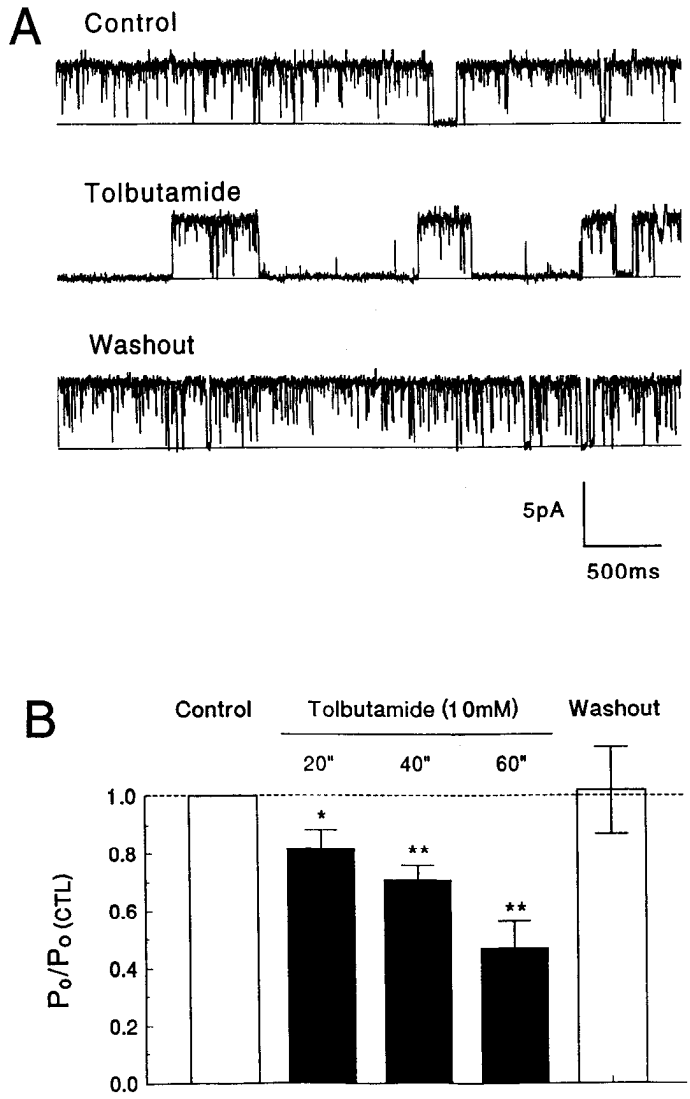

Figure 5. The effect of tolbutamide on the hypoxia-activated K^+ channel. *A,* Continuous recordings from an inside-out patch with equal K^+ (150 mM) on both sides of the membrane and a V_m of 20 mV. *Straight lines,* channel closure. Note that the channel activity was markedly reduced after 60 sec exposure to tolbutamide (10 mM). *B,* Averaged time profile (from five patches) of P_{open} before, during, and after tolbutamide exposure. *Numbers under solid bars* indicate 20, 40, and 60 sec into tolbutamide exposure, respectively. *Open bars* represent control (*left*) and washout (*right*). *Asterisks* represent statistical confidence levels (*, $P < 0.05$; **, $P < 0.01$). Data are presented as means ± SE.

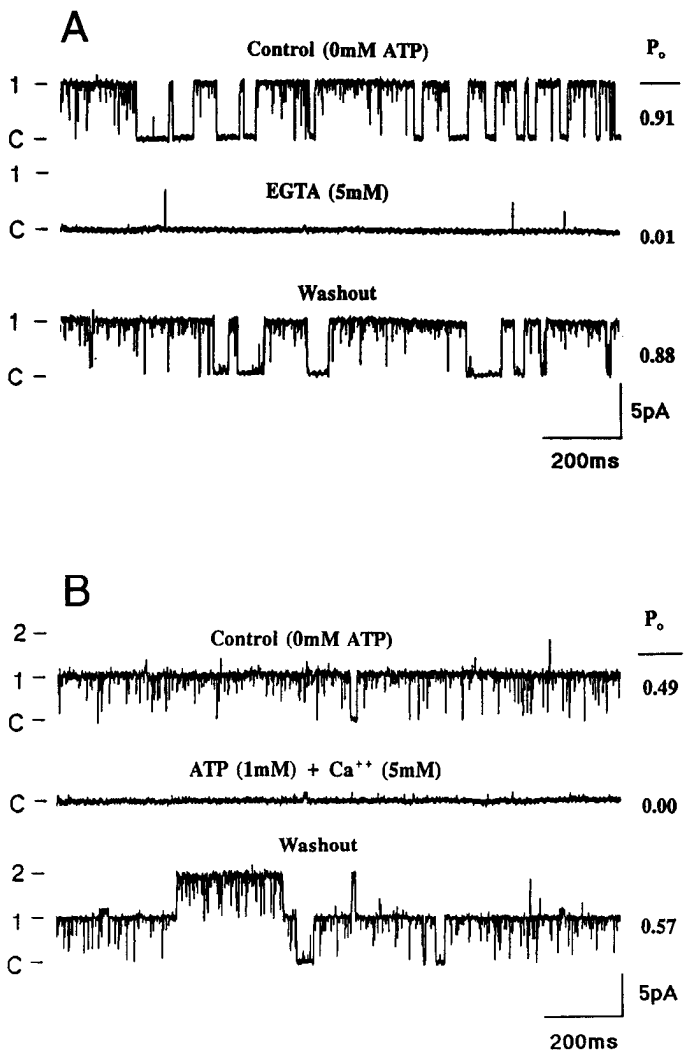

Figure 6. Ca^{2+} dependence of the hypoxia-activated channels. *A,* Single-channel current recorded from an inside-out patch with equal concentrations of K^+ in both internal and external solutions (V_m = 20 mV, 0 mM ATP). The channel was almost totally closed when EGTA (5 mM, pH 7.4) was added to the bath solution (*middle*). The channel opened again after washout of EGTA (*bottom*). P_{open} values were reversibly decreased with EGTA from 0.91 to 0.01 and recovered to 0.88 after washout. *B,* The inhibitory effect of ATP (1 mM) on channel activity was not removed by a high concentration of Ca^{2+}. The channel that was open at baseline (*top*) was completely inhibited by a solution containing ATP (1 mM) and Ca^{2+} (5 mM) (*middle*).

Single-channel kinetics

Baseline open and closed kinetics. Channel open and closed kinetics were studied using open and closed dwell time histograms from patches that showed activity of only a single channel. Figure 7 shows histograms (log scale) of the dwell times of the open and closed states of an ATP- and Ca^{2+}-sensitive K^+ channel. The open dwell times were well described by two distinguishable exponential components (τ_{o1} and τ_{o2}) (Fig. 7*A*). At a V_m of 20 mV, τ_{o1} averaged 8.9 ± 0.3 msec and τ_{o2} 38.6 ± 0.8 msec (n = 34). The relative areas of these components (a_{o1}, a_{o2}) were 0.18 and 0.82 (means, n = 34), respectively. Closed times could be well described by three distinct exponential components (Fig. 7*B*) with time constants τ_{c1} = 0.4 ± 0.0 msec, τ_{c2} = 4.7 ± 0.2 msec, and τ_{c3} = 75.0 ± 2.8 msec, and relative areas a_{c1} = 0.75, a_{c2} = 0.15, and a_{c3} = 0.10 (n = 34). It should be noted that the short closed time τ_{c1} is on the order of our time resolution (150 μsec) and therefore is not characterized well by the fit.

Effects of ATP on channel kinetics. The effect of ATP on P_{open} was mediated by a decrease in the channel mean open time and an increase in mean closed time. At a concentration of 1.0 mM, the mean open time was reduced by 61% (from 30.8 ± 0.2 msec to 11.9 ± 0.1 msec, n = 32), while the mean closed time increased greatly from a mean of 18.6 ± 0.2 msec to 612 ± 1 msec (n = 31). The effect of ATP on kinetics of open and closed times was studied with 200 μM ATP to maintain a low channel activity level (Fig. 7*C,D*). This was characterized by a marked decrease in τ_{o2} (28 ± 5 msec, n = 6) and an increase in both τ_{c2} and τ_{c3} (91 ± 32 msec, 426 ± 115 msec, n = 6) without significantly changing τ_{o1} and τ_{c1} ($P > 0.05$, n = 6). The fraction

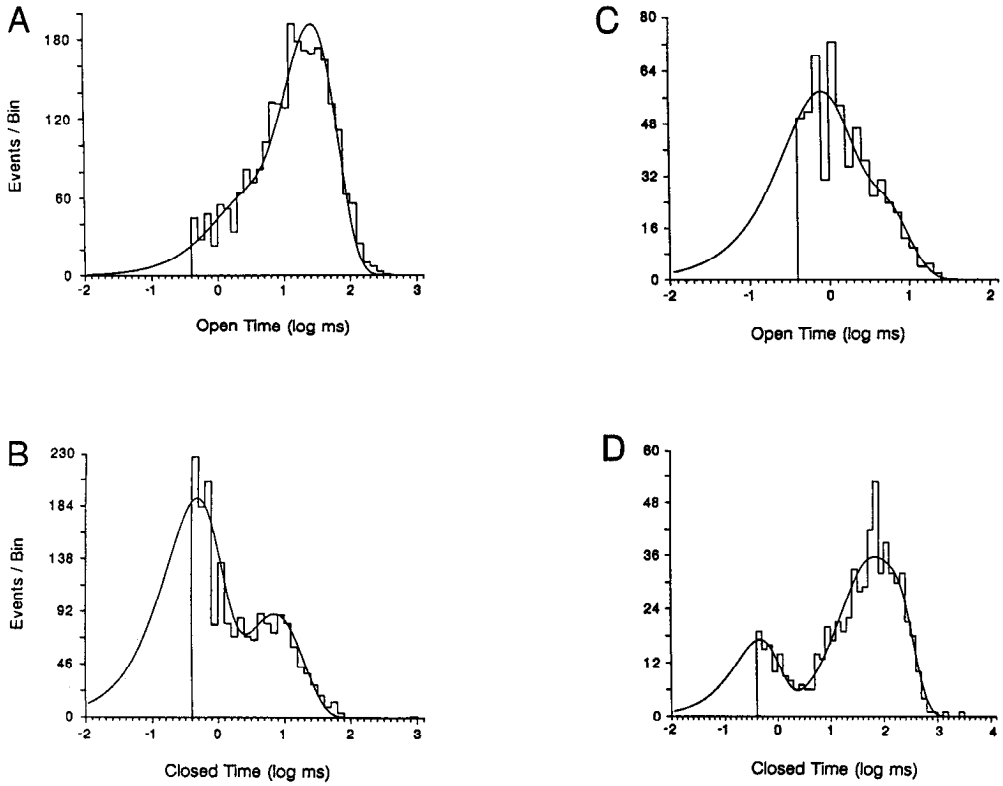

Figure 7. Single-channel kinetics of open and closed times. Channel activity was recorded with 0 mM ATP, 1.5 μM Ca^{2+} (measured with ion-selective electrodes), and equal concentrations of K^+ in both internal and external solutions (V_m = 20 mV). *A*, The open time histogram was fitted with the sum of two exponential terms. The relative area of shorter time constant (τ_{o1} = 1.4 msec) component (a_{o1}) is 0.12 of the fitted area, and the longer-duration component (τ_{o2} = 27.0 msec) has a fractional area (a_{o2}) of 0.88 based on 72.6 sec of recordings. *B*, Closed time histogram obtained from the same data of *A* was fitted by the sum of three exponentials (τ_{c1} = 0.4 msec, τ_{c2} = 4.3 msec, and τ_{c3} = 8.9 msec; a_{c1} = 0.65, a_{c2} = 0.09, and a_{c3} = 0.26). *C* and *D*, Effects of ATP on single-channel kinetics from another inside-out patch. Channel activity was partially inhibited (P_{open} = 0.20) using the same conditions as in *A* and *B* but with a low ATP concentration (200 μM) in the internal solution. At this concentration ATP suppressed the longer duration component of open times (τ_{o1} = 0.7 msec, τ_{o2} = 3.7 msec; a_{o1} = 0.64, a_{o2} = 0.36) (*C*) and enhanced the intermediate and the longest components of closed times (τ_{c1} = 0.4 msec, τ_{c2} = 28.8 msec, and τ_{c3} = 138.0 msec; a_{c1} = 0.25, a_{c2} = 0.25, and a_{c3} = 0.50) (*D*).

of brief open times was markedly increased with ATP (from 0.18 to 0.53), while the fraction of long closures was significantly higher with ATP than during control (a_{c1} = 0.49, a_{c2} = 0.24, a_{c3} = 0.27). The unitary current amplitude, however, did not show any significant change ($P > 0.05$, n = 31) (Table 1).

Voltage sensitivity. Figure 8 shows the relationship between channel activity and V_m. When symmetric concentrations of K^+ were applied to both sides of membranes, the channel started to open noticeably at about −40 mV and reached a maximal activation at about 20 mV. V_m for half-activation was −13 mV. Using much more physiological K^+ concentrations (i.e., internal 150 mM K^+, external 5 mM K^+), P_{open} began to increase near −60 mV and reached a maximum level at about −20 mV with 50% maximal activation at −42 mV. Since the resting V_m of SN neurons is about −50 to −60 mV (Grace and Onn, 1989; Murphy and Greenfield, 1992), these data suggest that the hypoxia-activated K^+ channel can be activated at resting V_m.

The time constant of the long-duration component of open times was greatly increased, while the time constant of both the intermediate and long duration components of closed times was reduced, when depolarizing from −60 mV to 60 mV (Fig. 9*A*,*B*). However, the brief components of both open and closed times were little changed by depolarization.

The relative area of the long open time component increased with depolarization, while the fraction of long closed time decreased with depolarization. Therefore, the increase in P_{open} with depolarization in the K_{ATP} channel is a result of an increase in longer duration openings and a shortening of the longer period of closures (Fig. 9*C*,*D*).

Contribution to whole-cell macroscopic outward currents

If hypoxia leads to activation of ATP- and Ca^{2+}-sensitive K^+ channels in SN neurons, then whole-cell outward currents should show an increase during O_2 deprivation. To test this hypothesis, we recorded membrane currents using the whole-cell patch configuration in 17 SN neurons and perforated patches in six cells. In these experiments, Na^+ current was eliminated using an extracellular solution containing 0 mM Na^+ or 5 mM Na^+ plus 1 μM TTX (holding potential = −70 mV). In eight of these cells we also added 0.5 mM Co^{2+} in these solutions to block Ca^{2+} currents. During the first 2–3 min of hypoxia, the outward current increased by 17 ± 4% in the whole-cell configuration ($P < 0.001$, n = 17) and 33 ± 17% in the perforated patches ($P < 0.05$, n = 6). Figure 10 shows a representative result from a perforated patch. The time profile of this increase in outward current amplitude (Fig. 10*B*) was similar to that obtained from cell-attached patches showing the increase in single-channel activity during hypoxia (Fig. 2*C*). Interestingly, when hypoxia was maintained for several more minutes, a marked reduction in outward currents followed, even to below baseline level.

Evidence supporting the presence of K_{ATP} channels in SN neurons at the whole-cell level was also obtained from data using ligands in bath solutions. Glibenclamide (10 μM, n = 8) and BRL 38227 (4–6 μM, n = 7) significantly decreased and increased the outward currents by 15–25% ($P < 0.01$), respectively, a result that was consistent with our single-channel observations.

Discussion

One of the outstanding unresolved questions that pertains to ischemic/hypoxic brain injury is whether neurons activate certain mechanisms that could allow them to survive when deprived of O_2 or nutrient. If such mechanisms existed, their detailed study would have broad implications since therapeutic strategies designed to take advantage of these inherent mechanisms could then be possibly formulated. In central neurons, such mechanisms have been described (Haddad and Jiang, 1993).

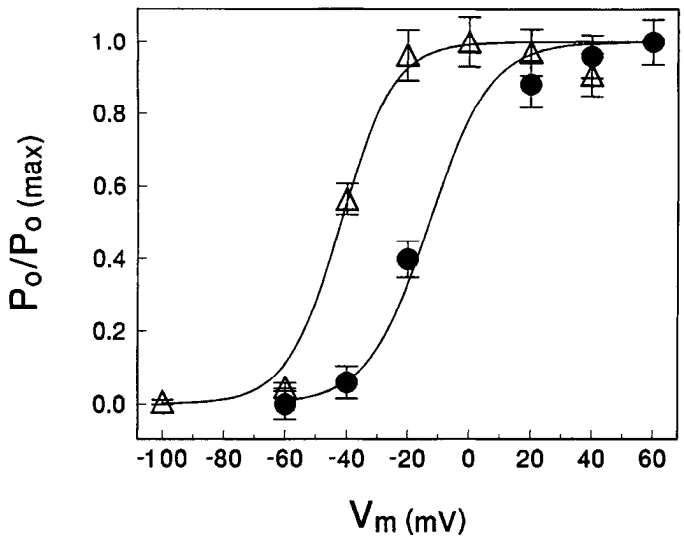

Figure 8. The effect of V_m on P_{open} at two different K^+ concentrations. Single-channel current was recorded from inside-out patches with 0 mM ATP, 1.5 μM Ca^{2+}. When equal concentrations of K^+ were applied to both sides of cell membranes (●, means ± SE, n = 8), the channel activity (P_{open}, normalized to the maximum level) started to increase near −40 mV. Full activation was reached when V_m was above 20 mV. The relation of P_{open} to V_m can be described with the Boltzmann expression $P_{open} = 1/\{1 + \exp[(K_v - V_m)/k]\}$, where P_{open} = open-state probability, V_m = membrane potential, K_v = −13 mV (V_m at 50% of P_{open}), and k = 10. With physiological concentrations of K^+ (internal 150 mM, external 5 mM), this channel was activated from a threshold of about −60 mV and reached a maximum activation when V_m was more depolarized than −20 mV (△, n = 6). The relation of P_{open} to V_m in this case can also be described with the Boltzmann expression, with K_v = −42 mV and k = 8.7. P_{open} values were normalized to the maximum value. Note that the 0 mV point on the symmetric K^+ curve and −80 mV in the physiological K^+ curve are missing, since these potentials are very close to the reversal potentials.

A shift in intermediary metabolic pathways (Hochachka, 1986; Xia et al., 1992), a decrease in neuronal excitability through inactivation of Na^+ channels (Cummins et al., 1991, 1993), an activation of certain K^+ conductances (Leblond and Krnjević, 1989; Jiang and Haddad, 1992), and expression of cell-protective proteins (Gonzalez et al., 1991; Lowenstein et al., 1991; Rordorf et al., 1991) are only some of the interesting adaptive alterations that can occur in central neurons during ischemia or hypoxia. Another fairly recent discovery is the presence of K_{ATP} channels in neurons. These channels may have a potential role in a number of neuronal functions as well as in pathophysiological conditions such as O_2 or glucose deprivation (Ashford et al., 1988, 1990; Grigg and Anderson, 1989; Mourre et al., 1989, 1990; Amoroso et al., 1990; Röper et al., 1990; Schmid-Antomarchi et al., 1990; Häusser et al., 1991; Jiang and Haddad, 1991; Jiang et al., 1992; Murphy and Greenfield, 1992; Patel et al., 1992; Riepe et al., 1992; Tromba et al., 1992; Xia and Haddad, 1992).

Our studies to date have been done on SN neurons since the substantia nigra contains high quantities of glibenclamide binding sites, as we and others have previously shown (Mourre et al., 1989; Jiang et al., 1992). In order to appreciate the characteristics of the neurons we dissociated, electrophysiological (current-clamp) and cytochemical studies have been performed. Using FICF, a well-established technique for studies of catecholamine neurons (Furness et al., 1977), we demonstrate that most (>95%) of our dissociated neurons are catecholaminergic. This is consistent with our current-clamp data showing that our dissociated SN neurons do not express bursting activity in response to depolarization, but have postinhibitory rebound (with smaller prehyperpolarizing pulses) and delayed excitation (with larger prehyperpolarizations), properties that have been well documented for catecholaminergic neurons in the SN area (Grace and Onn, 1989; Yung et al., 1991) and for type 2 "rhythmic" cells described by Murphy and Greenfield (1992).

Using indirect pharmacological approaches such as sulfonylurea receptor agonists or antagonists, we and others have previously suggested that activation of K_{ATP} channels occurs during hypoxia or ischemia (Mourre et al., 1989; Jiang and Haddad, 1991; Jiang et al., 1992; Murphy and Greenfield, 1992; Riepe et al., 1992). For example, glibenclamide has been shown to decrease significantly the hypoxia-induced hyperpolarization in hippocampal and neocortical (Grigg and Anderson, 1989; Mourre et al., 1989; Riepe et al., 1992) neurons, or enhance the anoxia-induced depolarization in hypoglossal neurons (Jiang and Haddad, 1991; Jiang et al., 1992). In the substantia nigra nucleus, the target neurons of glibenclamide are not clear. Murphy and Greenfield (1992) have found that tolbutamide and diazoxide affect noncatecholaminergic cells. However, Roeper et al. (1990) and Hausser et al. (1991) have shown that the functional target neurons are catecholaminergic cells. Since most of these experiments depended on the use of ligands such as glibenclamide or tolbutamide, these studies could only suggest or indirectly show that K_{ATP} channels might be involved during hypoxia. In contrast to these previous studies, we have, in these experiments, directly recorded and identified ATP-sensitive K^+ currents using cell-attached patches from SN neurons. We believe, for several reasons, that these channels, activated during hypoxia in the cell-attached mode, are the same channels we studied in excised membranes. First, the outward currents activated by hypoxia have a conductance (220 pS) identical to the ATP-sensitive K^+ current identified in excised patches. Second, these currents are also sensitive to conventional K^+ channel blockers such TEA, as virtually no channel activity was recorded in cell-attached patches when the K^+ channel blocker was present in the pipette solution. Furthermore, K_{ATP} channel blockers glibenclamide or tolbutamide reversibly suppressed these currents.

It is worth mentioning that our cell-attached patch experiments were performed using a high (nonphysiological) concentration of K^+ in the extracellular solution. We wished in these experiments to have a stable V_m and to eliminate any V_m change, especially during hypoxia. Indeed, this study demonstrates that a K^+ channel inhibited by ATP is activated during O_2 deprivation or metabolic inhibition (cyanide exposure). Although this current may not be the only K^+ channel involved, our data indicate that activation of this K^+ channel at least contributes to the hypoxia-induced whole-cell outward current.

This hypoxia-activated channel has similarities with K_{ATP} channels of other tissues. Like K_{ATP} channels in peripheral tissues, the hypoxia-activated channel in SN neurons is K^+ selective and very sensitive to intracellular ATP and ADP. The concentration of ATP for half-inactivation of the channel activity is similar to values previously reported in peripheral tissues (Ashcroft, 1988; Standen et al., 1989; Takano and Noma, 1993). This ATP sensitivity is not mediated by protein phosphorylation because the nonhydrolyzable ATP analog AMP-PNP also inhibits this channel, a property that all K_{ATP} channels in other tissues have (Takano and Noma, 1993). In SN neurons, the channel is also inhibited by glibenclamide (Ashcroft, 1988;

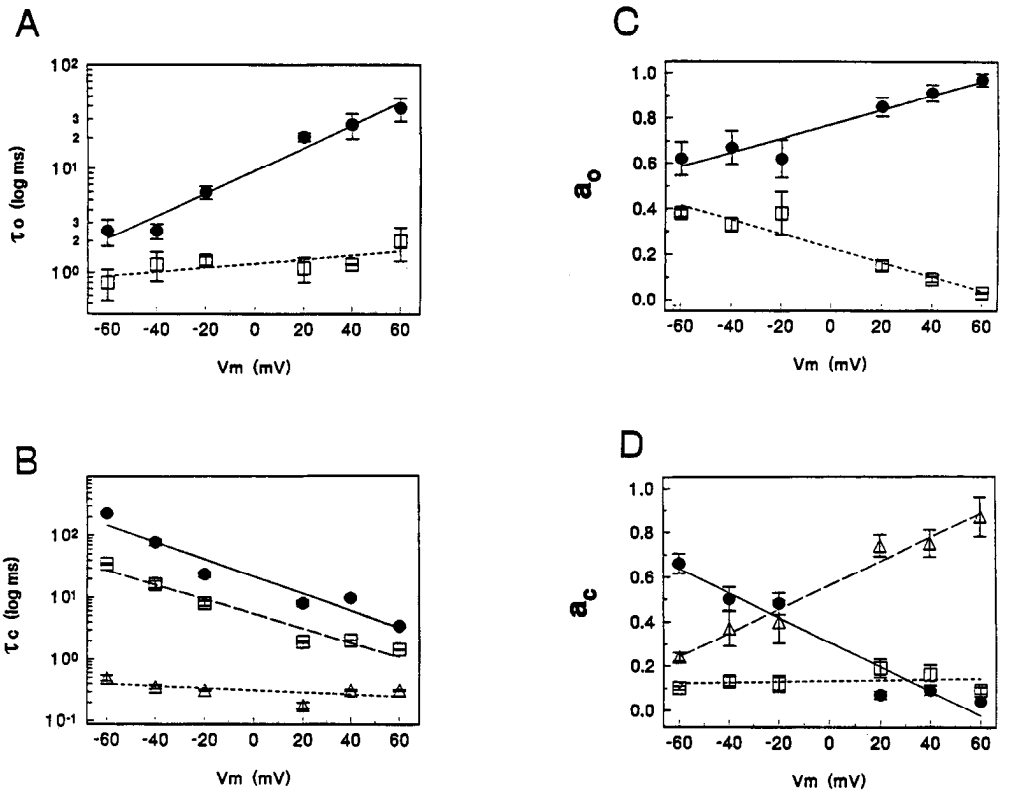

Figure 9. Changes in single-channel kinetics with V_m. *A*, τ_{o2} (*solid line*, ●) increases with depolarization, while τ_{o1} (*dashed line*, □) does not change with V_m. *B*, Although there is an evident difference among the three components of closed time with hyperpolarization, this difference becomes smaller when V_m is depolarized (τ_{c1} = △ and *short-dashed line*, τ_{c2} = *long-dashed line* and □, τ_{c3} = *solid line* and ●). Note the log scale of ordinates of *A* and *B*. *C*, The proportional weights of τ_{o1} and τ_{o2} components are similar at −60 mV. The percentage of the τ_{o2} component, however, greatly increases, and the τ_{o1} component decreases with depolarization. *D*, Depolarization increases and decreases, respectively, the short (τ_{c1}) and longer closing time constants (τ_{c3}) without significantly affecting the weight of τ_{c2} components. Data are presented as means ± SE (n = 6).

Takano and Noma, 1993) and other nonspecific K^+ channel blockers such as Cs^+ and several divalent cations, as reported previously (Quayle et al., 1988; Davies et al., 1991). The hypoxia-activated K^+ channel was blocked by sulfonylurea K_{ATP} channel blockers. Tolbutamide reversibly inhibited P_{open} but not unitary conductance. Glibenclamide also inhibited the channel activity in the majority of patches studied. A possible reason for the ineffectiveness of glibenclamide in two out of eight patches (see Results) would be that the channel and the glibenclamide binding sites may not form a single physical moiety, and therefore this blocker may not have reached receptor binding sites.

The single-channel conductance and the extent of rectification of K_{ATP} currents vary among tissues and cell types. For instance, with symmetrical K^+ concentrations on both sides of the plasma membranes, the unitary conductance is about 50 pS in pancreatic β-cells (Ashcroft et al., 1984; Cook and Hales, 1984), 80–90 pS in cardiac myocytes (Noma, 1983; Kirsch et al., 1990), and 135 pS in vascular smooth muscle cells (Standen et al., 1989). Tromba et al. (1992) have reported a K_{ATP} channel with a conductance of 100 pS in hippocampal neurons using asymmetrical K^+ concentration across the membrane. Under a similar condition of asymmetric K^+ concentrations across the membrane, the hypoxia-activated K^+ channel in SN neurons was also ~100 pS. Also, the hypoxia-activated K^+ channels show inward rectification with 50–100 mM Na^+ or 1–2 mM Mg^{2+} in the internal solution, whereas a linear conductance is seen when 150 mM K^+ with low Mg^{2+} (200 μm) and low Na^+ is present on both sides of the cell membrane, suggesting that the inward rectification of this channel is likely mediated by a voltage-dependent blockade of the outward current. This is also consistent with K_{ATP} channels described in cardiac myocytes (Noma, 1983; Tung and Kurachi, 1991; Fan et al., 1992), β-cells (Cook

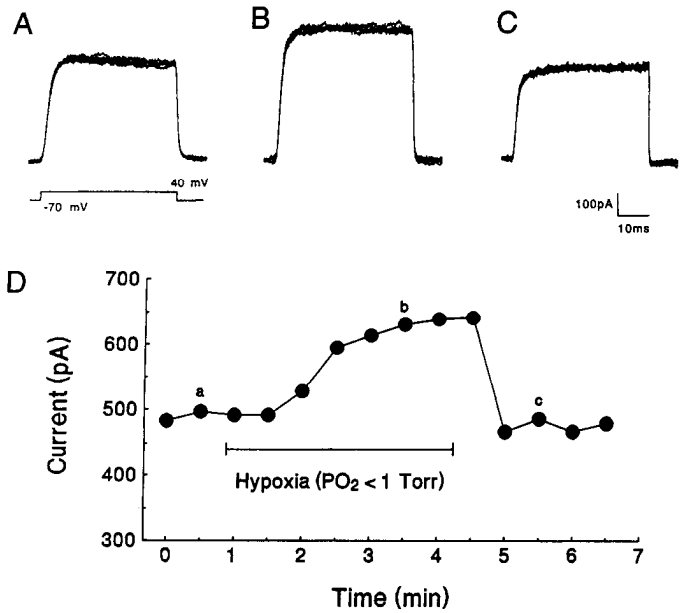

Figure 10. Whole-cell outward currents recorded from an SN neuron. The standard internal solution (K^+ = 150 mM) plus nystatin (0.1 mg/1 ml) was applied to the recording pipette and an external solution containing 5 mM Na^+ plus 1 μM TTX and 2.5 mM K^+ was used in the bath. *A*, Current traces (eight superimposed) recorded during baseline. V_m was held at −70 mV and depolarized to 40 mV (*lower trace*). *B*, The macroscopic outward currents were enhanced during hypoxia (2.5 min) induced by a solution bubbled with 100% N_2 plus $Na_2S_2O_4$ (2 mM). *C*, The current amplitude returned to the baseline level after washout. *D*, Time profile of current amplitudes measured near the end of the outward current (*a*, *b*, and *c* correspond to *A*, *B*, and *C*, respectively).

and Hales, 1984; Niki et al., 1989; Standen et al., 1989), and cortical and hypothalamic neurons (Ashford et al., 1988, 1990).

It is clear, however, that there are a number of differences between the ATP-sensitive K^+ channels we found in SN neurons and those in other tissues. First, the ATP-sensitive K^+ channels in SN cells have a clear voltage-gating property that has not been consistently demonstrated in peripheral tissue. Although Trube and Hescheler (1984), Spruce et al. (1985), and Standen et al. (1989) have shown that depolarization increases K_{ATP} channel mean open time in cardiac, skeletal, and smooth muscles, a number of other studies have also suggested that K_{ATP} channels in these tissues as well as in β-cells are voltage independent (Ashcroft, 1988; Takano and Noma, 1993). Second, activation of the ATP-sensitive K^+ channel in SN neurons depends on the presence of Ca^{2+}. Recent studies from several laboratories have also shown that K_{ATP} channels in skeletal and smooth muscles are stimulated by free Ca^{2+} in the cytosol (Silberberg and van Breemen, 1990; Krippeit-Drews and Uönnendonker, 1992), but a large number of other studies have described K_{ATP} channels that are either not sensitive to cytosolic free Ca^{2+} (Standen et al., 1989) or inhibited by Ca^{2+} (Kakei and Noma, 1984; Findlay, 1988).

It is also clear from our experiments that in spite of the fact that the ATP-sensitive K^+ channels in SN neurons are sensitive to Ca^{2+} and have a large conductance, they are different from Ca^{2+}-activated K^+ (BK) channels in several ways. (1) K_{ATP} channels are not known to be sensitive to charybdotoxin, a BK channel blocker (Latorre et al., 1989). (2) Of major importance is that Chung et al. (1991) have recently demonstrated that BK channel activity is increased by ATP (rather than decreased) in a Mg^{2+}-dependent manner, and nonhydrolyzable ATP analogs such as AMP-PNP do not have any effect. These properties are clearly different from those of the hypoxia-activated K^+ channel in SN neurons. (3) Several recent reports have shown that BK channels in smooth muscles are inhibited by ATP (Gelband et al., 1990; Groschner et al., 1992) as a result of Ca^{2+} chelation (Klöckner and Isenberg, 1992). This is unlike the behavior of the channel we found in our studies, since Mg·ATP in a high Mg^{2+} background and K_2·ATP in a high Ca^{2+} concentration *still* showed an inhibitory effect.

In the present study, we observed that the channel activity (P_{open}) changed with different K^+ concentrations. It is important to note that at the more physiologic concentrations of K^+ across the cell membrane, the activation curve is shifted to the left and this channel is more likely to be activated. Although it is not clear what causes the shift of the voltage-dependent curve of P_{open} with different concentrations of K^+, there are several possible explanations, such as a change in ionic strength of Na^+ or K^+ that can itself play a role in channel activity. We would like to emphasize that since this channel is activated at about -50 mV, the resting V_m, whether measured in slice or in dissociated SN neurons, is near this activation threshold. The reason for the small difference in SN resting V_m found in brain slice preparations (-55 to -60 mV) (Grace and Onn, 1989; Murphy and Greenfield, 1992) and in our present studies (i.e., -51 mV) is not clear but may be due to differences in temperature or recording techniques.

What are the functional implications of these hypoxia-activated K^+ channels and their modulation? In peripheral tissues, K_{ATP} channels are modulated by a large number of cytosolic factors such as pH (Davies, 1990; Cuevas et al., 1991; Davies et al., 1992; Findlay, 1992), Mg^{2+} (Findlay, 1987), glucose (Gillis et al., 1989; Ashford et al., 1990; Tromba et al., 1992), and aromatic aldehydes and ketones (Fan et al., 1992). In SN neurons, our results illustrate well the effect of ATP, ADP, Ca^{2+}, and V_m on this channel. It would be important therefore to ask how various factors interact intracellularly in modulating this channel. In this regard, we would like to emphasize that the interaction (which we describe in this article) among ATP, Ca^{2+}, and V_m is novel vis-à-vis the activity of this channel. As demonstrated by our results, a change in one of these factors alone, such as a decrease in ATP, increase in Ca^{2+}, or depolarization, does not open this channel. The prerequisite for activation of this channel seems to be a decrease in ATP *at a time* when an increase in local $Ca^{2+}{}_i$ as well as depolarization is occurring. We believe that this interaction between ATP, Ca^{2+}, and V_m is significant because the activation of this channel would be much less likely if only one of these variables changes in the cell. There are numerous conditions that can accompany changes in one of these cytosolic and membrane factors, such as a change in $Ca^{2+}{}_i$ or V_m, without involving any alteration in ATP. Since deprivation of O_2 or glucose generally induces changes in multiple factors concurrently, this channel then seems to be endowed with properties that would make it uniquely suitable for cell response to metabolic stress. Clearly, although the interaction among various factors may offer an explanation for activation of this current during hypoxia, further experiments are needed to demonstrate this speculation.

References

Amoroso S, Schmid-Antomarchi H, Fosset M, Lazdunski M (1990) Glucose, antidiabetic sulfonylureas and neurotransmitter release. Role of ATP-sensitive K^+ channels. Science 247:852–854.

Ashcroft FM (1988) Adenosine 5'-triphosphate–sensitive potassium channels. Annu Rev Neurosci 11:97–118.

Ashford MLJ, Sturgess NC, Trout NJ, Gardner NJ, Hales CN (1988) Adenosine-5-triphosphate–sensitive ion channels in neonatal rat cultured central neurons. Pfluegers Arch 412:297–304.

Ashford MLJ, Boden PR, Treherne JM (1990) Glucose-induced excitation of hypothalamic neurons is mediated by AT-sensitive K^+ channels. Pfluegers Arch 415:479–483.

Chung SK, Reinhart PH, Martin BL, Brautigan D, Levitan IB (1991) Protein kinase activity closely associated with a reconstituted calcium-activated potassium channel. Science 253:560–562.

Colquhoun D, Sigworth FJ (1983) Fitting and statistical analysis of single channel records. In: Single channel recordings (Sakmann B, Neher E, eds), pp 191–264. New York: Plenum.

Cook DL, Hales CN (1984) Intracellular ATP directly blocks K^+ channels in pancreatic β-cells. Nature 311:271–273.

Cuevas J, Bassett AI, Cameron JS, Furukawa T, Myerburg RJ, Kimura S (1991) Effect of H^+ on ATP-regulated K^+ channels in feline ventricular myocytes. Am J Physiol 261:H755–H761.

Cummins TR, Donnelly DF, Haddad GG (1991) Effect of oxygen deprivation on the excitability of isolated hippocampal CA1 neurons: developmental aspects. J Neurophysiol 66:1471–1482.

Cummins TR, Jiang C, Haddad GG (1993) Decrease in human neocortical excitability during anoxia via Na^+ channel modulation. J Clin Invest 91:968–915.

Daut J, Maier-Rudolph W, von Beckerath N, Mehrke G, Gunther K, Goedel-Meinen L (1990) Hypoxic dilation of coronary arteries is mediated by ATP-sensitive potassium channels. Science 247:1341–1344.

Davies NW (1990) Modulation of ATP-sensitive K^+ channels in skeletal muscle by intracellular protons. Nature 343:374–377.

Davies NW, Spruce AE, Standen NB, Stanfield PR (1989) Multiple blocking mechanisms of ATP-sensitive K^+ channels of frog skeletal muscle by tetraethylammonium ions. J Physiol (Lond) 413:31–47.

Davies NW, Pettit AI, Agarwal R, Standen NB (1991) The flickery block of ATP-dependent potassium channels of skeletal muscle by internal 4-aminopyridine. Pfluegers Arch 419:25–31.

Davies NW, Standen NB, Stanfield PR (1992) The effect of intracellular pH on ATP-dependent potassium channels of frog skeletal muscle. J Physiol (Lond) 445:549–568.

Domesick VB, Stinus L, Paskevich PA (1983) The cytology of dopaminergic and nondopaminergic neurons in the substantia nigra and ventral tegmental area of the rat: a light and electron-microscopic study. Neuroscience 8:743–765.

Dubinsky JM, Rothman SM (1991) Intracellular calcium concentrations during "chemical hypoxia" and excitotoxic neuronal injury. J Neurosci 11:2545–2551.

Fan Z, Nakayama K, Sawanobori T, Hiraoba M (1992) Aromatic aldehydes and aromatic ketones open ATP-sensitive K^+ channels in guinea-pig ventricular myocytes. Pfluegers Arch 421:409–415.

Findlay I (1987) The effects of magnesium upon adenosine triphosphate-sensitive potassium channels in a rat insulin-secreting cell line. J Physiol (Lond) 391:611–629.

Findlay I (1988) Calcium-dependent inactivation of the ATP-sensitive K^+ channel of rat ventricular myocytes. Biochim Biophys Acta 943: 297–304.

Findlay I (1992) Effects of pH upon the inhibition by sulphonylurea drugs of ATP-sensitive K^+ channels in cardiac muscle. J Pharmacol Exp Ther 262:71–79.

Friedman J, Haddad GG (1993) Major differences in Ca^{2+}_i response to anoxia between neonatal and adult rat CA1 neurons; role of Ca^{2+}_o and Na^+_o. J Neurosci 13:63–72.

Gelband CH, Silberger SD, Groschner K, van Breemen C (1990) ATP inhibits smooth muscle Ca^{++}-activated K^+ channels. Proc R Soc Lond [Biol] 242:23–28.

Gillis KD, Gee WM, Hammoud A, McDaniel ML, Falke LC, Misler S (1989) Effects of sulfonamides on a metabolite-regulated ATP_i-sensitive K^+ channel in rat pancreatic B-cells. Am J Physiol 257:C1119–C1127.

Gonzalez MF, Lowenstein D, Fernyak S, Hisanaga K, Simon R, Sharp FR (1991) Induction of heat shock protein 72-like immunoreactivity in the hippocampal formation following transient global ischemia. Brain Res Bull 26:241–250.

Grace AA, Onn SP (1989) Morphology and electrophysiological properties of immunocytochemically identified rat dopamine neurons recorded *in vitro*. J Neurosci 9:3464–3481.

Grigg JJ, Anderson EG (1989) Glucose and sulfonylurea modify different phases of the membrane potential change during hypoxia in rat hippocampal slices. Brain Res 489:302–310.

Groschner K, Silberger SD, Gelband CH, van Breemen C (1992) Ca^{++}-activated K^+ channels in airway smooth muscle are inhibited by cytoplasmic adenosine triphosphates. Pfluegers Arch 417:517–522.

Haddad GG, Donnelly DF (1990) O_2 deprivation induces a major depolarization in brain stem neurons in the adult but not in the neonatal rat. J Physiol (Lond) 429:411–428.

Haddad GG, Jiang C (1992) O_2 deprivation in the central nervous system: on mechanisms of neuronal response, differential sensitivity and injury. Prog Neurobiol 40:277–318.

Hamill OP, Marty A, Neher E, Sakmann B, Sigworth FJ (1981) Improved patch-clamp techniques for high-resolution current recording from cells and cell-free membrane patches. Pfluegers Arch 391:85–100.

Häusser MA, de Weille JR, Lazdunski M (1991) Activation of cromakalim of pre- and post-synaptic ATP-sensitive K^+ channels in substantia nigra. Biochem Biophys Res Commun 174:909–914.

Hochachka PW (1986) Defense strategies against hypoxia and hypothermia. Science 231:234–241.

Jiang C, Haddad GG (1991) Effect of anoxia on intracellular and extracellular potassium activity in hypoglossal neurons *in vitro*. J Neurophysiol 66:103–111.

Jiang C, Haddad GG (1992) Differential responses of neocortical neurons to O_2 and/or glucose deprivation in human and rat. J Neurophysiol 68:2165–2173.

Jiang C, Agulian S, Haddad GG (1991) O_2 tension in adult and neonatal brain slices under several experimental conditions. Brain Res 568:159–164.

Jiang C, Xia Y, Haddad GG (1992) Role of ATP-sensitive K^+ channels during anoxia: major differences between rat (newborn, adult) and turtle neurons. J Physiol (Lond) 448:599–612.

Kakei M, Noma A (1984) Adenosine 5'-triphosphate-sensitive single potassium channels in atrioventricular node cell of the rabbit heart. J Physiol (Lond) 352:265–284.

Kass IS, Lipton P (1989) Protection of hippocampal slices from young rats against anoxic transmission damage is due to better maintenance of ATP. J Physiol (Lond) 413:1–11.

Kay AR, Wong RKS (1986) Isolation of neurons suitable for patch-clamping from adult mammalian central nervous system. J Neurosci Methods 16:227–238.

Klöckner U, Isenberg G (1992) ATP suppresses activity of Ca^{++}-activated K^+ channels by Ca^{++} chelation. Pfluegers Arch 420:101–105.

Krippeit-Drews P, Uönnendonker U (1992) Dual effects of calcium on ATP-sensitive potassium channels of frog skeletal muscle. Biochim Biophys Acta 1108:119–122.

Latorre R, Oberhauser A, Labarca P, Alvarez O (1989) Varieties of calcium-activated potassium channels. Annu Rev Physiol 51:385–399.

Leblond J, Krnjević K (1989) Hypoxic changes in hippocampal neurons. J Neurophysiol 62:1–14.

Levitan ES, Kramer RH (1990) Neuropeptide modulation of single calcium and potassium channels detected with a new patch clamp configuration. Nature 348:545–547.

Lowenstein DH, Chan PH, Miles MF (1991) The stress protein response in cultured neurons: characterization and evidence for a protective role in excitotoxicity. Neuron 7:1053–1060.

Mourre C, Ben Ari Y, Bernardi H, Fosset M, Lazdunski M (1989) Antidiabetic sulfonylurea: location of binding sites in the brain and effects on the hyperpolarization induced by anoxia in hippocampal slices. Brain Res 486:159–164.

Mourre C, Widmann C, Lazdunski M (1990) Sulfonylurea binding sites associated with ATP-regulated K^+ channels in the central nervous system: autoradiographic analysis of their distribution and ontogenesis, and of their localization in mutant mice. Brain Res 519: 29–43.

Murphy KPS, Greenfield SA (1992) Neuronal selectivity of ATP-sensitive potassium channels in guinea-pig substantia nigra revealed by responses to anoxia. J Physiol (Lond) 453:167–183.

Nelson MT, Huang Y, Brayden JE, Hescheler J, Standen NB (1990) Arterial dilations in response to calcitonin gene-related peptide involve activation of K^+ channels. Nature 344:770–773.

Niki I, Kelly RP, Ashcroft SJH, Ashcroft FM (1989) ATP-sensitive K-channels in HIT T15 β-cells studied by patch-clamp methods, ^{86}Rb efflux and glibenclamide binding. Pfluegers Arch 415:47–55.

Noma A (1983) ATP-regulated K^+ channels in cardiac muscle. Nature 305:147–148.

Patel MN, Yim GKW, Isom GE (1992) Potentiation of cyanide neurotoxicity by blockade of ATP sensitive potassium channels. Brain Res 593:114–116.

Quayle JM, Standen NB, Stanfield PR (1988) The voltage-dependent block of ATP-sensitive potassium channels of frog skeletal muscle by cesium and barium ions. J Physiol (Lond) 405:677–697.

Riepe M, Hori N, Ludolph AC, Carpenter DO, Spencer PS, Allen CN (1992) Inhibition of energy metabolism by 3-nitropropiinic acid activates ATP-sensitive potassium channels. Brain Res 586:61–66.

Röper J, Hainsworth AH, Ashcroft FM (1990) ATP-sensitive K channels in guinea-pig isolated substantia nigra neurons are modulated by cellular metabolism. J Physiol (Lond) 430:130P.

Rordorf G, Koroshetz WJ, Ronventre JV (1991) Heat shock protects cultured neurons from glutamate toxicity. Neuron 7:1043–1051.

Rorsman P, Berggren PO, Bokvist K, Efendic S (1990) ATP-regulated K^+ channels and diabetes mellitus. News Physiol Sci 5:143–147.

Schmid-Antomarchi H, Amoroso S, Fosset M, Lazdunski M (1990) K^+ channel openers activate brain sulfonylurea-sensitive K^+ channels and block neurosecretion. Proc Natl Acad Sci USA 87:3489–3492.

Sigworth FJ, Sine SM (1987) Data transformations for improved display and fitting of single-channel dwell time histograms. Biophys J 52:1047–1054.

Silberberg SD, van Breemen C (1990) An ATP, calcium and voltage sensitive potassium channel in porcine coronary artery smooth muscle cells. Biochem Biophys Res Commun 172:517–522.

Silva NL, Pechura CM, Barker JL (1990) Postnatal rat nigrostriatal dopaminergic neurons exhibit five types of potassium conductances. J Neurophysiol 64:262–272.

Silver IA, Erecinska M (1990) Intracellular and extracellular changes of $[Ca^{2+}]$ in hypoxia and ischemia in rat brain *in vivo*. J Gen Physiol 95:837–866.

Spruce AE, Standen NB, Stanfield PR (1985) Voltage-dependent, ATP-

sensitive potassium channels of skeletal muscle membrane. Nature 316:734–738.

Standen NB, Quayle JM, Davis NW, Brayden JE, Huang Y, Nelson MT (1989) Hyperpolarizing vasodilators activate ATP-sensitive K^+ channels in arterial smooth muscle. Science 345:177–180.

Takano M, Noma A (1993) The ATP-sensitive K^+ channel. Prog Neurobiol 41:21–30.

Tromba C, Salvaggio A, Racagni G, Volterra A (1992) Hypoglycemia-activated K^+ channels in hippocampal neurons. Neurosci Lett 143: 185–189.

Trube G, Hescheler J (1984) Inward-rectifying channels in isolated patches of the heart cell membrane: ATP-dependence and comparison with cell-attached patches. Pfluegers Arch 401:178–184.

Tung RT, Kurachi Y (1991) On the mechanism of nucleotide diphosphate activation of the ATP-sensitive K^+ channel in ventricular cell of guinea-pig. J Physiol (Lond) 437:239–256.

Xia Y, Haddad GG (1991) Major differences in CNS sulfonylurea receptor distribution between rat (newborn, adult) and turtle. J Comp Neurol 314:278–289.

Xia Y, Jiang C, Haddad GG (1992) Oxidative and glycolytic pathways in rat (newborn, adult) and turtle brain: role during anoxia. Am J Physiol 262:R595–R603.

Yung WH, Hausser MA, Jack JJB (1991) Electrophysiology of dopaminergic and non-dopaminergic neurons of the guinea-pig substantia nigra pars compacta *in vitro*. J Physiol (Lond) 436:643–667.