

# Targeting Ischemic Stroke with a Novel Opener of ATP-Sensitive Potassium Channels in the Brain

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## ABSTRACT

During cerebral ischemia, the opening of neuronal ATP-sensitive potassium channels ( $K_{ATP}$  channels) affords intrinsic protection by regulating membrane potential. To augment this endogenous mechanism, we have synthesized iptakalim, a  $K_{ATP}$  opener. Through  $K_{ATP}$  channel activation, iptakalim affected multiple pathways of the glutamatergic system, limiting glutamate release and receptor actions, which are involved in excitotoxicity during ischemic damage. The molecule readily

penetrated the brain and showed low toxicity in animal experiments. In different animal models of stroke as well as in cell cultures, iptakalim provided significant neuroprotection, not only in promoting behavioral recovery but also in protecting neurons against necrosis and apoptosis. This compound thus has promise as a neuroprotective drug for the treatment of stroke and other forms of neuronal damage.

During ischemic insult, a high rate of neuronal death always leads to permanent functional loss. Protecting against the risk of neurons dying in the ischemic brain is thus still the main strategy for human stroke therapy. Years of animal research have resulted in a better understanding of the complex pathophysiology of ischemic brain injury, and this has led to drugs aimed at ameliorating neuronal death. Conventional neuroprotectants often target one specific receptor that participates in the neurotoxic cascade, such as the NMDA excitatory amino acid receptors. Other strategies such as the scavenging of free radicals target only one causal factor in neuronal death (Wahlgren and Ahmed, 2004). A valuable clue provided by an endogenous protective mechanism in the brain has probably been neglected. This involves positive reactions against the excess membrane depolarization that is initiated by reduced energy stores after lowered cerebral blood flow in cases of stroke. This process is often mediated by the ATP-sensitive  $K^+$  channel ( $K_{ATP}$  channel), the only channel protein that directly couples the metabolic state of a cell to its electrical activity (Ascroft and Ascroft, 1990). This channel is usually closed in normal conditions but is activated rapidly in response to de-

creases in intracellular ATP/ADP ratio under ischemic conditions, causing a  $K^+$  efflux. Channel opening seems to hyperpolarize the cell membrane, limiting neuronal excitability and  $Ca^{2+}$  influx and thus blocking the subsequent neurotoxic biochemical cascade (Seino and Miki, 2003). In view of this intrinsic protective role of the  $K_{ATP}$  channel, pharmacological activation of this channel should produce neuroprotection. Some  $K_{ATP}$  openers have been reported to be effective in preclinical experiments (Heurteaux et al., 1993; Lauritzen et al., 1997; Takaba et al., 1997; Wind et al., 1997). However, they lack clinical effectiveness, mainly because of strong detrimental side effects or poor brain penetration.

We hypothesized that the development of  $K_{ATP}$  channel openers with low toxicity and brain-permeable characteristics would be effective for stroke therapy. We have designed and synthesized thousands of compounds and have finally screened out the most promising candidate, 2,3-dimethyl-2-butylamine (iptakalim). It is remarkably different from other known  $K_{ATP}$  channel openers in that it has a fatty *para*-amine structure (Wang et al., CN1365967A). Herein, we present data supporting the neuroprotective role of this compound in the amelioration of experimental stroke.

## Materials and Methods

**Global Ischemic Model.** Mongolian gerbils ( $60 \pm 5$  g) of either sex were anesthetized, followed by bilateral carotid artery ligation

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**ABBREVIATIONS:** NMDA, *N*-methyl-D-aspartate; TUNEL, terminal deoxynucleotidyl transferase dUTP nick-end labeling; PI, propidium iodide; SHRsp, stroke-prone spontaneously hypertensive rats; HBSS, Hanks' balanced salt solution; TTX, tetrodotoxin; sEPSC, spontaneous excitatory postsynaptic current; TEA, tetraethylammonium; MK-801, dizocilpine maleate; CNS, central nervous system; SUR, sulfonylurea receptor.

for 5 min. Sham-operated gerbils were sacrificed just after exposing the carotid artery without clamping the vessels (Small et al., 1999). The animals were kept at normal body temperature before and after the operation by using heating lamps. Throughout the operation, rectal temperature was maintained at  $37 \pm 0.5^\circ\text{C}$  with a heat blanket with feedback control. Room temperature was regulated to  $22\text{--}25^\circ\text{C}$  by air conditioning. Drugs were administered i.p. 40 min before carotid artery ligation and each day up to day 7 after the operation. On days 1 and 5 after operation, all the gerbils were placed in an open field maze for 15 min while their locomotor activities were recorded. Locomotor activity included movements across squares and rears (rearing up on haunches), which represented the exploring activities in horizontal and vertical orientation, respectively. On days 2 and 3, the gerbils were trained in the T-maze (15 pairs of trials/day). On days 4 and 6, the gerbils were given 15 pairs of trials/day and the percentages of correct choices were determined to measure the working memory of the animals (Corbett and Nurse, 1998). For histological endpoints detected, global ischemic gerbils treated with drugs up to day 7 were anesthetized by an overdose of sodium amylbarbital and then were transcardially perfused with 50 ml of saline and 50 ml of 4% buffered polyformaldehyde. Brains were removed and stored in paraformaldehyde for 24 h. After fixation, the brains were coronally sectioned at  $6\text{ }\mu\text{m}$ . One set of sections was stained with hematoxylin and eosin, and cells in the CA1 region of hippocampus were counted. Another set of section was used for terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL).

**Salt Load SHRsp Stroke Model.** Stroke-prone spontaneously hypertensive rats (SHRsp; 10 weeks old,  $150 \pm 30\text{ g}$ ) of either sex were given a 1% NaCl solution as a substitute for drinking water every day to accelerate the onset of stroke. The trial group was given oral iptakalim or nimodipine (the positive control drug) each day for 12 weeks. The onset of stroke in each animal was recorded, and succeeding neurological deficits were observed every day for 14 days. The neurological deficits were evaluated by a specially designed scoring system described previously (Zhang and Feng, 1996): 0, no deficit; 1, mild stress; 2, forelimb or head twitch or with severe stress; 3, hemi-paralysis, body inclined or disabled; and 4, paralysis, tremor or convulsion. Systolic blood pressure and heart rate were measured once every 2 weeks by standard tail cuff technique (BP recorder, RBP-I, China). All animal procedures were performed in accordance with the National Institutes of Health's Guide For the Care and Use of Laboratory Animals.

**Primary Neuron Culture and Apoptosis Assay.** Neurons were obtained from the cortex of newborn rats, as described previously (Black et al., 1995). For flow cytometry, the primary culture medium was replaced on 12-day-cultured neuronal cells with low-glucose Dulbecco's modified Eagle's medium without serum. After culturing in a hypoxic container (filled with 95% N<sub>2</sub> + 5% CO<sub>2</sub>) for 16 h, the cells were transferred to normal environments for 24 h. Drugs were added before the hypoxic treatment. After reoxygenation for 24 h, cells were collected, dyed with 100  $\mu\text{g/ml}$  PI, and detected by flow cytometry (FACScalibur; BD Biosciences, San Jose, CA). For electron microscopy, cells were examined using a Philips TECHAI-10 transmission electron microscope.

**Glutamate Release and Uptake Assay.** PC-12 cells were cultured in Dulbecco's modified Eagle's medium containing 10% fetal cattle serum at  $37^\circ\text{C}$  in a CO<sub>2</sub> incubator. For glutamate release, the cells were plated on to 24-well plates, high-K<sup>+</sup> solution (80 mM) was added, and culture medium was collected after 12-h incubation. Proteins were precipitated using 0.2 M HClO<sub>4</sub> and the supernatants after centrifugation were mixed and vibrated with derived solution (20 mM *ortho*-phthalaldehyde, 2 mM  $\beta$ -mercaptoethanol, 25 mM sodium tetraborate, and 50% carbinol, pH 9.6) of equal volume for 3 min. Glutamate was measured by high-performance liquid chromatography combined with fluorescent detector analysis. For measurement of [<sup>3</sup>H]glutamate uptake, the culture medium was replaced by Hanks' balanced salt solution (HBSS) for 30 min. L-[<sup>3</sup>H]Glutamate (1

$\mu\text{Ci/ml}$ ) was added for 7 min at  $37^\circ\text{C}$ , then ice-cold HBSS was added to end the reaction. After three washes in HBSS, 200  $\mu\text{M}$  NaOH was added to the cells, and radioactivity was measured by liquid scintillation counting. The protein contents of the cells were determined by Lowry's assay.

**Patch-Clamp Recording.** The whole-cell configuration was conformed by sucking the neuronal membrane into the pipette when the resistance was more than 1 G $\Omega$ . The electric signals were acquired by an Axopatch-200B amplifier and analyzed using the pClamp 7.0 program (Axon Instruments, Union City, CA). Drugs were administered by a pneumatic ejector (Picospritzer II; Parker Hannifin Co.). For potassium currents or glutamate currents recording, the extracellular bathing solution contained 140 mM NaCl, 1 mM MgCl<sub>2</sub>, 5 mM KCl, 3 mM CaCl<sub>2</sub>, 10 mM HEPES, 10 mM glucose, and 0.001 mM TTX. For spontaneous excitatory postsynaptic current (sEPSC) recording, TTX was omitted from the extracellular bathing solution. For sodium current recording, the extracellular bathing solution contained 140 mM NaCl, 1 mM MgCl<sub>2</sub>, 5 mM KCl, 3 mM CaCl<sub>2</sub>, 10 mM TEA, 1 mM 4-aminopyridine, 0.2 mM CdCl<sub>2</sub>, 10 mM HEPES, and 10 mM glucose. For calcium current recording, the extracellular bathing solution contained 140 mM NaCl, 1 mM MgCl<sub>2</sub>, 5 mM KCl, 3 mM CaCl<sub>2</sub>, 10 mM TEA, 1 mM 4-aminopyridine, 0.001 mM TTX, 10 mM HEPES, and 10 mM glucose. For potassium currents, glutamate currents or sEPSC recording, the pipette solution contained 140 mM KF, 10 mM EGTA, and 10 mM HEPES. For sodium current recording, the pipette solution contained 140 mM CsCl, 10 mM TEA, 10 mM EGTA, and 10 mM HEPES. For calcium current recording, the pipette solution contained 140 mM CsCl, 10 mM TEA, 2 mM Na<sub>2</sub>ATP, 10 mM EGTA, and 10 mM HEPES.

## Results

**Profiles on Brain Potassium Channels.** Using the whole-cell, patch-clamp technique, we investigated the effects of iptakalim on potassium channels in cultured rat hippocampus neurons. Iptakalim enhanced the potassium currents at 1, 10, or 100  $\mu\text{M}$  (Fig. 1A, left). The effect of 1  $\mu\text{M}$  iptakalim on potassium currents was blocked by coapplication of the specific K<sub>ATP</sub> blocker glibenclamide (Fig. 1A, left). Thus, iptakalim seems to open and activate the neuronal K<sub>ATP</sub> channels in brain cells.

A conventional membrane ligand-receptor saturation and competitive binding assay was used to explore the possibility that specific iptakalim binding sites are present in the rat brain. We labeled binding sites in the rat hippocampus, striatum and cortex membranes with [<sup>3</sup>H]iptakalim. The K<sub>d</sub> values were 2.30, 2.45, and 2.16 nM, respectively, and the corresponding B<sub>max</sub> values were 1073, 858, and 715 fmol/mg of protein (Fig. 1, B–D). Unlabeled iptakalim, or the specific K<sub>ATP</sub> opener pinacidil, was incubated with rat cortex membrane for 75 min at  $25^\circ\text{C}$ . Both iptakalim and pinacidil inhibited the binding of [<sup>3</sup>H]iptakalim in dose-dependent manners, with IC<sub>50</sub> values of 7.08 and 6.19 nM, and K<sub>i</sub> values of 3.18 nM and 2.72 nM, respectively (Fig. 1E). Thus, iptakalim has a high affinity to neuronal K<sub>ATP</sub> channels in brain.

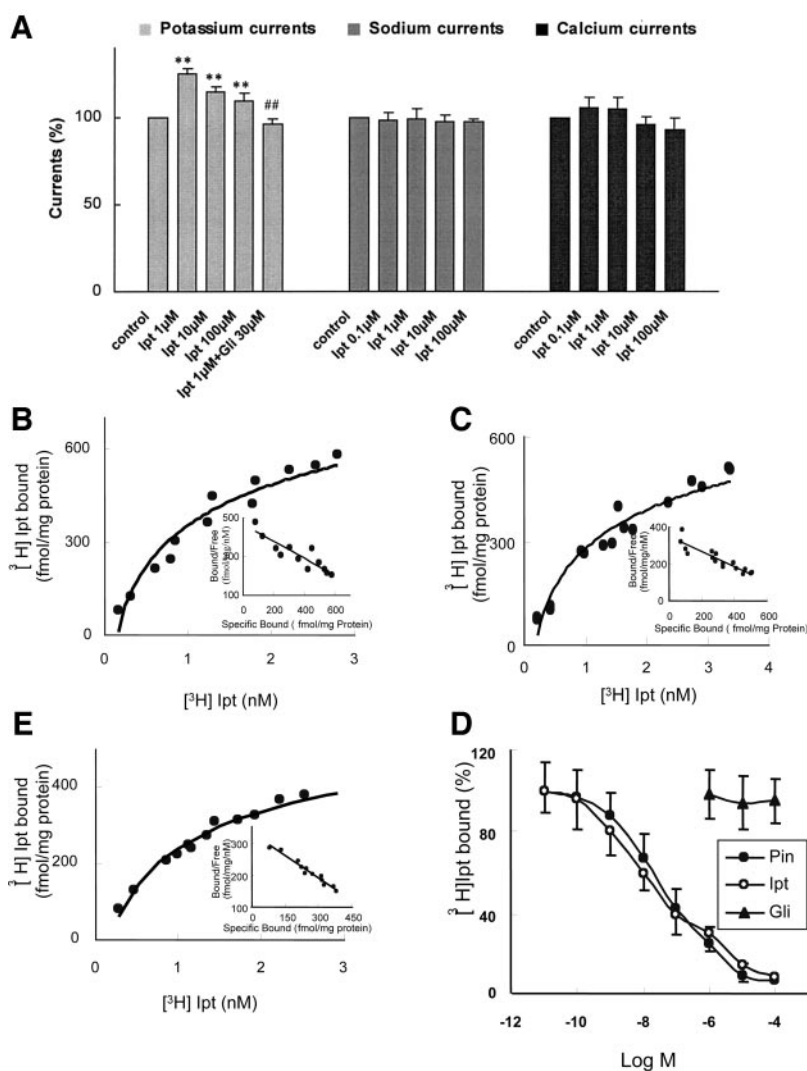
**Effects against the Brain Glutamatergic System.** Glutamate has been implicated as a mediator of neuronal injury in many neurological disorders, so interrupting the glutamatergic system in the brain (for example by inhibition of glutamate release or blockade of glutamate receptors) has been widely evaluated for stroke therapy. We therefore investigated the effects of iptakalim on glutamate release and uptake process. Iptakalim dose-dependently inhibited the glutamate release stimulated with 80 mM KCl solution that could be prevented by glibenclamide. Iptakalim at concentra-

tions of 0.1, 1, 10, and 100  $\mu\text{M}$  inhibited glutamate release by 19.8, 36.8, 50.5, and 60.3, respectively, and they were decreased to 3.6, 20.0, 36.4, and 48.9% ( $n = 6$ ) correspondingly in the presence of glibenclamide at 1  $\mu\text{M}$ . In the glutamate uptake experiment, 5 and 10  $\mu\text{M}$  iptakalim significantly increased the uptake of [ $^3\text{H}$ ]glutamate into PC-12 cells from  $2.12 \pm 0.16$  to  $2.72 \pm 0.24$  pmol/mg of protein/min ( $P < 0.05$ ,  $n = 6$ ) and  $2.82 \pm 0.24$  pmol/mg of protein/min ( $P < 0.05$ ,  $n = 6$ ), respectively. Simultaneous treatment with 10  $\mu\text{M}$  iptakalim and 20  $\mu\text{M}$  glibenclamide resulted in no change in [ $^3\text{H}$ ]glutamate uptake compared with controls ( $P > 0.05$ ,  $n = 6$ ). Thus, iptakalim inhibits glutamate release and enhances glutamate uptake in brain through activated  $\text{K}_{\text{ATP}}$  channels.

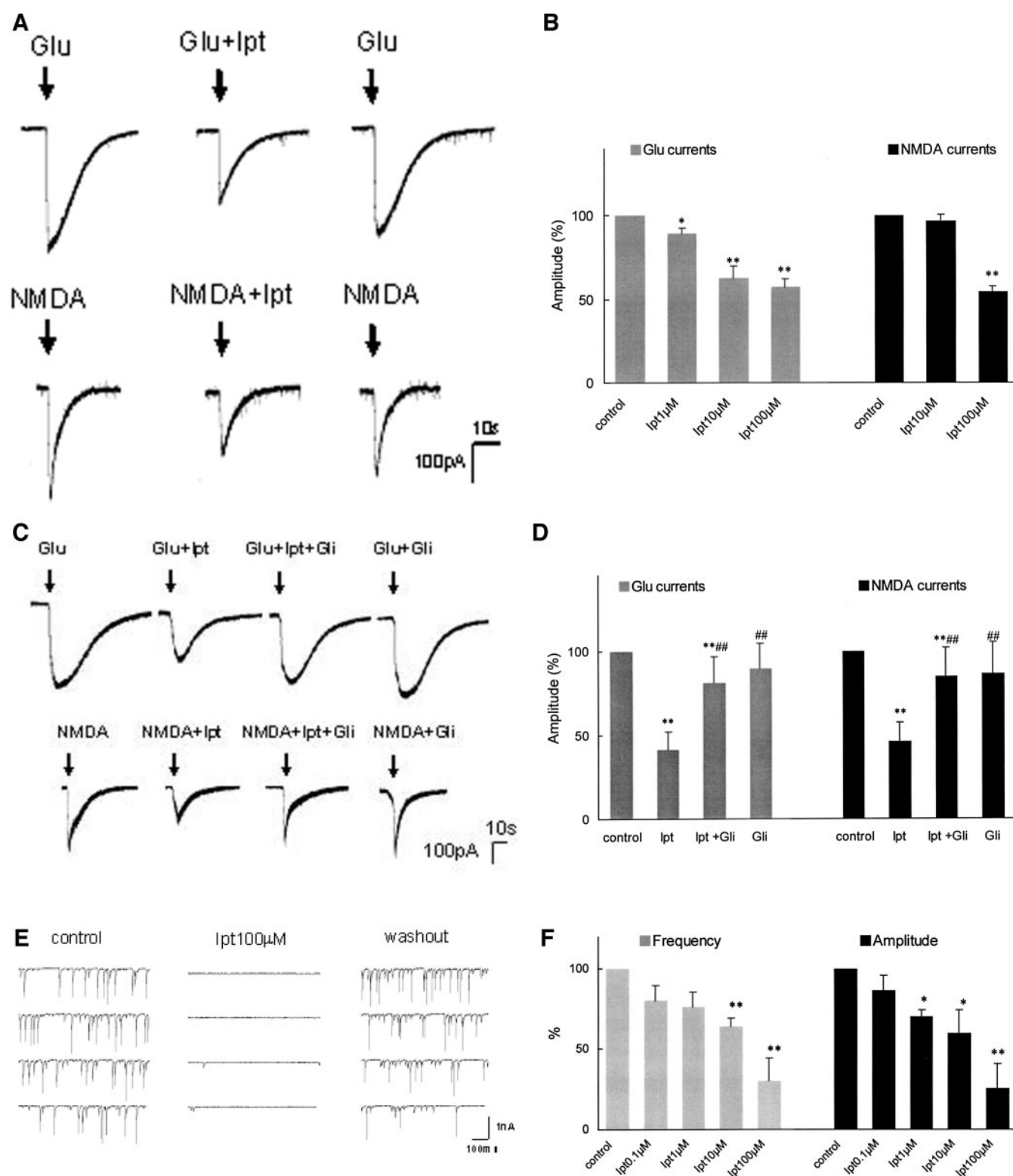
To determine whether iptakalim influences glutamate receptor function, we examined its effects on glutamate-evoked currents in cultured rat hippocampus neurons. Using voltage clamp at a holding potential of  $-70$  mV, flow pipette application of glutamate (100  $\mu\text{M}$ ) for 1 s evoked an inward current. Coapplication of 1, 10, and 100  $\mu\text{M}$  iptakalim decreased the current amplitudes significantly. When 30  $\mu\text{M}$  glibenclamide was applied simultaneously, the inhibition of 100  $\mu\text{M}$  iptakalim was attenuated significantly (Fig. 2, A–D). However, application of glibenclamide alone had no significant effect on glutamate-induced currents ( $P > 0.05$ ). We also

tested the effect of iptakalim on currents evoked by 100  $\mu\text{M}$  NMDA, an agonist for the NMDA glutamate receptor. Iptakalim reduced the NMDA-currents and glibenclamide also antagonized this effect of iptakalim (Fig. 2, A–D). Thus, iptakalim might suppress the responsiveness of hippocampal neurons to glutamate.

Last, we tested the effects of iptakalim on spontaneous synaptic activity in cultured rat hippocampus neurons. Spontaneous events were recorded at a holding potential of  $-70$  mV. The events detected under these conditions were probably sEPSCs mediated by spontaneous glutamate release, because the glutamate receptor antagonists MK-801 (10  $\mu\text{M}$ ) and 2,3-dihydroxy-6-nitro-7-sulfamoylbenzo(f)quinoxaline (10  $\mu\text{M}$ ) abolished them. Iptakalim dose-dependently inhibited the sEPSCs by decreasing both current frequency and amplitude, reversibly (Fig. 2, E and F). Because these two indications reflect transmitter release from presynaptic terminals and drug action to postsynaptic sites, respectively, these results supported the idea that iptakalim down-regulates the glutamatergic system. In separate studies, we also noted that iptakalim had no significant effects on sodium and calcium channels, the two other fundamental ion channels in neurons, which are also important for glutamate release and receptor activity (Fig. 1A).



**Fig. 1.** Effects of iptakalim (Ipt) on potassium channels. A, effects of iptakalim alone or in combination with glibenclamide (Gli) on potassium currents (left) in cultured rat hippocampus neurons. Data are expressed as means  $\pm$  S.D. ( $n = 9$ ). \*\*,  $P < 0.01$  versus control. ##,  $P < 0.01$  versus iptakalim 1  $\mu\text{M}$  alone. Iptakalim had no significant effects on sodium currents (middle,  $n = 5$ ) or calcium currents (right,  $n = 6$ ) in cultured rat hippocampus neurons. B–D, saturation curves of binding of [ $^3\text{H}$ ]Ipt to sulfonylurea receptors of K<sub>ATP</sub> channels from rat cerebral cortex (B), hippocampus (C), and striatum (D) membrane preparations. Inset, Scatchard plot for saturation binding. e, competitive inhibition of iptakalim, pinacidil (Pin) and glibenclamide on the specific binding of [ $^3\text{H}$ ]Ipt in rat cerebral cortex membrane preparations. Data are expressed as means  $\pm$  S.D. ( $n = 4$ ).



**Fig. 2.** Effects of iptakalim (Ipt) on glutamate (Glu) receptors and transmission. a, iptakalim (100  $\mu$ M) inhibited glutamate and NMDA-evoked currents in cultured rat hippocampus neurons. Glutamate or NMDA were used at 100  $\mu$ M. b, different doses of iptakalim on the relative amplitudes of glutamate and NMDA-evoked currents ( $n = 6$ ). \*\*,  $P < 0.01$  versus control. c and d, antagonism of glibenclamide (Gli) against the inhibitory effects of iptakalim in glutamate and NMDA-evoked currents. Iptakalim or glibenclamide were used at 100 and 30  $\mu$ M, respectively. Data were recorded from six neurons. \*\*,  $P < 0.01$  versus controls; #,  $P < 0.05$ ; ##,  $P < 0.01$  versus iptakalim treatment. e and f, spontaneous excitatory postsynaptic currents from six neurons. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$  versus controls. All data are expressed as means  $\pm$  S.D.

**Protective Effects against Neuronal Ischemic Injury.** To test the neuroprotective effects of iptakalim *in vitro*, we used high concentrations of glutamate to mimic ischemic injury in PC-12 cells. After treatment with 5 mM glutamate for 24 h, cell viability was assessed by an MTT assay. With increased doses, iptakalim significantly attenuated glutamate-induced cytotoxicity; this effect was reversed by coapplication of glibenclamide (Fig. 3A).

We also explored the effects of iptakalim on neuronal apoptosis using cultured cortical neurons subjected to hypoxia-hypoglycemia. Flow cytometry was used to measure the DNA content of the neurons and to assess the percentage that was apoptotic. Neurons exposed to hypoxia appeared in the 'G<sub>1</sub> subpeak', and the proportion of apoptosis was  $23.8 \pm 7.4\%$  after 16 h of hypoxia, higher than in the control ( $2.3 \pm 1.2\%$ ). Iptakalim at concentrations of 0.1, 1, and 10  $\mu\text{M}$  decreased the proportion of apoptosis dose dependently, and this effect was blocked by simultaneous application of glibenclamide (Fig. 3B). Transmission electron microscopy showed that the neurons in the model group displayed typical apoptotic features such as shrinking of the cell body, chromatin condensation, and a crescent structure. Iptakalim thus significantly attenuated apoptotic cell death after hypoxia (Fig. 3C).

**Protective Effects against Brain Ischemic Injury.** At first, we used the gerbil bilateral carotid artery occlusion model of global ischemia to investigate the potential efficacy of iptakalim. In behavioral measurements, treatment with iptakalim dose-dependently reduced the increases of square crossings and rears counted that were evoked by global cerebral ischemia. Ketamine, the positive control drug, significantly decreased only the rears counted (Figs. 4, A and B). The T-maze task revealed significant ischemic working-memory impairments that were not improved by ketamine, whereas iptakalim markedly increased the percentage of correct choices made by the ischemic gerbils to obtain a food reward (Fig. 4C). Thus, intraperitoneally administered iptakalim reduces the increase of locomotor activity evoked by ischemia and alleviates ischemia-induced working-memory impairments. We also tested the effects of iptakalim histologically. Hematoxylin and eosin staining showed that extensive losses (overall counted averaged  $\sim 15\%$  of sham group) of hippocampus CA1 zone pyramidal neurons in gerbils were induced by global ischemia for 5 min. Treatment with iptakalim dose-dependently suppressed ischemia-evoked hippocampus damage and increased the viable CA1 neurons counted of hippocampus. Treatment with ketamine significantly decreased the number of necrotic neurons and increased the remaining number of healthy neuron in hippocampus CA1 zone ( $\sim 79\%$  of sham group) (Fig. 4E). A TUNEL test showed that ischemia-evoked apoptotic neuronal death in the CA1 zone of the hippocampus was attenuated by ketamine and by an increased dose of iptakalim (Fig. 4f). Once ischemic damage occurs, various transmitters in brain often change accordingly. To investigate the effects of iptakalim on hippocampal amino acid transmitters during global cerebral ischemia, the levels of amino acids were assayed by high-performance liquid chromatography analysis 60 min after operation. Iptakalim had no effect on the concentrations of these transmitters under nonischemic conditions (data not shows), but it reversed the ischemia-evoked increases in glutamate, aspartic acid, glutamine, and glycine. However, it did not reverse the ischemia-induced increases in

$\gamma$ -aminobutyric acid or taurine (representative results are shown in Fig. 4D).

We also evaluated the therapeutic effects of iptakalim on the model of stroke induced by salt load in stroke-prone spontaneously hypertensive rats (SHRsp). Iptakalim dose-dependently decreased the incidence and mortality of stroke and delayed the onset and prolonged survival time after onset; nimodipine had similar effects (Figs. 5, A and B). The neurological deficit scores within 14 days after the onset of stroke showed that iptakalim and nimodipine alleviated these deficits significantly compared with vehicles (Fig. 5C). The systolic blood pressure of SHRsp in vehicle group increased continuously during the experimental period. Under the same experimental conditions, 0.25, 1.0, and 4.0 mg of iptakalim/kg/day decreased SBP significantly by around 20, 32, and 41 mm Hg, respectively ( $P < 0.01$ ). The heart rates remained unchanged by iptakalim at doses of 0.25 and 1.0 mg/kg/day ( $P > 0.05$ ) but were decreased markedly by around 40 beats/min when treated with 4.0 mg of iptakalim/kg/day ( $P < 0.01$ ).

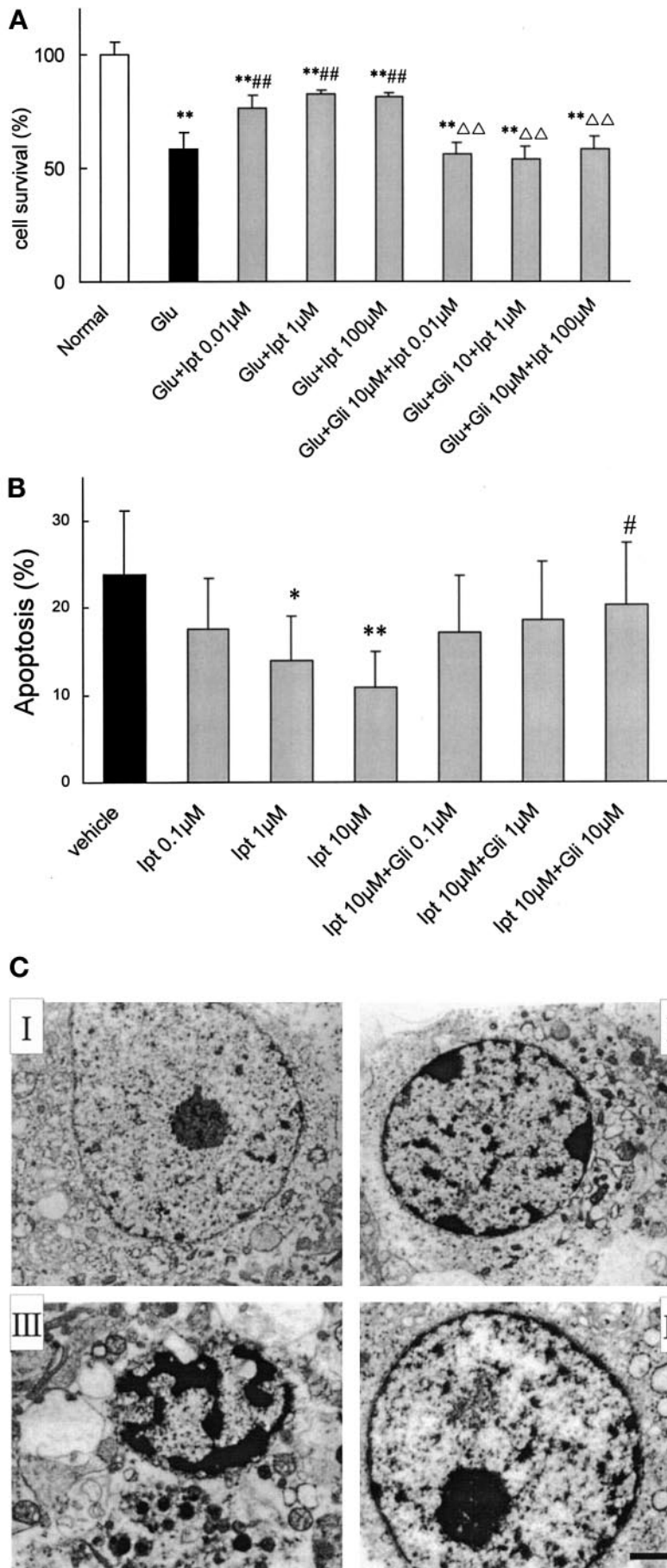
## Discussion

Potassium channels, including K<sub>ATP</sub> channels, mediate the actions of some classic drug targets, such as antidiabetic drugs (Kazic and Gojkovic-Bukarica, 1999). According to the development of central nervous system (CNS) therapeutics, the specific targeting of potassium channels is relatively new. Herein, we suggest a new antistroke approach by the augmentation of an endogenous regulator, the K<sub>ATP</sub> channel. Iptakalim has specific binding sites in the brain and is believed to open this channel moderately. Iptakalim also regulates the mRNA expression of inwardly rectifying K<sup>+</sup> channel 6.x and SUR, two major subunits of the K<sub>ATP</sub> channels (Liu et al., 2003). In this study, we found that iptakalim suppressed neuronal necrosis and apoptosis induced by hypoxia and by high glutamate concentrations in cell culture; moreover, in an animal model, iptakalim not only inhibited neuronal necrosis and apoptosis but also definitely protected brain function. These *in vivo* and *in vitro* results suggest that iptakalim may be neuroprotective in ischemic stroke and in other short- or long-term neurodegenerative diseases.

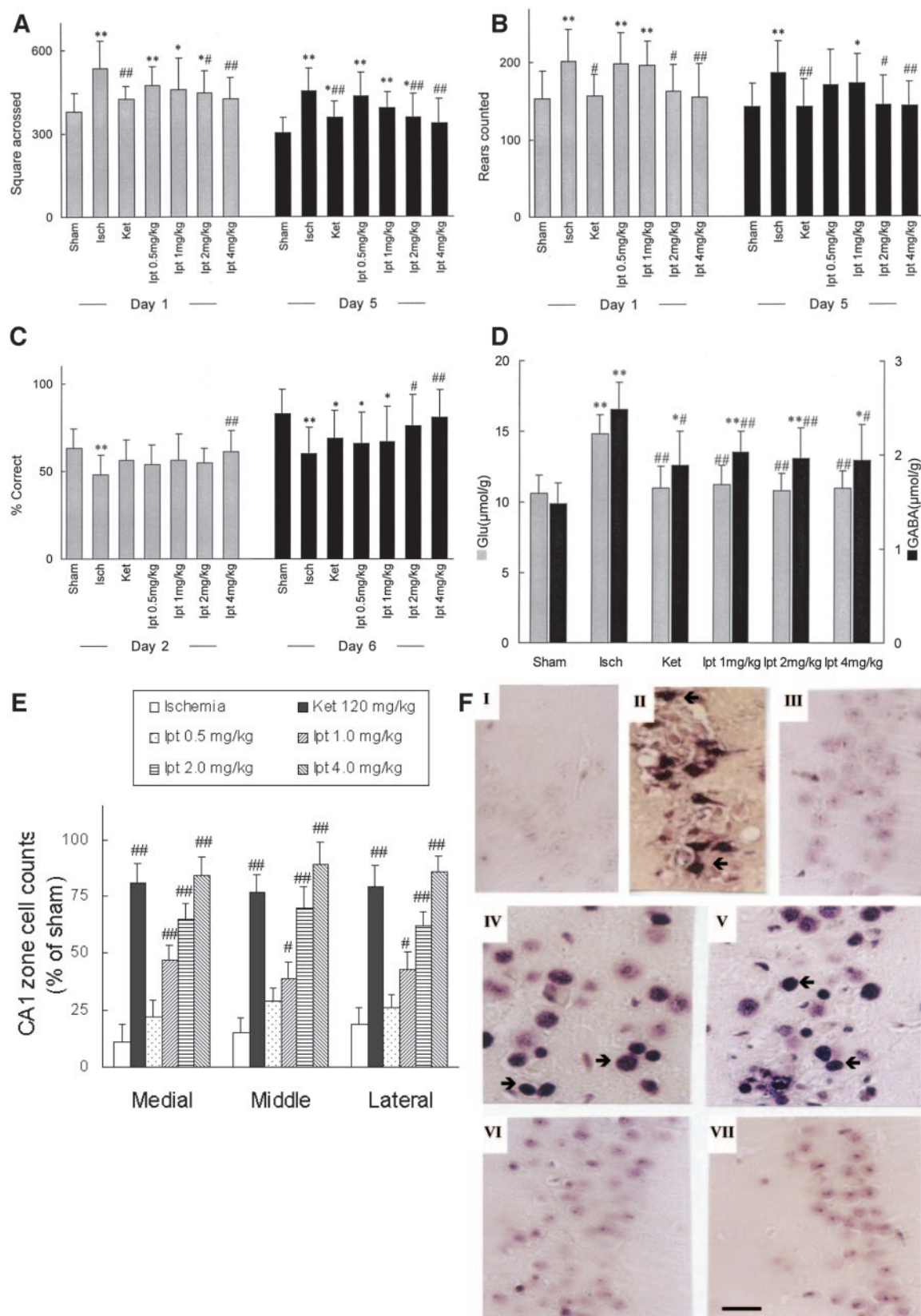
Besides nerve tissues, K<sub>ATP</sub> channels are also found in other tissues, such as pancreatic islets, cardiac muscles, and vascular smooth muscles (Fujita and Kurachi, 2000). Iptakalim shows no significant or detectable effects on heart rates, insulin, and glucagon excretion (H. Wang, unpublished observations). However, it demonstrated selective effects on reducing blood pressure in hypertensive animals. (Wang, 2003) Because hypertension is the major risk factor of stroke, this aspect of iptakalim is likely to expand its therapeutic range.

Application of iptakalim regulated the pathology of multiple neurotransmitter release, inhibiting the excess release of excitotoxic amino acids and glycine from sensitive brain regions induced by acute global ischemia in gerbils. Nevertheless, it never affected these in normal animals. This selectivity implies that the action of iptakalim may be selective for ischemic cells, with little influence on normal ones.

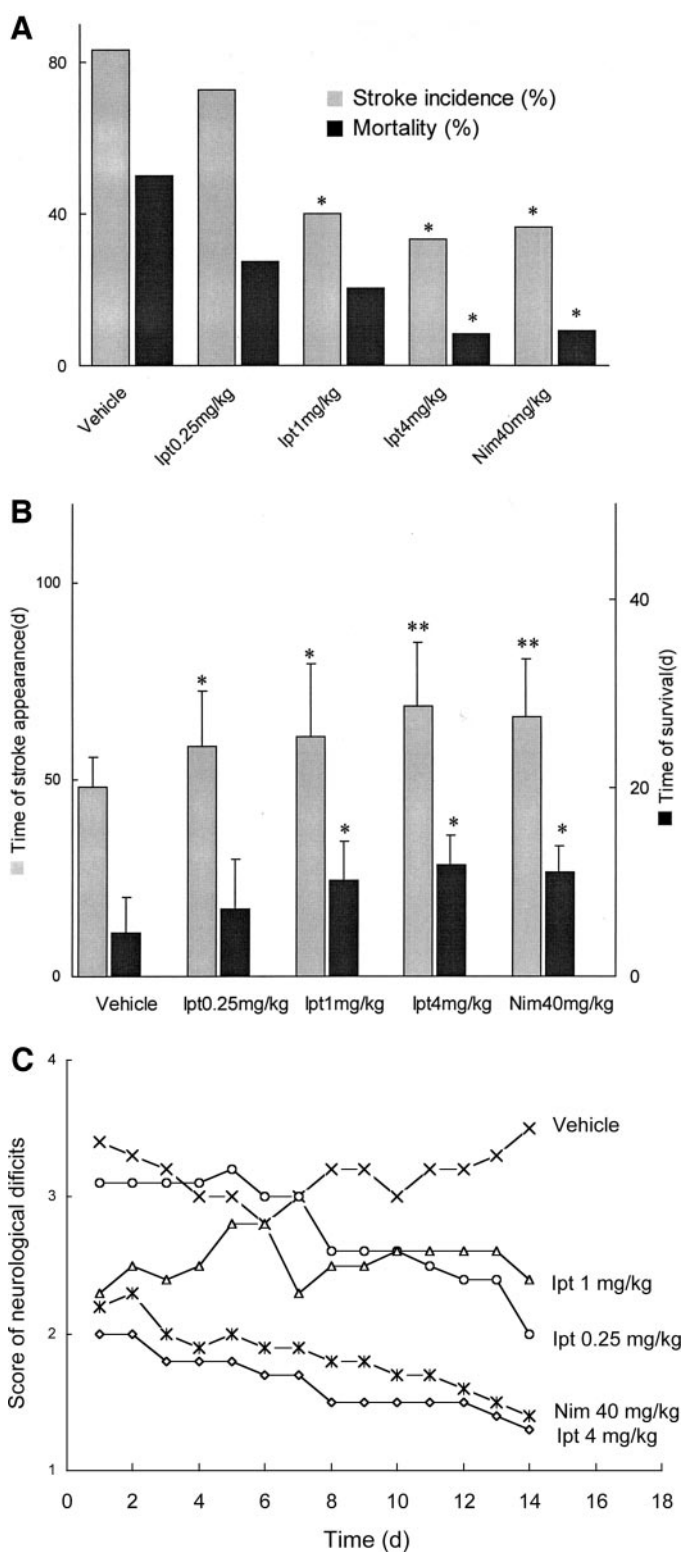
In addition to neuroprotective virtues, effective neuroprotectants should enter the brain easily. After oral (p.o.) administration, iptakalim quickly enters the mouse brain at



**Fig. 3.** Neuroprotective properties of iptakalim (Ipt) in cell culture. A, effects of iptakalim and glibenclamide (Gli) against glutamate (Glu)-induced cytotoxicity in PC12 cells ( $n = 6$ ). \*\*,  $P < 0.01$  versus normal; ###,  $P < 0.01$  versus glutamate treatment;  $\triangle\triangle$ ,  $P < 0.01$  versus treatment with glutamate and iptakalim. B, effects on apoptosis induced by hypoxia in rat cortical neurons. Data are expressed as means  $\pm$  S.D. Ten thousand neurons were counted in each group. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$  versus the hypoxic vehicle group. #,  $P < 0.05$  versus the 10  $\mu$ M Ipt group. C, apoptosis shown by transmission electron microscopy. I, normal neurons; II, III, hypoxic neurons; IV, the neurons were treated with Ipt at 10  $\mu$ M under hypoxic conditions. Bar, 1  $\mu$ m.



**Fig. 4.** Neuroprotective properties of iptakalim (Ipt) in globally ischemic gerbils. A–C, open field locomotion scores (a and b) and working memory activity in T-maze (C) ( $n = 8$ ). D, contents of glutamate (Glu) and GABA in hippocampus ( $n = 7$ ). Data are expressed as means  $\pm$  S.D. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$  versus sham-operated group; #,  $P < 0.05$ ; ##,  $P < 0.01$  versus ischemia group. E, normal cell counts (percentage of sham) in medial, middle, and lateral sector of CA1 zone in dorsal hippocampus of gerbils with global cerebral ischemia. Data are expressed as mean  $\pm$  S.D.,  $n = 6$ . #,  $P < 0.05$ ; ##,  $P < 0.01$  versus ischemia group. F, TUNEL staining of CA1 pyramidal neurons in dorsal hippocampi of sham-operated, ischemic, and ketamine (Ket)-treated gerbils treated with iptakalim at 0.5, 1.0, 2.0, and 4.0 mg/kg (I–VII). Short arrowheads indicate TUNEL-positive neurons. Bar, 15  $\mu\text{m}$ .



**Fig. 5.** Neuroprotective properties of iptakalim (Ipt) in SHRsp. A, incidence and mortality rates caused by stroke. B, time of stroke appearance and survival. There were 12 animals in each group. Data are expressed as means  $\pm$  S.D. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$  versus vehicle-treated group. C, scores of neurological deficits after the onset of stroke.

stable levels ( $T_{\max} = 30$  min;  $C_{\max} = 2.25$   $\mu\text{g/g}$  in brain; 3 mg/kg, p.o.) with a brain: plasma ratio of 3.2. The drug concentration in brain decreased to 18% of  $C_{\max}$  within 3 h

and reduced to a very low level at 6 h after administration (H. Wang, unpublished observations). The blood-brain barrier is usually the rate-limiting step in the translation of many neuroprotective molecules, such as neurotrophin, into clinically effective neurotherapeutics (Pardridge, 2002). Iptakalim penetrates the brain quickly and freely, even when taken orally. This profile may be attributed to its unusual chemical structure and low molecular weight.

Glutamate, the major neurotransmitter in the CNS, is the critical cause of excitotoxicity in CNS pathology (Choi, 1988). We have shown that iptakalim, via its action on neuronal K<sub>ATP</sub>, decreases the toxicity of glutamate and exerts protective effects. The evidence comes from three sets of results: a) inhibition of glutamate release by opening the presynaptic K<sub>ATP</sub> channels both in vivo and in vitro; b) inhibition of glutamate receptor activity by opening the postsynaptic K<sub>ATP</sub> in patch-clamp experiments; and c) enhancement of uptake by the glutamate transporter, and decrease in glutamate concentration in the synaptic cleft by opening the K<sub>ATP</sub>. Because glutamate transporter functional deficiency is found in many neurodegenerative diseases (Vandenberg, 1998; Ferrarese et al., 2000), researchers are looking for drugs that effectively stimulate this transporter. That glutamate uptake can be enhanced through K<sub>ATP</sub> channel opening suggests a clue for developing novel drugs.

In the binding test, pinacidil competed with [<sup>3</sup>H]iptakalim, with regular inhibition curves extrapolating to complete inhibition at saturation, which is compatible with a direct competition for a homogeneous class of binding sites of iptakalim. The fact that glibenclamide did not inhibit [<sup>3</sup>H]iptakalim binding indicated that it was binding to a class of site that is different from iptakalim. Glibenclamide is a specific blocker of K<sub>ATP</sub> channels and has a specific binding site for SUR subunits of K<sub>ATP</sub>. The opening of K<sub>ATP</sub> by KCO or other modulators could be significantly inhibited by glibenclamide, but the blocker may not occupy the same binding site of some kind of KCO directly, just like iptakalim. The possible explanation may be that glibenclamide binds to a different binding site negatively allosterically coupled to the iptakalim site. It will change the conformation of SUR and thus regulate the specific binding of iptakalim in the negative allosteric manner in brain membranes. This hypothesis could be supported by our results in cardiac membranes (Cui et al., 2004). In that study, the specific bindings of [<sup>3</sup>H] glibenclamide could not be displaced by iptakalim or pinacidil, and iptakalim could accelerate the dissociation kinetic process of [<sup>3</sup>H] glibenclamide binding with SUR without affecting the association kinetic process. That indicated the allosteric modulation of iptakalim on the binding sites of K<sub>ATP</sub> blocker glibenclamide.

Iptakalim is relatively safer with few side effects (H. Wang, unpublished observations). In short-term toxicity studies, iptakalim has shown low toxicity in mice, rats and dogs. The LD<sub>50</sub> values are 63 mg/kg (i.v.) and 338 mg/kg (p.o.) in mice and 413 mg/kg (p.o.) in rats. In dogs, the highest nonfatal dose is 205 mg/kg (p.o.). In long-term toxicity studies, no iptakalim-related cytotoxic effects were observed after oral administration for 180 days at doses up to 64 mg/kg/day in rats. Iptakalim was not genotoxic when evaluated in a battery of in vitro and in vivo assays, and was not teratogenic in mice. In another series of experiments, at the therapeutic doses, iptakalim exhibits no side effects on the central ner-

vous, respiratory, digestive, and endocrine systems. Therefore, this compound has wide potential safe dose range if used for antistroke therapy.

In conclusion, iptakalim influences the multiple-pathway glutamatergic system via  $K_{ATP}$  channel activation and produces definite neuroprotection in vivo and in vitro. In addition, this compound passes the blood-brain barrier readily and shows less adverse effects in animals. Iptakalim can be expected to develop into a potential candidate compound against stroke.

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