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INCREASE OF DELAYED RECTIFIER POTASSIUM CURRENTS IN LARGE ASPINY NEURONS IN THE NEOSTRIATUM FOLLOWING TRANSIENT FOREBRAIN ISCHEMIA

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Abstract —Large aspiny (LA) neurons in the neostriatum are resistant to cerebral ischemia whereas spiny neurons are highly vulnerable to the same insult. Excitotoxicity has been implicated as the major cause of neuronal damage after ischemia. Voltage-dependent potassium currents play important roles in controlling neuronal excitability and therefore influence the ischemic outcome. To reveal the ionic mechanisms underlying the ischemia-resistance, the delayed rectifier potassium currents (I_k) in LA neurons were studied before and at different intervals after transient forebrain ischemia using brain slices and acute dissociation preparations. The current density of I_k increased significantly 24 h after ischemia and returned to control levels 72 h following reperfusion. Among currents contributing to I_k, the margatoxin-sensitive currents increased 24 h after ischemia while the KCNQ/M current remained unchanged after ischemia. Activation of protein kinase A (PKA) down-regulated I_k in both control and ischemic LA neurons, whereas inhibition of PKA only up-regulated I_k and margatoxin-sensitive currents 72 h after ischemia, indicating an active PKA regulation on I_k at this time. Protein tyrosine kinases had a tonic inhibition on I_k to a similar extent before and after ischemia. Compared with that of control neurons, the spike width was significantly shortened 24 h after ischemia due to facilitated repolarization, which could be reversed by blocking margatoxin-sensitive currents. The increase of I_k in LA neurons might be one of the protective mechanisms against ischemic insult.

Key words: striatum, cerebral ischemia, neuronal death, interneuron, membrane excitability, potassium channel.

The neostriatum is one of the highly vulnerable regions in the brain to cerebral ischemia. Unlike small- to medium-sized spiny neurons, which die 24 h after 25–30 min of transient forebrain ischemia (Pulsinelli et al., 1982), large aspiny (LA) neurons in the striatum are resistant to ischemic insults (Francis and Pulsinelli, 1982; Chesselet et al., 1990). The mechanisms of this selective neuronal damage after cerebral ischemia are still unclear. Excitotoxicity has been widely accepted as one of the major causes of neuronal death after ischemia (Rothman and Olney, 1986; Choi and Rothman, 1990). Recent studies have shown that, after transient forebrain ischemia, the evoked fast excitatory postsynaptic currents in LA neurons were suppressed, suggesting that the depression of excitatory neurotransmission might be involved in post-ischemic neuroprotection (Pang et al., 2002). Decrease in membrane excitability is also believed to be neuroprotective because the membrane potentials of spiny neurons were depolarized during *in vitro* hypoxia/hypoglycemia, whereas that of LA neurons was hyperpolarized (Calabresi et al., 1997; Pisani et al., 1999; Centonze et al., 2001). While the activation of voltage-dependent Na^+ channel and Ca^{2+} entry contribute to the generation of the ischemia-induced membrane depolarization in spiny neurons, the activation of ATP- and Ca^{2+}-dependent potassium channel is responsible for the hyperpolarization in LA neurons (Centonze et al., 2001).

Voltage-dependent potassium (Kv) channels play important roles in the maintenance of neuronal excitability by regulating resting membrane potential, interspike membrane potential and spike frequency (Rudy, 1988). In particular, delayed rectifier potassium currents (I_k) are critical for controlling the action potential repolarization and spike duration (Rudy, 1988; Storm, 1990). It has been demonstrated that potassium channel activity in neurons is changed during hypoxia (Jiang and Haddad, 1994; Jiang et al., 1994; Gebhardt and Heinemann, 1999) or after ischemia (Chi and Xu, 2000, 2001). Evidence from CA1 pyramidal neurons in the hippocampus suggests that the up-regulation of potassium conductance might contribute to the decrease of neuronal excitability following ischemic insults (Chi and Xu, 2000). The post-ischemic changes of potassium currents in striatal neurons remain to be elucidated. LA neurons possess both A-type potassium currents and I_k (Song et al., 1998; Tkatch et al., 2000). Since I_k regulates Ca^{2+} influx during action potential (Du et al., 2000), alteration of I_k after ischemia might affect the intracellular Ca^{2+} concentration and the pathological process associated with intracellular Ca^{2+} (Choi, 1995). To reveal the temporal changes of I_k in LA neurons after transient forebrain ischemia and the possible mechanisms underlying such changes, whole-cell voltage-clamp recordings were performed on acutely dissociated neurons and brain slices. Dissociated neurons are generally the first choice for voltage-clamp recording because most of the dendrites have been removed and the space clamp errors are minimal. However, the mechanical and enzymatic procedures during dissociation might have adverse affects on channel

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Abbreviations: ACSF, artificial cerebrospinal fluid; H-89, *N*-[2-((*p*-bromocinnamyl)amino)ethyl]-5-isoquinolinesulfonamide; I_k, delayed rectifier potassium current; ISI, interspike interval; LA, large aspiny; MgTX, margatoxin; OGD, oxygen/glucose deprivation; TEA, tetraethylammonium; TTX, tetrodotoxin; 4-AP, 4-aminopyridine; 8-Br-cAMP, 8-bromoadenosine 3′,5′-cyclic monophosphoate.

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properties. These traumatic effects could be more severe in neurons after ischemia. On the other hand, neurons in the brain slice preparation are preserved relatively better than the dissociated neurons. But the extended dendrites of neurons in the slice compromise the voltage clamping of the cell and might result in a space clamp error. Therefore, both techniques were used in the present study to validate the results.

EXPERIMENTAL PROCEDURES

Male Wistar rats (100–180 g; Charles River Laboratories, Wilmington, MA, USA) were used in the present study. Experimental protocols were approved by the Institutional Animal Care and Use Committee (IACUC) of Indiana University School of Medicine in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. All efforts were made to minimize the suffering and the number of animals used.

Transient forebrain ischemia

Transient forebrain ischemia was induced using the four-vessel occlusion method (Pulsinelli and Brierley, 1979) with modifications (Ren et al., 1997). Briefly, the animals were fasted overnight to provide uniform blood glucose levels. For surgical preparation, the animals were anesthetized with a mixture of 1–2% halothane, 33% O_2 and 66% N_2 via a gas mask placed around the nose. The common carotid arteries were isolated after which a silicon-tube loop was placed loosely around each common carotid artery to allow subsequent occlusion of these vessels. The animal was then placed on a stereotaxic frame, and the vertebral arteries were electrocauterized. A very small temperature probe (0.025-inch diameter; Physitemp, Clifton, NJ, USA) was inserted beneath the skull in the extradural space, and the brain temperature was maintained at 37 °C with a heating lamp using a temperature control system (BAT-10; Physitemp). Glass microelectrodes (5–8 μm in diameter of tip) filled with 2 M NaCl were used to record ischemic depolarization, which is an indication of complete ischemia (Ren et al., 1997). A burr hole was drilled at 9.5 mm anterior to the interaural line, 3.0 mm from the midline. The microelectrode was advanced 3.0 mm below dura into the neostriatum. The recordings were performed with a neuroprobe amplifier (Model 1600; A-M Systems, Carlsborg, WA, USA). The duration of ischemic depolarization was determined by measuring the period from the beginning of the extracellular direct current potential reaching −20 mV to the point where the potential started to repolarize after recirculation. Transient forebrain ischemia was produced by occluding both common carotid arteries to induce ischemic depolarization for approximately 22 min. Cerebral blood flow resumed immediately upon release of the carotid artery clasps. Animals were returned to the cages after recovering from ischemia and allowed free access to water and food.

Brain slice and acute dissociation preparation

Brain slices were prepared from animals before ischemia and at 6 h, 24 h, 48 h and 72 h after reperfusion using procedures similar to those previously described (Pang et al., 2002). Briefly, the animals were anesthetized with ketamine–HCl (80 mg/kg, i.p.) and decapitated. The brains were quickly removed and immersed in ice-cold artificial cerebrospinal fluid (ACSF), which was composed of the following (in mM): 130 NaCl, 3 KCl, 2 $CaCl_2$, 2 $MgCl_2$, 1.25 NaH_2PO_4, 26 $NaHCO_3$, and 10 glucose, pH 7.4, 295–305 mOsm/L. Transverse striatal slices of 280–300 μm thickness were cut using a vibratome (VT 1000; Leica, Nussloch, Germany) and incubated in ACSF for ≥1 h at room temperature (approximately 24 °C) before being transferred to the recording chamber. The slice was submerged beneath the fluid surface and superfused continuously with oxygenated ACSF. The flow rate was adjusted to 2–3 ml/min. Unless otherwise stated, recordings were carried out at room temperature.

Acutely dissociated neurons were prepared from rats before ischemia and at 24 h and 72 h after ischemia using procedures similar to those previously reported (Yan and Surmeier, 1997). In brief, rats were anesthetized and decapitated. The brains were quickly removed and immersed in an ice-cold low Ca^{2+} solution containing (in mM): 140 Na isethionate, 2 KCl, 4 $MgCl_2$, 0.1 $CaCl_2$, 23 glucose, 15 HEPES, pH 7.4, 300–305 mOsm/L. The brain tissue containing neostriatum was cut in 400 μm slices while bathed in the low Ca^{2+} solution. Slices were incubated at room temperature in oxygenated ACSF. Then, the slices were transferred into the low Ca^{2+} solution and regions of striatum were dissected and placed in an oxygenated Hanks' balanced salt solution containing 1 to approximately 3 mg/ml protease. After approximately 30 min of enzyme digestion at 35 °C, tissue was rinsed three times in the low Ca^{2+} buffer and mechanically dissociated with a graded series of fire-polished Pasteur pipettes. The cell suspension was plated onto a 12 mm coverslip (Fisherbrand Coverglass, Pittsburgh, PA, USA), which was then placed in the recording chamber.

Whole-cell patch-clamp recording

Recording electrodes were prepared from borosilicate glass (Warner Instruments, Hamden, CT, USA) using a horizontal electrode puller (P-97; Sutter Instruments, Novato, CA, USA) to produce tip openings of 1 to approximately 2 μm (3–5 MΩ). Electrodes were filled with an intracellular solution containing (in mM): 145 KCl, 1 $MgCl_2$, 10 EGTA, 0.2 $CaCl_2$, 10 HEPES and 2% neurobiotin (Vector Laboratories, Burlingame, CA, USA), pH 7.4, 290–295 mOsm/l. Neurons were visualized with an infrared-differential interference contrast microscope (BX50WI; Olympus Optical, Tokyo, Japan) and a CCD camera. Only those cells with large somata (>20 μm in diameter) were selected for recording. Whole-cell patch-clamp recordings were performed with an Axopatch 200B amplifier (Axon Instruments, Foster City, CA, USA). After tight-seal (>1 GΩ) formation, the electrode capacitance was compensated. Immediately after establishment of whole-cell configuration, the resting membrane potential was obtained by direct reading from the amplifier. The membrane capacitance, series resistance and input resistance of the recorded neurons were measured by applying a 5 mV (10 ms) hyperpolarizing voltage pulse from a holding potential of −60 mV. The series resistance was 8–12 MΩ. Neurons with a series resistance >10% of the input resistance were discarded. The membrane capacitance reading was used as the value for whole cell capacitance. For all measurements, capacitance and series resistance compensation (60–80%) were used to minimize voltage errors. During the experiment, the membrane capacitance and series resistance were periodically monitored. Neurons with a series resistance change >20% during the experiment were excluded from the analysis. Signals were filtered at 2 kHz and digitized at a sampling rate of 5 kHz using a data-acquisition program (Axograph 4.6; Axon Instruments). To isolate the voltage-dependent outward potassium currents, tetrodotoxin (TTX; 1 μM) and $CdCl_2$ (300 μM) were added in the perfusate to block voltage-activated Na^+ and Ca^{2+} currents, as well as Ca^{2+}-activated potassium currents. In some experiments, 4-aminopyridine (4-AP, 1 mM) and tetraethylammonium (TEA, 20 mM) were applied to examine the pharmacological characteristics of I_k. The chemical agents were obtained from Sigma (St. Louis, MO, USA).

The current density of I_k for each neuron was obtained by dividing the total current by the membrane capacitance. The current amplitude of I_k was measured as an average amplitude at 340–390 ms after the onset of the command voltage pulses. The steady-state activation or inactivation curves were established

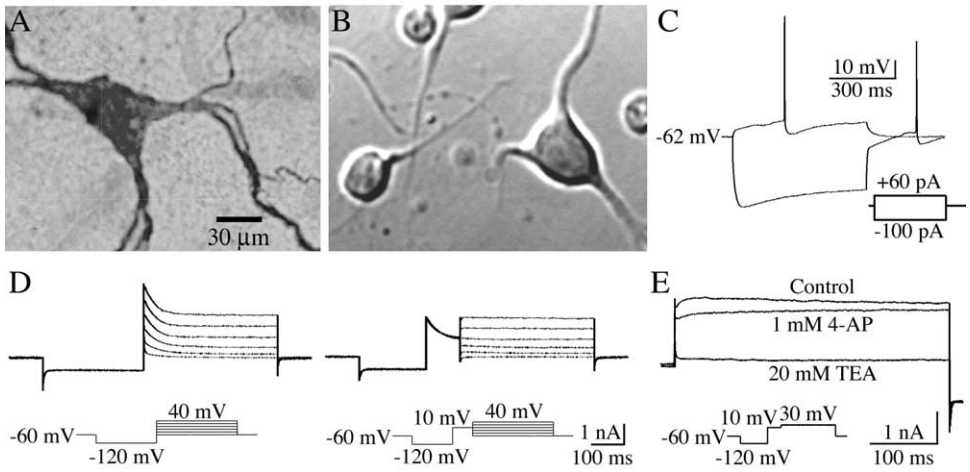

Fig. 1. I_k in LA neurons. (A) Photomicrograph of an intracellularly stained LA neuron in brain slice. This neuron has large somata with three primary dendrites bearing no spines. (B) Photomicrograph of acutely dissociated neurons from the neostriatum. The dissociated neurons have a few short dendrites. Among neurons with smaller somata (probably medium spiny neurons), the LA neuron is easily identified due to its large soma. (C) Representative traces showing the responses of LA neurons in brain slice to intracellular current injection. Injection of a negative current produced prominent sag following the initial hyperpolarization. Depolarizing current pulse induced regular firing followed by large-amplitude and long-duration afterhyperpolarization. (D) Representative traces of whole-cell potassium currents evoked in LA neuron in brain slice by a series of depolarizing steps (left panel). To isolate I_k, the A-type current was inactivated by a 100 ms prepulse at +10 mV following a conditioning voltage step (right panel). (E) Effects of potassium channel blockers on I_k. Bath application of 1 mM 4-AP partially reduced the amplitude of I_k. I_k was almost completely blocked by 20 mM TEA.

similarly to those previously reported (Chi and Xu, 2000). Briefly, the conductance (G) was calculated using the following equation: $G=I/(V_m-V_k)$, where I was the current amplitude, V_m was the command potential and V_k was the reversal potential of potassium ($V_k=-98$ mV). The conductance was then normalized with respect to the maximum value and plotted as a function of the Boltzmann distribution: $G/G_{max}=1/[1+\exp(V_m-V_{1/2})/V_c]$, where G_{max} was the maximum conductance at +70 mV, $V_{1/2}$ was the membrane voltage at which the current amplitude was half-maximum and V_c was the slope factor at $V_{1/2}$. The inactivation curves were fitted with a normalized Boltzmann distribution: $I/I_{max}=1/[1+\exp(V_{1/2}-V_m)/V_c$, where I_{max} was the maximum current at +70 mV.

In current-clamp recording, fast I-clamp mode was used, which is comparable to that obtained with conventional current-clamp amplifiers (Bennett et al., 2000). Depolarizing and hyperpolarizing current pulses were applied for characterizing the electrophysiological responses of LA neurons. The spike widths were measured at half-amplitude on the first action potentials evoked by threshold current pulses (600 ms). The interspike interval (ISI) was calculated from peak to peak of spikes evoked with two times the threshold current.

Neurobiotin was iontrophoresed into the neuron by passing depolarizing current pulses after successful recording on brain slices. The slice was then fixed overnight with 4% paraformaldehyde at 4 °C and incubated in 0.1% horseradish peroxidase-conjugated avidin D (Vector Laboratories) in 0.01 M potassium phosphate buffer saline (pH 7.4) with 0.5% Triton X-100 for 24 h at room temperature. After the detection of peroxidase activity with 3,3′-diaminobenzidine as chromogen, the sections were examined under a light microscope. Sections containing the labeled neurons were mounted on gelatin-coated slides for histological process.

Drug application

N-[2-((*p*-bromocinnamyl)amino)ethyl]-5-isoquinolinesulfonamide (H-89), 8-bromo-adenosine 3′,5′-cyclic monophosphoate (8-Br-cAMP), genistein, sodium orthovanadate, margatoxin (MgTX) and linopirdine were purchased from Sigma. Drugs were made up as concentrated stocks and stored at −20 °C. Experimental solutions were prepared immediately before use. The drugs were applied via bath superfusion.

Data analysis

The values were presented as means±S.E.M. Analysis of variance followed by post hoc Scheffe's test was used for statistical analysis (StatView 5.0; Abacus Concepts, Berkeley, CA, USA). Changes were considered significant if $P<0.05$.

RESULTS

LA neurons were easily identified based on their large somata (>20 μm in diameter) in brain slices and after dissociation (Fig. 1A, B). Intracellular staining with neurobiotin demonstrated that these neurons had large somata (337.1±19.6 μm^2 in somatic area, $n=22$) with three to five extended primary dendrites bearing few spines. Moreover, injection of a negative current pulse produced a prominent sag following the initial hyperpolarization (Fig. 1C), indicating the presence of a hyperpolarization-activated cation current. Depolarizing pulses (30–100 pA) induced repetitive spiking followed by large-amplitude and long-duration afterhyperpolarization (Fig. 1C). These morphological and electrophysiological features are characteristic of LA neurons (Wilson et al., 1990; Kawaguchi, 1993). Transient forebrain ischemia that produces approximately 22 min ischemic depolarization consistently caused >90% of cell death in the dorsal neostriatum (Ren et al., 1997). At 24 h and 72 h after ischemia, it was difficult to find small- to medium-sized neurons, whereas LA neurons remained intact.

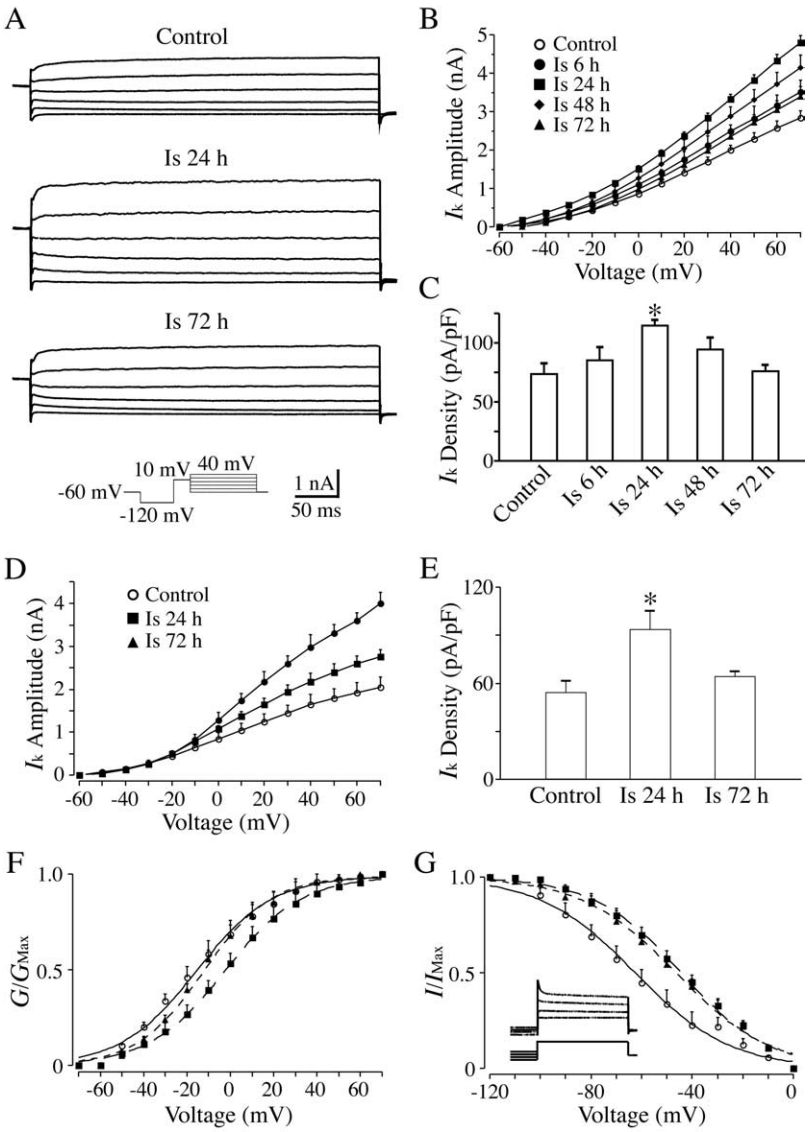

Fig. 2. Post-ischemic increase of I_k in LA neurons. (A) Representative recordings of I_k from LA neurons before and after ischemia. (B, C) Pooled data from brain slice preparation showing post-ischemic changes of I_k. (B) Plot showing the current amplitude of I_k as a function of command voltage. The amplitude increased significantly at 24 h ($P<0.01$) and returned to control levels 72 h after ischemia. (C) Histograms showing the increase of the current density of I_k evoked at +30 mV voltage step. (D, E) Pooled data from acutely dissociated neurons showing post-ischemic increase of amplitude (D) and current density (E) of I_k. The current density was obtained at +30 mV voltage step. (F) Voltage dependence of activation of I_k in acutely dissociated LA neurons. The activation curve shifted in the depolarizing direction at 24 h ($P<0.01$) and returned to control level 72 h following ischemia. (G) The steady-state inactivation of I_k in dissociated neurons. The inactivation curve shifted in depolarizing direction after ischemia ($P<0.01$). A representative recording of the inactivation of I_k is shown in the insert. The symbols for different groups in D apply to F, G. * $P<0.01$.

Post-ischemic changes of I_k in LA neurons

For whole-cell voltage-clamp recordings from LA neurons in brain slices, the membrane potentials were held at −60 mV, which are close to their resting membrane potentials (Wilson et al., 1990; Kawaguchi, 1993). In the presence of TTX and $CdCl_2$, voltage-dependent outward potassium currents were evoked by voltage steps (from −80 to +70 mV in 10 mV increments, 400 ms) following a 300 ms hyperpolarizing pulse of −120 mV. The evoked currents became detectable at about −55 mV, and the amplitude increased with more depolarizing voltage steps (Fig. 1D). Similar to previous reports (Song et al., 1998), these currents mainly consisted of rapidly inactivating A-type currents and sustained currents with little or no decay during the 400 ms voltage pulse. To isolate I_k, the A-type currents were inactivated by a 100 ms prepulse of +10 mV following a 200 ms hyperpolarizing pulse of −120 mV (Fig. 1D). Almost all of the early transient currents were removed, leaving relatively slowly activating and very slowly inactivating currents. To examine the pharmacological characteristics of I_k, 4-AP and TEA were applied via the bath solution. While 4-AP (1 mM) only partially reduced I_k,

Table 1. Voltage dependence of the activation and inactivation of *I*k[a]

	Control (n=17)	Is 24 h (n=14)	Is 72 h (n=15)
$V_{1/2}$ (Act)	−15.5±1.4	−2.4±0.8*	−12.1±0.9
V_c (Act)	17.5±1.2	17.7±0.6	15.4±0.8
$V_{1/2}$ (Inact)	−62.5±1.4	−44.4±0.9*	−46.5±1.0*
V_c (Inact)	18.8±1.2	17.3±0.8	18.6±0.9

[a] Values are mean ± S.E.M.: with number of neurons in parentheses. $V_{1/2}$: the potential (mV) of half-maximal activation (Act) or inactivation (Inact); V_c: proportional to the slope at $V_{1/2}$ (mV); * P<0.01.

TEA (20 mM) blocked most of these currents (Fig. 1E). These results indicate that most of I_k in LA neurons are TEA-sensitive and some components are 4-AP-sensitive.

To examine the post-ischemic changes of I_k in LA neurons, the current density was compared before and after ischemia. At a depolarizing step of +30 mV, the current density of I_k was 73.3±8.7 pA/pF (n=18) in control, and 111.2±4.2 pA/pF (n=14, P<0.01) and 75.9±5.5 pA/pF (n=15, P=0.59) at 24 h and 72 h after ischemia, respectively (Fig. 2A–C). Due to the extended dendrites of LA neurons in brain slices, the currents might be affected by space clamp error during whole-cell voltage-clamp recording. To verify the results obtained from brain slice preparation, potassium currents from acutely dissociated LA neurons were recorded using the same protocols as those in the brain slice preparation. Consistent with that in brain slice, the current density of I_k was significantly increased 24 h after ischemia. At +30 mV, the current density was 54.5±6.8 pA/pF (n=11) in control, and 93.8±10.9 pA/pF (n=6, P<0.01) and 64.1±3.2 pA/pF (n=7, P=0.45) at 24 h and 72 h after ischemia, respectively (Fig. 2D, E). These findings indicate that the patterns of the post-ischemic changes of I_k are about the same in brain slices and in dissociated neurons, despite the absolute values are slightly different between the preparations.

To further characterize the post-ischemic changes of I_k, the voltage dependence of these currents was investigated in dissociated LA neurons by comparing the activation and inactivation curves before and after ischemia. The activation curve shifted in depolarizing direction at 24 h after ischemia and returned to control levels at 72 h following reperfusion (Fig. 2F). The $V_{1/2}$ of the activation curve was −15.5±1.4 mV (n=17) in control and increased significantly to −2.4±0.8 mV at 24 h after ischemia (n=14, P<0.01). The V_c of the activation curve remained about the same after ischemia (Table 1). The steady-state inactivation properties of I_k were determined by measuring the current availability following 2 s prepulse steps of voltages between −120 mV and 0 mV with a testing pulse of +70 mV (Fig. 2G). While there were no significant changes in the V_c of the inactivation curve, the $V_{1/2}$ of the inactivation curve shifted significantly from −62.8±1.4 mV (n=17) in control to −44.4±0.9 mV at 24 h (n=14, P<0.01) and −46.5±1.0 mV at 72 h after ischemia (n=15, P<0.01, Table 1). These data indicate that the voltage dependence of the activation and inactivation of I_k in LA neurons were differentially altered following ischemia.

One of the possible causes of the post-ischemic increase of I_k is the enhanced expression of functional Kv channels. It has been shown that LA neurons express Kv1.1 and Kv1.2 mRNA, with low levels of Kv2.1 mRNA (Song et al., 1998; Baranauskas et al., 1999). MgTX, a potassium channel blocker that blocks Kv1.1, Kv1.2 and Kv1.3 channels in neurons (Koch et al., 1997; Kaczorowski and Garcia, 1999), was used to investigate if the post-ischemic increase of I_k was due to the up-regulation of Kv1 channels. The MgTX-sensitive currents were obtained by subtracting the currents evoked in the presence of MgTX from that without MgTX (Fig. 3A). In the presence of MgTX (5 nM), the amplitude of I_k was reduced by approximately 15% in control, approximately 30% and approximately 16% at 24 h and 72 h after ischemia, respectively (Fig. 3B). At a +30 mV voltage step, the current density of the MgTX-sensitive component was 8.9±1.3 pA/pF (n=9) in control, 38.3±1.5 pA/pF (n=7, P<0.01) at 24 h, and 10.6±1.0 pA/pF (n=6, P=0.63) at 72 h after ischemia, respectively (Fig. 3C). In this experiment, a 100 ms prepulse of +10 mV was used to isolate I_k. This prepulse might affect MgTX-sensitive currents evoked during depolarizing pulses, raising a possibility that the stronger blockade of I_k at 24 h after ischemia might be due to the rightward shifts of activation and inactivation curves (Fig. 2F, G) rather than the increased functional channels. To clarify this issue, Kv currents were evoked without prepulse, and the amplitudes were measured using the same methods as those for I_k. Consistent with the above results, 5 nM MgTX blocked 14.6% (n=9), 31.5% (n=7) and 17.3% (n=6) currents in control, 24 h and 72 h after ischemia (Fig. 3D), respectively. These results show that MgTX-sensitive current is increased after ischemia, which might contribute to the post-ischemic increase of I_k.

LA neurons also express KCNQ/M channels (Pusch et al., 1998; Cooper et al., 2001) that might be involved in the increase of I_k after ischemia. Application of 10 μM linopirdine (approximately 20 min) specifically inhibits approximately 90% of KCNQ/M currents in native neurons (Schnee and Brown, 1998; Yue and Yaari, 2004). The KCNQ/M currents were isolated by subtracting currents recorded in the presence of linopirdine from that without linopirdine (Fig. 4A). In control LA neurons, application of linopirdine blocked 40.7% of I_k (n=8). However, no significant changes in KCNQ/M currents were detected after ischemia. At a +30 mV voltage step, the current density of KCNQ/M currents was 32.4±4.7 pA/pF (n=8) in control and 30.8±4.5 pA/pF (n=6) at 24 h after ischemia (Fig. 4B). These data indicate that KCNQ/M currents are unlikely to have significant contributions to the post-ischemic increase of I_k in LA neurons.

Differential modulation of I_k by protein kinases in LA neurons

Accumulating evidence has demonstrated that protein kinases modulate ion channels. To reveal whether tyrosine kinases were involved in the post-ischemic changes of I_k in LA neurons, the effects of genistein, a broad-spectrum inhibitor of tyrosine kinases, were examined in control and

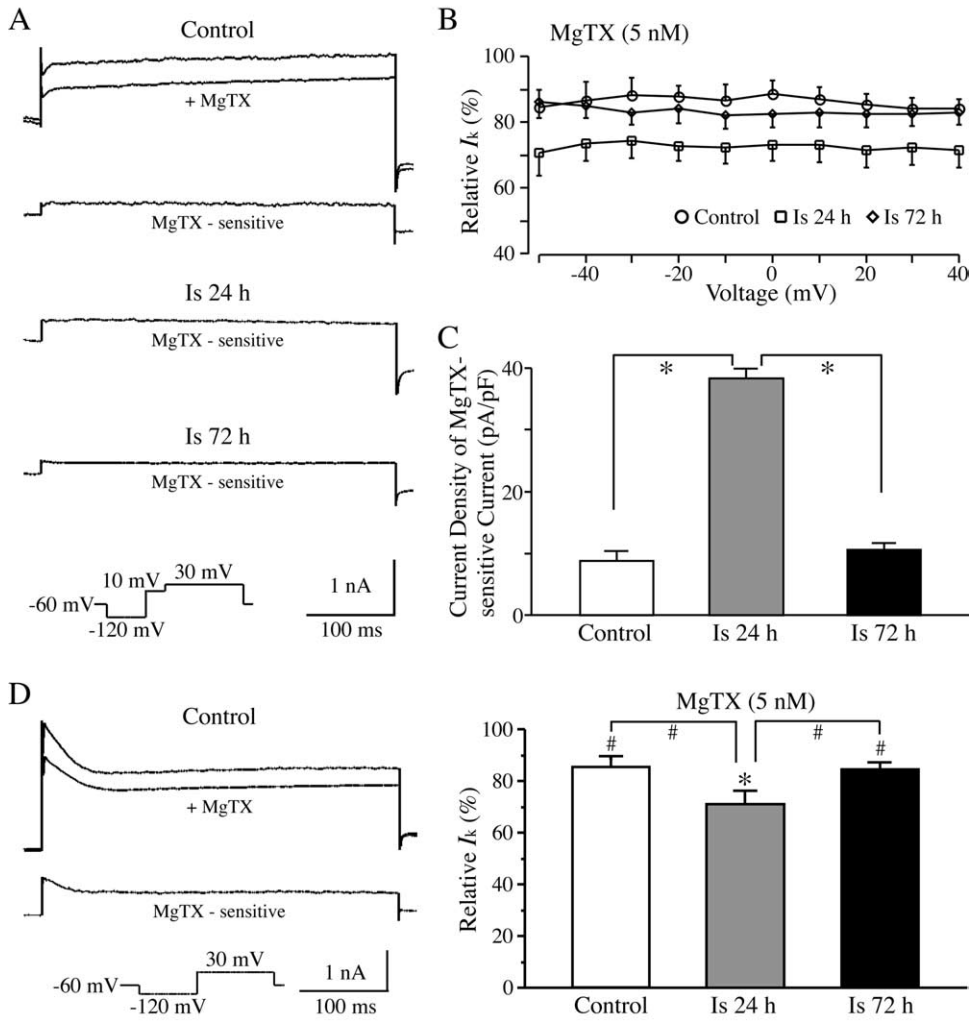

Fig. 3. The increase of MgTX-sensitive currents in LA neurons after ischemia. (A) Representative recordings showing a dramatic increase of MgTX-sensitive currents 24 h after ischemia. The MgTX-sensitive currents were isolated by subtracting the currents recorded in the presence of 5 nM MgTX from that without MgTX. (B) Plot showing the effects of MgTX on I_k in control and ischemic LA neurons evoked at different depolarizing steps. The currents recorded in the presence of MgTX were normalized to that without blockers. In control LA neurons and at 72 h after ischemia, MgTX blocked approximately 15% of I_k. However, about 30% of I_k were blocked by application of MgTX 24 h after ischemia. (C) Histograms showing the increase of MgTX-sensitive currents 24 h after ischemia. The currents were evoked by a depolarizing step to +30 mV. (D) MgTX-sensitive currents evoked by a depolarizing step to +30 mV following a conditioning voltage step (−120 mV, 300 ms; without 100 ms prepulse at +10 mV). Left panel showing an example of the isolation of MgTX-sensitive currents. Right panel showing that 5 nM MgTX blocked 14.6%, 31.5% and 17.3% of steady-state currents in control, at 24 h and 72 h after ischemia, respectively. * $P<0.01$; # $P<0.05$.

in ischemic neurons. When genistein (100 μM) was applied to control neurons, the amplitude of I_k was dramatically increased. Similar effects were observed in ischemic neurons (Fig. 5). The amplitude of I_k evoked at +30 mV was increased significantly by 25.3% ($n=6$) in control, 20.1% ($n=5$) and 29.2% ($n=5$) at 24 h and 72 h after ischemia ($P<0.01$), respectively. To further investigate whether the modulation of I_k by tyrosine kinases was fully activated, the effects of tyrosine phosphatase inhibitor sodium orthovanadate were also examined. Sodium orthovanadate (1 mM) significantly decreased I_k in both control and ischemic LA neurons ($P<0.01$, Fig. 5). At +30 mV depolarizing steps, the amplitude of I_k was inhibited by 35.5% ($n=6$) in control, 28.1% ($n=5$) and 23.8% ($n=5$) at 24 h and 72 h after ischemia, respectively. No significant difference was detected between control and ischemic groups. These data indicate that tyrosine kinases had a tonic inhibition on I_k to a similar extent before and after ischemia, and also suggest that tyrosine kinase modulation of Kv channels might not contribute to the increase of I_k after ischemia.

PKA has also been demonstrated to regulate I_k (Cole et al., 1996; Murakoshi et al., 1997). In the present experiments, bath application of membrane permeable 8-Br-cAMP (100 μM) led to a dramatic down-regulation of I_k in both control and ischemic LA neurons (Fig. 6A, B). When evoked at a +30 mV depolarizing step, 8-Br-cAMP inhibited I_k amplitude by 32.7% ($n=6$) in control, 16.2% ($n=6$) and 21.4% ($n=5$) at 24 h and 72 h after ischemia ($P<0.01$, Fig. 6B), respectively. Given that 8-Br-cAMP inhibits ion

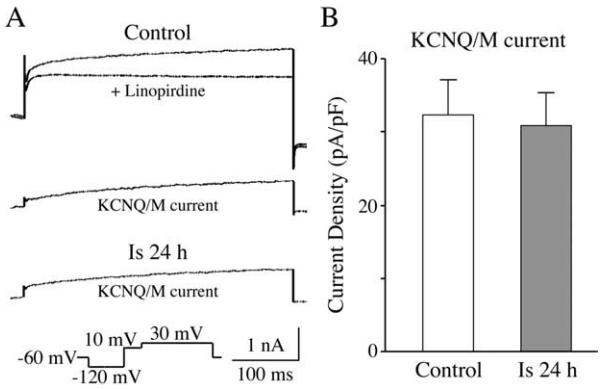

Fig. 4. KCNQ/M currents in LA neurons before and after ischemia. (A) Representative recordings showing KCNQ/M currents in LA neurons before and 24 h after ischemia. The KCNQ/M currents were isolated by subtracting the currents recorded in the presence of 10 μM linopirdine from those without linopirdine. (B) Histograms showing no significant changes in current density of KCNQ/M current before and after ischemia.

currents by phosphorylating Kv channel through PKA pathway, PKA inhibitors would be expected to have opposite effects on I_k. When H-89 (5 μM) was applied to LA neurons before ischemia (n=6) and 24 h after reperfusion (n=5), no obvious changes in I_k amplitude were observed (Fig. 6A, B), suggesting no basal PKA regulation on I_k under these conditions. However, when applied 72 h after ischemia, the same concentration of H-89 significantly increased I_k amplitude by 35.4% (at +30 mV depolarizing step, n=11, P<0.01, Fig. 6B). Considering the fact that the activities of Kv channels are highly sensitive to temperature (Singleton et al., 1999) and therefore may influence PKA modulation of Kv currents (Yuan et al., 2002), the effects of H-89 on I_k were also examined at 32 °C. As shown in Fig. 6C, although the current density of I_k recorded at 32 °C was greater than that at 24 °C (93.4±9.7 pA/pF vs 65.7±6.7 pA/pF in control, n=4; 142.0±11.3 PA/pF vs 119.5±10.2 pA/pF at 24 h after ischemia, n=5; P<0.01; at +30 mV depolarizing step), H-89 still had no effect on I_k before ischemia (n=8) and 24 h after reperfusion (n=6). These data demonstrate that PKA activation down-regulates I_k in both control and ischemic LA neurons. The tonic inhibition of endogenous PKA activity on I_k might be responsible for the decrease of I_k 72 h after ischemia.

Our data revealed that MgTX-sensitive current increased remarkably at 24 h and returned to control levels at 72 h after ischemia. The changes occurred at 72 h after ischemia might be due to the basal down-regulation of PKA on MgTX-sensitive currents. To test this hypothesis, MgTX-sensitive currents were analyzed in the presence of H-89 at 72 h after ischemia. Consistent with the above data, application of 5 μM H-89 significantly increased I_k amplitude (n=8, P<0.01). After being fully up-regulated (approximately 15 min of application of H-89), the I_k amplitude was significantly reduced by 5 nM MgTX. At a +30 mV depolarizing step, 37.3% of I_k was blocked by 5 nM MgTX (n=7, P<0.01, Fig. 6D). These results strongly suggest that endogenous PKA activity is involved in the decrease of I_k at 72 h after ischemia by inhibiting MgTX-sensitive currents.

Alterations of spike properties after ischemia

Changes in the Kv current undoubtedly lead to alterations of firing patterns. Particularly, I_k is required for keeping single spikes short and permitting high-frequency trains of spikes (Rudy, 1988; Lien and Jonas, 2003). The spike properties of LA neurons were therefore examined before and after ischemia. The spike width was shortened significantly from 0.78±0.02 ms in control (n=14) to 0.65±0.01 ms at 24 h after ischemia (n=12; P<0.01; Fig. 7). While no obvious alterations occurred in the spike upstroke (127.2±5.1 mV/ms in control, n=14; 133.2±5.9 mV/ms at 24 h after ischemia, n=12), the spike repolarization was changed from −38.9±1.7 mV/ms in control (n=14) to −61.8±3.2 mV/ms at 24 h after ischemia (n=12, P<0.01; Fig. 7A, C). Moreover, the spike duration was dramatically broadened after application of 5 nM MgTX 24 h after ischemia (0.65±0.01 ms vs 0.87±0.01 ms, n=12, P<0.01, Fig. 7A, C).

The MgTX-sensitive channel has also been suggested to influence firing frequency (Fadool et al., 2004). How-

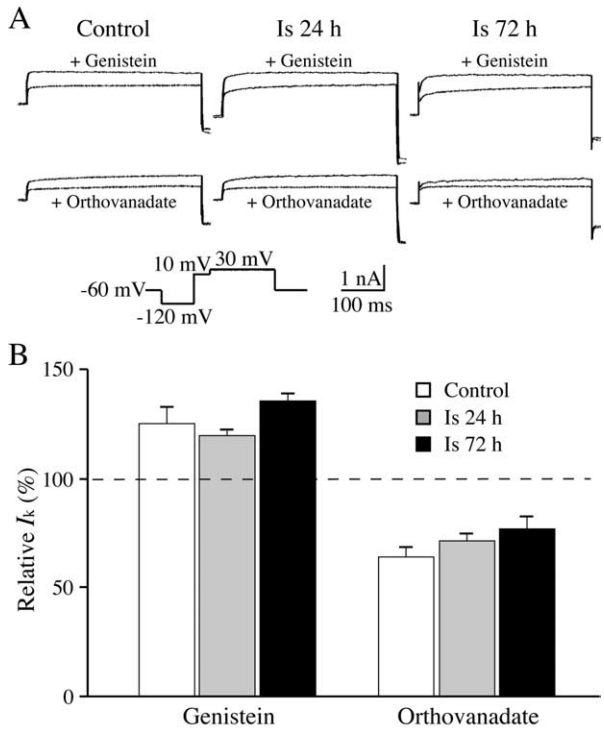

Fig. 5. Tonic inhibitory action of protein tyrosine kinase on I_k before and after ischemia. (A) Representative traces showing the effects of tyrosine kinase inhibitor genistein (100 μM) and tyrosine phosphatase inhibitor sodium orthovanadate (1 mM) on I_k. (B) Histograms showing that, in both control and ischemic LA neurons, sodium orthovanadate caused a decrease in I_k amplitude whereas genistein dramatically increased the currents. No significant difference was detected between the control and ischemic neurons in either experimental group. The currents recorded in the presence of genistein or sodium orthovanadate were normalized to those before application of these drugs, respectively.

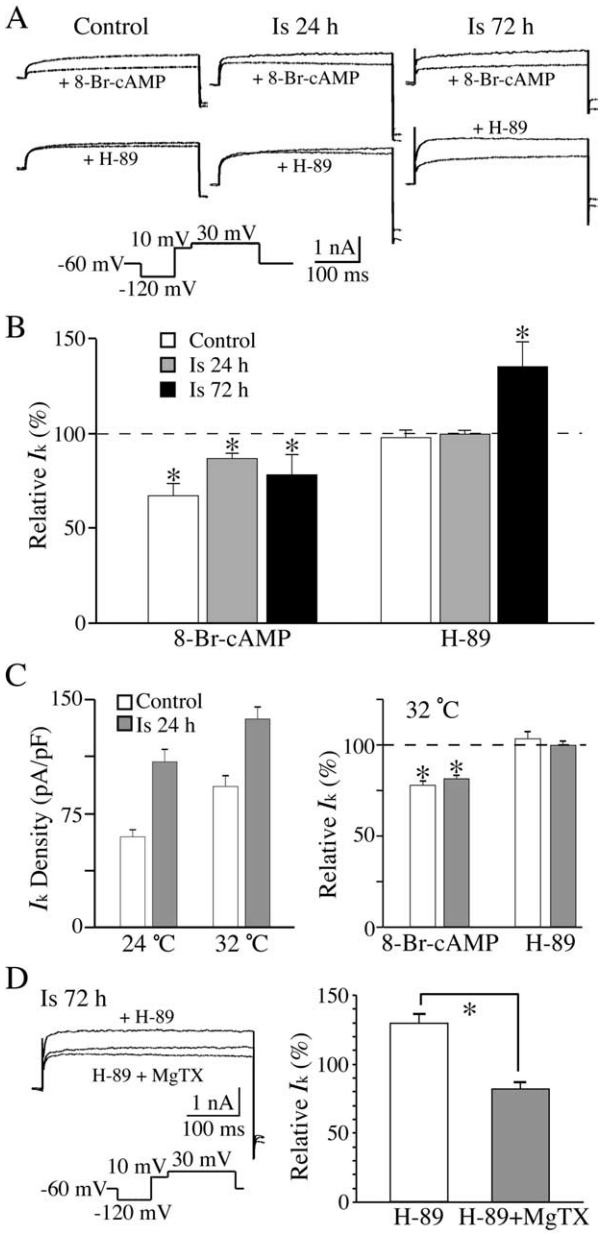

Fig. 6. Differential effects of PKA on I_k before and after ischemia. (A) Representative traces showing the effects of 8-Br-cAMP (100 μM) and H-89 (5 μM) on I_k. (B) Histograms showing that 8-Br-cAMP inhibited I_k in both control and ischemic LA neurons. H-89 had no effect on I_k before and 24 h after ischemia, but up-regulated I_k 72 h following reperfusion. The currents recorded in the presence of 8-Br-cAMP or H-89 were normalized to those before application of these drugs, respectively. (C) Compared with those at 24 °C, the current densities of I_k in control and ischemic neurons were increased at 32 °C ($P<0.01$, left panel). However, even at 32 °C, H-89 had no effect on I_k in control and 24 h after ischemia (right panel). (D) In the presence of H-89, MgTX blocked 37.3% of I_k in LA neurons 72 h following ischemia. Left panel showing representative traces recorded before and after application of drugs. Right panel showing the blockade of MgTX on I_k in the presence of H-89. The currents recorded in the presence of H-89 or H-89 plus MgTX were normalized to those before application of these drugs, respectively. * $P<0.01$.

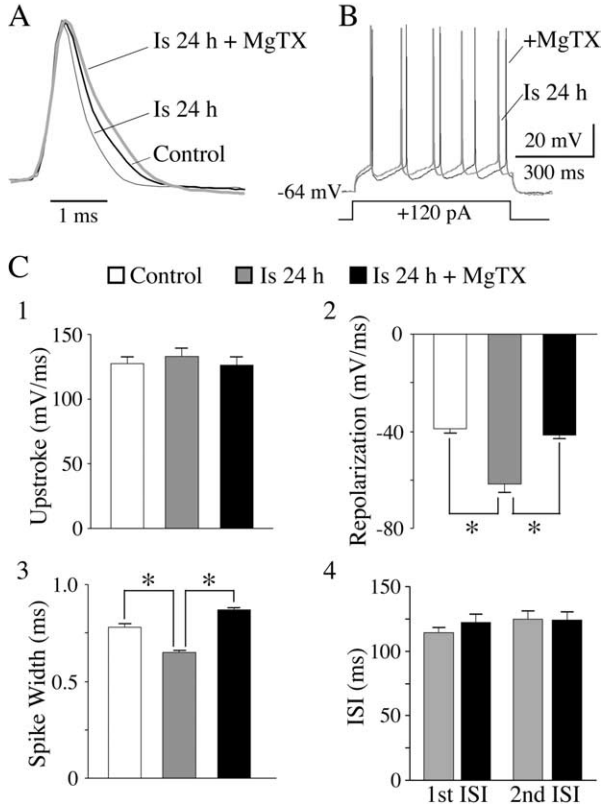

Fig. 7. Contribution of MgTX-sensitive currents to spike properties. (A) Normalized traces showing the differences in spike duration and action potential repolarization in neurons before and after ischemia, and the effects of MgTX. (B) Representative traces showing the firing frequency 24 h after ischemia. (C) Pooled data for action potential upstroke (C1) and repolarization (C2), spike width (C3) and ISI (C4). Comparing to that of control LA neurons, the spike width was shortened significantly and the spike repolarization was facilitated 24 h after ischemia. In the presence of 5 nM MgTX, the spike width was dramatically broadened and the repolarization was much slower. No significant differences were detected in either action potential upstroke or ISI. * $P<0.01$.

ever, application of 5 nM MgTX had no obvious effect on both the first and second ISI 24 h after ischemia ($n=10$, Fig. 7B, C). These results are not surprising because the ISI in LA neurons is mainly determined by hyperpolarization-activated cation current and small-conductance Ca^{2+}-activated potassium current (Bennett et al., 2000).

DISCUSSION

The present study has demonstrated that I_k in LA neurons is increased after transient forebrain ischemia. Such changes result from the alterations of Kv channel function and protein kinase modulation. While the up-regulation of MgTX-sensitive currents accounts for the increase of I_k 24 h after ischemia, the tonic down-regulation of MgTX-sensitive currents by endogenous PKA activity is responsible for the recovery of I_k 72 h following reperfusion. Because LA neurons are resistant to ischemia, the increase of I_k might be one of the mechanisms protecting these neurons from ischemic insults.

Changes of potassium currents after cerebral ischemia

Many studies have shown that potassium currents are altered during hypoxia or oxygen/glucose deprivation (OGD; Krnjevic and Leblond, 1989; Jiang and Haddad, 1993; Fujimura et al., 1997; Gebhardt and Heinemann, 1999). The investigation on potassium currents after reperfusion/re-oxygenation is less intensive due to the technical difficulties. Using brain slices and dissociated neurons prepared at different intervals after ischemia *in vivo*, the present study has shown that the I_k in LA neurons was enhanced 24 h after ischemia and then returned to control levels 72 h after reperfusion. Consistent with our findings, it has been reported that both A-type currents and I_k in CA1 pyramidal neurons were increased at 12 h and 36 h after reperfusion, respectively (Chi and Xu, 2000). Single channel recording from acutely dissociated CA1 neurons has indicated that the unitary conductance of large-conductance Ca^{2+}-dependent potassium channel in CA1 neurons 2 h after reperfusion was higher and the open time was longer than those in control neurons (Gong et al., 2000). Fewer studies on post-ischemic changes of potassium currents have been conducted on striatal neurons. It has been suggested that the activities of ATP- and Ca^{2+}-dependent potassium channels in LA neurons are enhanced following OGD, leading to a membrane hyperpolarization of these cells (Calabresi et al., 1997; Centonze et al., 2001). It is conceivable that the increase of potassium conductance after ischemia/hypoxia could reduce neuronal excitability and therefore lessen the excitotoxic damage.

One possible mechanism underlying the post-ischemic alteration of potassium currents is the changes in the number of functional channels. Studies have shown that the expression of functional Kv channels is altered by ischemic insults (Conforti and Millhorn, 1997; Conforti et al., 2000; Platoshyn et al., 2001). I_k could be mediated by Kv1, Kv2 and Kv3 channels (Pongs, 1992). The present study has indicated that MgTX-sensitive currents are the major contributor to the increase of I_k in LA neurons after ischemia. MgTX-sensitive currents includes currents mediated by Kv1.1, Kv1.2 and Kv1.3 channels (Koch et al., 1997; Kaczorowski and Garcia, 1999). It has been shown that the expression of Kv1.2 subunit is increased following transient focal ischemia (Chung et al., 2001). In addition, chronic hypoxia induces up-regulation of Kv1.2 subunit gene, which is correlated with an increased oxygen-sensitivity of the potassium current (Conforti and Millhorn, 1997; Conforti et al., 2000). Therefore, the increase of I_k in LA neurons after ischemia might be due to the up-regulation of Kv channels, such as kv1.2 subunits, that mediate MgTX-sensitive currents. LA neurons also express low levels of Kv2.1 mRNA (Baranauskas et al., 1999). The present study could not rule out that other Kv subunits, such as kv2.1, might also contribute to the post-ischemic increase of I_k.

It is well established that changes in Kv channel properties, such as the probability of channel opening and the voltage-dependence of activation and inactivation, are associated with the alterations of current amplitude. In fact, the voltage-dependence of potassium channels is altered after ischemia (Chi and Xu, 2000) or oxidative stress (Muller and Bittner, 2002). The shift of the activation curve to the hyperpolarization direction and the increased channel open probability are believed to be responsible for the enhancement of I_k in CA1 pyramidal neurons following ischemic insults (Chi and Xu, 2000, 2001). However, it seems unlikely that the post-ischemic increase of I_k in LA neurons is due to the changes in voltage-dependence of the channel. The activation curve in LA neurons shifted to depolarizing direction after ischemia and the depolarizing shift will reduce the channel open probability at a given membrane potential, resulting in a decrease of current amplitude (Hoffman and Johnston, 1998).

Kv channels are modulated by protein kinases (Jonas and Kaczmarek, 1996), which is of fundamental importance for the regulation of neuronal excitability and the response to synaptic inputs (Hoffman and Johnston, 1998; Yuan et al., 2002; Hu et al., 2003a; Varga et al., 2004). Protein kinase modulation of potassium channels can regulate the gating kinetics and current amplitude, and influence the number of functional channels on plasmic membrane (Xu et al., 2002; Hu et al., 2003b). Studies have shown that the activity of several Kv1 channels that mediate I_k is down-regulated by non-receptor and receptor tyrosine kinases (Nitabach et al., 2001, 2002). In the present study, the amplitude of I_k in both control and ischemic LA neurons was tonically inhibited by endogenous tyrosine kinase activity. These inhibitions are indistinguishable before and after ischemia. Besides protein tyrosine kinases, the Kv channels are also regulated by PKA (Jonas and Kaczmarek, 1996). The binding activity of PKA to cAMP, which reflects the functional integrity of the kinase, is profoundly altered in neurons after cerebral ischemia (Tanaka et al., 1997; Tanaka, 2001), raising a possibility that PKA might differentially regulate Kv channels before and after ischemia. The present study indicates that PKA activation down-regulates I_k in LA neurons, and I_k is constitutively inhibited by endogenous PKA activity 72 h after ischemia, which might contribute to the recovery of I_k at late time points following ischemia. To support this notion, the current density of MgTX-sensitive currents, which are the major contributor to the increased I_k after ischemia, was enhanced significantly in the presence of PKA inhibitor.

Functional implications of increased I_k in LA neurons

Potassium currents have great impacts on neurons in physiological and pathological conditions. Previous work has shown that potassium channels exert great influence on the neuronal outcomes following ischemia. Excessive K^+ efflux and intracellular K^+ depletion mediated by I_k have been implicated in triggering neuronal apoptosis (Yu et al., 1997, 1999). The potassium channel blocker TEA attenuates hypoxia- and ischemia-in-

duced damage in ischemia-vulnerable neurons (Huang et al., 2001; Wei et al., 2003). Further studies suggest that the activation of Kv2.1 channels is necessary for neuronal apoptosis and is sufficient to increase sensitivity to a sublethal dose of apoptotic stimulus (Pal et al., 2003). In contrast, activation of ATP-sensitive potassium channels is neuroprotective against ischemia, which involves membrane hyperpolarization, inhibition of harmful gene expression and activation of Ras/MAP kinase (Heurteaux et al., 1993; Yamada et al., 2001; Lin et al., 2004). It is believed that the up-regulation of A-type currents and large-conductance Ca^{2+}-activated potassium currents is also neuroprotective, presumably as a result of the increase of spike threshold and the decrease of membrane excitability (Chi and Xu, 2000; Runden-Pran et al., 2002). In LA neurons, both ATP-sensitive and Ca^{2+}-activated potassium currents are increased following ischemia, leading to a hyperpolarization of membrane potential (Centonze et al., 2001), which might help to protect these neurons. Apparently, the impact of enhanced potassium currents on the ischemic outcome depends on the temporal profile and the extent of enhancement. For instance, CA1 pyramidal neurons die 2 to approximately 3 days after transient cerebral ischemia (Pulsinelli et al., 1982) and I_k is increased progressively after ischemia with the highest levels at 3 days after reperfusion (Chi and Xu, 2000), suggesting that the enhanced I_k might be associated with the post-ischemic cell death by inducing apoptosis (Yu et al., 1999). Whereas a transient increase of I_k in ischemia-resistant LA neurons was observed in the present study at 24 h after ischemia. The increase of I_k in LA neurons dramatically shortened the spike duration. Given that Ca^{2+} entry is primarily triggered by spikes in LA neurons (Bennett et al., 2000), the shortened spike duration will certainly limit Ca^{2+} influx, and hence prevent the activation of cell death pathways that is due to the intracellular Ca^{2+} overload (Choi, 1995).

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