Brief Ischemia Decreases Large Conductance Ca^{2+}-activated K^+ Channel Activity in CA1 Pyramidal Neurons From Rat Hippocampus*

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Abstract Preconditioning of the brain with brief ischamia induces tolerance to subsequent lethal periods of ischemia. It has been suggested that the enhancement in large conductance Ca^{2+}-activated potassium (BK_{Ca}) channel activity is involved in the pathogenesis of ischemic neuronal injury. Inside-out configuration of patch clamp techniques were used to investigate the temporal changes in BK_{Ca} channel activity in CA1 pyramidal neurons acutely dissociated from rat hippocampus at 6 h, 24 h and 48 h following 3 min of brief ischemia. There were no changes in channel unitary conductance and reversal potential after brief ischemia. In contrast, a significant decrease in the channel open probability was observed during the first 24 h following brief ischemia. Kinetic analyses showed that the postischemic suppression of BK_{Ca} channel activity was due to a prolongation of the closed time since there was no significant change in open time after brief ischemia. It is suggested that the brief ischemia-induced suppression of BK_{Ca} channel activity may be associated with ischemic tolerance.

Key words K^+ channel, brief ischemia, hippocampus, patch clamp, rat

Brief periods of severe cerebral ischemia cause cellular degeneration of specific neuronal population in both humans and experimental animals[1,2]. These selectively vulnerable neurons are found in the hippocampus, striatum, thalamus, layers 2 and 5 of cerebral cortex and the cerebellar cortex. The CA1 region of the hippocampus is particularly sensitive to ischemic insult among these selectively vulnerable regions. However, preconditioning of the rat brain with a sublethal cerebral ischemia induces resistance to a subsequent lethal ischemia (ischemic tolerance)[3]. A 3-min period of forebrain ischemia in rats produces no appreciable neuronal damage in the CA1 subfield of the hippocampus but can protect against neuronal damage following subsequent longer periods of ischemia, which normally kills CA1 neurons in the hippocampus. Preconditioning-mediated neuroprotection has been shown to initiate several defense mechanisms such as up-regulation of heat shock proteins[3], bcl-2 protein[4], reactive oxygen species[5], as well as an activation of NF-κB[5] while the precise mechanism remains to be elucidated.

In the hippocampus, BK_{Ca} channels are largely responsible for action potential repolarization and generation of the fast afterhyperpolarization (fAHP)[6]. It is also suggested that BK_{Ca} channels may play an important role in regulating neuronal excitability at the resting membrane potential[7]. Recently, there appears to be strong correlations between the enhancement in activities of BK_{Ca} channel and the delayed neuronal death (DND) in CA1 hippocampal neurons after 15-min lethal forebrain ischemia[8~10]. Therefore, we assumed that the suppression of BK_{Ca} channel activity might be involved in ischemic tolerance induced by a sublethal ischemia. To address this question, we used a rat model of forebrain ischemia and examined the temporal changes in activities of BK_{Ca} channels in CA1 pyramidal neurons following 3-min ischemia.

1 Materials and methods

1.1 Animal treatments

Male adult Wistar rats weighing 200~250 g were subjected to transient forebrain ischemia by use of the four-vessel occlusion method[1] with some modifications. In brief, rats were anesthetized with chloral hydrate (i.p., 40 mg/100 g mass) and both common carotid arteries were exposed for subsequent occlusion of these vessels. Both vertebral arteries were electrocauterized permanently. On the following day, the fully awake rats were restrained and the carotid clasps were tightened to produce four-vessel occlusion. Sublethal transient forebrain ischemia was induced by occluding both common carotid arteries for

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3 min. Rectal temperature of the animals was maintained at 37 ℃ during surgery and ischemia with a heating lamp. Rats exposed to ischemia were allowed to survive for 6 h, 24 h, and 48 h respectively, and used for later cell isolation.

1.2 Pyramidal neurons preparation

Neurons were isolated as previously described[11,12]. In brief, rats were anesthetized with chloral hydrate (i.p., 40 mg/100 g mass) and then decapitated. Brains were quickly removed, iced, and blocked for slicing. The blocked tissue was cut into 400 μm slices with a Vibroslice whilst bathed in a low Ca^{2+}, HEPES-buffered salt solution containing 140 mmol/L sodium isethionate, 2 mmol/L KCl, 4 mmol/L $MgCl_2$, 0.1 mmol/L $CaCl_2$, 23 mmol/L glucose, 15 mmol/L HEPES, pH 7.4 (300~305 mOsm/L). Slices were then incubated for 1~6 h at room temperature (20~22 ℃) in a $NaHCO_3$-buffered saline bubbled with 95% O_2-5% CO_2 containing 126 mmol/L NaCl, 2.5 mmol/L KCl, 2 mmol/L $CaCl_2$, 2 mmol/L $MgCl_2$, 26 mmol/L $NaHCO_3$, 1.25 mmol/L NaH_2PO_4, 1 mmol/L pyruvic acid, 0.005 mmol/L glutathione, 0.1 mmol/L Nω-nitro-L-arginine, 1 mmol/L kynurenic acid, 10 mmol/L glucose, pH 7.4 with NaOH (300~305 mOsm/L). Slices were then removed into the low Ca^{2+} buffer, and CA1 region of hippocampus was dissected out under a dissecting microscope and placed into an oxygenated chamber containing pronase (Sigma protease TypeX Ⅳ, 1~1.5 g/L) in HEPES-buffered HBSS (Sigma) at 33 ℃. After 30~45 min of enzyme digestion, tissue was rinsed three times in the low Ca^{2+}, HEPES-buffered saline and dissociated mechanically with a graded series of fire-polished Pasteur pipettes. The cell suspension was then plated into a 35 mm Lux petri dish mounted on the stage of an inverted microscope containing HEPES-buffered HBSS saline. After allowing the cell to settle, the solution bathing the cells was changed to our recording solution.

1.3 Inside-out configuration recording

Gigaseal patch recording using the inside-out configuration (the feed-back resistor was 50 GΩ) were performed on neurons with pyramidal shape. The pipette resistance was 8~12 MΩ and the seal resistance was in excess of 5 GΩ. The composition of the bathing solution was 140 mmol/L Glu-K, 10 mmol/L NaCl, 10 mmol/L HEPES. The desired free calcium of 2 μmol/L was obtained by addition of 485 μmol/L $CaCl_2$ to a 500 μmol/L EGTA solution[9]. The composition of the pipette solution was 140 mmol/L Glu-K, 10 mmol/L NaCl, 10 mmol/L HEPES. $MgCl_2$ at 0.5 mmol/L was routinely added to the pipette solution purely for the convenience of easier seal formation in the absence of Ca^{2+}. Solutions were adjusted to a final pH of 7.4 with KOH. All reagents were obtained from Sigma (St. Louis, MO).

The single-channel currents were recorded using a Nihon Kohden CEZ-2300 patch clamp amplifier with the current filtered (-3 dB, four-pole Bessel filter) at 5 KHz. Data were digitized at sampling rates of 10 KHz using TL-125 KHz interface (Scientific Solution). The analysis routines used Pclamp (version 8.0., Axon Instruments) to determine distributions for channel amplitudes, and open and closed times. An automated 50% threshold crossing routine was used to detect channel transition. The ignored level for detecting events was limited 300 μs. NP_o was determined from data samples of 15-second duration and defined as: $NP_o = \Sigma\{t_1 + 2t_2 + 2t_3 + \ldots + nt_n\}$, where N is channel number, P_o is open probability, and t_1, t_2, t_n are the ratios of open time to total time of measurement for each channel at each of the current levels. All experiments were conducted at room temperature (20~22 ℃).

1.4 Statistical analysis

The data were expressed as $\bar{x} \pm s$ (n = number of experiments) and one-way ANOVA was used for statistical analysis. Statistical significance was at $P < 0.05$.

2 Results

The characteristics of BK_{Ca} channel in inside-out patches from both control and ischemic neurons were consistent with those of reported previously[8,9,11]: voltage dependence, high selectivity to K^+, high unitary conductance, and sensitivity to extracellular TEA and intracellular Ca^{2+}. Figure 1 shows original traces of single-channel current from CA1 neurons

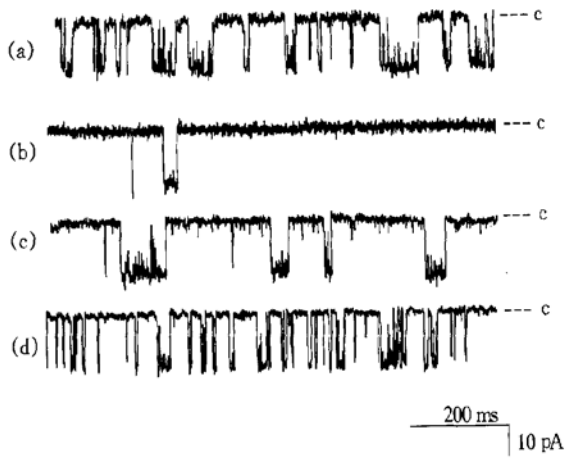

Fig. 1 Original current traces of BK_{Ca} channel

Comparison of BK_{Ca} channel activities recorded from excised, inside-out patches from hippocampal CA1 neurons of rats before and after 3-min ischemia. Traces of single channel current showed a decrease in BK_{Ca} channel activities in postischemic neurons at 6 h (b) and 24 h (c), and returned to control level (a) at 48 h (d) following reperfusion with a holding potential of -60 mV and 2 μmol/L $[Ca^{2+}]_i$. Inward currents evoked are shown as downward deflection. "c" indicate the current level at which all channels were closed.

before and 6 h, 24 h and 48 h after ischemia. There were no differences in the amplitude of single channel currents at a given holding potential among the groups. To determine unitary conductance and reversal potential of BK_{Ca} channels, we measured amplitudes of the single channel currents at different holding potentials and the unitary conductance was determined by fitting a regression line through the data. It was clearly shown from data illustrated in figure 2 that the unitary conductance and reversal potential of BK_{Ca} channels in postischemic neurons remained unchanged within 48 h following reperfusion

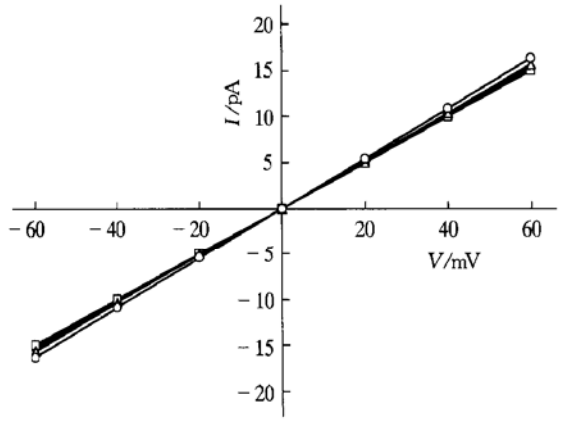

Fig. 2 Unitary conductance and reversal potential of BK_{Ca} channel of subgroup

Comparison of unitary conductance of BK_{Ca} channels in hippocampal CA1 neurons of rats before and after 3-min ischemia. Plots of amplitude (I) of BK_{Ca} channel against holding potentials (mV) in CA1 neurons before (◇) and 6 h (□), 24 h (△) and 48 h (○) after ischemia, showing no apparent changes in channel unitary conductance and reversal potential.

compared with those of control neurons. In contrast, as shown in Figure 1, the activity of BK_{Ca} channels at a holding potential of − 60 mV with 2 μmol/L $[Ca^{2+}]_i$ was obviously lower in postischemic neurons within 24 h reperfusion than that of control neurons and then returned to control level at 48 h after reperfusion. The values of channel open probability in CA1 neurons before and 6 h, 24 h, 48 h after ischemia were 0.23 ±0.05 (n = 13), 0.028 ±0.019 (P< 0.05, n= 7), 0.055 ± 0.022 (P < 0.05, n = 11), 0.11 ±0.025 (P > 0.05, n= 10), respectively . A similar change in open probability was also found at all membrane potentials tested following ischemia (Figure 3).

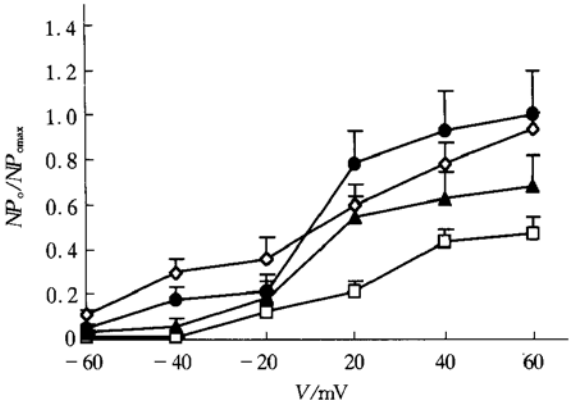

Fig. 3 Comparison of open probability of BK_{Ca} channels of subgrounp

Comparison of open probability of BK_{Ca} channels in hippocampal CA1 neurons of rats before and after 3-min ischemia. Plots of normalized NP_o of BK_{Ca} channel against holding potentials (V) in CA1 neurons before (◇) and 6 h (□), 24 h (▲), 48 h (●) after ischemia, showing a significant decrease in sum of open probability of postischemic BK_{Ca} channels within 24 h following reperfusion at varied holding potentials tested with 2 μmol/L $[Ca^{2+}]_i$ (P< 0.01 for 6 h, P< 0.05 for 24 h, ANOVA).

Kinetic analysis of BK_{Ca} channels was obtained from patches in which single-channel activities were observed. At a given membrane potential and fixed $[Ca^{2+}]_i$, open probability is determined by channel open and closed times. To understand which one is the major component attributing to the decrease in open probability of BK_{Ca} channels after ischemia, channel kinetics were compared between control and postischemic neurons. The distributions of open and closed times of BK_{Ca} channels of each group at − 60 mV could be fitted well by a two-exponential function. It was shown clearly from the data summarized in table 1 that there was a significant prolonged closed time in postischemic neurons within the first 24 h following reperfusion, while no apparent change in open time was detected, indicating a major contribution of prolonged close-state duration to the decrease in open probability caused by sublethal ischemia.

Table 1 Changes in kinetics of BK_{Ca} channels in CA1 neurons after 3-min ischemia

Reperfusion			Open time constants/ms		Closed time constants/ms	
			τ_{o1}	τ_{o2}	τ_{c1}	τ_{c2}
Before		(n= 8)	0.51 ±0.01	2.40 ±0.36	2.30 ±0.23	37.11 ±9.64
6 h	after	(n= 7)	0.94 ±0.19	2.60 ±0.53	4.45 ±0.42[1)]	168.23 ±36.69[1)]
24 h	after	(n= 10)	0.88 ±0.11	3.14 ±0.57	4.68 ±0.58[1)]	102.05 ±13.94[2)]
48 h	after	(n= 10)	0.82 ±0.16	3.35 ±0.65	3.15 ±0.18	30.21 ±7.00

[1)] P< 0.01, [2)] P< 0.05 compared with control (ANOVA), Values are $\bar{x} \pm s$.

3 Discussion

In supporting our hypothesis, the study found a decrease of BK_{Ca} channel activity in CA1 pyramidal neurons from rat hippocampus during the first 24 h following 3-min ischemia. Results are consistent with previous studies showing that BK_{Ca} channel is a target for the modulation by ischemia/hypoxia although the underlying mechanisms remained to be clarified. It has been reported that acute hypoxia inhibits BK_{Ca} channel activity in the dissociated neurons from mice neocortex via some cytosolic factors[13]. Changes in these cytosolic factors such as intracellular pH, protein kinases for phosphorylation[13], or nitric oxide levels for S-nitrosylation[14] may also contribute to the preconditioning-induced decrease in the BK_{Ca} channel activity observed in the study. Moreover, the redox mechanism may be involved in the modulation of BK_{Ca} channel activity by preconditioning ischemia. In consistent with this assumption, recent studies have shown that the reactive oxygen species are common mediators in the preconditioning-induced rescue pathways[5] and the alterations of BK_{Ca} channels in CA1 pyramidal neurons following lethal ischemia is due to an oxidation modulation of the channels[9]. It appears that the modulation by single or multiple factors may maintain the closed status of the channel. Yet, further studies are needed to clarify the detail of the mechanisms.

Pyramidal neurons in the CA1 subfield of the hippocampus occur DND after lethal forebrain ischemia. Recently, it has been shown that such severe ischemia induces a persistent enhancement in BK_{Ca} channel activity in CA1 neurons long before cell death[8,9] and BK_{Ca} channel blockers TEA and charybdotoxin protect ischemia-induced CA1 neuronal damage[10], indicating that the increase in BK_{Ca} channel activity plays important roles in the pathogenesis of CA1 neuronal death after severe ischemia. Caspases and nucleases are major inducers of apoptosis[15]. It has been shown that decrease in $[K^+]_i$, due to elevated K^+ efflux through opened K^+ channels, removes the inhibitory effect of cytoplasmic K^+ on caspase-3-like protease and the internucleosomal DNA cleavage nuclease, and consequently results in cell apoptosis[16~18]. Perhaps it is one of the underlying mechanisms that enhancement in BK_{Ca} channel activity leads to the DND of CA1 pyramidal neurons after lethal ischemia.

It is well known that 3-min ischemia produces no death in rat hippocampal CA1 pyramidal neurons but induces resistance to subsequent lethal ischemic insult. Interestingly, the present study found that such a brief ischemia caused a decrease, rather than an increase observed after lethal ischemia, in BK_{Ca} channel activity in CA1 neurons. Thus, it is speculated that the decreased basal activity of BK_{Ca} channels induced by brief ischemia would greatly counteract the subsequent enhancing effect on the channel activity produced by lethal ischemia and thereby result in a neuroprotection by preventing K^+ efflux and the activation of caspase-3 and nucleases.

In summary, the results of the present study not only support the conclusion that the enhancement in activities of BK_{Ca} channel may be involved in the delayed neuronal cell death after lethal ischemia, but also suggest that the down-regulation of the channel may be a neuroprotective factor against subsequent lethal ischemia.

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短暂脑缺血大鼠海马 CA1 区锥体细胞大电导 Ca^{2+} 依赖 K^+ 通道活动降低*

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摘要 短暂脑缺血可对随后的损伤性脑缺血表现出明显的耐受. 有研究表明大电导 Ca^{2+} 依赖 K^+ (BK_{Ca}) 通道活动增强参与了缺血性脑损伤. 采用膜片钳的内面向外式，观察了 3 min 短暂脑缺血后 6 h、24 h 以及 48 h 大鼠海马 CA1 区锥体细胞上 BK_{Ca}通道活动的动态变化. 短暂脑缺血后 BK_{Ca}通道的单通道电导和翻转电位均未见明显变化，但通道的开放概率则在缺血预处理后的前 24 h 内显著降低. 通道动力学分析显示通道关闭时间变长是短暂脑缺血后通道活动降低的主要原因，因为通道的开放时间未发生明显变化. 结果提示短暂脑缺血所致的 BK_{Ca}通道活动降低可能与缺血耐受的产生有关.

关键词 钾通道，短暂脑缺血，海马，膜片钳，大鼠
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