Sodium Influx Plays a Major Role in the Membrane Depolarization Induced by Oxygen and Glucose Deprivation in Rat Striatal Spiny Neurons

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Background and Purpose—Striatal spiny neurons are selectively vulnerable to ischemia, but the ionic mechanisms underlying this selective vulnerability are unclear. Although a possible involvement of sodium and calcium ions has been postulated in the ischemia-induced damage of rat striatal neurons, the ischemia-induced ionic changes have never been analyzed in this neuronal subtype.

Methods—We studied the effects of in vitro ischemia (oxygen and glucose deprivation) at the cellular level using intracellular recordings and microfluorometric measurements in a slice preparation. We also used various channel blockers and pharmacological compounds to characterize the ischemia-induced ionic conductances.

Results—Spiny neurons responded to ischemia with a membrane depolarization/inward current that reversed at approximately $-40 \text{ mV}$. This event was coupled with an increased membrane conductance. The simultaneous analysis of membrane potential changes and of variations in $[\text{Na}^+]_i$ and $[\text{Ca}^{2+}]_i$ levels showed that the ischemia-induced membrane depolarization was associated with an increase of $[\text{Na}^+]_i$ and $[\text{Ca}^{2+}]_i$. The ischemia-induced membrane depolarization was not affected by tetrodotoxin or by glutamate receptor antagonists. Neither intracellular BAPTA, a Ca$^{2+}$ chelator, nor incubation of the slices in low-Ca$^{2+}$–containing solutions affected the ischemia-induced depolarization, whereas it was reduced by lowering the external Na$^+$ concentration. High doses of blockers of ATP-dependent K$^+$ channels increased the membrane depolarization observed in spiny neurons during ischemia.

Conclusions—Our findings show that, although the ischemia-induced membrane depolarization is coupled with a rise of $[\text{Na}^+]_i$ and $[\text{Ca}^{2+}]_i$, only the Na$^+$ influx plays a prominent role in this early electrophysiological event, whereas the increase of $[\text{Ca}^{2+}]_i$ might be relevant for the delayed neuronal death. We also suggest that the activation of ATP-dependent K$^+$ channels might counteract the ischemia-induced membrane depolarization. (Stroke. 1999;30:171-179.)

Key Words: brain ■ ion channels ■ oxygen ■ glucose ■ neuroprotection ■ rats

Severe cerebral ischemia results in a rapid loss of ATP production that initiates a cascade of changes including a major increase in $[\text{Ca}^{2+}]_i$. This large accumulation of Ca$^{2+}$ has received considerable attention as a possible critical change in determining neuronal damage after short-term cerebral ischemia. Two main sources for the increase in $[\text{Ca}^{2+}]_i$ during ischemia have been postulated. First, it has been hypothesized that Ca$^{2+}$ influx might occur via high voltage-activated Ca$^{2+}$ channels. Accordingly, we have recently shown that in cortical neurons, blockers of L-type Ca$^{2+}$ channels such as nifedipine and nimodipine reduce the membrane depolarization and the rise in $[\text{Ca}^{2+}]_i$ caused by oxygen and glucose deprivation (in vitro ischemia). Second, the activation of the $N$-methyl-$D$-aspartate (NMDA) glutamate receptor–channel complex has been considered the other major cause of the ischemia-induced membrane depolarization and rise in $[\text{Ca}^{2+}]_i$. In agreement with this hypothesis, cerebral ischemia results in a large increase in the extracellular concentration of glutamate, and excitotoxic mechanisms have been commonly proposed as the cause of postschismic neuronal loss.

Recent experimental findings have shown that Na$^+$-channel blockers reduce ischemia-induced cortical neuronal injury when combined with glutamate receptor antagonists. Moreover, microfluorometric studies have demonstrated that anoxia induces an increase in $[\text{Na}^+]_i$, in rat CA1 hippocampal neurons. The mechanisms underlying the sustained neuronal penetration of Na$^+$ during ischemia are still unclear. In fact,

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the classic voltage-gated Na$^+$ channels that initiate the action potential rapidly inactivate during ischemic depolarization, suggesting that their contribution to the ischemic event is unlikely. Alternatively, it is possible that the rise in [Na$^+$] during ischemia is due either to the activation of non-NMDA glutamate receptors or to the opening of non-inactivating Na$^+$ channels.

Despite this large body of evidence, the relative contribution of Na$^+$ and Ca$^{2+}$ ions to the ischemic depolarization has never been simultaneously investigated in the same neuronal subtype. Moreover, although several pharmacological compounds that act on Na$^+$ and Ca$^{2+}$ channels have been tested in experimental models of ischemia, the effects of these putative neuroprotective agents have never been analyzed at the cellular level on the electrophysiological events caused by ischemia in brain slices. In the present study, we used a brain slice preparation to characterize the effects of in vitro ischemia on striatal spiny neurons intracellularly recorded. In some experiments, the electrophysiological recordings have been combined with microfluorometric measurements of [Na$^+$] and [Ca$^{2+}$]. To address the differential contribution of these ions to the ischemia-induced depolarization, various experimental approaches have been used: (1) ionic substitutions in the external medium, (2) application of selective blockers of Na$^+$ and Ca$^{2+}$ channels, (3) intracellular injection of a Ca$^{2+}$-chelating agent, (4) application of ionotropic glutamate receptor antagonists, (5) application of drugs that in other experimental models of ischemia have shown a neuroprotective action via the inhibition of Na$^+$ and Ca$^{2+}$ channels, and (6) use of blockers of ATP-dependent K$^+$ channels to test the possible involvement of other ionic conductances in this electrophysiological event.

Materials and Methods

Preparation and Maintenance of the Slices

Wistar rats (150 to 250 g) were used. The preparation and maintenance of coronal slices have been described previously. Briefly, corticostratal coronal slices (200 to 300 μm) were prepared from tissue blocks of the brain with the use of a Vibratome (Pelco International). A single slice was transferred to a recording chamber and submerged in a continuously flowing Krebs’ solution (35°C, 2 to 3 mL/min) gassed with 95% O$_2$/5% CO$_2$ and maintained at 34°C. In some experiments, the osmolarity of this solution was adjusted by replacing NaCl with choline chloride (300 mM). The pH of the solution was adjusted to 7.4 with NaHCO$_3$. In some experiments choline chloride was used to replace NaCl, since this solution was gassed with N$_2$/5% CO$_2$ instead of the normal gas mixture. In some experiments, the osmolarity of this solution was adjusted by replacing NaCl with choline chloride (300 mM). The pH of the solution was adjusted to 7.4 with NaHCO$_3$. In some experiments choline chloride was used to replace NaCl, since this solution was gassed with N$_2$/5% CO$_2$ instead of the normal gas mixture.

Preparation and maintenance of the slices were carried out according to published protocols. After incubation in oxygenated Krebs’ solution for 30 minutes, the slices were transferred to a recording chamber mounted on an upright microscope (Axioskop FS; Zeiss), equipped with a 60× immersion objective (Olympus). Fluorescence of SBFI or fura-2 was excited by epi-illumination with light provided by a 75 W xenon lamp bandpass-filtered alternatively at 340 and 380 nm. Emission light passed a barrier filter (500 nm) and was detected by a charge-coupled device camera (Photonic Science). Pairs of 340 and 380 nm images were acquired at intervals of 12 seconds and analyzed off-line with software (IonVision; ImproVision) running on a PowerMac 8100 (Apple Computer). Ratio images were calculated from pairs of 340 and 380 nm images corrected for background fluorescence (measured from regions free of dye fluorescence). Time courses of ratio values were calculated from regions that include the cell bodies (with “regions of interest” defined as those pixels that exhibit at least 20% to 30% of maximal specific fluorescence). Ratio values were transformed into ion concentration with the method of Grynkiewicz et al. The calibration parameters R$_{max}$ and R$_{min}$ for the transformation of SBFI and fura-2 signals were obtained in situ by bathing perforated (nystatin and gramicidin D or ionomycin, respectively) cells in Na$^+$-free or Ca$^{2+}$-free (50 mM EGTA) solution containing 140 mM/L Na$^+$ and 1 mM/L Ca$^{2+}$, respectively, containing solution. In 3 perforated cells, extracellular Na$^+$ concentration was systematically changed by substitution of NaCl with KCl to obtain ratio values corresponding to known Na$^+$ concentrations. The apparent K$_J$ values for fura-2 are estimated by measuring fluorescence ratios obtained from a solution containing fura-2 and known concentrations of free Ca$^{2+}$ (Fura-2 Calcium Imaging Calibration Kit, Molecular Probes) trapped between 2 coverslips spaced by pieces of coverslips, and imaged with the water immersion objective. A corresponding approach was used to estimate the K$_J$ of SBFI with calibration solution containing 1 mM/L EGTA, 0.1 mM/L CaCl$_2$, 2 mM/L MgCl$_2$, 10 mM/L HEPES, 0 to 145 mM/L NaCl, and 145 to 0 mmol/L potassium gluconate (K$^+$) + [Na$^+$] = 145 mmol/L, pH 7.4). This in vitro apparent K$_J$ was consistent with the K$_J$ estimated from perforated cells.

Recording Technique

In most of the experiments, the intracellular recording electrodes were filled with 2 mol/L KCl (30 to 60 MΩ). In other experiments, 200 mmol/L BAPTA was added to the solution of the intracellular pipette to buffer intracellular Ca$^{2+}$. In a third group of experiments, the pipettes were filled with 2 mol/L potassium acetate. An Axoclamp 2A amplifier (Axon Instruments) was used for recordings, either in current-clamp or in voltage-clamp mode. In single-electrode voltage-clamp mode the switching frequency was 3 kHz. The head-stage signal was continuously monitored on a separate oscilloscope. Traces were displayed on an oscilloscope and stored on a digital system. For synaptic stimulation, bipolar electrodes were used. These stimulating electrodes were located either in the cortical areas close to the recording electrode or in the white matter between the cortex and the striatum to activate corticostriatal fibers. In some experiments, biocytin was used in the intracellular electrode to stain the neurons. In these cases, biocytin at a concentration of 2% to 4% was added to a 0.5 mol/L KCl pipette solution. Slices containing neurons stained with biocytin were fixed in paraformaldehyde (in 0.1 mol/L phosphate buffer at pH 7.4) overnight and processed according to published protocols. After incubation in phosphate buffer containing 30% sucrose for 3 hours, the slices were frozen and further resectioned in a cryostat at a thickness of 40 μm. In several cases, sections were further processed to make permanent the staining of biocytin-loaded cells.

Another set of experiments for combined optical and electrical recordings, the tip of the recording electrode was filled either with a solution of 2 mmol/L fura-2 (pentapotassium salt; Molecular Probes) in 1 mol/L KCl or with 1 containing 5 mmol/L SBFI (tetratrammonium salt; Molecular Probes) in 1 mol/L KC1. The shank of the recording electrode was backfilled with a 2-mol/L KCl solution. After cell impalement, cells were loaded with fura-2 or with SBFI by injecting 0.1 to 0.5 nA negative current for 10 to 15 minutes. An Axoclamp 2A amplifier was used for electrophysiology. Traces were displayed on an oscilloscope and stored on a digital system. In the single-electrode voltage-clamp mode, switching frequency is 3 kHz. The head-stage signal was continuously monitored on a separate oscilloscope. The recording chamber was mounted on the stage of an upright microscope (Axioskop FS; Zeiss), equipped with a 60× water immersion objective (Olympus). Fluorescence of SBFI or fura-2 was excited by epi-illumination with light provided by a 75 W xenon lamp bandpass-filtered alternatively at 340 or 380 nm. Emission light passed a barrier filter (500 nm) and was detected by a charge-coupled device camera (Photonic Science). Pairs of 340 and 380 nm images were acquired at intervals of 12 seconds and analyzed off-line with software (IonVision; ImproVision) running on a PowerMac 8100 (Apple Computer). Ratio images were calculated from pairs of 340 and 380 nm images corrected for background fluorescence (measured from regions free of dye fluorescence). Time courses of ratio values were calculated from regions that include the cell bodies (with “regions of interest” defined as those pixels that exhibit at least 20% to 30% of maximal specific fluorescence). Ratio values were transformed into ion concentration with the method of Grynkiewicz et al. The calibration parameters R$_{max}$ and R$_{min}$ for the transformation of SBFI and fura-2 signals were obtained in situ by bathing perforated (nystatin and gramicidin D or ionomycin, respectively) cells in Na$^+$-free or Ca$^{2+}$-free (50 mM EGTA) solution containing 140 mmol/L Na$^+$ and 1 mmol/L Ca$^{2+}$, respectively, containing solution. In 3 perforated cells, extracellular Na$^+$ concentration was systematically changed by substitution of NaCl with KCl to obtain ratio values corresponding to known Na$^+$ concentrations. The apparent K$_J$ values for fura-2 are estimated by measuring fluorescence ratios obtained from a solution containing fura-2 and known concentrations of free Ca$^{2+}$ (Fura-2 Calcium Imaging Calibration Kit, Molecular Probes) trapped between 2 coverslips spaced by pieces of coverslips, and imaged with the water immersion objective. A corresponding approach was used to estimate the K$_J$ of SBFI with calibration solution containing 1 mmol/L EGTA, 0.1 mmol/L CaCl$_2$, 2 mmol/L MgCl$_2$, 10 mmol/L HEPES, 0 to 145 mmol/L NaCl, and 145 to 0 mmol/L potassium gluconate (K$^+$) + [Na$^+$] = 145 mmol/L, pH 7.4). This in vitro apparent K$_J$ was consistent with the K$_J$ estimated from perforated cells.
Data Analysis and Drug Applications

Values given in the text and in the figures are mean±SEM of changes in the respective cell populations. Student’s t test (for paired and unpaired observations) was used to compare the mean values. The characteristics of action potentials and current-voltage curves in different experimental conditions were studied with the use of a fast strip-chart recorder and a digital system (Nicolet System 400: Benchtop Waveform Acquisition System; Sekonic). Drugs were applied by dissolving them to the desired final concentration in the saline and by switching the perfusion from control saline to drug-containing saline. 6-Cyano-7-nitroquinoline-2,3-dione (CNQX) was from Tocris. D-2-amino-5-phosphonovalerate (D-APV), BAPTA, tetrodotoxin (TTX), and phenytoin were from Sigma. Gibenclamide, nifedpine, riluzole, saxitoxin, and tolbutamide were from RBI. Lamotrigine and gabapentin were from Glaxo-Wellcome and Parke-Davis, respectively.

Results

Physiological and Morphological Properties of the Recorded Neurons

The neurons included in the present study (n=213) were striatal spiny neurons. These cells were identified by means of an electrophysiological analysis. In 72 cells the electrophysiological characterization was confirmed by a morphological analysis obtained by biocytin staining (data not shown). Striatal spiny neurons had a small soma (10 to 18 μm) and an extensive dendritic tree studded densely with spines. These cells had high resting membrane potentials (–84±5 mV), relatively low apparent input resistances (spines. These cells had high resting membrane potentials (38±4 mV), relatively low apparent input resistances (85 mV), and a decrease of the input resistance (Figure 1A, trace b). This effect persisted also when the membrane was manually clamped to the resting potential (data not shown). The amplitude of the ischemia-induced membrane depolarization in striatal spiny neurons was –5 mV and was constant throughout the experiment. The reversal potential obtained in these experiments was not significantly different from the 1 measured with potassium chloride–filled electrodes.

Figure 1. Ischemia induces a membrane depolarization/inward current in striatal spiny neurons. A, Depolarizing pulse induced tonic firing discharge in a striatal spiny neuron (resting membrane potential, –85 mV). The lower trace indicates the current

Effects of Ischemia in Current-Clamp and Voltage-Clamp Experiments

As shown in Figure 1A (trace b), glucose and oxygen deprivation induced a membrane depolarization in striatal spiny neurons. The amplitude of the ischemia-induced membrane depolarization was time-dependent. When the slice was exposed to ischemia for relatively short periods (<6 minutes in most of the cells, 30 of 33), the membrane depolarization was fully reversible. Repetitive, brief periods (2 minutes) of ischemia (a 5- to 10-minute interval between each application) caused reproducible membrane potential changes. The ischemia-induced membrane depolarization was coupled with a decrease of the input resistance (Figure 1A, trace b). This effect persisted also when the membrane was manually clamped to the resting potential (data not shown). The amplitude of the ischemic depolarization was time-dependent (2 minutes: 18±3 mV, n=22; 4 minutes: 45±4 mV, n=25; 6 minutes: 80±2 mV, n=12). Exposure to ischemia for 10 minutes caused an irreversible membrane depolarization in all the cells tested (15 of 15; data not shown).

The effect of ischemia on striatal spiny neurons was also investigated with the single-microelectrode voltage-clamp technique. In voltage-clamp recordings, glucose and oxygen deprivation induced an inward current that was coupled with an increase in membrane conductance as detected by the application of constant hyperpolarizing voltage steps (1 to 3 seconds’ duration, 5 to 15 mV amplitude; Figure 1B). These events had a time course similar to that observed in current-clamp experiments. As shown in Figure 1C, the extrapolated reversal potential for the ischemia-induced inward current was –40±4 mV (n=7) with potassium chloride–filled electrodes. This value was obtained in voltage-clamp experiments. The cells were clamped at –80 mV, and voltage steps (1 to 3 seconds’ duration) were applied every 10 mV in depolarizing and hyperpolarizing directions (usually from –110 mV to –30 mV) under control and ischemic conditions. In 4 experiments we measured the reversal potential of this current by using potassium acetate–filled electrodes to investigate the possible contribution of intracellular chloride. The reversal potential obtained in these experiments was not significantly different from the 1 measured with potassium chloride–filled electrodes (–39±4 mV).

Effects of Ischemia on [Na\(^{+}\)]\(_{i}\) and [Ca\(^{2+}\)]\(_{i}\)

In some experiments (n=7) the cells were injected with fura-2, and electrical recordings were combined with mea-
Measurements of $[Ca^{2+}]_i$. In these experiments a fixed period (4 minutes) of ischemia was applied. As shown in Figure 2A, the ischemia-induced membrane depolarization started 30 to 60 seconds after the interruption of the oxygen- and glucose-containing solution and increased progressively during the period of ischemia. After interruption of the ischemic period the membrane potential returned to the control level within 2 to 3 minutes. Figure 2B shows that the rise in $[Ca^{2+}]_i$ was slow and progressive. The peak $[Ca^{2+}]_i$ after 4 minutes of ischemia was $127 \pm 22.3$ nmol/L. In 8 other experiments, the cells were injected with SBFI for combined electrophysiological and $[Na^+]_i$ analysis. The time course of the changes in $[Na^+]_i$, was similar to the time course observed for the membrane depolarization (Figure 2C). The peak $[Na^+]_i$, after 4 minutes of ischemia, was $36.5 \pm 5$ mmol/L. It is noteworthy that on washout of the ischemic solution, $[Ca^{2+}]_i$ levels returned promptly to the basal values within 2 minutes, whereas $[Na^+]_i$ was still significantly increased after the same period of washout (Figure 2B, 2C). The return of $[Na^+]_i$, to resting values was closely related to the time course of membrane repolarization (Figure 2A, 2C). Because the amplitude and time course of the ischemia-induced membrane depolarizations measured in the experiments with the 2 dyes were similar, the data were pooled and plotted as shown in Figure 2A.

Figure 2. The ischemic depolarization is coupled with a rise of $[Ca^{2+}]_i$ and $[Na^+]_i$. The graph (A) shows the time course of the depolarization induced by 4 minutes of ischemia in several experiments ($n=15$) in which the electrophysiological recordings were coupled with microfluorometric measurements of either $[Ca^{2+}]_i$ ($n=7$; B) or $[Na^+]_i$ ($n=8$; C). See text for further details.

Figure 3. Ionic mechanisms underlying ischemia-induced membrane potential changes and lack of effect of putative neuroprotective drugs on the ischemic depolarization recorded in striatal spiny neurons. A, TTX (1 $\mu$mol/L; $n=6$), low-Ca$^{2+}$/high-Mg$^{2+}$-containing solutions ($n=5$), and intracellular BAPTA ($n=5$) failed to affect the ischemia-induced membrane potential changes observed in spiny neurons. The lowering of the extracellular sodium ($n=6$) significantly reduced the ischemia-induced membrane depolarization. B, CNQX (10 $\mu$mol/L) plus APV (50 $\mu$mol/L; $n=6$), nifedipine (10 $\mu$mol/L; $n=4$), gabapentin (300 $\mu$mol/L; $n=4$), lamotrigine (100 $\mu$mol/L; $n=5$), phenytoin (100 $\mu$mol/L; $n=5$), and riluzole (50 $\mu$mol/L; $n=5$) failed to affect the ischemia-induced membrane depolarization. C, Most of these drugs at the concentrations used were able to reduce the EPSPs mediated by the activation of glutamatergic corticostriatal fibers. Nifedipine at the concentration used did not reduce the corticostriatal EPSP.

Effects of Low-Ca$^{2+}$/High-Mg$^{2+}$ Solutions and Intracellular BAPTA on Ischemia-Induced Membrane Depolarizations

Because the ischemic depolarization was coupled with a significant increase in $[Ca^{2+}]_i$, we analyzed the effect of ischemia in a medium containing low Ca$^{2+}$ (0.5 mmol/L) and high Mg$^{2+}$ (10 mmol/L), which is known to reduce the influx of Ca$^{2+}$ into the recorded cell by decreasing the entry of Ca$^{2+}$ via voltage-activated channels and by blocking the excitatory synaptic transmission. Incubation of the slices in low-Ca$^{2+}$/high-Mg$^{2+}$ containing solutions ($n=5$) failed to reduce the ischemia-induced membrane depolarization ($n=5$, $P>0.05$; Figure 3A). Moreover, the dependence of the ischemia-induced membrane depolarization/inward current on $[Ca^{2+}]_i$ was studied with microelectrodes filled with the Ca$^{2+}$-chelating agent BAPTA (200 mmol/L). In BAPTA-injected spiny neurons, the ischemic depolarization was not affected ($n=5$, $P>0.05$; Figure 3A). Because spiny neurons do not possess a prominent Ca$^{2+}$-activated afterhyperpolarization, the effectiveness of the BAPTA treatment was confirmed by the finding that the...
tetanus-induced long-term depression was blocked in these cells.20

Effects of TTX and Low-Na\(^{+}\)-Containing Solutions on Ischemia-Induced Depolarization

Because TTX blocks voltage-activated Na\(^{+}\) channels and the synaptic transmission mediated by action potential discharge, we measured the effects of ischemia on the membrane potential of spiny neurons before and after the incubation of the slice with this toxin. In the presence of 1 \(\mu\)mol/L TTX, corticostriatal synaptic transmission was completely abolished, but this Na\(^{+}\)-channel blocker did not affect the membrane depolarization/inward current described in spiny cells under ischemic conditions (85 mV in a and b; \(P<0.05\); Figure 3A). We also found that 0.3 to 1 \(\mu\)mol/L saxitoxin, an extremely potent Na\(^{+}\)-channel blocker in nanomolar concentrations,25 did not alter the ischemic depolarization (n=3; data not shown). The possible involvement of a TTX-resistant Na\(^{+}\) influx in the ischemic depolarization/inward current was studied in low-Na\(^{+}\)-containing solutions (38 mmol/L; see Materials and Methods). The lowering of the extracellular Na\(^{+}\) significantly reduced the ischemia-induced membrane depolarization and inward current (n=6; \(P<0.01\); Figure 3A).

Antagonists of Glutamate Receptors and Neuroprotective Drugs on Ischemic Depolarization and Corticostriatal Transmission

During ischemia the release of excitatory amino acids may also occur via a “nonsynaptic” mechanism that is Ca\(^{2+}\)-independent and insensitive to TTX.26 Thus, we also tested whether the direct blockade of postsynaptic glutamate receptors could affect ischemia-induced membrane depolarization. We incubated (10 minutes before the onset of ischemia) the slices in 10 mmol/L CNQX, an antagonist of AMPA glutamate receptors, plus 50 \(\mu\)mol/L APV, an antagonist of NMDA glutamate receptors. Even under this experimental condition, ischemia caused membrane depolarizations whose amplitude and duration were similar to those observed in control medium (n=6; \(P>0.05\); Figure 3B). These concentrations of glutamate antagonists fully blocked excitatory postsynaptic potentials (EPSPs) evoked by the activation of glutamatergic corticostriatal fibers in brain slice preparations (Figure 3C).11,20

Blockers of high voltage-activated Ca\(^{2+}\) channels have been reported to reduce the neuronal vulnerability to ischemia20 and the ischemic depolarization in cortical neurons.2 Thus, we also studied the effect of preincubation of the slices in 10 mmol/L nifedipine on the ischemic depolarization. This L-type Ca\(^{2+}\)-channel blocker was not able to affect the membrane depolarization/inward current observed under ischemic conditions (n=4; \(P>0.05\); Figure 3B) or the corticostriatal EPSP (Figure 3C). It has recently been proposed that antiepileptic drugs may protect central neurons during ischemic/hypoxic insults because it has been shown that these drugs inhibit voltage-dependent Na\(^{+}\) and Ca\(^{2+}\) channels.3,28–32 For this reason, we tested the effects of the following antiepileptic drugs on ischemia-induced membrane depolarization: gabapentin (300 \(\mu\)mol/L; n=4), lamotrigine (100 \(\mu\)mol/L; n=5), and phenytoin (100 \(\mu\)mol/L; n=5). None of these drugs reduced ischemic depolarization (\(P>0.05\); Figure 3B), but they reduced the corticostriatal EPSPs (Figure 3C). We also tested the effect of riluzole, a drug that has been shown to block voltage-dependent Na\(^{+}\) channels and to act as a neuroprotective agent in neurodegenerative disorders and in some experimental models of in vivo and in vitro ischemia,33–36 on ischemic depolarization. Riluzole (50 \(\mu\)mol/L; n=5) reduced the EPSP amplitude (Figure 3C) and failed to affect the depolarization caused by ischemia (Figure 3B).

Effects of ATP-Dependent K\(^{+}\) Channel Blockers on Ischemic Depolarization

To investigate the possible involvement of ATP-dependent K\(^{+}\) conductances in the membrane potential changes caused by energy failure in spiny neurons, we studied the effects of ischemia after the preincubation of the slices either in tolbutamide or in glibenclamide, blockers of ATP-dependent K\(^{+}\) channels.5,57 Preincubation in tolbutamide significantly increased the membrane depolarization/inward current after ischemic exposure (Figure 4A, 4C, traces a and b). As shown in Figure 4A, this effect was dose-dependent (100 \(\mu\)mol/L, n=5, \(P<0.05\); 300 \(\mu\)mol/L, n=5, \(P<0.01\); 1000 \(\mu\)mol/L, n=5, \(P<0.001\)). Similar findings were obtained with glibenclamide, which also enhanced ischemic depolarization in a dose-dependent manner (30 \(\mu\)mol/L, n=4, \(P<0.05\); 100 \(\mu\)mol/L, n=4, \(P<0.01\); 300 \(\mu\)mol/L, n=4, \(P<0.001\); Figure 4B, 4C, traces c and d). The resting membrane potential and the input resistance of the cells were not affected by tolbutamide (n=18; \(P>0.05\)) or glibenclamide (n=15; \(P>0.05\)) at the concentrations used in the aforemen-
tions. Moreover, the effect of ATP-dependent K⁺-channel blockers was not coupled to significant changes of the I-V relationship measured in voltage-clamp experiments. In these experiments, the cells were clamped at –85 mV, and voltage-steps (0.5 to 3 seconds’ duration) were applied every 10 mV in depolarizing and hyperpolarizing directions (usually from –115 to –55 mV) under control conditions and during the drug administration (n=4, Figure 5A and n=4, Figure 5B, respectively).

**Figure 5.** Tolbutamide and glibenclamide do not alter the current-voltage relationship of striatal spiny neurons under control conditions. A and B. Plots show the lack of effects of tolbutamide and glibenclamide, respectively, on the current-voltage relationship of 2 striatal spiny cells. The plots were obtained from voltage-clamp experiments by holding the cells at –85 mV and applying positive and negative voltage steps of progressively increasing amplitudes (duration: 0.5 to 3 seconds).

**Discussion**

The present study shows that in vitro ischemia produced an early membrane depolarization in striatal spiny neurons. The amplitude of this membrane depolarization was time-dependent. The time course of the ischemic depolarization is much faster than that observed for aglycemia and hypoxia, suggesting that even brief periods of energy deprivation (aglycemia) may dramatically enhance the depolarizing effect of hypoxia. Voltage-clamp experiments showed that this event was due to an inward current that was coupled with an increased conductance. Moreover, in this study we measured the rise in [Na⁺], and [Ca²⁺], during ischemic depolarization using microfluorometric analysis from single neurons coupled with electrophysiological recordings. This analysis has shown that ischemic depolarization was associated with a significant increase in [Na⁺], and [Ca²⁺]. It should be noted that the time course of [Na⁺], and [Ca²⁺], elevation was not dissimilar. However, the recovery appeared significantly slower for [Na⁺], when compared with [Ca²⁺]. In addition, although the [Ca²⁺], elevation reached at 4 minutes of ischemia was in the range expected during physiological neuronal activity, it seems unlikely that the concomitant rise in somatic [Na⁺], could be produced by physiological spiking activity. Indeed, we failed to induce [Na⁺], elevations comparable to those induced by 4 minutes of ischemia even by driving the cell to maximal action potential firing for periods of tenths of a second (data not shown).

Moreover, neither the lowering of Ca²⁺ in the external medium nor the intracellular injection of BAPTA, a Ca²⁺-chelating agent affected the ischemic membrane depolarization. These observations suggest that the rise in [Ca²⁺], might have an important function in ischemia-induced “delayed neuronal death” but not in the ischemic depolarization observed in striatal spiny neurons. We have recently reported that nifedipine, an L-type Ca²⁺-channel blocker, significantly decreased the membrane depolarization and the rise in [Ca²⁺], measured in cortical neurons during ischemia. This drug failed to alter the ischemic depolarization recorded in striatal spiny neurons, indicating that the mechanisms underlying the ischemic depolarization are cell-type specific. On the other hand, we observed that the lowering of Na⁺ in the external medium produced a significant reduction of ischemic depolarization, suggesting that Na⁺ influx makes a major contribution to this early electrophysiological event. Blockade of voltage-gated Na⁺ channels either by TTX or by saxitoxin did not mimic the effect of low Na⁺. The latter finding indicates that in spiny neurons the rise in [Na⁺], induced by ischemia was not due to toxin-sensitive non-inactivating Na⁺ channels. Accordingly, pretreatment with various putative neuroprotective agents such as phenytoin, lamotrigine, gabapentin, and riluzole, which have been shown to act as blockers of Na⁺ channels, significantly decreased synaptic transmission but failed to reduce ischemic depolarization.

The positive effect of low extracellular Na⁺ and the lack of effect of TTX, low extracellular Ca²⁺, and glutamate receptor antagonists on membrane depolarization in striatal spiny neurons are consistent with previous observations in other neuronal cell types during anoxia. The experiments with TTX and low external Ca²⁺ seem to rule out the involvement of a “classic synaptic” release of excitatory transmitters in the ischemic depolarization. Nevertheless, a “non-synaptic” release of glutamate that is insensitive to TTX and Ca²⁺-independent has been described during ischemia. Such a release might contribute to the ischemic depolarization. However, the lack of effect of ionotropic glutamate receptor antagonists on this electrophysiological effect makes this possibility unlikely.

On the basis of the extrapolated I-V curve, the reversal potential for the ischemia-induced responses in spiny neurons is approximately –40 mV (KCl electrodes). This value is very close to the reversal potential for aglycemia and hypoxia in these cells and suggests that common ionic mechanisms underlie these events. Accordingly, a nonselective cation conductance with the contribution of Na⁺ and K⁺ ions might be responsible for the ischemia-induced electrical changes recorded in striatal spiny neurons and for those induced by aglycemia. It is also possible that a critical contribution to the
rise of [Na\(^+\)], during oxygen and glucose deprivation is made by an impaired activity of Na\(^+\),K\(^-\)-ATPase. In fact, this electrogenic pump extrudes 3 Na\(^+\) ions for 2 K\(^+\) ions and for this activity requires an adequate ATP supply whose production is defective during ischemia.

A further piece of evidence in favor of a K\(^+\) conductance taking part in this electrophysiological phenomenon is represented by the effects of tolbutamide and glibenclamide. These blockers of ATP-dependent K\(^+\) channels\(^6,37\) significantly increase the membrane depolarization/inward current after ischemic exposure. The I-V relationship of the recorded cells was not altered by the concentrations of blockers used in the present experiments. Thus, we can argue that the facilitatory effects of tolbutamide and glibenclamide on the ischemic depolarization are related to the blockade of ATP-dependent K\(^+\) channels rather than to an aspecific increase of the neuronal input resistance. The ATP-dependent K\(^+\) channels are activated by the depletion of ATP that occurs during energy failure. During the ischemic insult, these conductances allow an efflux of K\(^+\) ions that counterbalance the main driving force of Na\(^+\) ions that by entering the cell cause the recorded membrane depolarization. Thus, the blockade of ATP-dependent K\(^+\) conductances would enhance the ischemic depolarization in striatal spiny neurons. It should be noted, however, that high concentrations of tolbutamide (100 to 1000 \text{mol/L}) and glibenclamide (30 to 300 \text{mol/L}) are required to achieve this effect. In peripheral tissue (i.e., pancreas, heart, and vascular smooth muscle) glibenclamide in the low micromolar range is generally adequate to inhibit ATP-dependent K\(^+\) channels. Both high- and low-affinity binding sites for glibenclamide have been described in the rat brain.\(^41\) Compared with peripheral tissue, relatively high concentrations of glibenclamide and tolbutamide have been used by others to study oxygen-deprived neurons.\(^42,43\) Thus, the ATP-dependent K\(^+\) channel in neurons is presumably different from that in peripheral tissue. Nevertheless, the effect of glibenclamide on rubidium efflux and on extracellular K\(^+\) activity is maximum at 10 to 20 \text{mol/L}.\(^42,44\) The present observations that augmentation of membrane depolarization does not start until 30 \text{mol/L} glibenclamide raises the possibility of a nonspecific effect on other K\(^+\) channels.

Future studies are in progress in our laboratory to further characterize the ionic changes underlying the ischemic depolarization in striatal spiny neurons and in other neuronal subtypes within this structure. These studies might help to understand the mechanisms responsible for cell-type–specific vulnerability to ischemia in the striatum.

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References

Ionic Mechanisms of Ischemia


**Editorial Comment**

Complete cerebral ischemia causes a complex sequence of electrophysiological changes that are not fully understood. Within the first few minutes of ischemia, neuronal ionic membrane conductance increases, extracellular potassium activity increases slightly, membrane excitability decreases, and spontaneous electroencephalographic activity disappears. Activation of calcium-sensitive K+ channels has been postulated to contribute to these early electrophysiological changes. If oxygenation is not rapidly restored, ATP is soon depleted to a level approaching the rate of ATP depletion may be slower and the sequence of depolarization-time trajectory is required.

Another finding of the present study is that nifedipine failed to attenuate the depolarization amplitude in striatal spiny neurons. This result is in contrast to previous work from this laboratory in which L-type Ca2+ channel blockers reduced the increase in intracellular Ca2+ and the magnitude of anoxic depolarization in cortical pyramidal neurons. Together, these results emphasize the diversity of involvement of different ionic channels in anoxic depolarization among different cell types.

A third finding is that ATP-sensitive K+ (KATP)-channel antagonists augment anoxic depolarization. These results imply that opening of KATP channels during ischemia limits the magnitude of depolarization. However, some caution should be exercised because rather high concentrations of glibenclamide and tolbutamide were required, thereby raising the concern of nonspecificity. Nevertheless, others have shown that high-dose glibenclamide limits the maximum increase in extracellular K+ in anoxic cortex in vivo.

Last, there are some potential limitations in applying the results from the brain slice preparation to the in vivo situation. First, spontaneous electrical activity is suppressed in brain slices. Thus, some of the changes in ionic conductance normally associated with the loss of electroencephalographic activity before anoxic depolarization may be absent in the slice preparation. Moreover, because of the lower metabolic rate associated with a loss of electrical activity, the rate of ATP depletion may be slower and the sequence of ionic channel opening may be altered. Second, the bathing...
solution surrounding the slice provides an additional source of Na\(^+\), Ca\(^{2+}\), and water and an additional sink for K\(^+\) channels that are not normally present in vivo. Third, harvesting tissue for the brain slice preparation in itself produces transient ischemia. Because a change in phospholipids and ATP may alter the characteristics of ionic channels,\(^6,8\) their functioning during subsequent oxygen-glucose deprivation may be modified. Thus, some caution should be taken in interpreting the lack of effect of Na\(^+\)-channel blockers in the present study.

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References


Sodium Influx Plays a Major Role in the Membrane Depolarization Induced by Oxygen and Glucose Deprivation in Rat Striatal Spiny Neurons
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