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Authors: Takagi, Hiroshi, Kodama, Kunihiko, Saito, Minoru, and Suzuki, Hideo

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Presynaptic K^+ Channel Modulation is a Crucial Ionic Basis of Neuronal Damage Induced by Ischemia in Rat Hippocampal CA1 Pyramidal Neurons

Hiroshi Takagi[1], Kunihiko Kodama[2], Minoru Saito[3*] and Hideo Suzuki[4]

[1]*Second Department of Physiology, Shinshu University School of Medicine,*
[2]*Department of Neurosurgery, Shinshu University School of Medicine,*
Matsumoto, Nagano 390-8621, Japan
[3]*Department of Applied Physics, College of Humanities and Sciences,*
Nihon University, Setagaya-ku, Tokyo 156-8550, Japan
[4]*Department of Physics, School of Science and Engineering,*
Waseda University, Shinjuku-ku, Tokyo 169-8555, Japan

ABSTRACT—The ischemia-induced synaptic potentiation (ISP) during and/or after brain ischemia has been suggested to be one of the crucial factors responsible for irreversible neuronal damage of hippocampal CA1 pyramidal neurons. However, the presynaptic modulation mechanism that leads to neuronal damage during and/or after ischemia was still unknown. By combining electrophysiological methods and infra-red differential interference contrast (IR-DIC) imaging procedures, we showed for the first time that ISP is the result of extraordinary presynaptic depolarization in association with the suppression of 4-aminopyridine (4-AP) sensitive K^+ channels at the presynaptic sites. Furthermore, we also showed that the 4-AP sensitive presynaptic K^+ channels played a crucial role in inducing neuronal damage at a very acute phase of ischemia-induced neuronal damage and would be a therapeutic target against the neuronal damage after brain ischemia.

Key words: ischemia, presynaptic K^+ channel, 4-aminopyridine, hippocampus, delayed neuronal death

INTRODUCTION

The excessive glutamate release during brain anoxia or ischemia triggers the death of neurons, causing mental and physical handicaps (Choi and Rothman, 1990; Nishizawa, 2001). The hippocampal CA1 region is highly vulnerable to ischemia, which induces a selective and delayed degeneration of pyramidal neurons. This phenomenon is called delayed neuronal cell death (DND) (Kirino, 1982; Szatokowski and Attwell, 1994). During ischemia, glutamate is released by a reversal operation of glutamate uptake carriers at CA1 pyramidal neurons (Rossi *et al*., 2000). This glutamate release induces a rise in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) in CA1 pyramidal neurons in an NMDA-receptor dependent manner (Silver and Erecinska, 1990). Following the ischemia, $[Ca^{2+}]_i$ almost returns to its basal level within 5 min, whereas the neurons exposed to excessive glutamate die within a few hr (Silver and Erecinska, 1992).

Ischemia also generates a novel form of long-term potentiation (LTP), which is called anoxic long-term potentiation (A-LTP) (Hammond *et al*., 1994). The synaptic mechanisms underlying A-LTP in the hippocampal CA1 regions have been considered to be due to an enhancement of presynaptic glutamate release (Hammond *et al*., 1994). The enhancement of glutamate release leads to DND coupled with postsynaptic NMDA receptor activation (Hammond *et al*., 1994). This postsynaptic event is defined, but the presynaptic ion channel modulation mechanisms underlying the excessive glutamate release were still unknown. In the present study, we applied a combined technique of electrophysiological and infra-red differential interference contrast (IR-DIC) procedures to hippocampal slices in order to determine a presynaptic ionic basis of A-LTP, which induces necrosis like neuronal cell death.

* Corresponding author: Tel. +81-3-3329-1151;
FAX. +81-3-5317-9432.
E-mail: msaito@chs.nihon-u.ac.jp

MATERIALS AND METHODS

Hippocampal slices (300 μm) were prepared from 4-week-old

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Wistar rats using standard procedures (Takagi *et al.*, 1992a, b; Watanabe *et al.*, 1998). Animal care and experimental procedures were performed according to the guidelines of Shinshu University. Briefly, 300 μm thick hippocampal slices were prepared using a microslicer (DTK-1500, Dosaka-EM, Japan) in a submerged holding chamber containing ice-cold standard extracellular solution (sES) (mM: 124 NaCl, 2.5 KCl, 1.0 NaH_2PO_4, 26 $NaHCO_3$, 2.0 $CaCl_2$, 1.0 $MgCl_2$ and 10 dextrose, bubbled with 95% O_2/ 5% CO_2), and then stored at room temperature for the experiments (<6 hr). After 60 min of pre-incubation, the slices were mounted in a recording chamber and perfused with an external solution (32°C) at a constant rate of about 3–4 ml/min. The field excitatory postsynaptic potential (fEPSP) was recorded from the stratum radiatum in the CA1 region by a glass microelectrode filled with extracellular solution (tip diameter, about 1 μm; DC resistance, 4–8 MΩ). We delivered the orthodromic stimuli of 100 μs square pulses at 0.03 Hz frequency to the orientation of the tips in the CA3 region (the Schaffer collateral) throughout the experiment using a monopolar glass electrode filled with extracellular solution. We set the stimulus intensity (5–50 V) in order to evoke a value of 40–60% of the maximum fEPSP slope. Electrophysiological recordings were performed through a patch clamp amplifier head stage (EPC-7, HEKA, Germany) interfaced with a personal computer (Aptiva J33, IBM, Japan), and the data were acquired, digitized, stored and analyzed using pCLAMP software (ver. 6.0.2, Axon Instruments, CA, USA). A microscope (Axioscope FS2, Carl Zeiss, Germany) equipped with a x40 water immersion lens and a CCD camera (C2400-50, Hamamatsu Photonics, Japan) was used to monitor morphological changes in the CA1 pyramidal neurons in hippocampal slices using an IR-DIC procedure.

Morphological images were recorded on a VCR, and these images were digitized and stored on a personal computer (Dell V333c, Dell, Japan) through a video capture cable system (USB-CAP, I.O data, Japan). Stored morphological images were contrasted by Photoshop software (ver. 4, Adobe Systems, Japan).

Ischemia for hippocampal slices was applied for 5 min by the replacement of extracellular solution from sES to ischemia extracellular solution (iES) (mM: 124 NaCl, 2.5 KCl, 1.0 NaH_2PO_4, 26 $NaHCO_3$, 2.0 $CaCl_2$, 1.0 $MgCl_2$ and 10 Deoxy-glucose, bubbled with 95% N_2/ 5% CO_2) at 10 min after the onset of experiment. Here, A-LTP was induced by the replacement from O_2 to N_2 and from dextrose to deoxy-glucose. To establish a real time monitoring system for ischemia-induced DND, we monitored fEPSP slope and paired pulse facilitation (PPF) ratio for about 3 hr after ischemia.

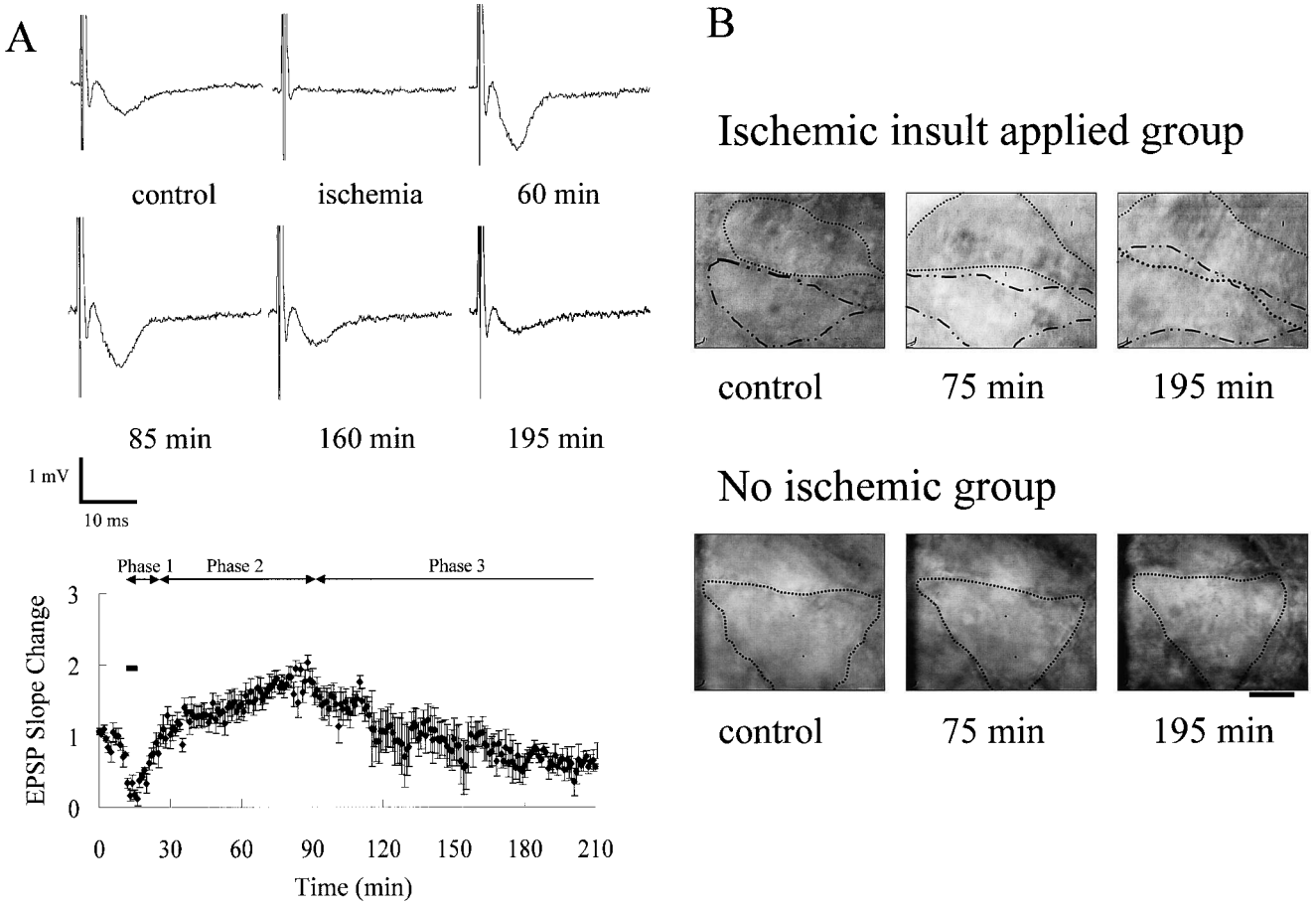


Fig. 1. Ischemia-induced fEPSP slope and morphological changes in pyramidal neurons in the hippocampal CA1 region. (A) EPSP traces recorded from CA1 regions are expressed before ischemia (control), during ischemia, and at 60, 85, 160 and 195 min after ischemia (upper column). The time course of the ischemia-induced fEPSP slope change is shown (lower column). 60, 85, 160 and 195 min after ischemia in upper column correspond to 75, 100, 175 and 210 min in lower column. Data are expressed as mean±SE. The control level was normalized to 1. The period of ischemia is expressed as a solid black bar. (B) Morphological images of hippocampal CA1 pyramidal neurons through IR-DIC are expressed at the control condition, 75 min and 195 min after ischemia (upper column: ischemic group; lower column: no ischemic group) (bar: 10 μm). 75 min and 195 min after ischemia correspond to 90 min and 210 min in lower column of (A). Dashed lines indicate the outline changes of the pyramidal neurons.

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Throughout the experiment, morphological changes in the CA1 pyramidal neurons in hippocampal slices were monitored for 1 min at 0, 10, 15, 20, 30, 45, 60, 75, 90, 105, 120, 135, 150, 165, 180, 205 and 210 min after the onset of experiment.

The data were expressed as mean±SE. Statistical significance was examined by Student *t*-test.

RESULTS

After replacement of sES to iES, the fEPSP slope changed in 3 phases (Fig. 1A). After the replacement, the fEPSP slope suddenly decreased to zero within 3 min (Phase 1). The fEPSP slope gradually recovered within 5-10 min after ischemia, and then significantly increased to 2.04±0.89 (n=3) compared to the control level that was normalized to 1 (p<0.001) (Phase 2). The ischemia-induced synaptic potentiation (ISP) lasted for 30–60 min. Then the fEPSP slope gradually decreased to a lower level (0.81± 0.06, p<0.005) than the control level within 135–145 min after ischemia (Phase 3). Furthermore, the pyramidal neurons became swelling at 75 min after ischemia (Fig. 1B). The plasma membranes of the pyramidal neurons became unclear at 195 min after ischemia (Fig. 1B). In no ischemic group, no morphological changes of the pyramidal neurons appeared even at 195 min after ischemia (Fig. 1B). Therefore, these neuronal damages were caused not by artificial infra-red effects, but mainly by ischemia.

The ISP was observed in each of experiments (n=3) under the normal extracellular $CaCl_2$ concentration (2 mM) condition (Fig. 2A). The PPF ratio also changed in every experiment (n=6). During ischemia, the PPF ratio elevated suddenly (Fig. 2B). Then the PPF ratio gradually and significantly decreased after ischemia, and remained at a significantly lower level (1.31±0.003, n=6) compared to the basal (control) PPF ratio level (1.58±0.57, n=6, p<0.001) (Fig. 2B). We thus concluded that the ISP resulted from presynaptic modulation and that this modulation of the presynaptic releasing site was induced in phases 1 and 2.

To elucidate the presynaptic modulation mechanisms in phases 1 and 2, possible contributions of K^+ channels, Ca^{2+} channels and releasing machineries in the presynaptic sites were studied. The ISP was not observed under a higher extracellular $CaCl_2$ concentration (10 mM) condition (n=4) (Fig. 2C). The decline of the fEPSPs slope during ischemia (Phase 1) was not observed, but the fEPSP slope slightly increased under the higher extracellular $CaCl_2$ condition. During and after ischemia, the PPF ratio did not change under the higher extracellular $CaCl_2$ condition. Furthermore, the basal (control) PPF ratio was significantly lower (0.86±0.12, n=4) (Fig. 2D) than the normal condition (1.58±0.57, n=6, p<0.001) (Fig. 2B). In addition, 2 mM 4-aminopyridine (4-AP), an A- and D-type K^+ channel-selective blocker (Storm, 1990; Takagi *et al*., 1998; Takagi *et al*., 2000), inhibited the ISP induction (n=4) (Fig. 2E). After ischemia, the PPF ratio did not change under the 2 mM 4-AP condition (n=4) (Fig. 2F). The basal (control) PPF ratio under the 2 mM 4-AP condition (0.57±0.01, n=4) was signif-

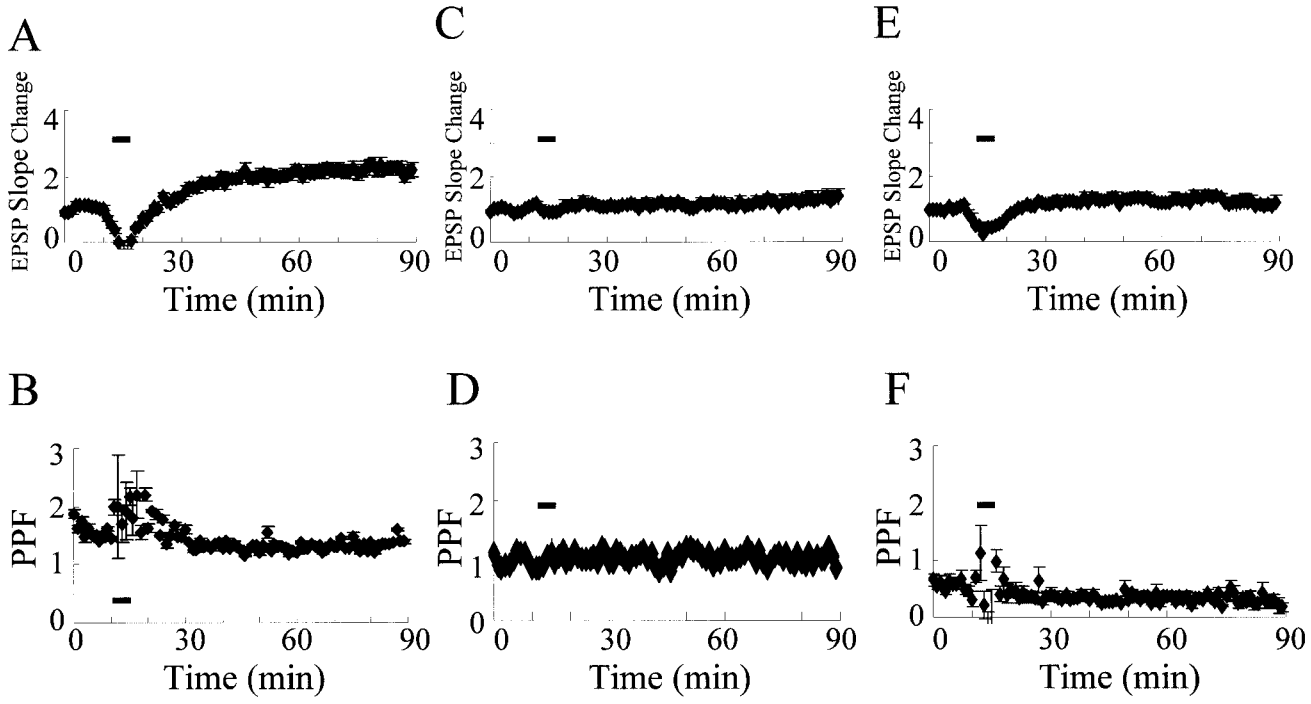


Fig. 2. Ischemia-induced changes of fEPSP slope and PPF ratio. (A and B) Time-dependent changes of fEPSP slope and PPF ratio of the normal condition ($[Ca^{2+}]_o$=2 mM) group, (C and D) time-dependent changes of fEPSP slope and PPF ratio of the higher concentration Ca^{2+} ($[Ca^{2+}]_o$= 10 mM) applied group, and (E and F) time-dependent changes of fEPSP slope and PPF ratio of the 2 mM 4-aminopyridine (4-AP) added group are shown. Data are expressed as mean±SE. In each case, the control level of fEPSP was normalized to 1. The period of ischemia is expressed as a solid black bar.

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icantly lower than the normal condition (1.58±0.57, n=6, $p<0.001$).

DISCUSSION

Neurotransmitter release is triggered by membrane depolarization and regulated by sequential Ca^{2+} influx which is dependent on the concentration gradient of Ca^{2+} between the extracellular space and the intracellular space (Llinás *et al.*, 1976). 4-AP sensitive K^+ channels and Ca^{2+}-dependent K^+ channels have been reported to regulate neurotransmitter release at presynaptic nerve terminals (Weeler *et al.*, 1996; Hu *et al.*, 2001). The present results suggest that the ISP results from 4-AP sensitive presynaptic K^+ channel modulation (maybe 'suppression'), but not from the modulation of presynaptic Ca^{2+} channels and Ca^{2+}-dependent K^+ channels, causing a more excitable state in presynaptic nerve terminals than that observed under no ischemic conditions.

In this study, we obtained important new findings. (1) The ISP was not observed under a higher extracellular $CaCl_2$ concentration (10 mM), where the Ca^{2+} influx through presynaptic Ca^{2+} channels and Ca^{2+}-dependent K^+ channels was enhanced. (2) The 4-AP, an A- and D-type K^+ channel-selective blocker, also inhibited the ISP induction. These results suggest that the modulation of 4-AP sensitive presynaptic K^+ channels plays a crucial role in the ISP induction. Neurotransmitter overdose results in an overexcitation of postsynaptic membrane, detected as ISP, and then induces cell damage or cell death. The absence of ischemia-induced fEPSP decline under the higher $CaCl_2$ concentration does not indicate a protective procedure against neuronal cell damage, but it rather means the presynaptic membrane is controlled at a more excitable state than under the normal condition due to higher $CaCl_2$ pretreatment before ischemia (Llinás *et al.*, 1976). The suppression of ISP under the higher $CaCl_2$ concentration proves that the amplitude of fEPSP is controlled at a relatively higher level than under the normal condition in the experiments. Furthermore, the ISP is produced only under the condition where synapses can work more efficiently.

We established the best *in vitro* real-time monitoring system for acute neuronal cell death for the first time, and we also found that 4-AP sensitive presynaptic K^+ channel modulation (maybe 'suppression') is the crucial mechanism of neuronal cell damage and/or death. Our results will provide useful therapeutic procedures against acute neuronal cell death if the modulation of 4-AP sensitive presynaptic K^+ channels can be reset to normal, since 4-AP also inhibited the ISP induction. Further studies are needed as follows for the establishment of therapeutic procedures against ischemia-induced neuronal cell damage and/or death. (1) To determine whether or not a 4-AP sensitive presynaptic K^+ channel opener (Pena and Tapia, 2000; Ortiz *et al.*, 2002) can protect against acute neuronal cell damage. (2) To elucidate the cellular mechanisms (eg, IL-1β/nitric oxide system, MAP kinase system, Estrogen system) (Togashi *et al.*, 2001; Nozaki *et al.*, 2001; Jover *et al.*, 2002) of ischemia-induced modulation of 4-AP sensitive presynaptic K^+ channels. (3) To elucidate the time and spatial dependent correlation between ISP and DND.

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