Ca$^{2+}$-Activated K$^+$ Currents in Rat Locus Coeruleus Neurons Induced by Experimental Ischemia, Anoxia, and Hypoglycemia

YOSHINAKA MURAI, HITOSHI ISHIBASHI, SUSUMU KOYAMA, AND NORIO AKAIKE
Department of Physiology, Faculty of Medicine, Kyushu University, Fukuoka 812-82, Japan

Murai, Yoshinaka, Hitoshi Ishibashi, Susumu Koyama, and Norio Akaike. Ca$^{2+}$-activated K$^+$ currents in rat locus coeruleus neurons induced by experimental ischemia, anoxia, and hypoglycemia. J. Neurophysiol. 78: 2674–2681, 1997. The effects of metabolic inhibition on membrane currents and N-methyl-D-aspartic acid (NMDA)-induced currents were investigated in dissociated rat locus coeruleus (LC) neurons by using the nystatin perforated patch recording mode under voltage-clamp conditions. Changes in the intracellular Ca$^{2+}$ concentration ([Ca$^{2+}$]) during the metabolic inhibition were also investigated by using the microfluorimetry with a fluorescent probe, Indo-1. Removal of both the oxygen and glucose (experimental ischemia), deprivation of glucose (hypoglycemia), and a blockade of electron transport by sodium cyanide (NaCN) or a reduction of the mitochondrial membrane potential with carbonyl cyanide-p-trifluoromethoxyphenyl-hydrazone (FCCP) as experimental anoxia all induced a slowly developing outward current (I$_{OUT}$) at a holding potential of -40 mV. The application of $10^{-4}$ M NMDA induced a rapid transient peak and a successive steady state inward current and a transient outward current immediately after washout. All treatments related to metabolic inhibition increased the NMDA-induced outward current (I$_{NMDA-OUT}$) and prolonged the one-half recovery time of I$_{NMDA-OUT}$. The reversal potentials of both I$_{OUT}$ and I$_{NMDA-OUT}$ were close to the K$^+$ equilibrium potential ($E_k$) of -82 mV. Either charybdotoxin or tolbutamide inhibited the I$_{OUT}$ and I$_{NMDA-OUT}$, suggesting the contribution of Ca$^{2+}$-activated and ATP-sensitive K$^+$ channels, even though the inhibitory effect of tolbutamide gradually diminished with time. Under the metabolic inhibition, the basal level of [Ca$^{2+}$], was increased and the one-half recovery time of the NMDA-induced increase in [Ca$^{2+}$], was prolonged. The I$_{OUT}$ induced by NaCN was inhibited by a continuous treatment of thapsigargin but not by ryanodine, indicating the involvement of inositol 1,4,5-trisphosphate (IP$_3$)-induced Ca$^{2+}$ release (IICR) store. These findings suggest that energy deficiency causes Ca$^{2+}$ release from the IICR store and activates continuous Ca$^{2+}$-activated K$^+$ channels and transient ATP-sensitive K$^+$ channels in acutely dissociated rat LC neurons.

INTRODUCTION

Brain ischemia has a strong influence on the mammalian central nervous system (CNS). During brain ischemia extracellular concentrations of excitatory amino acids glutamate and aspartate have been reported to increase (Silver and Erecinska 1990). The elevated levels of these amino acids cause an increase in the cytosolic-free intracellular Ca$^{2+}$ concentration ([Ca$^{2+}$]) by way of activating Ca$^{2+}$ influxes through N-methyl-D-aspartate (NMDA) and Ca$^{2+}$ permeable non-NMDA receptors (Nellgard and Wieloch 1992; Siomion et al. 1984; Szatkowski and Attwell 1994). An excessive increase in [Ca$^{2+}$] leads finally to neuronal damage or death (Beal 1992; Choi 1988; Tymianski et al. 1994). The critical role of [Ca$^{2+}$], in ischemic neurotoxicity is supported by the fact that removal of Ca$^{2+}$ from the extracellular fluid during ischemia or the blockage of glutamate receptors prevented the delayed neuronal death (Choi 1987; Rothman et al. 1987).

Hypoxia-induced changes in membrane potential were studied in various slice preparations (Dool et al. 1991; Fujiwara et al. 1987; Hansen et al. 1982; Leblond and Krnjevic 1989; Nieber et al. 1995). In hippocampal slices short-term hypoxic stimuli produce a small transient depolarization followed by more pronounced hyperpolarization lasting ~2–10 min, accompanying a decrease in membrane resistance. Thereafter the hyperpolarization changed to depolarization, which is initially slow but subsequently accelerates, ultimately leading to a complete loss of membrane potential (Fujiwara et al. 1987; Reiner et al. 1990). The initial small depolarization is thought to be due to the inhibition of Na$^+-K^+$-ATPase, and the long-term hypoxia-induced depolarization is due to a large increase in the extracellular K$^+$ concentration that is caused by a reduction of the Na$^+-K^+$-ATPase activity (Hansen 1985). Although the short-term hypoxia-induced hyperpolarization is due to an increase in K$^+$ conductance (Fujiwara et al. 1987; Hansen et al. 1982; Leblond and Krnjevic 1989), the exact nature of the K$^+$ current remains controversial. In isolated mouse dorsal root ganglion neurons, the hyperpolarization during hypoxia is due to the increased K$^+$ conductance through Ca$^{2+}$-activated K$^+$ channels, because the hyperpolarization is blocked by the intracellular application of Ca$^{2+}$ chelators (Duchen 1990). The activation of Ca$^{2+}$-activated K$^+$ currents (I$_{Ca}$) during hypoxia was also reported in rat hippocampal neurons (Krnjevic and Xu 1991; Leblond and Krnjevic 1989; Zhang and Krnjevic 1993). On the other hand, a fall in intracellular ATP concentrations during anoxia leads to activation of ATP-sensitive K$^+$ currents (I$_{KATP}$), as was demonstrated in mammalian heart and pancreatic β-cells (Ashcroft and Ashcroft 1990, 1992). In rat dorsal vagal neurons, blockers of ATP-sensitive K$^+$ channels such as tolbutamide and glibenclamide suppress anoxia-induced hyperpolarization, and activators of ATP-sensitive K$^+$ channels mimic the response to metabolic blockade, suggesting the involvement of I$_{KATP}$ (Trapp and Ballanyi 1995). The activation of I$_{KATP}$ during hypoxia was also reported in rat hippocampal and substantia nigra neurons (Jiang et al. 1994; Moure et al. 1989).

The nucleus locus coeruleus (LC) is known to be a noradrenergic region that provides extensive projections to both the cortical and subcortical structures and may serve a number of cognitive and emotional processes including attention and anxiety (Foote et al. 1983). The LC neurons have been

2674

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reported to discharge spontaneous action potentials with a pacemaker-like regularity (Cedarbaum and Aghajanian 1977; Henderson et al. 1982). Recently, such spontaneous activities were reported to be reduced during hypoxia (Nieber et al. 1995). However, it was difficult to delineate the mechanism of hypoxic action in detail using brain slices, because the hypoxic effects on the presynaptic terminals, postsynaptic somata, and glial cells are intermingled while a complex intercellular network exist among them. To clarify the effect of hypoxia on postsynaptic somata, therefore, it is indispensable to use single neurons isolated from the surrounding brain structures. In the present study the effects of ischemia, anoxia, and hypoglycemia on membrane currents, NMDA-induced currents, and $[\text{Ca}^{2+}]_i$, were investigated and compared in the LC neurons acutely dissociated from the rat.

**METHODS**

**Preparation**

LC neurons were acutely dissociated from 10- to 14-day-old Wistar rats as described previously (Shirasaki et al. 1990). Briefly, rats were anesthetized with pentobarbital sodium (50 mg/kg ip) and 400-mm-thick coronal sections of brain were cut with a microslicer (DTK-1000, Dosaka, Kyoto, Japan). The slices were preincubated in standard external solution saturated with 100% O$_2$ at room temperature for 30–60 min. They were then treated with 0.1 mg/ml pronase at 31°C for 20–30 min and subsequently with 0.1 mg/ml thermolysin under the same conditions for 20–30 min. Thereafter the LC region was micropunched out and neurons were mechanically dissociated with fire-polished Pasteur pipettes in a small plastic culture dish (35 mm diam; Falcon, NJ). The dish used for the measurement of $[\text{Ca}^{2+}]_i$ had a central hole (19 mm id) that was covered with a coverslip (24 × 24 mm with a thickness of 0.12–0.17 mm; Matsunami, Tokyo, Japan) attached to the bottom of the dish with a high-vacuum silicone grease (Dow Corning). The dissociated LC neurons adhered to the bottom of the dish within 20–30 min.

**Solutions**

The standard external solution contained (in mM) 150 NaCl, 5 KCl, 1 MgCl$_2$, 2 CaCl$_2$, 10 N-(2-hydroxyethyl)piperazine-N'-2-ethanesulfonic acid (HEPES), and 10 glucose. The pH was adjusted to 7.4 with tris (hydroxymethyl) aminomethane (Tris-base). For recording the NMDA-induced currents, MgCl$_2$ was removed from the standard external solution and 10 $-^6$ M glycine added. The glucose-free solution was prepared by replacing glucose with equimolar mannitol or 2-deoxy-D-glucose. The composition of the patch-pipette (internal) solution was (in mM) 150 KCl and 10 HEPES (pH 7.2 with Tris-base). A stock solution of 10 mg/ml nystatin in methanol was prepared and kept at −20°C. This solution was then dissolved into the pipette solution to produce a final nystatin concentration of 400 mg/ml. A rapid application of external solution was performed using the “Y-tube” technique as described previously (Nakagawa et al. 1990). Using this technique, the external solution could be completely exchanged within 20 ms. The duration of NMDA application was fixed at 20 s for electrical recording.

**Electrical measurements**

Electrical measurements were performed with the nystatin perfused patch recording mode (Akaike and Harata 1994). Patch-pipettes were prepared by a vertical micropipette puller (PB-7, Narishige, Tokyo). The resistance of the patch-pipette filled with the internal solution was 4–6 MΩ. Ionic currents were measured with a patch-clamp amplifier (TM-1000, Act ME, Tokyo), low-pass filtered at 1 kHz (E-3201A, NF Electronic Instruments, Tokyo), and monitored on both a digital storage oscilloscope (VC-6025, Hitachi, Tokyo) and a pen recorder (Linearcorder F, Graphtec, Tokyo). The data were stored on magnetic tape by using a digital audiotape recorder (RE-130TE, TEAC, Tokyo). The ramp voltage commands were generated by a function generator (FG-122, NF Electronic Instruments) and consisted of a linear hyperpolarizing voltage command of −70 mV with frequency of 0.5 Hz. All experiments were performed at room temperature (22–25°C).

**Fluorometric analysis of $[\text{Ca}^{2+}]_i$**

The method for fluorescence measurements was essentially the same as that described elsewhere (Nordvig et al. 1991). Briefly, neurons were incubated in the standard external solution with $5 \times 10^{-6}$ M Indo-1 octoethylamlylester form (Dojindo, Kumamoto, Japan) at room temperature for 30 min. The $[\text{Ca}^{2+}]_i$ measurements were performed on an inverted microscope with a quartz epi-fluorescence attachment (Diaphot TMD, Nikon, Tokyo). The illumination was obtained from a 100 W xenon arc lamp through a 360-nm band-pass filter for 300 ms every 6 s and directed onto the specimen by a Nikon 390-nm diachronic mirror through Fluor 40 oil objective (NA 1.3, Nikon). The fluorescence signals were directed to two photomultiplier tubes (P1, Nikon) to give a fluorescence ratio (405:480 nm). The signals from the photomultiplier tubes were stored in a computer (PC98VL, NEC, Tokyo) to calculate $[\text{Ca}^{2+}]_i$. The $[\text{Ca}^{2+}]_i$ was estimated according to the following equation (Gryniewicz et al. 1985)

$$[\text{Ca}^{2+}]_i = K_o (R - R_{\text{min}})/(R_{\text{max}} - R)$$

where $K_o$ is the dissociation constant for the Ca$^{2+}$-Indo-1 complex, $\beta$ is the maximum:minimum fluorescence ratio measured at a wavelength of 480 nm, $R$ is the fluorescence ratio (i.e., the fluorescence intensity at 405 nm divided by that at 480 nm), and $R_{\text{min}}$ and $R_{\text{max}}$ are the minimum and maximum fluorescence ratios measured in Ca$^{2+}$-free and Ca$^{2+}$-saturated conditions, respectively. We assume 250 nM for $K_o$ (Gryniewicz et al. 1985) and 4.18 for $\beta$ from our calibration. To obtain $R_{\text{min}}$ and $R_{\text{max}}$, we used Indo-1 in saline containing 3 M sucrose and Ca$^{2+}$-ethylene glycol-bis(β-aminoethyl ether)-$\gamma$-N,N',N''-tetraacetic acid (EGTA) buffers to provide free and saturated concentrations of Ca$^{2+}$ (Poenie 1990). $R_{\text{min}}$ and $R_{\text{max}}$ values were 0.041 ± 0.002 (mean ± SE; n = 6) and 1.39 ± 0.024 (n = 6), respectively.

**Measurements of partial pressure of oxygen**

The partial pressure of oxygen ($P_{O_2}$) of the external solutions was measured with the blood gas analysis system (288 Blood Gas System, Chiron Diagnostics).

**Drugs**

The following drugs were used in the present study: 2-deoxy-D-glucose, carbonyl cyanide-3-trifluoromethoxyphenyl-hydrazone (FCCP), glibenclamide, NMDA, nystatin, thapsigargin and tetrodotoxin (Sigma, St. Louis, MO), pronase (Calbiochem, San Diego, CA), ryanodine and tolbutamide (Wako, Tokyo), apamin, charybdotoxin (ChTX) andiberiotoxin (ITX, Peptide Institute, Osaka, Japan), and dantrolene (Funakoshi, Tokyo). FCCP and glibenclamide were dissolved in dimethyl sulfoxide (DMSO), resulting in $10^{-5}$ M of stock solution. Tolbutamide was dissolved in 0.1 N NaOH, resulting in $10^{-1}$ M of stock solution. The final DMSO concentration never exceeded 0.01%, a concentration that did not affect the membrane currents and $[\text{Ca}^{2+}]_i$, by itself. These
Statistics

The results are expressed as means ± SE. Statistical significance was determined by one-way analysis of variance (ANOVA) followed by the Fisher post hoc test for multiple comparisons.

RESULTS

Responses of LC neurons to NMDA and metabolic arrest

Figure 1A shows 10⁻⁴ M NMDA-induced currents and a holding current in the dissociated rat LC neurons at a holding potential (Vₜₗ) of −40 mV before and during experimental ischemia, which was made by superfusing with a glucose-free solution equilibrated with 100% N₂. NMDA induced a rapid transient and a steady state inward current in normal external solution. After washing out the agonist, a transient (IOUT) was clearly observed. Because an application of control solution never evoked any currents, Iₓ, was not an artifact of our perfusion system. The membrane conductance increased during the peak and steady state inward currents and during the transient outward current (data not shown). Experimental ischemia gradually shifted the holding current in an outward direction, and the application of NMDA during ischemia prominently increased the amplitude and one-half recovery time (T₁/₂) of Iₓ with changing the peak inward current (Figs. 1 and 3). After withdrawal of ischemia, the shifted outward holding current (IOUT) and the prolonged T₁/₂ of Iₓ returned to the control level (data not shown). In the present experiments, partial pressures of oxygen (Pₐₗ) under normal and hypoxic (100% N₂) conditions were 160 ± 10 (n = 6) and 40 ± 5 (n = 6) mmHg, respectively.

The exposure to a glucose-free solution as a model of hypoglycemia (Fig. 1B) to 10⁻⁷ M FCCP, a mitochondrial uncoupler (Fig. 1C), and to 10⁻⁴ M sodium cyanide (NaCN), an inhibitor of oxidative phosphorylation (Fig. 1D), all induced outward currents; these treatments also increased the amplitude and T₁/₂ of Iₓ. The amplitude of IOUT became stable ~3 min after application of the metabolic inhibitors and then continued at almost the same level during the treatment with these agents. The IOUT values (at 3 min) induced by experimental ischemia, hypoglycemia, FCCP, and NaCN were 47.0 ± 2.4 (n = 4), 23.0 ± 2.4 (n = 4), 94.0 ± 6.0 (n = 5), and 69.6 ± 3.2 (n = 5) pA, respectively (Fig. 2). The amplitude of Iₓ was significantly increased under ischemia, hypoglycemia, FCCP, and NaCN, whereas the NMDA-induced peak inward current was not affected by these treatments (Fig. 3). T₁/₂ of Iₓ was also significantly prolonged under these metabolic inhibitions (Fig. 3).

Ionic nature of outward currents

To determine the ionic basis of the NMDA- and NaCN-induced outward currents, current-voltage (I-V) relationships were examined by using the ramp-wave method. As shown in Fig. 4 the ramp waves consisting of −70 mV hyperpolarizing voltage pulses from a Vₜₗ of −40 to −110 mV at 0.5 Hz were applied before the application of NMDA and immediately after washing out NMDA in the presence or absence of 10⁻⁴ M NaCN. The reversal potentials of Iₓ at control condition (Fig. 4, bottom left panel) and during application of 10⁻⁴ M NaCN (Fig. 4, bottom right panel) were −80 ± 2 mV (n = 8) and −77 ± 2 mV (n = 4), respectively. The reversal potential of the NaCN-induced IOUT (Fig. 4, bottom right panel) was −81 ± 1 mV (n = 4). These values were close to the theoretical K⁺ equilibrium potential (Eᵥ) of −82 mV calculated from the given extracellular K⁺ concentrations considering ionic activities. Similar results were also obtained in the case of experimental ischemia, hypoglycemia, and FCCP application (data not shown), thus indicating that both Iₓ and IOUT are passing through K⁺ channels.

High Ca²⁺ permeability through the NMDA receptor channel complexes (Mayer and Westbrook 1987) raises the possibility that Iₓ is a Ca²⁺-activated K⁺ current. To explore this possibility, the effects of various K⁺ channel blockers were examined. As shown in Fig. 5A, 3 × 10⁻⁷ M ChTX, a blocker of the intermediate conductance type of Ca²⁺-activated K⁺ channels, inhibited the Iₓ by 67.3 ± 8.5% (n = 5) in control condition. On the other hand, 3 × 10⁻⁷ M iberiotoxin (n = 4; Fig. 5B) as a blocker of high-conductance type of Ca²⁺-activated K⁺ channels, 3 × 10⁻⁷ M amamine (n = 5; not shown) as a blocker of small conductance type of Ca²⁺-activated K⁺ channels, and 3 × 10⁻⁴ M tolbutamide (n = 7; Fig. 5C) and 3 × 10⁻⁷ M...
glibenclamide \( (n = 5); \) not shown \) as a blocker of ATP-sensitive K\(^+\) channels had no significant effect on \( I_{\text{NMDA-OUT}} \) in control condition. These results suggest that \( I_{\text{NMDA-OUT}} \) in control conditions are due to the activation of Ca\(^{2+}\)-activated K\(^+\) channels.

The effects of K\(^+\) channel blockers on \( I_{\text{NMDA-OUT}} \) and \( I_{\text{OUT}} \) during anoxia were also examined about 10 min after beginning the application of \( 10^{-4} \) M NaCN, at which time an NMDA response having a stable \( I_{\text{NMDA-OUT}} \) was observed in the experimental anoxia. As shown in Fig. 5A, ChTX inhibited the NaCN-induced \( I_{\text{OUT}} \) by 63.4 ± 8.6% \( (n = 5) \), and inhibited \( I_{\text{NMDA-OUT}} \) by 86.7 ± 8.9% in the presence of \( 10^{-4} \) M NaCN. On the other hand, \( 3 \times 10^{-7} \) M iberiotoxin \( (n = 4) \), \( 3 \times 10^{-7} \) M apamine \( (n = 5) \), \( 3 \times 10^{-4} \) M tolbutamide \( (n = 7); \) Fig. 5C), and \( 3 \times 10^{-7} \) M glibenclamide \( (n = 5) \) had no significant effects on either \( I_{\text{OUT}} \) or \( I_{\text{NMDA-OUT}} \). Figure 6 summarizes the effects of these K\(^+\) channel blockers on the two kinds of outward currents. These results suggest the involvement of Ca\(^{2+}\)-activated K\(^+\)-channel in both \( I_{\text{OUT}} \) and \( I_{\text{NMDA-OUT}} \).

Recently, the contribution of ATP-sensitive K\(^+\) channels to anoxia-induced K\(^+\) currents was reported in adult rat LC neurons (Nieber et al. 1995). In the present study, therefore, the effect of tolbutamide on \( I_{\text{OUT}} \) was investigated more carefully. As shown in Fig. 7 the inhibitory effect of tolbutamide on \( I_{\text{OUT}} \) was decreased time-dependently. Three minutes after the application of NaCN, tolbutamide blocked \( I_{\text{OUT}} \) by 73.7 ± 9.5% and ChTX blocked \( I_{\text{OUT}} \) by 21.1 ± 11.0% \( (n = 6) \). Ten minutes after the application of NaCN, however, tolbutamide only slightly inhibited the \( I_{\text{OUT}} \), and ChTX blocked \( I_{\text{OUT}} \) by 81.0 ± 6.0% \( (n = 6) \). Thus these results indicate that the ATP-sensitive K\(^+\) channels are activated.
during the initial phases of experimental anoxia and that the activity of ATP-sensitive K\(^+\) channels shows a rapid rundown during the metabolic deprivation in acutely dissociated rat LC neurons.

**Changes in \([Ca^{2+}]_i\).**

The effects of NMDA on \([Ca^{2+}]_i\), during experimental hypoglycemia or anoxia induced by NaCN were examined by fluorometric recording by using Indo-1. The application of 10\(^{-4}\) M NMDA increased \([Ca^{2+}]_i\), which reached a steady state level within 18 s under control condition. In eight cells, the basal \([Ca^{2+}]_i\) level was 133 \(\pm\) 8 nM and the NMDA-induced rises in \([Ca^{2+}]_i\), at 6, 12, 18, and 24 s after application of the agonist was 566 \(\pm\) 59, 633 \(\pm\) 31, 650 \(\pm\) 26, and 648 \(\pm\) 20 nM, respectively. After washing out the agonist, the elevated \([Ca^{2+}]_i\), almost returned to the basal level within 2 min (Fig. 8). Hypoglycemia itself also increased the basal \([Ca^{2+}]_i\), which approached a new steady state \([Ca^{2+}]_i\) level within 3 min. The increased \([Ca^{2+}]_i\) during hypoglycemia recovered to the basal level after withdrawal of glucose-free solution (Fig. 8A). In four neurons, hypoglycemia increased basal \([Ca^{2+}]_i\) by 66 \(\pm\) 8 nM. The peak amount of the NMDA-induced \([Ca^{2+}]_i\), increase was not influenced by hypoglycemia. NMDA raised \([Ca^{2+}]_i\) by 636 \(\pm\) 5 and 646 \(\pm\) 11 nM \(n = 4\) in the presence and absence of glucose, respectively. These values were not significantly different. Under hypoglycemia, however, the NMDA-induced \([Ca^{2+}]_i\) increase was long-lasting, and persisted even after NMDA was washed out and required \(>2\) min for a complete recovery to the previous steady state level (Fig. 8). Hypoglycemia prolonged the one-half recovery time of the NMDA-induced \(Ca^{2+}\) transients by 141 \(\pm\) 12% \(n = 4\).

The application of 10\(^{-4}\) M NaCN increased the basal \([Ca^{2+}]_i\), and also made a new steady state \([Ca^{2+}]_i\), level as in the case of hypoglycemia (Fig. 8B). During exposure to 10\(^{-4}\) M NaCN, the NMDA-induced changes in \([Ca^{2+}]_i\), was state \([Ca^{2+}]_i\) level within 3 min. The increased \([Ca^{2+}]_i\) during hypoglycemia recovered to the basal level after withdrawal of glucose-free solution (Fig. 8A). In four neurons, hypoglycemia increased basal \([Ca^{2+}]_i\) by 66 \(\pm\) 8 nM. The peak amount of the NMDA-induced \([Ca^{2+}]_i\), increase was not influenced by hypoglycemia. NMDA raised \([Ca^{2+}]_i\) by 636 \(\pm\) 5 and 646 \(\pm\) 11 nM \(n = 4\) in the presence and absence of glucose, respectively. These values were not significantly different. Under hypoglycemia, however, the NMDA-induced \([Ca^{2+}]_i\) increase was long-lasting, and persisted even after NMDA was washed out and required \(>2\) min for a complete recovery to the previous steady state level (Fig. 8). Hypoglycemia prolonged the one-half recovery time of the NMDA-induced \(Ca^{2+}\) transients by 141 \(\pm\) 12% \(n = 4\).

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also long lasting and persisted even after NMDA was washed out. The increased value of the basal [Ca\(^{2+}\)], by NaCN was 141 ± 13 nM (n = 5). The NMDA-induced increase in [Ca\(^{2+}\)], was not influenced by the addition of NaCN, resulting in 486 ± 34 and 447 ± 18 nM (n = 5) in the absence and presence of 10^{-4} M NaCN, respectively. In addition, NaCN also increased the one-half recovery time of the NMDA-induced Ca\(^{2+}\) transients by 317 ± 27% (n = 4). The increased [Ca\(^{2+}\)], in the presence of NaCN recovered to the basal level after withdrawal of NaCN.

**Contribution of intracellular Ca\(^{2+}\) stores**

The [Ca\(^{2+}\)], is known to be greatly influenced by Ca\(^{2+}\)-release from intracellular Ca\(^{2+}\) stores in neurons (Henzi and MacDermott 1992). In the present study, therefore, the involvement of intracellular Ca\(^{2+}\) stores was investigated. Figure 9 shows the effects of ryanodine, a blocker of a Ca\(^{2+}\)-induced Ca\(^{2+}\) release (CICR), and thapsigargin, a blocker of inositol 1,4,5-trisphosphate (IP\(_3\))-induced Ca\(^{2+}\) release (IICR), on the NMDA- and anoxia-induced outward currents. Caffeine is known to release Ca\(^{2+}\) from a CICR pool in excitable cells (McPherson and Campbell 1993) and deplete Ca\(^{2+}\) in the intracellular Ca\(^{2+}\) pool in the presence of ryanodine, which then almost irreversibly binds to CICR channels in the open state and prevents the closure of the channels (Henzi and MacDermott 1992). As shown in Fig. 9A, the caffeine-induced outward currents (Ca\(^{2+}\)-activated K\(^{+}\) currents) were evoked by the first application of 10^{-6} M caffeine. During a prolonged application of 10^{-6} M ryanodine, which initially induced the small outward currents, the caffeine response decreased with the successive application. Even in this condition, I\(_{\text{NMDA-OUT}}\) and the NaCN-induced I\(_{\text{OUT}}\) were still observed (n = 4). On the other hand, 10^{-6} M thapsigargin inhibited the NaCN-induced I\(_{\text{OUT}}\) and the development of I\(_{\text{NMDA-OUT}}\) during anoxia (n = 4; Fig 9B). These results suggest the involvement of the ICR pool in the experimental anoxia-induced outward currents in acutely dissociated rat LC neurons.

**DISCUSSION**

The I\(_{\text{NMDA-OUT}}\) was previously reported in cultured rat cortical neurons and dissociated rat Meynert neurons (Akaike and Harata 1994; Mistry and Hablitz 1990). Similar outward currents were activated in acutely dissociated cerebral cortical neurons and nucleus tractus solitarii neurons by kainic acid and ATP, respectively (Omura et al. 1993; Ueno et al. 1992). In the present study, I\(_{\text{NMDA-OUT}}\) was clearly observed immediately after NMDA was washed out. The I\(_{\text{NMDA-OUT}}\) had a reversal potential near the E\(_{\text{K}}\) (Fig 4) and was strongly blocked by ChTX (Figs. 5 and 6). The amplitude and one-half recovery time of I\(_{\text{NMDA-OUT}}\) and NMDA-induced rises in [Ca\(^{2+}\)], were increased and prolonged under metabolic deprivations, respectively (Figs. 1, 3, and 8). These results suggest the possible involvement of Ca\(^{2+}\)-activated K\(^{+}\) channels in I\(_{\text{NMDA-OUT}}\) during both normal and ischemic conditions in the dissociated rat LC neurons.

In the present study a continuous application of metabolic inhibitors induced I\(_{\text{OUT}}\) and produced a raise in [Ca\(^{2+}\)], (Fig. 8). Hypoxia- or metabolic inhibitor-induced increase in [Ca\(^{2+}\)], was also observed in various rat CNS neurons (Dubinski and Rothman 1991; Uematsu et al. 1988). The increase in [Ca\(^{2+}\)], could be caused by an inability of neurons to maintain proper operation of the Ca\(^{2+}\)-buffering system (Blaustein 1988), because a decrease in energy levels curtails Ca\(^{2+}\)-sequestration mechanisms in endoplasmic reticulum (Tanford 1981) and mitochondria (Carafoli 1979) and also limits the activity of the Ca\(^{2+}\) pump in the plasma membrane (Carafoli 1987). The present result with ChTX suggests the involvement of Ca\(^{2+}\)-activated K\(^{+}\) channels during hypoxia is also in accordance with reports in rat hippocampal neurons (Leblond and Krmjevic 1989) and mouse sensory neurons (Duchen 1990). In the present study the metabolic deprivation also induced the tolbutamide-sensitive I\(_{\text{OUT}}\) (Fig. 7), suggesting the contribution of ATP-sensitive K\(^{+}\) channels. The hypoxia-induced increase in ATP-sensitive K\(^{+}\) conductance was also reported in adult rat LC neurons (Nieber et al. 1995).

In the present study, however, the tolbutamide-sensitive K\(^{+}\) current component decreased time-dependently during a continuous application of the metabolic inhibitors. A marked reduction in the activity of ATP-sensitive K\(^{+}\) channels was also observed in dissociated rat substantia nigra neurons when hypoxia was maintained for several minutes (Jiang et al. 1994). Cerebral ischemia leads to a number of metabolic changes that include an increase in Ca\(^{2+}\), free fatty acid, and diacylglycerides (Katsura et al. 1993). The activity of the cardiac ATP-sensitive K\(^{+}\) channel was shown to be influenced by these metabolites (Kim and Duff 1990; Kirsch et al. 1990), suggesting that the changes of cellular metabolism during ischemia modulate ATP-sensitive K\(^{+}\) channel activity. The mechanism underlying the regulation of ATP-sensitive K\(^{+}\) channel activity in rat LC neurons requires further investigation in a future study.

In mammalian CNS neurons, at least two Ca\(^{2+}\) release mechanisms from intracellular Ca\(^{2+}\) pools were established. One involves the binding of the second messenger IP\(_3\) to specific IP\(_3\) receptors on the surface of intracellular organelas whereas the other involves the activation by cytoplasmic Ca\(^{2+}\) of Ca\(^{2+}\)-induced Ca\(^{2+}\) release channels (Henzi and MacDermott 1992). In the present study ryanodine did not affect NaCN-induced I\(_{\text{OUT}}\), whereas thapsigargin, which se-
lectively depletes Ca$^{2+}$ in the IP$_3$-sensitive pool by inhibiting the ATPase responsible for Ca$^{2+}$-sequestration (Robinson and Burgoyne 1991), inhibited the NaCN-induced I$_{KATP}$. Therefore the IICR pool plays an important role in the rise in [Ca$^{2+}]_i$ during metabolic inhibition in rat LC neurons. The Ca$^{2+}$-releasing mechanisms during metabolic deprivations, however, still remain to be clarified.

In the present study experimental ischemia, anoxia, and hypoglycemia increased the level of [Ca$^{2+}]_i$, thus resulting in the activation of Ca$^{2+}$-activated K$^+$ channels throughout a continuous inhibition of neuronal metabolisms. The transient activation of ATP-sensitive K$^+$ channels was also observed at the beginning of the metabolic inhibition. The hyperpolarization of the neuronal membrane induced by the increase in both the Ca$^{2+}$-activated and ATP-sensitive K$^+$ conductances may reduce further energy consumption by suppressing neuronal activity. Consequently, these two kinds of K$^+$ outward currents observed in the present preparation might be useful as negative feedback systems in reducing the NMDA- and ischemia-induced neurotoxicity.

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Address reprint requests to N. Akaike.

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REFERENCES


SHIRASAKI, T., NAGAKAWA, T., WAKAMORI, T., TATEISHI, N., FUKUDA, A., MURASE, K., AND AKAIKE, N. Glycine-insensitive desensitization of N-


