Enhancement in activities of large conductance calcium-activated potassium channels in CA1 pyramidal neurons of rat hippocampus after transient forebrain ischemia

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Abstract

It has been reported previously that the neuronal excitability persistently suppresses and the amplitude of fast afterhyperpolarization (fAHP) increases in CA1 pyramidal cells of rat hippocampus following transient forebrain ischemia. To understand the conductance mechanisms underlying these post-ischemic electrophysiological alterations, we compared differences in activities of large conductance $\text{Ca}^{2+}$-activated potassium (BK) channels in CA1 pyramidal cells acutely dissociated from hippocampus before and after ischemia by using inside-out configuration of patch clamp techniques. (1) The unitary conductance of BK channels in post-ischemic neurons (295 pS) was higher than that in control neurons (245 pS) in symmetrical 140/140 mM K in inside-out patch; (2) the membrane depolarization for an $e$-fold increase in open probability ($P_o$) showed no significant differences between two groups while the membrane potential required to produce one-half of the maximum $P_o$ was more negative after ischemia, indicating no obvious changes in channel voltage dependence; (3) the $[\text{Ca}^{2+}]$, required to half activate BK channels was only 1 $\mu$M in post-ischemic whereas 2 $\mu$M in control neurons, indicating an increase in $[\text{Ca}^{2+}]$, sensitivity after ischemia; and (4) BK channels had a longer open time and a shorter closed time after ischemia without significant differences in open frequency as compared to control. The present results indicate that enhanced activity of BK channels in CA1 pyramidal neurons after ischemia may partially contribute to the post-ischemic decrease in neuronal excitability and increase in fAHP.

1. Introduction

Pyramidal neurons in the hippocampal CA1 region are particularly vulnerable to ischemic insult and display a delayed cell death fashion after transient cerebral ischemia [22,35]. Neuronal hyperactivity induced by excessive accumulation of extracellular glutamate during ischemia was hypothesized to trigger the process of neuronal degeneration [7,36]. However, the electrophysiological evidence for hyperactivity in hippocampus after ischemia remains controversial. Using extracellular recording techniques in vivo, some investigators reported an increased [6,42] while others showed a decreased [5,12,20] firing rate in the CA1 region following ischemia. Recently, by using in vivo [14] and in vitro [40] intracellular recording technique, a persistent reduction in spontaneous firing rate and a progressive suppression of excitability was observed in CA1 pyramidal neurons after severe forebrain ischemia while only a transient change in these properties was found in CA3 neurons and dentate granule cells [13]. The decrease in neuronal excitability after severe ischemia may be due to an increase in potassium conductances. In consistence, it was reported that the amplitude of fast afterhyperpolarization (fAHP), which is mediated by calcium-dependent potassium conductance, progressively increased in CA1 neurons following ischemia [14]. However, little information is available concerning the conductance mechanisms related to the decrease in neuronal excitability.

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excitability as well as the increase in fAHP in post-ischemic CA1 neurons.

Single-channel studies have identified at least two types of Ca\(^{2+}\)-activated K\(^+\) channels: large conductance (BK\(_{\text{Ca}}\)) and small conductance (SK\(_{\text{Ca}}\)) channels on membranes of pyramidal neurons from hippocampal CA1 region, on the basis of their pharmacological and biophysical properties [26,37]. BK\(_{\text{Ca}}\) channel is both voltage- and calcium-dependent. In hippocampal pyramidal neurons in vivo, BK\(_{\text{Ca}}\) channel is thought to be activated during an action potential by membrane depolarization, together with a rise in the intracellular Ca\(^{2+}\) concentration. The resulting K\(^+\) current is largely responsible for action potential repolarization and generation of fAHP [25,41]. It was also suggested recently that BK\(_{\text{Ca}}\) channels may play an important role in regulating neuronal excitability at the resting membrane potential [21,44]. Therefore, BK\(_{\text{Ca}}\) channels are critical in setting the degree of neuronal excitability, which in turn determines the rate of action potential firing and bursting patterns.

So we assumed that the post-ischemic electrophysiological changes mentioned above in CA1 pyramidal neurons may be partially due to the alterations in BK\(_{\text{Ca}}\) channel activity. To address this question, we examined the activity of BK\(_{\text{Ca}}\) channels in CA1 pyramidal neurons of rat hippocampus after transient forebrain ischemia using inside-out configuration of patch clamp techniques.

2. Materials and methods

2.1. Transient forebrain ischemia

Experimental procedures in this study were performed within National Institutes of Health guidelines (Guide for the Care and Use of Laboratory Animals, NIH publication 93-23, revised 1985). A total of 116 male adult Wistar rats weighing 200–250 g was used in the present study. The animals were divided into two groups with 46 rats as control and the remaining 70 rats subjected to transient forebrain ischemia.

Transient forebrain ischemia (15 min) was induced by the use of the four-vessel occlusion method [34] with some modification. Briefly, on the day prior to the experiment, rats were anesthetized with chloral hydrate (i.p., 40 mg/100 g weight) 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 saline solution (in mM): 140 sodium isethionate, 2 KCl, 4 MgCl\(_2\), 0.1 CaCl\(_2\), 23 glucose, 15 HEPES, pH 7.4 (300–305 mOsm/l). Slices were then incubated for 1–6 h at room temperature (20–22°C) in a NaHCO\(_3\)-buffered saline bubbled with 95% O\(_2\)/5% CO\(_2\) containing (in mM): 126 NaCl, 2.5 KCl, 2 CaCl\(_2\), 2 MgCl\(_2\), 26 NaHCO\(_3\), 1.25 NaH\(_2\)PO\(_4\), 1 pyruvic acid, 0.005 glutathione, 0.1 N ω-nitro-L-arginine, 1 kynurenic acid, 10 glucose, pH 7.4 with NaOH (300–305 mOsm/l). All reagents were obtained from Sigma (St. Louis, MO). Slices were then removed into the low Ca\(^{2+}\), and CA1 region of hippocampus was dissected out under a dissecting microscope and placed into an oxygenated chamber containing pronase (Sigma protease Type XIV, 1–1.5 mg/ml) in HEPES-buffered HBSS (Sigma) at 33°C. 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.

2.3. Single-channel current recording

Gigaseal patch recordings using the inside-out patch configuration as described by Hamill [16] (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. Single-channel currents were recorded in excised inside-out patches, which were prepared by pulling the patch electrode away from pyramidal cells. To remove inactivation of BK\(_{\text{Ca}}\) channels recorded at depolarization after formation of inside-out configuration [18], all recordings at membrane depolarization were preceded by a hyperpolarization step
of −60 mV for at least 1 min. The composition of the flow solution that bathed the intracellular face of the patch membrane after excision was (in mM): 140 KCl, 10 NaCl; 10 HEPES. Free Ca$^{2+}$ concentrations of 0.01, 0.1, 0.5 and 2 µM were obtained by adding a total of 55.7, 279, 435 and 485 µM of CaCl$_2$, respectively, to a 500 µM-EGTA (Sigma) solution [29]. For a solution with a desired free calcium of 5 µM or higher, EGTA was omitted and CaCl$_2$ was added as necessary. Solutions were adjusted to a final pH of 7.40 with KOH. Pipette solution consisted of either the bathed solution to give symmetrical charge-carrier distribution, or low K$^+$ solution (5 mM K$^+$ and 2 mM Ca$^{2+}$) to further examine channel selectivity for K$^+$. MgCl$_2$ at 0.5 mM was routinely added to the pipette solution purely for the convenience of easier seal formation in the absence of other divalent ions. All experimental solutions were made using deionized water.

Sensitivity of the channel recorded to the external tetraethylammonium (TEA, Sigma) was examined by using inside-out configuration when TEA was included in the pipette as described previously [26].

### 2.4. Data collection and analysis

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 1 kHz. Data were digitized at sampling rates of 10 kHz using a TL-125 kHz interface (Scientific Solutions). The analysis routines used PCLAMP (version 5.5.1, Axon Instruments) to determine distributions for channel amplitudes, and open and closed times. A 50% threshold criterion was used to determine the durations of open and closed events. Logarithmic distributions of open and closed durations were exponentially fitted with the use of the least-square algorithm method. The ignored level for detecting events was limited to 300 µs. Channel open probability ($P_o$) is obtained by dividing $NP_o$ by $N$ [43], and $NP_o$ was defined as: $NP_o = \Sigma t_1 + 2t_2 + 2t_3 + \cdots + nt_n$, where $N$ is the channel number, $t_1$, $t_2$, $t_3$, $t_n$ are the ratios of open time to total time of measurement for each channel at each of the current levels [28]. Single channel data were obtained at room temperature (20–22°C). The data in text are expressed as mean±S.D. and Student’s $t$-test was used for statistical analysis.

### 3. Results

Successful recordings were made in 217 membrane patches of CA1 pyramidal neurons obtained from 116 adult rats. Properties of BK$_{Ca}$ channels in adult CA1 pyramidal neurons were similar to those in other preparations: voltage dependence, high selectivity to K$^+$, high unitary conductance, and sensitivity to extracellular TEA and intracellular Ca$^{2+}$. The channels recorded were selective for K$^+$, as its channel current–voltage relations reverse at 3.17±2.3 mV ($n = 18$), very close to the equilibrium potential for potassium ion ($E_k$), with symmetrical K$^+$ (140/140 mM) in both the pipette and bathing solution. The reversal potential shifted to −62.7±5.9 mV ($n = 17$) with low concentration of 5 mM K$^+$ in pipette, indicating a high selectivity for K$^+$. Activity of BK$_{Ca}$ channels recorded was affected by the concentration of calcium ion at the intracellular side, [Ca$^{2+}$], of excised membrane patches (Fig. 2B). Whereas less than micromolar concentrations of [Ca$^{2+}$] were sufficient to activate BK$_{Ca}$ channels from the inner membrane surface, 100 µM Ca$^{2+}$ could not activate the channels when applied only at the extracellular membrane surface of excised membrane patches, and this was the case for all membrane potentials tested (from −50 mV to +50 mV). Current–voltage relations showed that the channels recorded had high unitary conductance (245.44±19.14 pS in symmetrical 140/140 mM K$^+$ in inside-out patch; $n = 15$). BK$_{Ca}$ channel in CA1 neuron was blocked by low concentration of external 0.5 mM TEA ($n = 23$) and was less sensitive to 5 mM TEA ($n = 21$) applied to the internal side of the patches. These points served as keys to the identification of the channels recorded as BK$_{Ca}$ channels.

In addition to BK$_{Ca}$ channels, the membrane patches typically contained other channels with smaller conductance than that of BK$_{Ca}$ channels. These channels with smaller conductance will not be considered in this paper, and we have typically selected records in which they are not obviously present.

#### 3.1. Comparison of channel unitary conductance before and after ischemia

Fig. 1A shows original traces of single-channel current from CA1 neurons before and after ischemia. Amplitude histograms constructed from Fig. 1A exhibited that single channel current was 4.89 pA in control neuron (Fig. 1B) and 5.94 pA in post-ischemic neuron (Fig. 1C) at 2 µM [Ca$^{2+}$] with membrane potential of +20 mV. Unitary current amplitude increased across the entire membrane voltage range tested in both neurons from two groups. To compare unitary conductance of BK$_{Ca}$ channels between two groups, amplitudes of the single-channel currents were measured at a number of different membrane potentials and unitary conductance of BK$_{Ca}$ channels was determined by fitting a regression line through the data (Fig. 1D). The mean slope conductance of BK$_{Ca}$ channels in post-ischemic neurons (291.35±11.48 pS, $n = 15$) was significantly higher than that in control neurons (245.44±19.14 pS, $n = 15$) ($P < 0.01$) in symmetrical 140/140 mM K$^+$, and the difference in unitary conductance of BK$_{Ca}$ channels showed no voltage dependence.
Fig. 1. Comparisons of conductance and open probability ($P_o$) of BK$_{Ca}$ channels in CA1 neurons before and after ischemia. (A) Traces of currents recorded showing a higher amplitude in neurons after ischemia (lower) as compared to control (upper) with membrane voltage held at $+20\, \text{mV}$ or $-20\, \text{mV}$ and 2 $\mu$M [Ca$^{2+}$]. Outward and inward currents evoked are shown as downward and upward deflections, respectively. The dotted lines indicate the current level at which all channels were closed; dashes on the sides of the records denote unitary current amplitudes. Note also that a larger open probability ($P_o$) after ischemia with two active BK$_{Ca}$ channels in post-ischemic neuron and one in control neuron. (B and C) Amplitude histograms constructed from (A) showing that single-channel current in post-ischemic neurons (5.93 pA, C) was higher than that in control (4.96 pA, B). (D) Plots of amplitude ($I$) of BK$_{Ca}$ channels against membrane potential ($V$) in control (●) and post-ischemic (▲) neurons showing a higher unitary conductance of BK$_{Ca}$ channels after ischemia as compared with control.

3.2. Comparison of voltage dependence and Ca$^{2+}$ sensitivity of BK$_{Ca}$ channels before and after ischemia

Fig. 1A shows that activity of BK$_{Ca}$ channels in post-ischemic neurons (lower trace) was greater than that in control neurons (upper trace) at fixed membrane potential with 2 $\mu$M [Ca$^{2+}$]. At depolarized membrane potential (+20 mV), BK$_{Ca}$ channels in both control and post-ischemic neurons exhibited long-lasting openings while multiple openings were frequently observed in neurons after ischemia; at negative membrane potential (−20 mV), more openings were seen in patches from post-ischemic than from control neurons. A larger open probability of BK$_{Ca}$ channels may be due to being more sensitive to
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brane patches held at 1 mem-
ponent contributing to the differences in open probability for an
frequency. To understand which one is the major com-
ability is determined by channels’ open time and open potential required to produce one-half of the maximum open probability
and after ischemia
Comparison of kinetics of BK channels before Ca
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channels in post-ischemic neurons. To analyze the differ-
[Ca ] and / or a sharper voltage dependence of BKiC a
3.3. Comparison of kinetics of BK Ca channels before
and after ischemia
Kinetic analysis of BK Ca channels was obtained from
patches in which single-channel activities were observed. At a given holding voltage and [Ca2+], the open prob-
ability is determined by channels’ open time and open
frequency. To understand which one is the major com-
ponent contributing to the differences in open probability
between two groups, kinetics of BK Ca channels were
compared between control and post-ischemic CA1 neu-
rons. Fig. 3 shows dwell time histograms constructed from
membrane patches held at +20 mV with 2 µM [Ca2+].
The distributions of open and closed times of BK Ca

[Ca2+] and/or a sharper voltage dependence of BK Ca
channels in post-ischemic neurons. To analyze the differences in voltage dependence of BK Ca channels between
two groups, individual P o-V curves were fitted by the
Boltzmann equation

\[ P_o = \frac{P_{o,\text{max}}}{1 + \exp\left(\frac{V_{1/2} - V}{K}\right)} \]

with 2 µM [Ca2+]. The equation was transformed into the logarithmic form,

\[ V = V_{1/2} + K \times \ln\left[\frac{P_o}{P_{o,\text{max}}(1 - P_o)}\right] \]

where K is the membrane depolarization for an e-fold increase in P o, and V 1/2 is the patch potential at which P o is one-half of the maximum P o (P o,\text{max}). V 1/2 and K could be obtained by plotting \( \ln\left[\frac{P_o}{P_{o,\text{max}}(1 - P_o)}\right] \) against voltage (Fig. 2A). The values of V 1/2 and K were 2.6±2.1 mV and 17.0±0.7 mV (n=10) for CA1 neurons in control and −12.3±1.7 mV and 16.6±1.1 mV (n=10) for CA1 neurons after ischemia, respectively. Statistical analysis showed that the K value was not significantly different (P>0.05) between two groups while the V 1/2 was more negative (P<0.01) after ischemia when compared with control.

To determine differences in sensitivity to [Ca2+] of
BK Ca channels between two groups, inside-out patch
recordings were obtained with different Ca concentrations at the cytosolic surface of the membrane. In constructing this plot, data with each [Ca2+] were averaged from 15 different membrane patches at fixed mem-
brane potential of +20 mV. A quantitative comparison of sensitivity to [Ca2+] of BK Ca channels is illustrated in
Fig. 2B, where P o is plotted as a function of [Ca2+]. BK Ca channels in CA1 neurons showed a concentration-depend-
ent increase in P o as [Ca2+] was raised from 0.01 µM to 100 µM. P o values in post-ischemic CA1 neurons were larger than those in control neurons, especially when the
[Ca2+] was between 0.5 and 2.0 µM (Fig. 2B). The
[Ca2+] required to half activate (P o=0.5) BK Ca channels
was 2 µM in control neurons while it was only 1 µM after
ischemia.

These results indicated that a larger open probability of
BK Ca channels from CA1 pyramidal neurons after is-
chemia mainly reflected more sensitivity to [Ca2+], rather than voltage dependence.
channels from two groups could be fitted well by a two-exponential function (Fig. 3). It was shown clearly from the data summarized in Table 1 that the open time of BK channels was longer with a shorter closed time after ischemia as compared with control, whereas no significant differences were detected in open frequency (before ischemia: 51.2±21.3 events/s, after ischemia: 52.8±22.3 events/s, n=15, P>0.05), indicating a major contribution of open time to difference in open probability before and after ischemia.

Table 1

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<th>Open time constants (ms)</th>
<th>Closed time constants (ms)</th>
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<tr>
<td></td>
<td>τ₁, τ₂</td>
<td>τ₁, τ₂</td>
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<tr>
<td>Before ischemia</td>
<td>2.76±2.11</td>
<td>19.20±10.10</td>
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<tr>
<td>After ischemia</td>
<td>11.80±6.38</td>
<td>51.97±32.29*</td>
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*Values are means±S.D. The number of neurons in each group was 15. *P<0.05, †P<0.01 compared with before ischemia (Student’s t-test).
4. Discussion

The principal finding of the present study is that the activity of $\text{BK}_{\text{Ca}}$ channels is enhanced in CA1 pyramidal neurons of rat hippocampus after transient forebrain ischemia. This functional enhancement in $\text{BK}_{\text{Ca}}$ channels may partially account for the post-ischemic changes in membrane properties of CA1 neurons following ischemia.

As shown in Fig. 1D, the conductance of $\text{BK}_{\text{Ca}}$ channels increased by about 20% after ischemia. Previous study in skeletal muscles [11] and vascular myocytes [32] showed that, under physiological conditions, internal Na$^+$ and Mg$^{2+}$ can reduce conductance of $\text{BK}_{\text{Ca}}$ channels by interacting with two independent binding sites closely associated with the permeation pathway of $\text{BK}_{\text{Ca}}$ channels. It might be possible that the modulation of internal Na$^+$ on conductance of $\text{BK}_{\text{Ca}}$ channels was changed after ischemia.

Calcium sensitivity is a critical property of $\text{BK}_{\text{Ca}}$ channels that affects their physiological function in neurons by determining the intracellular Ca$^{2+}$ level required for channel opening. After ischemia, the sigmoid curve describing sensitivity to [Ca$^{2+}$], of $\text{BK}_{\text{Ca}}$ channels as a function of membrane potential shifted to the left (Fig. 2B), indicating an increase in the sensitivity of $\text{BK}_{\text{Ca}}$ channels to [Ca$^{2+}$]. The negative shift of $V_{1/2}$ after reperfusion could be accounted for by the increased Ca$^{2+}$ sensitivity, since we did not find a significant change in the membrane depolarization for an $e$-fold increase in $P_o$. A change in Ca$^{2+}$ sensitivity was also observed in cultured spinal neurons of Xenopus during development [4]. In view of alternation in sensitivity to intracellular calcium after ischemia, we can only speculate as to the nature of this change. It seems to be possible that functional changes in the regulatory proteins of potassium channels after ischemia, e.g. $\beta$-subunits [23], which can modulate the calcium-sensitivity of $\text{BK}_{\text{Ca}}$ channels when expressed with the $\alpha$-subunit [30], may be one candidate in interpreting the alteration of [Ca$^{2+}$], sensitivity.

Changes in phosphorylation states may be another possible mechanism whereby $\text{BK}_{\text{Ca}}$ channel behavior can be profoundly altered [27]. Previous studies have shown modifications of $\text{BK}_{\text{Ca}}$ channels by protein kinases [8–10,31,33] and phosphatases [38,45] through phosphorylation and dephosphorylation. Indeed, a change in activities or translocations of protein kinases [1,19,39,46] and phosphatases [17] was found in hippocampal CA1 neurons after ischemia. So we assumed that increased [Ca$^{2+}$], sensitivity of $\text{BK}_{\text{Ca}}$ channels might be due to alterations in its phosphorylation states via protein kinases or phosphatases after ischemia. Heterologous expression study has shown that alternative splicing of a common RNA precursor produces functional diversity of the expressed Slowpoke channel, a Drosophila calcium-activated potassium channel, properties in terms of unit conductance, Ca$^{2+}$ sensitivity and gating [24]. These results in Drosophila suggest that one more explanation for the increased [Ca$^{2+}$], sensitivity of $\text{BK}_{\text{Ca}}$ channels in post-ischemic neurons may be due to changes in alternative splicing. Further experiments are needed to clarify these issues.

$\text{BK}_{\text{Ca}}$ channels have been demonstrated to contribute to the fAHP in hippocampal pyramidal neurons [25,41]. The augmented activity of $\text{BK}_{\text{Ca}}$ channels after ischemia found in the present study may account for the result of a previous report [14] showing that the amplitude of fAHP in CA1 neurons increased significantly from around 1 mV to ~5 mV following ischemia. More sensitive to intracellular calcium, more negative $V_{1/2}$, longer open time and reduced closed time, and also increased unitary conductance of $\text{BK}_{\text{Ca}}$ channels observed in post-ischemic CA1 neurons would all tend to make $\text{BK}_{\text{Ca}}$ channels more influential in affecting neuronal excitability. Therefore, in addition to an increase in fAHP, the enhanced activity of $\text{BK}_{\text{Ca}}$ channels may lead to a reduction in spontaneous firing rate and neuronal excitability observed in CA1 pyramidal neurons after transient forebrain ischemia [14,40].

In conclusion, the present results show that augmented activity of $\text{BK}_{\text{Ca}}$ channels reflects more sensitivity to intracellular calcium, more negative $V_{1/2}$, longer open time and reduced closed time, and also increased unitary conductance after transient forebrain ischemia, indicating that the enhanced $\text{BK}_{\text{Ca}}$ channels may contribute to the increase in fAHP and partially account for the decrease in neuronal excitability in CA1 pyramidal neurons after ischemia.

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References

[2] G. Baranauskas, T. Tkatch, D.J. Surmeier, Delayed rectifier currents and translocations of protein kinases [1,19,39,46] and phosphatases [17] was found in hippocampal CA1 neurons after ischemia. So we assumed that increased [Ca$^{2+}$], sensitivity of $\text{BK}_{\text{Ca}}$ channels might be due to alterations in its phosphorylation states via protein kinases or phosphatases after ischemia. Heterologous expression study has shown that alternative splicing of a common RNA precursor produces functional diversity of the expressed Slowpoke channel, a Drosophila calcium-activated potassium channel, properties in terms of unit conductance, Ca$^{2+}$ sensitivity and gating [24]. These results in Drosophila suggest that one more explanation for the increased [Ca$^{2+}$], sensitivity of $\text{BK}_{\text{Ca}}$ channels in post-ischemic neurons may be due to changes in alternative splicing. Further experiments are needed to clarify these issues.


