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Tetraethylammonium exacerbates ischemic neuronal injury in rat cerebrocortical slice cultures

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Abstract

We investigated potential contribution of K^+ channel activity to regulation of ischemia-induced neuronal injury, using cerebrocortical slice cultures. Exposure of cultures to a glucose-free conditioning solution containing sodium azide and 2-deoxyglucose caused neuronal cell death as assessed by cellular uptake of propidium iodide, which was prevented by MK-801, an *N*-methyl-D-aspartate (NMDA) receptor antagonist. Application of tetraethylammonium markedly exacerbated ischemic neuronal injury. Charybdotoxin, a blocker of large-conductance Ca^{2+}-activated K^+ (BK_{Ca}) channels, also augmented ischemic injury, whereas AM 92016, a blocker of delayed rectifier K^+ channels, and dequalinium, a blocker of small-conductance Ca^{2+}-activated K^+ channels, had no significant effect. In addition, tetraethylammonium and charybdotoxin were effective in augmenting NMDA-induced neuronal injury. These results present unprecedented evidence for the ability of tetraethylammonium to enhance ischemic neuronal death, and suggest that BK_{Ca} channels constitute an endogenous system to protect cortical neurons from ischemic injury, via prevention of NMDA receptor over-activation.

Keywords: BK_{Ca} (large-conductance Ca^{2+}-activated K^+ channel); Cerebral cortex; Chemical ischemia; Excitotoxicity; Neuronal death

1. Introduction

Due to great demand of oxygen and glucose in the maintenance of their integrity and functions, central nervous system neurons are particularly vulnerable to ischemic insults. Transient ischemia in vivo or simulated ischemic conditions in vitro is sufficient to induce robust injury on neuronal cells in the forebrain structures (Snider et al., 1999). Substantial efforts have been devoted to the development of effective means to protect central neurons from ischemic injury, yielding some promising yet unsatisfactory achievements (Lo et al., 2003).

The cascade originating from ischemic insults and leading eventually to neuronal cell death involves a wide variety of cellular events, some of which directly participate in the transduction of death-inducing signals, whereas others may modulate death signaling indirectly. During ischemic insults, massive amounts of excitatory amino acids such as glutamate are released either synaptically or non-synaptically (Nishizawa, 2001; Fujimoto et al., 2004). Excessive activation of glutamate receptors causes membrane depolarization as well as a large amount of Ca^{2+} influx into neuronal cytoplasm, which is directly coupled to neuronal death-inducing cascade. On the other hand, an increasing number of evidence indicates that central nervous system tissues do not merely surrender to ischemic insults, but rather, are able to mobilize diverse sets of endogenous mechanisms to protect neuronal cells (Sapolsky, 2001). Among the first line of defense mechanisms are ion channels located on neuronal plasma membranes that can regulate cell excitability.

K^+ channels play a major role in maintaining resting membrane potentials, causing membrane repolarization after neuronal firing, and mediating the actions of several neurotransmitters. In the context of ischemic neuronal

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injury, ATP-sensitive K^+ channels have received attention because they are expected to open in response to decreases in intracellular ATP levels that occur under ischemic conditions, and hyperpolarize neurons to prevent over-excitation and subsequent cellular injury. In fact, drugs that modulate the activities of ATP-sensitive K^+ channels affect ischemic and exicitotoxic neuronal injury both in vitro and in vivo (Heurteaux et al., 1993; Lauritzen et al., 1997). Contribution of other classes of K^+ channels to regulation of ischemic neuronal injury, however, remains largely unknown. It is also noteworthy that opening of K^+ channels may promote, rather than prevent, the induction of cell death under several circumstances. Yu et al. (1997, 1999) have demonstrated that tetraethylammonium (TEA)-sensitive delayed rectifier K^+ channels, by promoting efflux of cytosolic K^+, mediate the induction of apoptosis of cortical neurons. In the present study we investigated potential regulation of ischemic neuronal injury by K^+ channel activity, using cerebrocortical slice cultures. In contrast to the apoptosis model, we found that TEA exacerbates ischemic neuronal injury, possibly by blockade of large-conductance Ca^{2+}-activated K^+ (BK_{Ca}) channels.

2. Materials and methods

2.1. Drugs and chemicals

Minimal essential medium/HEPES (GIBCO 12360-038), Hank's balanced salt solution (GIBCO 24020-117), heat-inactivated horse serum (GIBCO 26050-088), and penicillin G potassium/streptomycin sulfate (GIBCO 15140-122) were obtained from Invitrogen Japan (Tokyo, Japan). Tetraethylammonium was obtained from Sigma-Aldrich Chemical (St. Louis, MO, USA). MK-801 and AM 92016 were from Tocris Cookson (Bristol, UK). Propidium iodide was from Wako Chemicals (Osaka, Japan), and toluidine blue was from Chroma (Kongen, Germany). Other chemicals were obtained from Nacalai Tesque (Kyoto, Japan).

2.2. Organotypic slice cultures

Organotypic cortical slice cultures were prepared essentially according to the methods described previously (Noraberg et al., 1999; Shirakawa et al., 2002). Briefly, we anesthetized postnatal day 2 or 3 Wistar rats (Nihon SLC, Shizuoka, Japan) by chilling them in ice for 2–3 min, decapitated them, and removed the brain from the skull and cut it into two hemispheres. Coronal slices (thickness at 300 μm) of each hemisphere at rostro-caudal levels containing the parietal cortex and the striatum were cut with a tissue chopper (Narishige, Tokyo, Japan) under sterile conditions. Cortico-striatal region was dissected out of each slice so that the cultivated tissue did not contain other brain regions such as the septum and the basal forebrain. Slices were transferred onto a Millicell-CM insert membrane (30 mm in diameter, Millipore, Bedford, MA, USA) in 6-well plates (Corning Costar, Tokyo, Japan). Six slices were placed on each culture insert. Culture medium, consisting of 50% minimal essential medium/HEPES, 25% Hanks' balanced salt solution and 25% heat-inactivated horse serum supplemented with 6.5 g/l glucose, 2 mM L-glutamine, 100 U/ml penicillin G potassium and 100 μg/ml streptomycin sulfate, was supplied at 700 μl/well. The 6-well plates were placed in a 34 °C, 5% CO_2 humidified atmosphere in an incubator. Culture medium was exchanged with fresh one on the next day of culture preparation, and thereafter, every 2 days.

2.3. Ischemic and excitotoxic insults, and evaluation of cell injury

On 11 days in vitro, culture medium was replaced with serum-free medium consisting of 75% minimal essential medium/HEPES and 25% Hanks' balanced salt solution supplemented with 6.5 g/l glucose, 2 mM L-glutamine, 100 U/ml penicillin G potassium and 100 μg/ml streptomycin sulfate. On 12 days in vitro, the medium was exchanged to Ringer's buffer (124 mM NaCl, 4.9 mM KCl, 1.3 mM $MgSO_4$, 2 mM $CaCl_2$, 1.2 mM KH_2PO_4, 25.6 mM $NaHCO_3$ and 10 mM glucose, pH 7.4). After incubation for 1 h, ischemic insult was applied to the cultures by transfer of culture inserts to the plates with the conditioning solution (700 μl/well). The conditioning solution was glucose-free Ringer's buffer supplemented with 3 mM sodium azide and 10 mM 2-deoxyglucose (Kume et al., 2002). After ischemic treatment for 0–120 min, culture inserts were transferred to the plates with serum-free culture medium containing 2 μM propidium iodide, and maintained for further 24 h. Drugs were added to the Ringer's buffer for 1 h pre-incubation, to the ischemic conditioning solution, and to the culture medium for 24 h post-incubation. In experiments on *N*-methyl-D-aspartate (NMDA) cytotoxicity, culture medium was replaced with serum-free medium on 11 days in vitro, and subsequent treatment with NMDA for 24 h was done with serum-free medium of the same composition but supplemented with 2 μM propidium iodide. Drugs were added to the culture medium 1 h prior to the application of NMDA, and were also present during treatment with NMDA.

At the end of the culture period, propidium iodide fluorescence of the parietal region of each slice was observed using an inverted fluorescence microscope (TE300, Nikon, Tokyo, Japan) with a rhodamine filter set (Nikon G-2A; EX 510–560 nm/BA 590 nm) and a 20× objective lens. Fluorescence images corresponding to a field of 512×480 μm were captured by ARGUS-20 image processing system (Hamamatsu Photonics, Hamamatsu, Japan) through a CCD camera equipped with an image intensifier (C-2400-87, Hamamatsu Photonics), and were stored as image files of 512×480 pixels. In each experiment, one culture insert was treated with 100 μM NMDA for 24 h to determine the degree of standard injury. The

sensitivity of the image intensifier was set on the basis of the fluorescence signal in cultures that underwent the standard injury, so that the signals did not reach saturated levels. Images of all experimental groups in one experiment were obtained at the same sensitivity of the image intensifier. Stored images were then analyzed with NIH image 1.62 software. The signal intensity of each pixel was expressed as an 8-bit value (0–255), and the average signal intensity in 200×200 pixels located at the center of the image was obtained as the fluorescence value of the respective slice. Fluorescence values of experimental groups were normalized with the mean of the fluorescence values obtained from six slices that received standard injury as 100%. Data shown in figures are from a representative set of experiments, and each column shows mean+S.E.M. of the values obtained from six slices in one culture insert. Reproducibility of the results was confirmed by two or three different sets of experiments.

In several experiments, Nissl staining was performed after observation of propidium iodide fluorescence. Cultures were fixed overnight with 4% paraformaldehyde solution containing 5% sucrose, and after rinsing with distilled water three times, they were immersed in 0.1% toluidine blue solution for 20 min. After thorough wash with distilled water, specimens were dehydrated with graded ethanol, mounted on slides with glycerol and observed with a bright-field upright microscope.

2.4. Statistical analysis

Statistical significance of differences was evaluated with one-way analysis of variance followed by Student–Newman–Keuls' test. Probability values less than 5% were considered significant.

3. Results

3.1. Chemical ischemia causes cell death in cortical slice cultures

Cortico-striatal slices were maintained for 11 days in culture. During cultivation the striatal region within each slice was gradually flattened, and often, monolayer of cells remained. On the other hand, the cortical region maintained much thicker appearance than the striatal region, and Nissl staining confirmed existence of multi-cellular (usually 4 to 5) layer throughout the cortical region of healthy slice cultures. Simulated ischemia was achieved by exposure of these slice cultures to a glucose-free conditioning solution containing sodium azide and 2-deoxyglucose. This treatment induced a remarkable cellular injury as assessed by Nissl staining and propidium iodide uptake after 24 h (Fig. 1A).

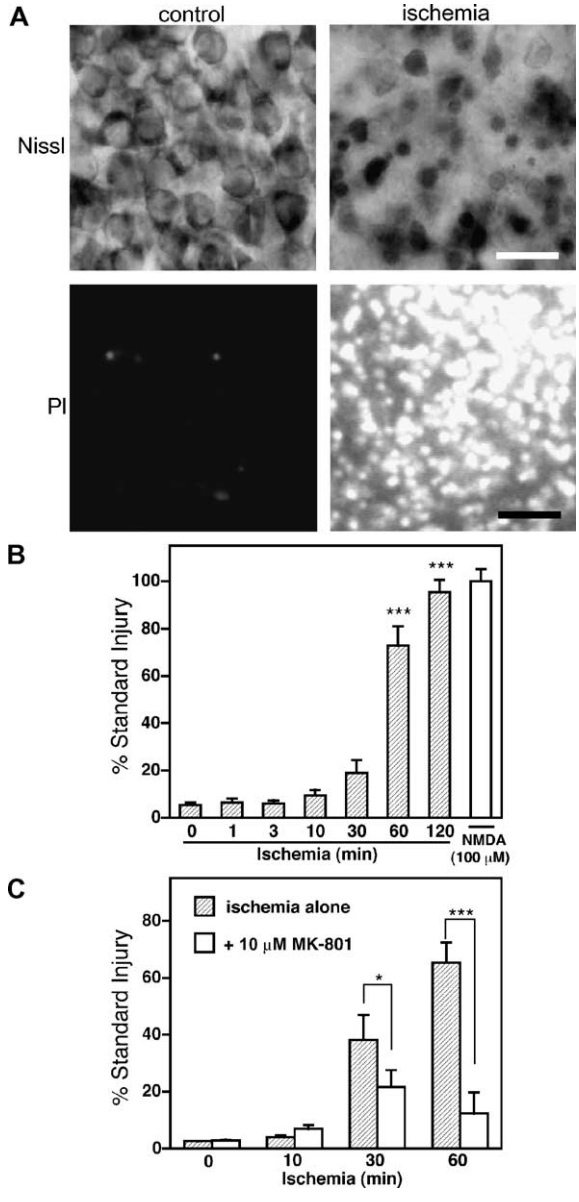

Fig. 1. Neuronal injury is induced by chemical ischemia in cortical slice cultures. (A) Typical images of cortical slice cultures. Top row, bright-field images where cell morphology was visualized by Nissl staining. Slices maintained under control conditions possessed numerous neuronal cells with round or oval cell bodies (left), whereas many pyknotic cells were observed in slices that underwent 60 min ischemia and 24 h post-incubation (right). Bottom row, fluorescence images after 24 h incubation of cortical slice cultures with propidium iodide (PI)-containing medium. Slices received treatment with conditioning solution to induce chemical ischemia for 0 min (left) or 60 min (right) before incubation with propidium iodide. Scale bars, 25 μm for Nissl images and 50 μm for propidium iodide images. (B) Relationship between the intensity of propidium iodide fluorescence and the duration of incubation with the conditioning solution. Slice cultures received treatment with the conditioning solution for indicated durations (0–120 min), and fluorescence intensity was measured after 24 h of incubation with propidium iodide. Data are normalized by setting the mean of the fluorescence intensities in six slices treated with 100 μM NMDA for 24 h (standard injury) as 100%. n=6 slices for each treatment group. ***P<0.001 vs. 0 min ischemia. (C) Slice cultures received treatment with the conditioning solution for indicated durations (10, 30 and 60 min), and fluorescence intensity was measured after 24 h of incubation with propidium iodide. MK-801 (10 μM) was applied 1 h before the treatment with the conditioning solution and was present during the entire period of incubation until observation of propidium iodide fluorescence. n=6 slices for each treatment group. *P<0.05, ***P<0.001.

Nissl staining revealed that ischemic treatment for 60 min resulted in appearance of numerous cells with pyknotic morphology, which were in contrast to healthy cells with round or oval cell bodies in control cultures. At the same time, robust increases in propidium iodide uptake were observed in slices that received 60 min ischemia.

The extent of cellular injury was dependent on the duration of exposure to the conditioning solution (Fig. 1B). Exposure time of less than 30 min caused no apparent injury as monitored by propidium iodide uptake, whereas marked increases in propidium iodide fluorescence were observed in cultures treated with the conditioning solution for 30 min or more. A significant increase in propidium iodide fluorescence was obtained with 60 or 120 min exposure.

Under ischemic conditions, the excitatory neurotransmitter glutamate is massively released either synaptically or non-synaptically (Nishizawa, 2001). To examine whether activation of NMDA subtype of glutamate receptors is involved in ischemic neuronal injury, MK-801 (10 μM), an non-competitive blocker of NMDA receptors, was applied 1 h before ischemic treatment and was present in culture medium until the time of observation of propidium iodide fluorescence. MK-801 markedly and significantly inhibited the increase in propidium iodide fluorescence induced by 30–60 min exposure to ischemic conditions (Fig. 1C), confirming that NMDA receptor-mediated events play a crucial role in neuronal cell injury.

3.2. TEA exacerbates ischemic injury

In an attempt to clarify the roles of K^+ channels in regulation of ischemic neuronal death, we examined the effect of TEA, a conventional K^+ channel blocker. TEA was applied 1 h before ischemic treatment and present during the entire incubation period thereafter. TEA at concentrations of 1 and 5 mM markedly exacerbated neuronal injury induced by 30 min of ischemic treatment (Fig. 2A and B). At a lower concentration of 500 μM, TEA showed a tendency to augment neuronal injury induced by 30 min ischemia, but the difference did not reach statistical significance (Fig. 2B). TEA showed no significant effect on neuronal injury induced by 60 min ischemia, which is most likely due to the nearly maximal injury caused by this prolonged period of ischemic insult (data not shown). Injury induced by 30 min ischemia plus 1 mM TEA was markedly attenuated by co-application of 10 μM MK-801 (Fig. 2C), indicating that NMDA receptor activation is also crucial for the exacerbated neuronal injury under these conditions.

3.3. BK_{Ca} channel blocker mimics the effect of TEA

The above results suggest that the activity of TEA-sensitive K^+ channels plays an important role in restricting ischemic neuronal injury. TEA has a broad spectrum of blocking actions on diverse sets of K^+ channels, but at relatively low concentrations (≤1 mM), it blocks only a few

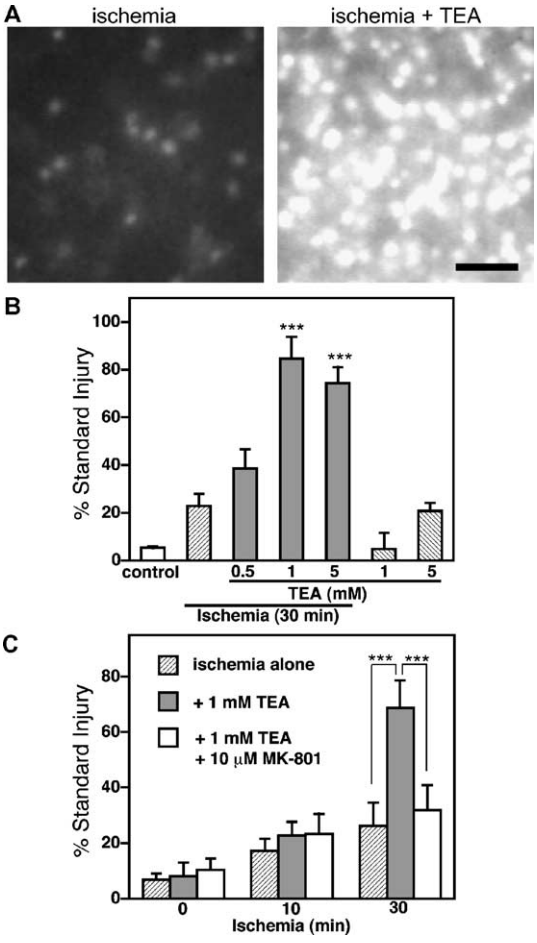

Fig. 2. TEA exacerbates ischemic neuronal injury. (A) Representative images of propidium iodide fluorescence obtained from a slice that received 30 min ischemia (left) and a slice that received 30 min ischemia plus 1 mM TEA (right). Scale bar, 50 μm. (B and C) Slice cultures received treatment with the conditioning solution for indicated periods (30 min in B; 10 and 30 min in C), and fluorescence intensity was measured after 24 h of incubation with propidium iodide. TEA at indicated concentrations (0.5, 1 and 5 mM in B; 1 mM in C) and MK-801 (10 μM) was applied 1 h before the treatment with the conditioning solution and was present during the entire period of incubation until observation of propidium iodide fluorescence. n=6 slices for each treatment group. ***P<0.001 (in B, vs. ischemia alone).

K^+ channel types. In particular, BK_{Ca} channels and delayed rectifier voltage-dependent K^+ channels are effectively blocked by TEA (Coetzee et al., 1999). To determine which type of K^+ channels was responsible for the effect of TEA on ischemic injury, we examined effects of drugs that selectively block either BK_{Ca} channels or delayed rectifier K^+ channels. We first examined the effect of charybdotoxin, a selective blocker of BK_{Ca} channels (Coetzee et al., 1999). Application of charybdotoxin at a concentration of 300 nM caused a remarkable enhancement of neuronal injury by 30 min ischemia (Fig. 3A).

AM 92016 is a sotalol analog that is devoid of β-adrenoceptor blocking actions and affords a nearly complete blockade of delayed rectifier K^+ current in heart cells at a concentration of 1 μM (Connors et al., 1992; Lei and Brown, 1998). To clarify the possible involvement of

delayed rectifier K^+ channels in regulation of ischemic neuronal injury, AM 92016 at 5 μM was applied 1 h before ischemic treatment and present in culture medium during the entire incubation period thereafter. We observed no significant effect of AM 92016 on ischemic neuronal injury (Fig. 3B). Additionally, we examined the effect of dequalinium, a potent blocker of small-conductance Ca^{2+}-activated K^+ channels (Strobaek et al., 2000), and found no significant effect of this drug on ischemic neuronal injury (Fig. 3C).

3.4. TEA exacerbates NMDA-induced cell death

As noted above (Figs. 1C and 2C), over-activation of NMDA receptors plays a significant role in induction of ischemic neuronal injury. BK_{Ca} channels may be located in the vicinity of extrasynaptic NMDA receptors in central nervous system neurons (Issacson and Murphy, 2001). It is likely, therefore, that BK_{Ca} channels open in response to Ca^{2+} influx through NMDA receptor channels and cause hyperpolarization, thereby providing a negative feedback to restrict the activation of NMDA receptor channels. These assumptions prompted us to examine the effects of BK_{Ca} channel blockade on neuronal injury induced by direct application of NMDA to cortical slice cultures (Fig. 4). Application of NMDA for 24 h at a concentration of 10 μM did not cause a significant injury, whereas NMDA at 15 μM caused a robust injury as demonstrated by a large increase in propidium iodide fluorescence. When 1 mM TEA was applied 1 h before application of 10 μM NMDA and present during the entire incubation period, a marked and significant enhancement of neuronal cell injury was observed (Fig. 4A). Injury induced by 15 μM NMDA was not significantly augmented by TEA, due to the nearly maximal injury induced by NMDA alone. Moreover, charybdotoxin at 300 nM produced a similar effect as that of TEA, significantly augmenting neuronal injury by 10 μM NMDA (Fig. 4B).

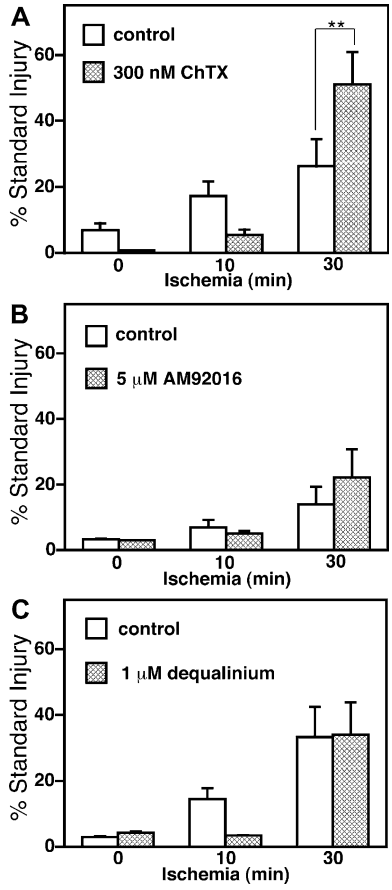

Fig. 3. Effects of K^+ channel blockers on ischemic neuronal injury. Slice cultures received treatment with the conditioning solution for indicated durations (10 and 30 min), and fluorescence intensity was measured after 24 h incubation with propidium iodide. Charybdotoxin (ChTX; 300 nM, A), AM 92016 (5 μM, B) or dequalinium (1 μM, C) was applied 1 h before the treatment with the conditioning solution and was present during the entire period of incubation until the observation of propidium iodide fluorescence. n=6 slices for each treatment group. $^{**}P<0.01$.

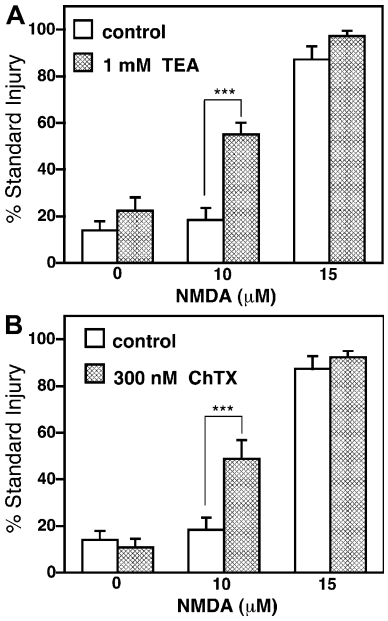

Fig. 4. Effects of K^+ channel blockers on NMDA-induced cell death. Slice cultures were incubated with medium containing NMDA at indicated concentrations (10 and 15 μM) and propidium iodide. Fluorescence intensity was measured after incubation for 24 h. TEA (1 mM, A) or charybdotoxin (ChTX; 300 nM, B) was applied 1 h before the treatment with NMDA and was present during the entire period of incubation until the observation of propidium iodide fluorescence. n=6 slices for each treatment group. $^{***}P<0.001$.

4. Discussion

Ischemic injury leading to neuronal death involves multiple cascades of events. One of the early events of these cascades is glutamate efflux from synaptic (Fujimoto et al., 2004) and/or non-synaptic (Rossi et al., 2000) origins. Excess amount of extracellular glutamate can trigger excitotoxicity by activating neuronal ionotropic glutamate receptors, and involvement of excitotoxic events in ischemic neuronal injury has been widely accepted (Lo et al., 2003).

Indeed, we reported previously that chemical ischemia caused an increase in extracellular glutamate content in dissociated cortical neurons in primary culture, and that NMDA receptor antagonists markedly attenuated neuronal injury in this model (Kume et al., 2002). This was also the case with the present study: neuronal injury was induced in cerebrocortical slice cultures by chemical ischemia, which was blocked by an NMDA receptor antagonist MK-801. These results confirm that NMDA receptors play a pivotal role in ischemia-induced neuronal damage in the cerebral cortex.

Opening of NMDA receptor channels requires removal of voltage-dependent blockade by Mg^{2+} in addition to agonist binding, and therefore, regulation of membrane potentials during early stage of ischemia may profoundly affect the degree of resultant neuronal injury. We found here that a K^+ channel blocker TEA markedly exacerbated ischemic neuronal injury. The concentration of TEA required to produce a significant effect was relatively low, and charybdotoxin mimicked the effect of TEA. These pharmacological profiles are consistent with the view that blockade of BK_{Ca} channels is responsible for exacerbation of ischemic neuronal injury. Expression of BK_{Ca} channel subunit *Slo* is detected in cortical pyramidal neurons (Knaus et al., 1996), and BK_{Ca} channels are implicated in regulation of excitability of cortical neurons such as spike repolarization and rhythmic bursting (Sun et al., 2003; Traub et al., 2003). Thus, blockade of BK_{Ca} channels during ischemic insults may promote membrane depolarization that enables robust activation of NMDA receptor-associated channels triggering excitotoxic events.

Although we did not address potential mechanisms of BK_{Ca} channel activation during ischemic insults, NMDA receptor stimulation per se is likely to recruit this endogenous protective mechanism. In rat olfactory bulb granule cells, stimulation of extrasynaptic NMDA receptors causes a large hyperpolarizing response that is attributable to the activation of BK_{Ca} channels (Issacson and Murphy, 2001). We demonstrated here that neuronal injury induced by application of NMDA was markedly augmented by TEA and charybdotoxin. Therefore, functional coupling between NMDA receptor channels and BK_{Ca} channels is likely to exist in rat cerebrocortical neurons. Indeed, activation of Ca^{2+}-dependent K^+ channels in response to NMDA receptor stimulation has been documented in rat cortical neurons (Backus and Trube, 1993). Coupling of NMDA receptors to hyperpolarizing responses may constitute an important regulatory mechanism that suppresses over-excitation of neuronal cells and protects them from ischemic and excitotoxic neuronal injury. It should also be noted that BK_{Ca} channels may be activated through the mechanisms other than elevation in cytosolic Ca^{2+}. In hippocampal CA1 pyramidal cells, activity of BK_{Ca} channels is enhanced after transient forebrain ischemia (Gong et al., 2000), which may involve oxidation-based changes in channel properties (Gong et al., 2002).

In addition to neuronal cell bodies, BK_{Ca} channels are also present in major fiber tracts and nerve terminals in rat brain (Knaus et al., 1996; Hu et al., 2001). Particularly, BK_{Ca} channels are concentrated in presynaptic compartment, where they may regulate depolarization-induced exocytotic release of neurotransmitters (Yazejian et al., 2000). Further studies are warranted to determine the relative contribution of presynaptic and somatic BK_{Ca} channels to the regulation of neuronal injury.

The present results appear to be in sharp contrast to previous studies showing that TEA prevents neuronal apoptosis induced by several insults including serum deprivation, staurosporine and NMDA (Yu et al., 1997, 1999). This apparent difference may be attributable to the fact that neuronal death in the present experimental model is not apoptotic in nature: our preliminary examinations indicate that caspase inhibitors do not block neuronal death in our ischemic model (data not shown). In fact, Yu et al. (1999) reported that NMDA induced necrosis in cortical neurons in medium containing physiological concentrations of Na^+ and Ca^{2+}, and that apoptosis was observed only under special compositions of extracellular cations (reduced Na^+ and Ca^{2+}). Moreover, the protective effect of TEA observed by Yu et al. (1997, 1999) was due to its blockade of delayed rectifier K^+ channels, and BK_{Ca} current was not detectable in their neuronal cultures (Yu et al., 1999).

On the other hand, TEA has been reported to prevent hippocampal injury following transient forebrain ischemia (Huang et al., 2001) and cortical damage following transient focal ischemia (Wei et al., 2003). Comparison of results from these in vivo experiments with our in vitro results is difficult, with respect to the severity of insults and the modes of cell death involved. However, one study (Huang et al., 2001) reported that TEA was effective in reducing ischemic injury of hippocampal CA1 region only when they were applied after reperfusion, which implies that the observed protective effect of TEA represents a different aspect of K^+ channel involvement from that of our findings. Finally, consistent with our results, protective role of BK_{Ca} channel activity has been demonstrated in ischemic model of hippocampal slice cultures based on oxygen glucose deprivation (Runden-Pran et al., 2002). Taken all of these observations into consideration, we propose that drugs interfering activation of delayed rectifier K^+ channels not only might be beneficial for preventing neuronal death under certain paradigms of ischemic insults, but may also be deleterious unless they are devoid of blocking activity against BK_{Ca} channels.

A number of diverse mechanisms are considered to regulate the induction of neuronal cell death in ischemic neuronal injury (Sapolsky, 2001). BK_{Ca} channels are emerging as a promising candidate of endogenous protective mechanisms to reduce neuronal hyperexcitability and resultant neuronal injury, as BK_{Ca} channel openers exert significant neuroprotective effects against in vivo ischemic neuronal injury (Gribkoff et al., 2001). The in vitro

experimental model used in the present study may provide a useful system to examine the detailed mechanisms of the involvement of endogenous protective mechanisms against ischemic neuronal injury, and may also serve as a fast and convenient experimental systems to screen the efficacy of K^+ channel-acting agents as neuroprotective drugs.

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