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Effects of Glucose Deprivation on NMDA-Induced Current and Intracellular Ca^{2+} in Rat Substantia Nigra Neurons

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SUMMARY AND CONCLUSIONS

1. The effects of glucose deprivation on *N*-methyl-D-aspartate (NMDA)-induced current (I_{NMDA}) and the intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) in the acutely dissociated rat substantia nigra neurons were investigated using the nystatin-perforated patch-clamp technique under voltage clamp and the microfluometry with a fluorescent probe, Indo-1.

2. Application of NMDA induced a peak and a successive steady-state inward current, and an outward current immediately after washout at a holding potential of −40 mV. The amplitudes of the three current components of I_{NMDA} were increased by increasing the concentrations of NMDA with half-maximum concentrations (EC_{50}s) of 1.1×10^{-4} M, 1.2×10^{-4} M, and 1.6×10^{-4} M, respectively.

3. The reversal potentials of the peak inward and outward currents were −4 ± 3 (SE) mV and −76 ± 2 mV, respectively. The latter was close to the theoretical K^+ equilibrium potential (−82 mV).

4. The outward current was potentiated by increase in extracellular Ca^{2+} concentration and was blocked by Cs^+ internal solution and suppressed by 5×10^{-3} M tetraethylammonium chloride and 10^{-7} M charybdotoxin, indicating that it was Ca^{2+}-activated K^+ current.

5. Application of NMDA increased $[Ca^{2+}]_i$ in a concentration-dependent manner with an EC_{50} of 3.9×10^{-5} M.

6. Depriving the external solution of glucose induced a slowly developing outward current and increased the basal level of $[Ca^{2+}]_i$. It also prolonged the NMDA-induced outward current without affecting the peak inward current, and prolonged the NMDA-induced increase in $[Ca^{2+}]_i$ without changing the peak $[Ca^{2+}]_i$.

7. These findings suggest that the deprivation of glucose did not affect the NMDA-induced influx of Ca^{2+} into the cells, but it inhibited Ca^{2+} clearance by affecting the efflux of Ca^{2+} to the extracellular space, reuptake into the intracellular Ca^{2+} stores, and/or active extrusion from intracellular stores.

INTRODUCTION

Excessive activation of glutamate receptors in neurons of the CNS is known to induce neurotoxicity (Olney 1969). The key element underlying the neurotoxicity is the intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$). During brain ischemia, release of glutamate and aspartate is increased three- to eightfold of the preischemia level, leading to excessive accumulation of glutamate and aspartate in the extracellular fluid in the affected areas of the CNS (Benveniste et al. 1984, 1989). The released excitatory amino acids increase $[Ca^{2+}]_i$ from 10^{-8}–10^{-7} M to 10^{-4} M under ischemia (Silver and Erecinska 1990, 1992) by way of activating Ca^{2+} influx through *N*-methyl-D-aspartate (NMDA) receptors (Choi 1987; Jensen et al. 1991; Salinska et al. 1991; Segal and Manor 1992; Simon et al. 1984; Szatkowski and Attwell 1994), non-NMDA receptors (Nellgård and Wieloch 1992), or both receptors (Andiné et al. 1992). The elevated $[Ca^{2+}]_i$ finally triggers acute and delayed neuronal death (Choi 1988; Rothman et al. 1987) by still-unknown mechanisms. The critical role of $[Ca^{2+}]_i$ in the ischemic neurotoxicity is supported by the facts that removal of Ca^{2+} from the extracellular fluid during ischemia, or the blockade of glutamate receptors, prevented the delayed neuronal death (Choi 1987; Rothman et al. 1987). However, the time course of the elevated $[Ca^{2+}]_i$ and its effects on neuronal activity at a single-cell level remain obscure.

The effects of ischemic changes in neural functions include depression of the excitatory postsynaptic current, resulting from paradoxical presynaptic inhibition of glutamate release (Hershkowitz et al. 1993), and depression of Ca^{2+}-mediated slow inward current and NMDA-evoked inward current (Krnjevic et al. 1989; Leblond and Krnjevic 1989) in CA1 pyramidal neurons in hippocampal slices. The sites of actions of ischemia are difficult to delineate in detail using brain slices, because the effects on presynaptic terminals, postsynaptic somata, and glial cells are intermingled, forming a complex intercellular network. To clarify the effect of ischemia on postsynaptic somata, it is indispensable to use single neurons isolated from the surrounding neural structures. The aim of the present study is to examine the ischemic changes in NMDA-induced currents (I_{NMDA}) and $[Ca^{2+}]_i$ using single intact neurons. So far, only an activation of ATP-sensitive K^+ current [$I_{K(ATP)}$] by hypoxia has been reported on isolated neurons to date (Jiang et al. 1994).

Two recent advances in experimental techniques made it possible to perform such experiments on single intact neurons. One is the acute dissociation technique of single neurons from the CNS (Kaneda et al. 1988). Another is the nystatin-perforated patch recording, which allows voltage clamp or current clamp of the neurons without disturbing the intracellular environment (see minireview in Akaike and Harata 1994).

Usually the deprivation of oxygen and/or glucose is used to obtain ischemia-like conditions (Alici and Heinemann 1995; Dubinsky and Rothman 1991; Silver and Erecinska 1990; Szatkowski and Attwell 1994). In this experiment we deprived the extracellular solution of glucose to introduce ischemia-like conditions.

METHODS

Preparation

Substantia nigra neurons were acutely dissociated enzymatically from 2-wk-old Wistar rats as described previously (Kaneda et al.

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1988; Nakagawa et al. 1990). Briefly, rats were anesthetized with intraperitoneal injection of pentobarbital sodium (50 mg/kg) and 400-μm-thick coronal sections of brain were cut with a microslicer (DTK-1000; Dosaka, Kyoto, Japan). The slices were treated with the well-oxygenated standard external solution containing 0.1–0.2 mg/ml pronase (Calbiochem) at 31°C for 20–40 min, followed by the standard external solution containing 0.1–0.2 mg/ml thermolysin (Sigma) under the same conditions. After the enzyme treatment, the substantia nigra region was micropunched out from the slices and neurons were dissociated mechanically with a fire-polished micropipette in the culture dish (Falcon). All experiments were carried out at room temperature (23–25°C).

Solutions and drugs

The standard external solution contained (in mM) 150 NaCl, 5 KCl, 1 $MgCl_2$, 2 $CaCl_2$, 10 *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES), and 10 glucose, pH adjusted to 7.4 with tris(hydroxymethyl)aminomethane (Tris) base. For analyzing the I_{NMDA} and $[Ca^{2+}]_i$ changes, $MgCl_2$ was simply removed from the standard external solution and 10^{-6} M glycine was added. In the glucose-free external solution, glucose was removed from the external solution and replaced by the same concentration of mannitol to maintain the osmolarity. In some experiments, 2×10^{-4} M $CdCl_2$, 3×10^{-7} M tetrodotoxin, or 5×10^{-3} M tetraethylammonium chloride (TEA) were added to the external solution to block the voltage-dependent Ca^{2+}, Na^+, or K^+ channels, respectively. To identify the specific K^+ channels, 10^{-7} M charybdotoxin (ChTX) or 2×10^{-5} M glibenclamide were also added to the control solution. The standard patch pipette (internal) solution contained (in mM) 150 KCl and 10 HEPES, pH adjusted to 7.2 with Tris base. In some experiments, Cs^+ internal solution was used, which contained (in mM) 150 CsCl and 10 HEPES, pH adjusted to 7.2 with Tris base. The internal solutions did not contain Na^+. Inclusion of Na^+ is expected to activate Na^+/K^+-ATPase, which would lead to postischemic aglycemic outward current (Siegmund et al. 1994), possibly obscuring the changes in the NMDA-induced outward current. NMDA (Sigma) was dissolved in distilled water at 10^{-1} M and was diluted accordingly by the control solution. ChTX was dissolved in distilled water at 10^{-4} M for stock solution. Glibenclamide was dissolved in dimethyl sulfoxide at 2×10^{-2} M for stock solution. Nystatin (Sigma) was dissolved in methanol, resulting in 10 mg/ml of stock solution, and added to the pipette solution at a final concentration of 400 μg/ml just before use.

A rapid application of external solution was performed with the "Y tube" technique described previously (Nakagawa et al. 1990). Using this technique, the external solution could be completely exchanged within 10–20 ms. Duration of NMDA application was fixed at 15 s for electrical recording and 30 s for fluorescence measurement to ensure constant outward current and $[Ca^{2+}]_i$ increase, respectively.

Electrical measurements

The single substantia nigra neurons were voltage-clamped using the nystatin-perforated patch-clamp technique described previously (Akaike and Harata 1994). Patch electrodes were fabricated from glass capillaries (G-1.5, Narishige, Tokyo, Japan) on a vertical puller (PP83, Narishige). The resistance of the patch pipette was between 4 and 10 MΩ when filled with the internal solution. After formation of a tight seal on the cell surface, no suction was applied to avoid ruptures of the patch membrane. The experiments were initiated after 10 min, when the series resistance had stabilized. The current and voltage were recorded using a patch-clamp amplifier (TM-1000, Act ME Laboratory, Tokyo, Japan) and stored on magnetic tapes with the use of a digital audiotape recorder (RE-130TE, TEAC, Tokyo, Japan) for subsequent analysis.

$[Ca^{2+}]_i$ measurement

The method for fluorescence measurements was essentially the same as that described elsewhere (Noma et al. 1991). In brief, cells were incubated in the standard external solution with 5×10^{-6} M Indo-1 acetoxymethylester form (Dojindo, Kumamoto, Japan) at room temperature for 30 min. The $[Ca^{2+}]_i$ measurements were performed on an inverted microscope with a quartz epifluorescence attachment (Diaphot TMD, Nikon, Tokyo, Japan). A cell adhering to the coverglass in the dish was illuminated by a 100-W xenon arc lamp with the light passing through a 360-nm bandpass filter for 300 ms every 6 s and directed on to the specimen by a Nikon 390-nm dichroic mirror through a Fluor ×40 oil objective (NA 1.3, Nikon). The fluorescence signals (emission at 405 and 480 nm) were directed to the two photomultiplier tubes (P1, Nikon) through a beam splitter. Signals from photomultiplier tubes were stored in the computer (PC98XL, NEC, Tokyo, Japan) for the calculation of $[Ca^{2+}]_i$. The $[Ca^{2+}]_i$ was estimated according to the following equation (Grynkiewicz et al. 1985)

$$[Ca^{2+}]_i = K_D\beta(R - R_{min})/(R_{max} - R) \quad (1)$$

where K_D is the dissociation constant for the Ca^{2+}-Indo-1 complex; β is the fluorescence ratio of Ca^{2+}-free and Ca^{2+}-saturated (10^{-2} M) conditions 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_{min} and R_{max} are the minimum and maximum fluorescence ratios measured in Ca^{2+}-free and Ca^{2+}-saturated conditions, respectively. We assumed 250 nM for K_D (Grynkiewicz et al. 1985) and 4.18 for β from our calibration. To obtain R_{min} and R_{max}, we used Indo-1 in saline containing 3 M sucrose and 10^{-2} M bis-(*o*-aminophenoxy)-*N,N,N',N'*-tetraacetic acid with or without 10^{-2} M $CaCl_2$ to provide free or saturated concentrations of Ca^{2+}. R_{min} and R_{max} values were 0.041 ± 0.002 (mean ± SE; $n = 6$) and 1.39 ± 0.024 ($n = 6$), respectively.

High resting $[Ca^{2+}]_i$ ($\geq$ 300 nM) might indicate that the cells are damaged by dissociation or loading with Indo-1. Because all these neurons with high resting $[Ca^{2+}]_i$ showed trivial changes in $[Ca^{2+}]_i$ on application of NMDA, they were excluded from the present study.

Statistics

The data are shown as means ± SE. Differences in the numerical values between two groups were evaluated using a paired *t*-test.

The concentration-response curves for amplitudes of I_{NMDA} and amplitudes of NMDA-induced $[Ca^{2+}]_i$ changes were drawn according to the following equation

$$I = I_{max} \cdot C^{n_H}/(C^{n_H} + K_D{}^{n_H}) \quad (2)$$

where I is the observed I_{NMDA} or NMDA-induced $[Ca^{2+}]_i$ change and C is the NMDA concentration. K_D and n_H denote the dissociation constant and the Hill coefficient, respectively.

RESULTS

Concentration dependence of three I_{NMDA} components

Figure 1*A* shows the original current traces induced by different concentrations of NMDA [10^{-5} M (*left*), 3×10^{-5} M (*middle*), 10^{-4} M (*right*)] at a holding potential (V_H) of −40 mV by the use of nystatin-perforated patch-clamp technique. Application of NMDA induced a rapidly desensitizing peak inward current and a following steady-state inward current. After withdrawal of NMDA, a transient outward current was observed. Both the current noise level and membrane conductance were increased during the steady-state inward and transient outward currents (data not

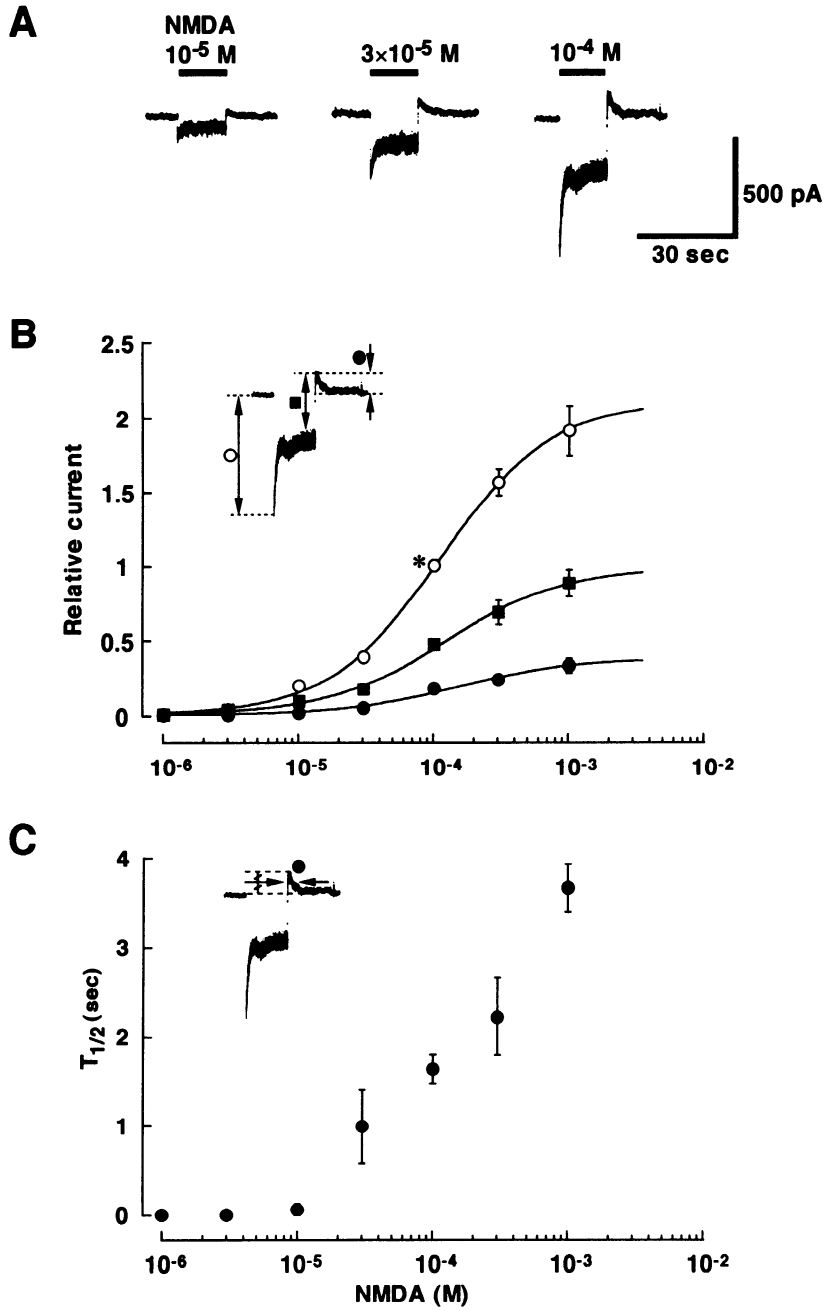

FIG. 1. Concentration dependence of *N*-methyl-D-aspartate (NMDA)-induced current (I_{NMDA}). *A*: I_{NMDA} elicited by 10^{-5} M NMDA (*left*), 3×10^{-5} M NMDA (*middle*), and 10^{-4} M NMDA (*right*) at a holding potential (V_H) of −40 mV. *B*: concentration-response relationship of I_{NMDA}. The amplitudes of the peak inward current (○), the steady-state inward current (■), and the outward current (●) are plotted against NMDA concentration. The amplitudes are normalized to the absolute amplitude of the peak inward current induced by 10^{-4} M of NMDA (*). *Inset*: method of measuring the amplitudes. Smooth curves were drawn according to *Eq. 2*. Each point is the average of 3–6 neurons. Vertical bar: mean ± SE. *C*: concentration-response relationship of the half-decay time ($T_{1/2}$) of the outward current (●). Each point is the average of 3–6 neurons. Vertical bar: mean ± SE.

shown). To characterize the three components of I_{NMDA}, the concentration-response relationships of I_{NMDA} were determined as shown in Fig. 1*B*. The *inset* shows the measurement of the three components. For clarity, the amplitude of the steady-state inward current was defined as the difference between the peak outward current and the apparent steady-state current. All the amplitudes were normalized by the absolute value of the peak inward current induced by 10^{-4} M NMDA for each cell. All the currents were increased by increasing the concentration of NMDA with half-maximum concentrations (EC_{50}s) of 1.1×10^{-4} M, 1.2×10^{-4} M, and 1.6×10^{-4} M and with Hill coefficients of 1.06, 0.98, and 1.02 for the peak inward current, the steady-state inward current, and the outward current, respectively.

The present results on the concentration-response relationship are approximately in agreement with those reported previously for the peak and steady-state inward currents in acutely dissociated Meynert neurons of the rats (Akaike et al. 1991). However, the outward current was not observed in the previous study, in which the conventional whole cell patch recording was employed to clamp $[Ca^{2+}]_i$ near 1.7×10^{-8} M by ethylene glycol-bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid (EGTA). Thus the outward current was characteristic of nystatin-perforated patch recording, and the duration of the outward current was further analyzed for the characterization. The duration was assessed by a half-decay time ($T_{1/2}$) as shown in Fig. 1*C*. The $T_{1/2}$ was increased by increasing the concentration of NMDA, with a threshold of 10^{-5} M and an approximate EC_{50} of 10^{-4} M, assuming the maximal $T_{1/2}$ within the examined

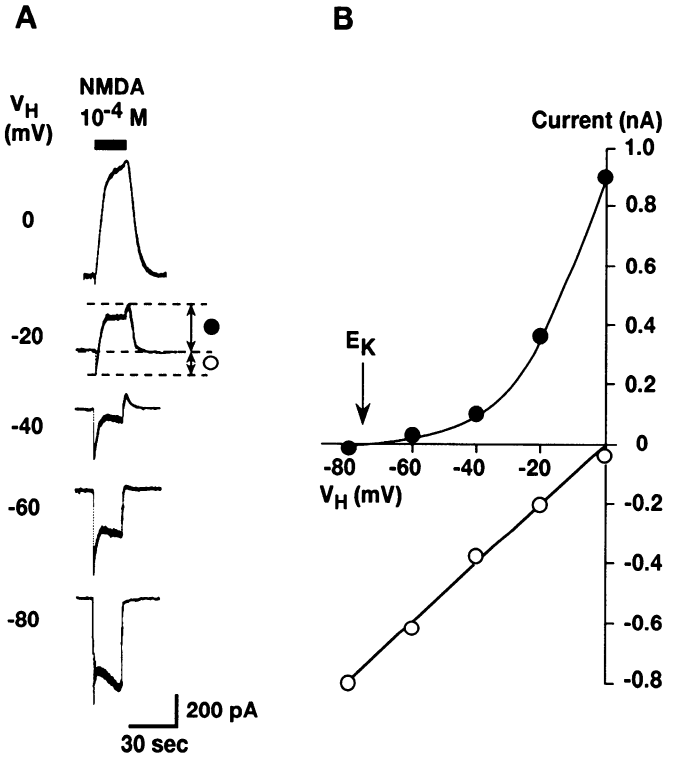

FIG. 2. Voltage dependence of I_{NMDA}. *A*: current responses induced by 10^{-4} M NMDA (indicated by bar) at various V_Hs (0, −20, −40, −60, and −80 mV). *B*: current-voltage curves of the peak inward current (○) and the outward current (●). The figure is representative of 12 reproducible experiments.

range. Because the maximal $T_{1/2}$ did not reach the steady state, the real EC_{50} could not be determined. However, this approximation is useful for comparison with other data.

Current-voltage relationship of NMDA-induced outward current

Because the outward current was consistently observed with the nystatin-perforated patch recording, the current-voltage relationship was examined to determine the ionic basis of the NMDA-induced outward current. The I_{NMDA} was recorded at various V_Hs from −80 to 0 mV in 20-mV steps as shown in Fig. 2*A*. At a V_H of −80 mV, application of 10^{-4} M NMDA induced a large inward current, whereas an outward current after withdrawal of NMDA was hardly discernible. Between V_Hs of −80 and −20 mV, the amplitudes of the peak and steady-state inward currents as we defined in Fig. 1*B* decreased, whereas the amplitude of the outward currents increased. At a V_H of 0 mV, the peak and steady-state inward current was almost abolished and the outward current made up most of the I_{NMDA}. The current-voltage plots showed a linear relationship for the peak inward current and a marked outward rectification for the outward current (Fig. 2*B*). The reversal potentials of the peak inward and outward currents were −4 ± 3 mV (n = 12) and −76 ± 2 mV (n = 9), respectively. These reversal potentials of the inward and outward currents were in good agreement with that of a nonselective cation channel (Mayer and Westbrook 1987b) and the theoretical K^+ equilibrium potential of −82 mV calculated with the Nernst equation from the given external and internal concentrations of K^+, indicating that they are mediated by a nonselective cation channel and a K^+ channel, respectively.

K^+ as the charge carrier of the outward current

Two other experiments were performed to ascertain that the outward current is carried by K^+. Figure 3*A* shows the current induced by 10^{-4} M NMDA with the Cs^+ internal solution at a V_H of −40 mV. The outward current observed with the standard internal solution (Figs. 1 and 2) was completely suppressed with the Cs^+ internal solution. Figure 3*B* shows the effect of 5×10^{-3} M TEA on the I_{NMDA} recorded with the K^+ internal solution. The holding current shifted inwardly with the application of TEA. The peak inward current of I_{NMDA} was not affected by TEA, whereas the outward current was reversibly blocked. The sensitivity to internal Cs^+ and external TEA is suggestive of the K^+ channels involved in the outward current. The gradual decrease in the inward current during a continuous application of NMDA (Fig. 3*B, left*) disappeared in the presence of TEA, concomitant with the disappearance of the outward current (Fig. 3*B, right*), indicating that the apparent desensitization of I_{NMDA} was partly attributable to the overlying outward current.

Identification of the outward current as Ca^{2+}-activated K^+ current

High Ca^{2+} permeability of the NMDA channels (Mayer and Westbrook 1987a) raises a possibility that the outward K^+ current is a Ca^{2+}-activated K^+ current ($I_{K(Ca)}$). To confirm this view, we performed the following three experiments. First, the effect of different concentrations of extracellular Ca^{2+} ($[Ca^{2+}]_o$) was assessed (Fig. 4*A*). When the outward current of the I_{NMDA} was abolished by the Cs^+ internal solution (Fig. 4*Aa*), the amplitudes of the peak inward current were increased by increasing the $[Ca^{2+}]_o$. In four cells, the amplitudes of the peak inward current with 2, 5, and 10 mM of $[Ca^{2+}]_o$ were 246 ± 17, 276 ± 18, and 300 ± 17 pA, respectively. All the values were significantly different from each other ($P < 0.05$). With the K^+ internal solution (Fig. 4*Ab*), the amplitude and duration of the outward currents were obviously increased in a concentration-dependent manner. Second, the outward current was not observed when the conventional whole cell patch recording was performed in the internal solution containing 10 mM

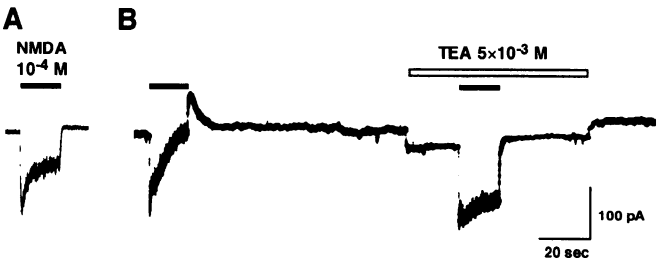

FIG. 3. Effects of Cs^+ internal solution and tetraethylammonium chloride (TEA) on the NMDA-induced outward current at a V_H of −40 mV. *A*: current response induced by 10^{-4} M NMDA (indicated by bar) when the internal solution contained 150 mM CsCl. The figure is representative of 4 reproducible observations. *B*: current responses induced by 10^{-4} M NMDA (indicated by bar). TEA (5×10^{-3} M) was applied extracellularly during the period indicated by the open bar. The figure is representative of 4 reproducible experiments.

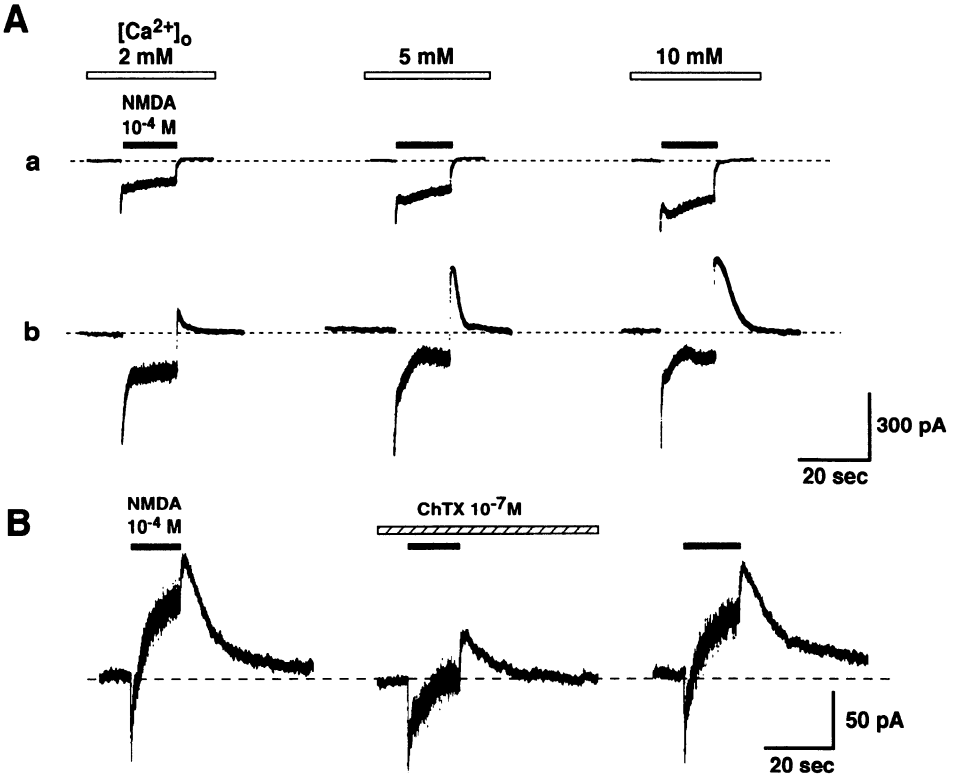

FIG. 4. Effects of extracellular Ca^{2+} concentration ($[Ca^{2+}]_o$) and charybdotoxin (ChTX) on I_{NMDA}. *A*: original current traces induced by 10^{-4} M NMDA (indicated by bar) at a V_H of −40 mV under various concentrations of extracellular Ca^{2+} ($[Ca^{2+}]_o$) during the period indicated by open bar [2 mM (*left*), 5 mM (*middle*), 10 mM (*right*)] with the internal solution containing 150 mM CsCl (*Aa*) and 150 mM KCl (*Ab*). Dotted lines: control holding current level. *B*: current responses brought about by 10^{-4} M NMDA (indicated by bar) before (*left*), during (*middle*), and after (*right*) application of 10^{-7} M ChTX (indicated by hatched bar). *Aa, Ab,* and *B* are representative of 4 reproducible experiments.

EGTA (data not shown), indicating that the outward current is dependent on $[Ca^{2+}]_i$. Last, the effect of ChTX, a blocker of the intermediate conductance type of $I_{K(Ca)}$, was examined. As shown in Fig. 4*B*, the outward current, which had an amplitude of ~100 pA (Fig. 4*B, left*), was suppressed to ~40% in the presence of 10^{-7} M ChTX in the external solution (Fig. 4*B, middle*). The partially blocked outward current returned to the predrug level after withdrawal of ChTX (Fig. 4*B, right*). In four experiments, the amplitudes of the outward currents with or without ChTX were 58 ± 14 and 106 ± 15 pA, respectively. The difference was statistically significant ($P < 0.005$). In addition, the outward current was not affected by 2×10^{-5} M glibenclamide, a blocker of $I_{K(ATP)}$ ($n = 4$, data not shown). These experiments, in conjunction with the fact that the outward current of I_{NMDA} was Ca^{2+} dependent and showed marked outward rectification, strongly suggest that it is $I_{K(Ca)}$.

NMDA-induced changes in $[Ca^{2+}]_i$

For directly evaluating the changes in $[Ca^{2+}]_i$ accompanying NMDA receptor activation, microfluometry was performed with Indo-1. Figure 5*A* shows the $[Ca^{2+}]_i$ increase brought about by the application of different concentrations of NMDA [3×10^{-6} M (*left*), 10^{-4} M (*middle*), 10^{-3} M (*right*)]. The $[Ca^{2+}]_i$ increased rapidly and reached a steady state within ~30 s with application of NMDA, and returned to the control level within ~3 min after withdrawal of NMDA. Figure 5*B* shows a concentration-response curve of the rise in $[Ca^{2+}]_i$ ($\Delta[Ca^{2+}]$) normalized to that at 10^{-4} M NMDA. The EC_{50} and Hill coefficient were 3.9×10^{-5} M and 0.75, respectively. The values of the absolute peak $[Ca^{2+}]_i$ level were 387 ± 42 nM ($n = 8$) for 10^{-4} M NMDA.

For assessing the duration of changes in $[Ca^{2+}]_i$, we devised a new factor ΔS, which is calculated by the following equation

$$\Delta S = \int (\Delta[Ca^{2+}(t)])dt \qquad (3)$$

where $\Delta[Ca^{2+}(t)]$ represents $\Delta[Ca^{2+}]$ at time t after NMDA application. The ΔS was increased by NMDA in a concentration-dependent manner (Fig. 5*C*). Because the concentration-response relationships of $\Delta[Ca^{2+}]$ and ΔS are quantitatively in accordance with those of the amplitude of peak inward I_{NMDA} and $T_{1/2}$ of the outward I_{NMDA}, respectively (Figs. 1 and 5), the parameters $\Delta[Ca^{2+}]$ and amplitude of peak inward I_{NMDA} would reflect the amount of Ca^{2+} influx through NMDA receptors, and the parameters ΔS and $T_{1/2}$ of outward I_{NMDA} would represent the time course of Ca^{2+} clearance from the cytoplasm.

There remains a possibility that $\Delta[Ca^{2+}]$ was partly mediated by the Ca^{2+} influx through the voltage-dependent Ca^{2+} channels in addition to that through the NMDA channels, when the cell was not voltage-clamped (Mayer and Westbrook 1987a). To exclude this possibility, we performed the fluorescence measurements with or without 2×10^{-4} M Cd^{2+} and 3×10^{-7} M tetrodotoxin to block voltage-dependent Ca^{2+} channels (data not shown). In four cells, ΔS values were 12.6 ± 2.7 and 11.7 ± 2.2 $\mu M \cdot s$ with and without Cd^{2+} and tetrodotoxin, respectively, and were not significantly different. Furthermore, we performed simultaneous recording of the I_{NMDA} responses and the fluorescence measurements with or without glucose in the external solution at a V_H of −40 mV ($n = 5$; data not shown). The current responses and the $\Delta[Ca^{2+}]_i$ showed no difference with those recorded separately. Thus an interference from the voltage-dependent Ca^{2+} channels in the time course of the $[Ca^{2+}]_i$ is thought to be only minimal.

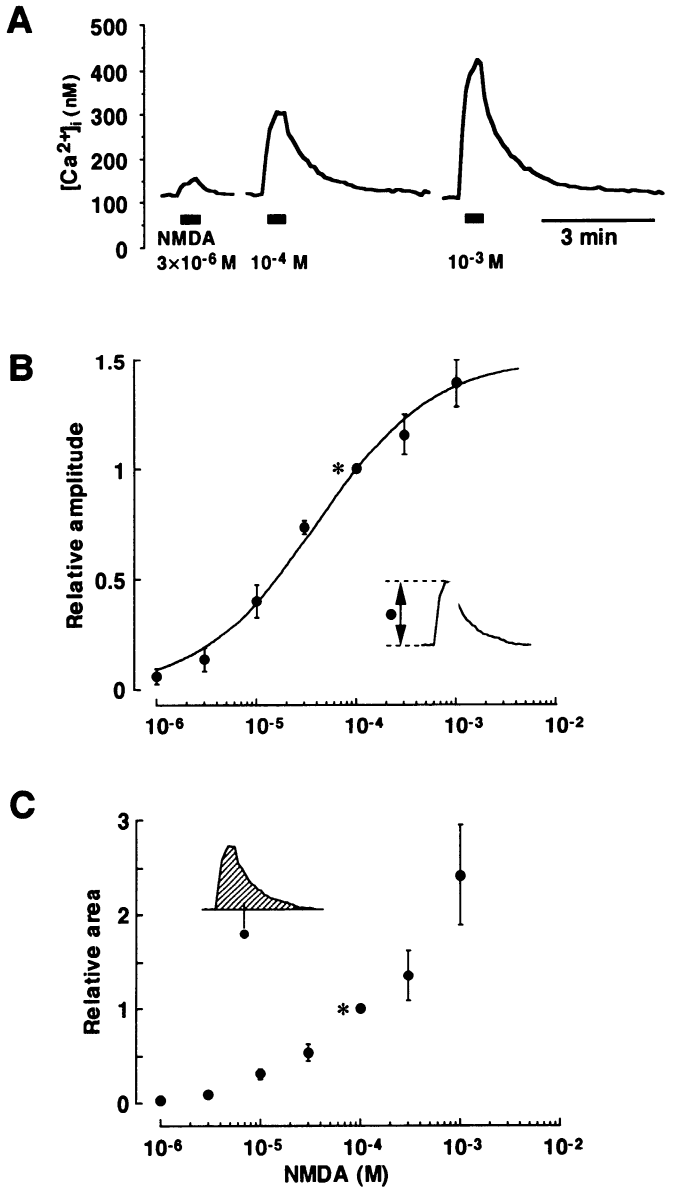

FIG. 5. Concentration dependence of intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$). *A*: increase in $[Ca^{2+}]_i$ brought about by 3×10^{-6} M NMDA (bar, *left*), 10^{-4} M NMDA (bar, *middle*), and 10^{-3} M NMDA (bar, *right*). *B*: concentration-response relationship of $[Ca^{2+}]_i$ increase ($\Delta[Ca^{2+}]$, ●). The change in $[Ca^{2+}]_i$ was normalized to that induced by 10^{-4} M NMDA (*). Each point is the average of 3–8 neurons. Vertical bar: mean ± SE. *C*: concentration-response relationship of the integrated $\Delta[Ca^{2+}]$ (ΔS, ●). ΔS was normalized to that induced by 10^{-4} M NMDA (*). Each point is the average of 3–6 neurons. Vertical bar: mean ± SE.

Effects of glucose deprivation on I_{NMDA} and $[Ca^{2+}]_i$

Effects of glucose-free conditions on I_{NMDA} and $[Ca^{2+}]_i$ were examined. As shown in Fig. 6*A*, application of the glucose-free external solution gradually shifted the holding current in an outward direction. Application of NMDA during glucose deprivation prominently increased the amplitude and the duration of the outward current without changing the amplitude of the peak inward current. The amplitude and the $T_{1/2}$ of the outward current in this neuron increased from 12 pA and 2.0 s under control conditions to 55 pA and 4.3 s under glucose-free conditions, respectively. After washout of the glucose-free solution, the shifted holding current gradually returned to the control level. The fluorescence measurements were performed with the same protocol for comparing the changes in I_{NMDA} and $[Ca^{2+}]_i$ (Fig. 6*B*). The $[Ca^{2+}]_i$ was 220 nM on the basal level, increased to 350–370 nM during the application of 10^{-4} M NMDA, and returned to the basal level within 3 min after withdrawal of NMDA. After exchange to the glucose-free solution, the basal $[Ca^{2+}]_i$ increased gradually and reached a new steady-state level at ~5 min. In five cells, the basal and the new steady-state $[Ca^{2+}]_i$ levels were 198 ± 27 and 261 ± 42 nM, respectively. The NMDA-induced $\Delta[Ca^{2+}]$ was long-lasting, persisting even after the withdrawal of NMDA and requiring >3 min for recovery to the previous steady-state level. The increased $[Ca^{2+}]_i$ with glucose deprivation completely returned to the basal level after washout of the glucose-free solution. Although the amplitudes of the peak inward current and the peak $\Delta[Ca^{2+}]$ under glucose deprivation were larger than those of control conditions for the cell in Fig. 6, it seems an unusual case. The changes were not statistically significant, as shown below.

The comparable changes in I_{NMDA} and $[Ca^{2+}]_i$ under glucose deprivation were evaluated statistically. The amplitude of the peak inward current induced by 10^{-4} M NMDA (Fig. 7*Aa*) under control and glucose-free conditions, respectively, was 109 ± 16 and 117 ± 25 pA in five cells, with no significant difference (Fig. 7*Ab*). The $T_{1/2}$ of the outward current under control and glucose-free conditions, respectively, was 2.7 ± 0.6 and 5.0 ± 1.3 s in seven cells, and was significantly different (Fig. 7*Ac*). The $\Delta[Ca^{2+}]$ induced by 10^{-4} M NMDA (Fig. 7*Ba*) under control and glucose-free conditions, respectively, was 210 ± 41 and 201 ± 30 nM in eight cells (Fig. 7*Bb*), without significant difference. The ΔS was measured for 180 s because the increased $[Ca^{2+}]_i$ returned to the control level within 180 s under physiological conditions (Figs. 5*A* and 6*B*), whereas it did not under glucose-free conditions (Fig. 6*B*). The ΔS under control and glucose-free conditions, respectively, was 7.8 ± 2.1 and 13.6 ± 2.5 $\mu M \cdot s$ in five cells, and was significantly different (Fig. 7*Bc*). Thus the $T_{1/2}$ and the ΔS were increased significantly by the glucose deprivation, whereas the peak inward current and the $\Delta[Ca^{2+}]$ were not affected. These findings suggest that the deprivation of glucose did not affect the influx of Ca^{2+} into the cells through the NMDA receptor, but it inhibited the clearance of Ca^{2+} either by efflux to the extracellular space, reuptake into the intracellular Ca^{2+} stores, and/or active extrusion from intracellular stores.

DISCUSSION

The NMDA-induced outward current was reported previously (Krnjevic et al. 1989; Mistry and Hablitz 1990; Seutin et al. 1994), yet its nature and characteristics remained unclear. Similar outward currents were also observed in CA1 pyramidal neurons (data not shown) and Meynert neurons (Akaike and Harata 1994) with NMDA, and were activated by both kainic acid and ATP in Meynert neurons, cerebral cortical neurons, and nucleus tractus solitarii neurons (Akaike and Harata 1994; Omura et al. 1993; Ueno et al. 1992). These outward currents were reported to be $I_{K(Ca)}$. In the present experiment, we characterized, for the first time, the NMDA-induced outward current, the effects of glucose deprivation on I_{NMDA}, and NMDA-induced changes

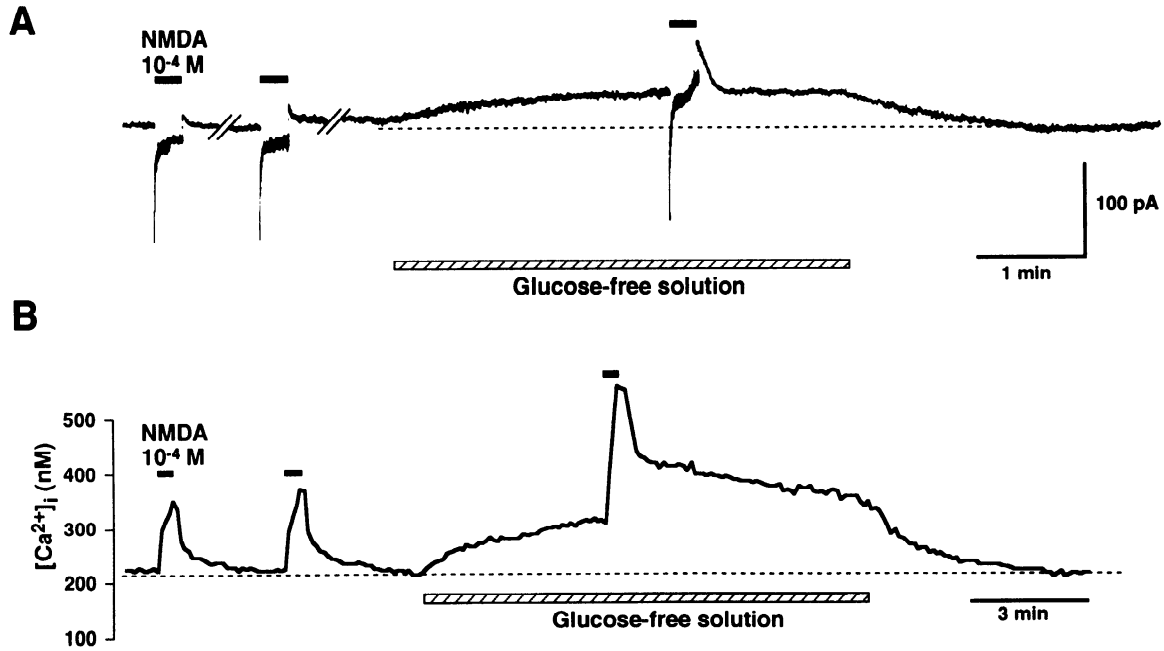

FIG. 6. Effects of glucose-free solution on I_{NMDA} and $[Ca^{2+}]_i$. *A*: original current traces at a V_H of −40 mV. Extracellular solution was rapidly switched to a solution containing 10^{-4} M NMDA during the periods indicated by the bar. After recording of control I_{NMDA}, the external solution was changed to glucose-free solution (hatched bar), followed by the application of the same concentration of NMDA. The figure is representative of 7 experiments. *B*: fluorescence measurements with the same protocol as in *A*. The figure is representative of 8 experiments.

in $[Ca^{2+}]_i$. The outward current induced by NMDA could be constantly observed after withdrawal of NMDA (Figs. 1–4, 6, and 7), and its duration was prolonged under glucose-free conditions (Figs. 6 and 7). The major component of the outward current was identified as $I_{K(Ca)}$ because it had a reversal potential near the theoretical K^+ equilibrium potential (Fig. 2), was blocked by internal Cs^+, external TEA, and ChTX (Figs. 3 and 4), and was dependent on $[Ca^{2+}]_i$ and $[Ca^{2+}]_o$ (Fig. 4). Insensitivity of the NMDA-induced outward current to glibenclamide indicates that it is not $I_{K(ATP)}$. A partial involvement of $I_{K(ATP)}$ cannot be completely excluded, because glibenclamide-insensitive, tolbutamide-sensitive $I_{K(ATP)}$ has been described in hippocampal neurons (Godfraind and Krnjevic 1993) and in pancreatic β cells (Williams et al. 1993). However, the effect of tolbutamide was not examined in the present study, because tolbutamide directly affects I_{NMDA} in isolated hippocampal neurons of the rat (Kwiecien et al. 1993).

In the rat brain slices and the cultured neurons, the hypoxic and/or glucose-free conditions elicited membrane hyperpolarization on the basis of the increase of the K^+ current (Knöpfel et al. 1990; Leblond and Krnjevic 1989; Mourre et al. 1989; Rader and Lanthorn 1989). However, the nature of the K^+ current is controversial. It was reported to be the $I_{K(ATP)}$ (Jiang et al. 1994; Mourre et al. 1989), the $I_{K(Ca)}$ (Leblond and Krnjevic 1989), and neither the $I_{K(ATP)}$ nor the $I_{K(Ca)}$ (Knöpfel et al. 1990). Our finding of the increase in the holding current during glucose-free conditions (Fig. 6*A*) is compatible with the hyperpolarization. However, the glucose-free-induced current was not examined in detail.

The intracellular Ca^{2+} acting as a second messenger plays a key role in neuronal functions and has been extensively studied in various types of neurons. The resting $[Ca^{2+}]_i$ was reported to be 10 nM in the rat hippocampus in vivo (Silver and Erecinska 1990, 1992), 30–50 nM in cultured embryonic neurons of the mouse (Murphy and Miller 1988), 71 ± 4 nM in cultured cerebral cortical neurons (Frandsen and Schousboe 1992), and 122 ± 14 nM in cultured neurons of the rat hippocampus (Oliver et al. 1990). The resting $[Ca^{2+}]_i$ in the present experiment was 100–280 nM and compatible with the above values. The $\Delta[Ca^{2+}]$ brought about by the application of 2×10^{-4} NMDA and peak value of $[Ca^{2+}]_i$ brought about by 3×10^{-4} M NMDA were reported to be 363 ± 13 nM (Oliver et al. 1990) and 890 ± 12 nM (Frandsen and Schousboe 1992), respectively. The $\Delta[Ca^{2+}]$ of 210 ± 41 nM (n = 8, Fig. 7) and peak $[Ca^{2+}]_i$ of 387 ± 42 nM (n = 8) brought about by 10^{-4} M NMDA in the present study were also compatible with the above values.

Changes in $[Ca^{2+}]_i$ during ischemia and/or hypoxia, which can induce neuronal damage or death, are very important issues. Hypoxia in rat hippocampal neurons induced the increase in the resting $[Ca^{2+}]_i$ in 5 min (Dubinsky and Rothman 1991). In gerbil hippocampal slices, ischemia-like conditions increased the resting $[Ca^{2+}]_i$ in 8 min with 4 min of latency (Mitani et al. 1993). In the present study, the rise in $[Ca^{2+}]_i$ reached the steady state in ~5 min, in good accordance with the previous studies. The obvious latency, which was reported in the study on hippocampal slices (Mitani et al. 1993), was not observed in the present study. The difference could be ascribed to the difference in the perfusion time, because the perfusion time on a single neuron is much faster (10–20 ms) than that on slices (minutes). The increase in the resting $[Ca^{2+}]_i$ during hypoxia and/or ischemia was reported to be ~1 μM in rat hippocampal neurons in culture (Dubinsky and Rothman 1991) and ~30 μM in rat hippocampus in vivo (Silver and Erecinska 1990, 1992). The changes in the resting $[Ca^{2+}]_i$ during glucose-free conditions were 10–100 nM in the present study. The higher

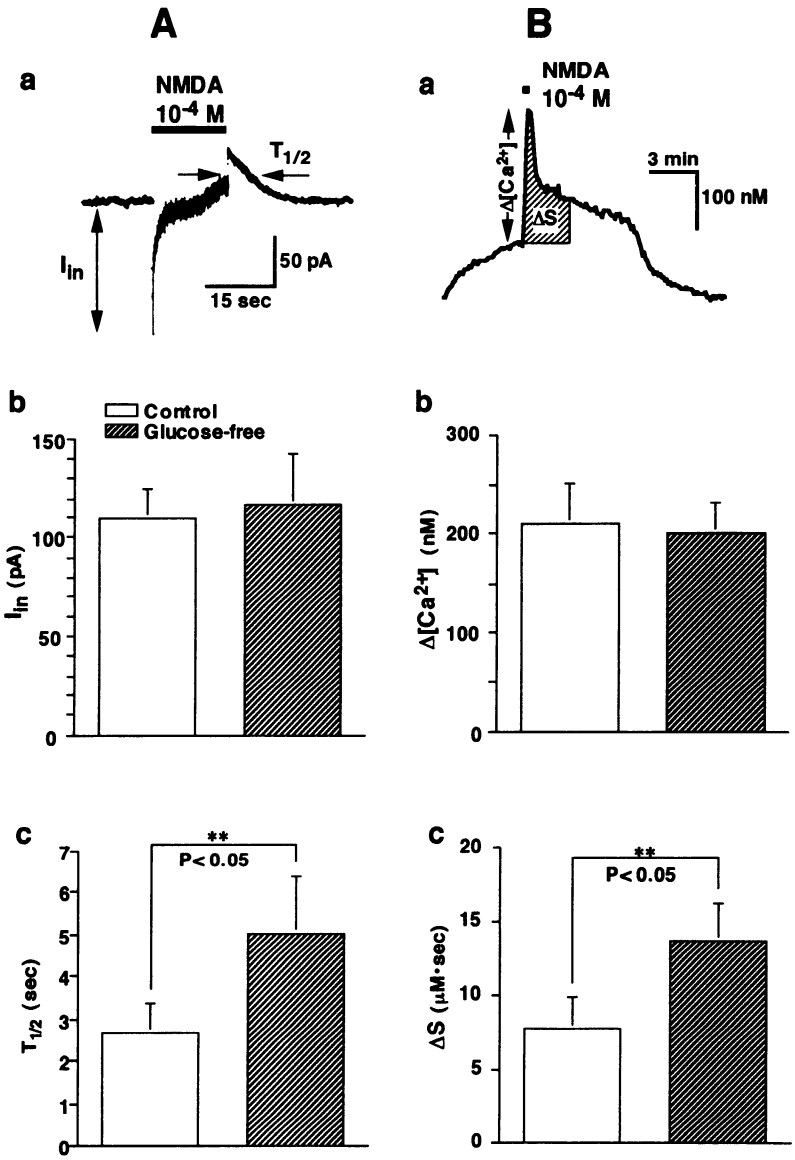

FIG. 7. I_{NMDA} response and $[Ca^{2+}]_i$ increase with and without glucose in the external solution. *Aa*: I_{NMDA} response during application of 10^{-4} M NMDA at a V_H of -40 mV (indicated by bar). I_{in} and $T_{1/2}$: amplitude of the peak inward current and the $T_{1/2}$ of the outward current, respectively. *Ab* and *Ac*: statistical evaluation of I_{in} and $T_{1/2}$ with or without glucose, measured at corresponding letters in *Aa*. *Ba*: changes in $[Ca^{2+}]_i$ during the application of 10^{-4} M NMDA (indicated by bar). $\Delta[Ca^{2+}]$ and ΔS: rise in $[Ca^{2+}]_i$ and the integration of $\Delta[Ca^{2+}]$, respectively. *Bb* and *Bc*: statistical evaluation of $\Delta[Ca^{2+}]$ and ΔS with or without glucose in the external solution, measured at the corresponding letters in *Ba*. Double asterisk: statistical significance at $P < 0.05$.

changes in the resting $[Ca^{2+}]_i$ in the previously reported cases are thought to arise from additional sources of Ca^{2+}. Because the cultured neurons and the brain slices are known to release glutamate and aspartate during ischemia (Benveniste et al. 1984, 1989; Choi 1987, 1988; Olney 1969), the rise in $[Ca^{2+}]_i$ during ischemia is also mediated by Ca^{2+} influx through glutamate receptors, which is excluded in the acutely dissociated single neurons.

There are accumulating lines of evidence suggesting that a preceding increase in $[Ca^{2+}]_i$ suppresses the peak amplitude of the response to NMDA. The increases in $[Ca^{2+}]_i$ are induced by activation of cytoplasmic ion channels/receptors, including voltage-dependent Ca^{2+} channels (Legendre et al. 1993; Medina et al. 1994; Vyklicky 1993), kainate receptors (Medina et al. 1994), and NMDA receptors themselves (Legendre et al. 1993; Medina et al. 1994; Vyklicky 1993), or by a constant control with the chelators in the whole cell patch pipette solution (Legendre et al. 1993; Rosenmund and Westbrook 1993). The studied range of $[Ca^{2+}]_i$ for the suppression was ~100 nM when kainate was applied to hippocampal neurons in culture (Medina et al. 1994) and 1 μM–1 mM when $[Ca^{2+}]_i$ was varied with intracellular chelators in the same preparation (Legendre et al. 1993; Rosenmund and Westbrook 1993). Although the increase in baseline $[Ca^{2+}]_i$ during ischemia-like conditions reached 261 ± 42 nM ($n = 5$), within the reported range for suppression, neither the peak amplitude of the I_{NMDA} nor ΔCa^{2+} brought about by NMDA was affected in the present experiment. One possible explanation involves a subcellular distribution of Ca^{2+}. The $[Ca^{2+}]_i$ was measured at the center of the soma in the present study, and the absolute values of the $[Ca^{2+}]_i$ measurement might not have accurately reflected the changes of $[Ca^{2+}]_i$ in the submembranous domain. Because the rise in $[Ca^{2+}]_i$ during ischemia partly reflects the release of Ca^{2+} from internal stores (Belousov et al. 1995; Mitani et al. 1993), it is conceivable that the changes in submembranous $[Ca^{2+}]_i$ were not as high as in the middle of the soma and were, therefore, not sufficient for suppressing NMDA receptors on the cytoplasmic membrane. Another possibility is the difference due to the preparations or the ages of the used animals. Using the hippocampal neurons in the slice, Krnjevic et al. (1989) showed that a brief anoxia

suppressed the NMDA-induced inward current in the adult rats, whereas it had less effect in immature animals. Thus the NMDA receptors in substantia nigra neurons from an immature rats could manifest different responses to intracellular Ca^{2+}.

The lack of the effect of glucose deprivation on the NMDA-induced Ca^{2+} influx suggests that the apparent prolongation of an outward current and $\Delta[Ca^{2+}]$ by NMDA was mediated by an abnormality in the intracellular Ca^{2+} homeostasis per se. The increased Ca^{2+} in the cytoplasm is normally removed by *1*) ATP-driven sequestration into intracellular organelles and extrusion to extracellular space, *2*) Na^+/Ca^{2+} exchange across the cytoplasmic membrane, and *3*) binding to intracellular Ca^{2+}-binding proteins. Dysfunction of any of these factors hampers the Ca^{2+} removal and leads to a prolonged time course of Ca^{2+} clearance. A decrease in the ATP content during ischemia suppresses the activity of Ca^{2+} ATPase required for pumping Ca^{2+} out of the cytosol. The decrease in ATP also suppresses the Na^+/K^+ ATPase (Hoffman et al. 1994) and disturbs the Na^+ gradient across the cytoplasmic membrane, leading to the suppression of Na^+/Ca^{2+} exchange (Koch and Barish 1994; Siegmund et al. 1994) or even to reversed direction of the exchange activity (Stys et al. 1992). Because the Ca^{2+}-binding proteins serve as an endogenous buffer of Ca^{2+}, a change in the amount of Ca^{2+}-binding proteins could affect the cellular ability to manipulate intracellular Ca^{2+}. Its content is known to be inversely correlated with the susceptibility to ischemic insults (Rami et al. 1992). Yet a change in the amount of Ca^{2+}-binding proteins is the most unlikely in the present study, because onset time of ischemic effect was short (not discernible), in contrast to the long onset of changes in protein content (days) (Yamada et al. 1995). In addition to possible abnormalities of these three factors that are operative in normal cells, an active extrusion from intracellular stores may also be involved in the prolonged time course of Ca^{2+} clearance under ischemia. Although a differentiation between the reduced uptake into intracellular stores and the increased release from the stores is technically difficult, the intracellular Ca^{2+} mobilization from a dantrolene-sensitive store has been associated with hypometabolic injury (Dubinsky and Rothman 1991; Mitani et al. 1993) and NMDA/glutamate excitotoxicity (Dubinsky and Rothman 1991; Frandsen and Schousboe 1992).

In the present study we show that the net effect of glucose deprivation is a deranged Ca^{2+} homeostasis. Because ischemic damage is based on intricate interactions of various factors, further studies should be performed to identify the critical factor involved in the abnormality of Ca^{2+} handling and its detailed mechanism.

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