Regulation of Presynaptic NMDA Responses by External and Intracellular pH Changes at Developing Neuromuscular Synapses

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NMDA receptors play important roles in synaptic plasticity and neuronal development. The functions of NMDA receptors are modulated by many endogenous substances, such as external pH (pH_e), as well as second messenger systems. In the present study, the nerve–muscle cocultures of *Xenopus* embryos were used to investigate the effects of both external and intracellular pH (pH_i) changes on the functional responses of presynaptic NMDA receptors. Spontaneous synaptic currents (SSCs) were recorded from innervated myocyte using whole-cell recordings. Local perfusion of NMDA at synaptic regions increased the SSC frequency via the activation of presynaptic NMDA receptors. A decrease in pH_e from 7.6 to 6.6 reduced NMDA responses to 23% of the control, and an increase in pH_e from 7.6 to 8.6 potentiated the NMDA responses in increasing SSC frequency. The effect of NMDA on intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) was also affected by pH_e changes: external acidification inhibited and alkalinization potentiated $[Ca^{2+}]_i$ increases induced by NMDA. Intracellular pH changes of single soma were measured by ratio fluorometric method using 2,7-bis (carboxyethyl)-5,6-carboxyfluorescein (BCECF). Cytosolic acidification was used in which NaCl in Ringer's solution was replaced with weak organic acids. Acetate and propionate but not methylsulfate substitution caused intracellular acidification and potentiated NMDA responses in increasing SSC frequency, intracellular free Ca^{2+} concentration, and NMDA-induced currents. On the other hand, cytosolic alkalinization with NH_4Cl did not significantly affect these NMDA responses. These results suggest that the functions of NMDA receptors are modulated by both pH_e and pH_i changes, which may occur in some physiological or pathological conditions.

Key words: NMDA receptor; NMDA-induced current; intracellular alkalinization; cytosolic acidification; extracellular pH change; developing motoneuron

L-Glutamate plays an important role as an excitatory synaptic neurotransmitter in the mammalian CNS (Watkins and Evans, 1981; Foster and Fagg, 1984). Evidence has accumulated that at least three types of ionotropic receptors for glutamate exist on neuronal postsynaptic membranes: AMPA, kainate, and NMDA receptors (Monaghan et al., 1989). NMDA receptors possess high Ca^{2+} permeability and serve many functions in neuronal development, synaptic plasticity, and acquisition of memory (Hollmann and Heinemann, 1994; McBain and Mayer, 1994). However, activation of these receptors also can contribute to the pathology of epilepsy (Dingledine et al., 1990; Meldrum, 1992), stroke (Choi, 1990, 1992), and neurodegenerative disorders such as Alzheimer's disease, amyotrophic lateral sclerosis, and Huntington's disease (Beal, 1992). Therefore, the regulation of NMDA receptor activity is important in the CNS.

NMDA receptors are controlled by many endogenous substances as well as second messenger systems (Hollmann and Heinemann, 1994; McBain and Mayer, 1994). Among the ionotropic glutamate receptors, the NMDA receptor displays a unique sensitivity to extracellular pH (pH_e). In mammalian central neurons, NMDA-evoked currents were significantly enhanced by extracellular alkaline shifts in pH of a few tenths. On

the contrary, a decrease in pH_e was shown to reduce NMDA receptor-mediated cation conductance dramatically in cultured central neurons (Tang et al., 1990; Vyklicky et al., 1990; Traynelis and Cull-Candy, 1991; Takahashi and Copenhagen, 1996). Both extracellular and intracellular pH are modulated in many physiological and pathological conditions. Excitatory synaptic transmission in the CNS has been reported to be associated with a rapid alkalinization of the extracellular space (Chesler, 1990; Chesler and Kaila, 1992). On the other hand, under normal conditions the pH_i is decreased after prolonged neuronal activity (Chesler and Kaila, 1992). In addition, NMDA is reported to reduce neuronal pH_i in central neurons and in spinal motoneurons (Edres et al., 1986; Dixon et al., 1993; Hartley and Dubinsky, 1993; Irwin et al., 1994). Whether the NMDA receptor is modulated by intracellular pH shifts is still unknown. Recently, we found that glutamate, which is also reported to be co-released from some cholinergic nerve terminals (Vyas and Bradford, 1987; Israel et al., 1993; Meister et al., 1993), markedly increased the frequency of spontaneous synaptic currents (SSCs) at embryonic neuromuscular synapses via the activation of NMDA and non-NMDA receptors (Fu et al., 1995). SSC frequency increased markedly in response to the local perfusion of glutamate at the synaptic regions, whereas only a slight increase was observed when perfusion was performed at the soma. Furthermore, local perfusion of NMDA to the growth cone induced an inward current (our unpublished observation). These results indicate the existence of NMDA receptors at the developing motor nerve terminals. In this study, we further investigated the functional regulation of presynaptic NMDA receptors by both pH_e and pH_i

changes. It was found that intracellular acidification potentiated the responses to NMDA in increasing SSC frequency, intracellular free Ca^{2+} concentration, and NMDA-induced currents, whereas intracellular alkalinization did not significantly affect these responses.

MATERIALS AND METHODS

Cell culture. Xenopus nerve–muscle cultures were prepared as reported previously (Tabti and Poo, 1991). Briefly, the neural tube and the associated myotomal tissues of 1-d-old *Xenopus* embryos (stages 20–22) were dissociated in Ca^{2+}- and Mg^{2+}-free Ringer's solution supplemented with EDTA. The cells were plated onto clean glass coverslips and were used for experiments after 24 hr at room temperature (20–22°C). The culture medium consisted of 50% (v/v) Ringer's solution (115 mM NaCl, 2 mM $CaCl_2$, 1.5 mM KCl, 10 mM HEPES, pH 7.6), 49% L-15 Leibovitz medium (Sigma, St. Louis, MO), 1% fetal bovine serum (Life Technologies, Grand Island, NY), and antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin).

Electrophysiology. Whole-cell patch-clamp recording methods followed those described by Hamill et al. (1981). Patch pipettes were pulled with a two-stage electrode puller (pp-83, Narishige, Tokyo, Japan), and the tips were polished immediately before the experiment using a microforge (MF-83, Narishige). SSCs were recorded from innervated myocytes by whole-cell recording in the voltage-clamp mode. Recordings were made at room temperature in the Ringer's solution. For whole-cell recordings of myocytes, the solution inside the recording pipette contained 150 mM KCl, 1 mM NaCl, 1 mM $MgCl_2$, and 10 mM HEPES, pH 7.2. The extent of the potentiation was measured by the frequency ratio of SSCs, which is defined as the ratio of SSC frequency at peak level observed during application of drugs compared with the mean frequency observed before drug treatment. NMDA-induced currents were recorded from soma. The solution inside the recording pipette contained 145 mM CsCl, 0.1 mM $CaCl_2$, 3 mM $MgCl_2$, 5 mM EGTA, 5 mM HEPES, pH 7.2, and 1 mM amphotericin B was added to internal solution to get perforated patch. The membrane currents passing through the patch pipette were recorded with a patch-clamp amplifier (Dagan 8900). The data were digitized using Neuro-corder (Neuro Data DR 390) and stored on a videotape for later playback with a storage oscilloscope (Tektronix 5113, Beaverton, OR) or an oscillographic recorder (Gould RS3200, Valley View, OH). The Data 6100 waveform analyzer (Data Precision, Danvers, MA) was used to analyze the frequency of SSCs. For analysis of spontaneous events at high frequency when overlaps of current events occurred, the events were counted visually from chart records of an oscillographic recorder driven at high chart speeds (25–100 mm/sec). Data are expressed as mean $\pm$ SEM. Statistical significance was evaluated by Student's *t* test.

Measurement of pH_i. Measurement of pH_i has been described in detail elsewhere (Wu et al., 1994). In brief, cultures were loaded with 5 μM 2,7-bis (carboxyethyl)-5,6-carboxyfluorescein (BCECF-AM, Molecular Probes, Eugene, OR) for 5–10 min at room temperature in Ringer's solution and then washed with the same solution three times. A single soma was isolated by adjusting the slit width of the photomultiplier tube and excited by alternate flashes of 490 and 440 nm wavelength light, using a filter wheel (Cairn Research, Kent, England) rotating at 32 Hz. The excitation light was transmitted to the cell under study using a 510 nm dichroic mirror under the microscope nosepiece, and the resulting fluorescence was collected via a 40× magnification oil-immersion lens. The overall sampling rate was 0.5 Hz. The 490/440 emission ratio from the intracellular BCECF was calculated and converted to a linear pH scale using *in situ* calibration data obtained at the end of the experiment by the nigericin technique. A calibration curve similar to that used by Wu et al. (1994) was established by measuring the fluorescence ratio values between pH_i 4.5 and 9.5. Between pH_i 5.5 and 9.0, the response is linear and fits the equation pH_i-pK + log $[(R_{max} - R)/(R - R_{min})]$ + log(F_{440min}/F_{440max}), where R is the ratio of 530 nm fluorescence at 490 nm excitation to 530 nm fluorescence at 440 nm excitation. R_{max} and R_{min} are the maximum and minimum ratio values from the data curve, and K is the dissociation constant for the dye, taken as 55 nM.

Measurement of intracellular Ca^{2+} levels. The intracellular concentration of free Ca^{2+} ($[Ca^{2+}]_i$) in the soma was measured using the Ca^{2+}-sensitive fluorescent dye fura-2. Cultures plated onto a 25 mm round coverslip (No. 1001, Assistent) were loaded with 2 μM fura-2 AM (Molecular Probes) in Ringer's solution for 1 hr at room temperature. The cells were then washed with the same solution. The fluorescence of

a single soma was measured as indicated above except that the cell was excited by alternate flashes of 340 and 380 nm wavelength. The 340/380 emission ratio from the intracellular fura-2 was calculated and converted to a linear Ca^{2+} scale by *in situ* calibration at the end of the experiment using the Ca^{2+} ionophore ionomycin (5 μM, Sigma). The following equation (Grynkiewicz et al., 1985) was used to convert the fluorescence ratio into the intracellular Ca^{2+} concentration $[Ca^{2+}]_i = K_d [(R - R_{min})/R_{max} - R](S_{f2}/S_{b2})$, where R is the ratio of 510 nm fluorescence at 340 nm excitation to 510 nm fluorescence at 380 nm excitation, R_{max} (10 mM Ca^{2+}) and R_{min} (10 mM EGTA in Ca^{2+}-free Ringer's solution) are the maximum and minimum ratio values from the data curve, K_d is the dissociation constant for the dye, taken as 135 nM at room temperature, and S_{f2}/S_{b2} is the ratio of the 380 nm signals determined at R_{min} and R_{max}.

Because Ca^{2+} and H^+ are presumably competitive, we have tested whether the 340/380 signal was affected by the change of pH_i because of the possible pH sensitivity of fura-2. Three levels of pH (6.8, 7.2, and 7.8) were tested in Ca^{2+}-free Ringer's solution supplemented with 2 mM EGTA using the free acid form of fura-2, and we found that there was little change in the 340/380 ratio when the pH was changed (6.8, 7.8, and 7.2); the first two points were the two extreme levels of pH_i we have observed in our experiments. We used Ca^{2+}-free Ringer's solution supplemented with 2 mM EGTA in the above test to mimic a similar level of resting $[Ca^{2+}]_i$ (~70 nM) in *Xenopus* cultured spinal neurons.

Chemicals. The following chemicals were used: NH_4Cl and sodium acetate (Sigma), sodium methylsulfate (Aldrich, Milwaukee, WI), sodium propionate (Hayashi, Osaka, Japan), and NMDA (RBI, Natick, MA).

RESULTS

Regulation of NMDA responses in increasing SSC frequency by pH_e changes

In nerve–muscle cultures prepared from 1-d-old *Xenopus* embryos, synaptic contacts were established between dissociated spinal neurons and myocytes within the first day of culture. SSCs were readily detectable from the innervated myocytes by whole-cell voltage-clamp recording. These currents have been shown to be caused by spontaneous ACh secretion from the neuron, because they are abolished by bath application of D-tubocurarine and unaffected by tetrodotoxin (Xie and Poo, 1986). It was found recently that there are NMDA receptors in the nerve terminals of developing motoneurons (Fu et al., 1995; Liou et al., 1996). We thus used these nerve–muscle cocultures to investigate the functional regulation of presynaptic NMDA receptors by pH changes. Figure 1 shows the recording of SSCs from an innervated myocyte. Local application of NMDA by pressure at synaptic regions with another glass microelectrode as indicated in the inset (pipette opening, 9 μm; pipette NMDA concentration, 150 μM) reversibly increased the SSC frequency (SSC frequency ratio was 13.7 $\pm$ 3.4; $n = 21$). The effects of changes in pH_e on the NMDA responses were investigated by replacing normal Ringer's solution with pH_e 6.6 or 8.6 Ringer's solution. The pH was adjusted before the experiment with 1N HCl or NaOH, respectively, and the pH value remained unchanged throughout the experiment (within 1 hr). The change in extracellular pH from 7.6 to 6.6 or 8.6 did not by itself significantly affect the frequency of spontaneous ACh secretion (SSC frequency ratio was 1.2 $\pm$ 0.1, $n = 5$, and 0.9 $\pm$ 0.1, $n = 6$, respectively). The mean SSC amplitude in pH 6.6 and 8.6 Ringer's solution also showed no significant change before NMDA application (146.5 $\pm$ 35.0 pA, $n = 5$, and 154.7 $\pm$ 19.3 pA, $n = 4$, respectively; control SSC amplitude was 167.3 $\pm$ 14.3 pA, $n = 21$). However, changes in pH_e range between 6.6 and 8.6 had different effects on the SSC potentiating action of NMDA. Alkalinization of external pH from 7.6 to 8.6 potentiated the NMDA effect by 195 $\pm$ 34% of the control (SSC frequency ratio was 26.5 $\pm$ 4.7, $n = 4$) (Figs. 1, 2*A*). On the other hand, acidification of external medium from pH 7.6 to 6.6 antagonized the NMDA action to 23 $\pm$ 12% of the control (SSC frequency ratio was 3.2 $\pm$
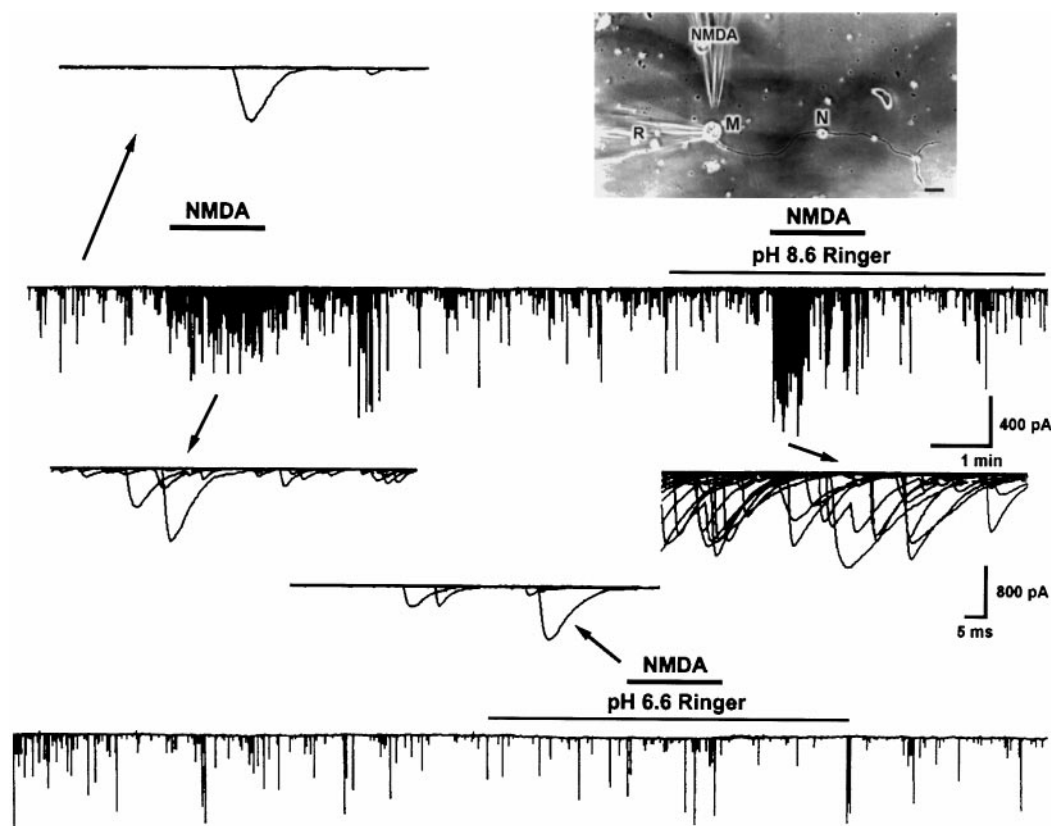
Figure 1. Effect of external pH changes on the SSC-increasing action of NMDA. Shown is continuous tracing of SSCs recorded from an innervated muscle cell in 1-d-old *Xenopus* culture. The myocyte was voltage-clamped at a potential of −60 mV. Downward deflections are SSCs (filtered at 150 Hz). The *top right panel* shows the relative position of patch pipette (*R*) and NMDA pipette (*NMDA*). Local perfusion of NMDA at the synaptic region with another micropipette increased SSC frequency. NMDA responses were potentiated in pH 8.6 Ringer's solution and antagonized in pH 6.6 Ringer's solution. Samples of superimposed SSCs in 1 sec before and after NMDA treatment are shown at higher time resolution. Scale bar, 40 μm. *M*, Myocyte; *N*, neuron.

1.7, $n = 4$) (Figs. 1, 2*A*). The time course-response curves of the NMDA action in different external pH are shown in Figure 2*B*.

Intracellular acidification enhances the responses to NMDA in increasing SSC frequency

Cytosolic acidification was induced by weak organic acid substitution. Weak organic acid with pK_a values in the range of 4–6 might allow selective acidification of cells. The protonated forms of such weak acids are more membrane permeable than their charged anionic forms and then dissociate intracellularly to liberate H^+. Exposure of cells to weak acids is thus expected to result in an acute acid load to the cytoplasm. For these experiments, all the extracellular NaCl was replaced by the sodium salts of organic acids in normal Ringer's solution. Cytosolic pH in neuronal soma was monitored by the BCECF fluorescence method. The baseline pH_i value was 7.17 ± 0.03 ($n = 46$). The change of external solution from pH_e 7.6 to 6.6 or 8.6 caused only a slight cytosolic acidification of 0.14 ± 0.02 units ($n = 6$) or intracellular alkalinization of 0.11 ± 0.04 units ($n = 4$), respectively (Fig. 3*A,D*). However, a rapid intracellular acidification occurred after exposure with either acetate or propionate at a fixed pH of external medium (pH_e 7.6) (Fig. 3*B,D*). The pH_i reached peak values of 6.86 ± 0.12 ($n = 8$) with acetate and 6.78 ± 0.15 ($n = 7$) with propionate. The intracellular fluid revealed an acidic shift of 0.2–0.4 pH units. Removal of acetate or propionate results in a rebound cytosolic alkalinization. On the other hand, the peak value of pH_i after exposure to methylsulfate

did not show significant change (pH_i was 7.09 ± 0.03; $n = 6$). The frequency and amplitude of SSC did not show significant change after exposure to organic acids alone for 1–2 min (SSC frequency ratio was 1.3 ± 0.2, $n = 6$, with acetate; 1.4 ± 0.3, $n = 6$, with propionate; and 1.1 ± 0.2, $n = 4$, with methylsulfate, respectively; mean SSC amplitude was 150.9 ± 21.6 pA with acetate; 186.3 ± 46.9 pA with propionate; and 142.2 ± 13.5 pA with methylsulfate, respectively). However, substitution with either acetate or propionate markedly potentiated SSC frequency, increasing the action of NMDA (SSC frequency ratio was 35.5 ± 7.2, $n = 6$, and 34.8 ± 10.9, $n = 5$, respectively) (Figs. 4*A,B*, 5*A*), while substitution of NaCl with strong organic acids such as methylsulfate resulted in no significant effect on the NMDA response (SSC frequency ratio was 8.5 ± 2.6, $n = 4$) (Fig. 5*A*). We further investigated the effect of intracellular alkalinization on the NMDA responses. Exposure of the soma to 15 mM NH_4Cl resulted in a rapid increase of pH_i ($\Delta pH = 0.77 \pm 0.04$; $n = 8$), and the pH_i in peak value was 7.67 ± 0.14 (Fig. 3*C,D*). The alkalinization seen during the first moments of exposure to NH_4^+ is presumably caused by the rapid, passive entry of NH_3 and its subsequent hydration to form NH_4^+ and OH^- (Roos and Boron, 1981). Cytosolic alkalinization with NH_4Cl alone for 2 min also did not significantly affect SSC frequency and amplitude (SSC frequency ratio was 0.9 ± 0.2; mean SSC amplitude was 145.4 ± 28.5 pA; $n = 6$). As shown in Figures 4*C* and 5*A*, the NMDA responses were not affected after intracellular alkalinization (SSC frequency ratio was 12.6 ± 3.6,
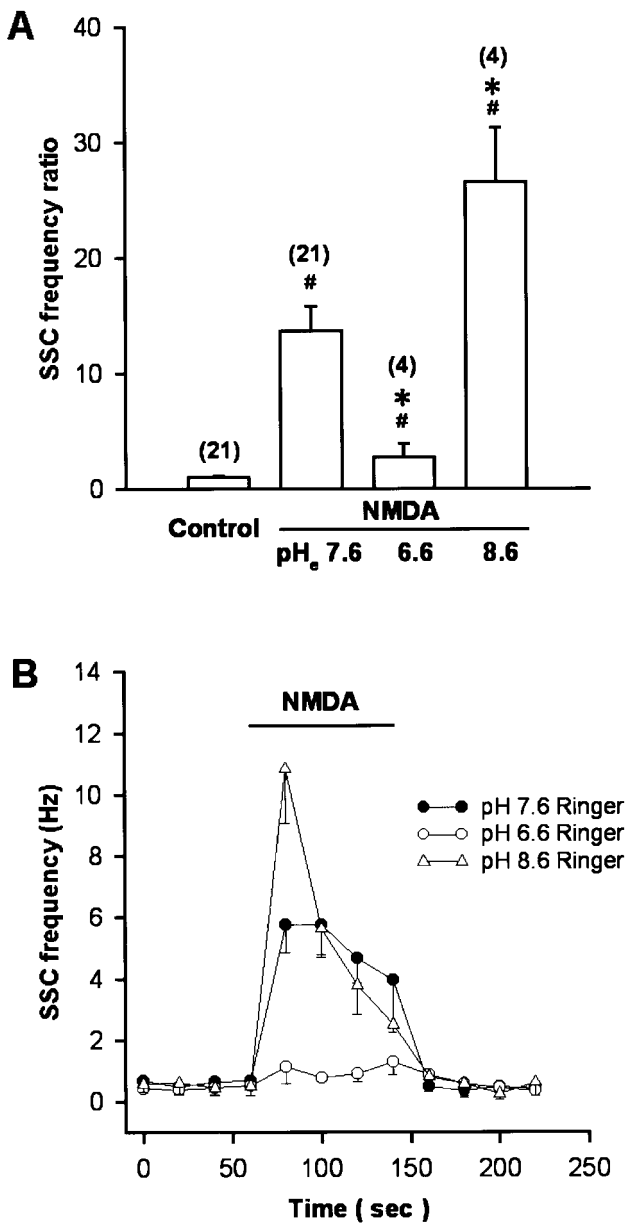
Figure 2. Summary of the effect of external pH changes on the SSC-increasing action of NMDA. Note that NMDA increased SSC frequency in normal pH 7.6 Ringer's solution and the change of external pH to 8.6 potentiated NMDA action, whereas the reduction of external pH to 6.6 antagonized the SSC-increasing action of NMDA (*A*). *B*, Time course-response curves in response to NMDA application. Data are presented as mean ± SEM (*n*). *$p < 0.05$ compared with control.

$n = 6$). The time course-response curves of the NMDA action on the cytosolic acidification and alkalinization are shown in Figure 5*B*.

Modulation of NMDA effects on neuronal cytoplasmic Ca^{2+} by the changes of pH_e and pH_i

The intracellular concentration of free Ca^{2+} ($[Ca^{2+}]_i$) in the soma was measured using the Ca^{2+}-sensitive fluorescent dye fura-2. The basal neuronal $[Ca^{2+}]_i$ value was 70.2 ± 4.6 nM ($n = 32$). Superfusion with 100 μM NMDA in pH 7.6 Ringer's solution increased $[Ca^{2+}]_i$ by 131.3 ± 26.9 nM ($n = 27$). Responses to NMDA were affected by changing the pH of external medium.

Lowering the pH_e from 7.6 to 7.0 or 6.6 decreased the $[Ca^{2+}]_i$ responses of NMDA to $64 \pm 10\%$ and $23 \pm 7\%$ of the control ($n = 4$ for each), respectively (Fig. 6*A,B*), whereas increasing the pH_e to 8.6 potentiated the $[Ca^{2+}]_i$ response of NMDA to $156 \pm 18\%$ of the control ($n = 4$) (Fig. 6*A,B*). By contrast, intracellular acidification by either acetate or propionate substitution enhanced the $[Ca^{2+}]_i$ response to NMDA. NMDA response was enhanced concentration-dependently by acetate substitution. Two-thirds of acetate substitution (two-thirds of the NaCl in Ringer's solution was replaced with equimolar sodium acetate) nonsignificantly increased NMDA response to $134 \pm 15\%$ of control ($n = 7$), whereas 100% acetate substitution significantly increased the NMDA response to $168 \pm 28\%$ ($n = 8$) (Fig. 7*A,D*). Substitution with another weak acid propionate (100% substitution) also increased NMDA response to $200 \pm 50\%$ ($n = 5$) (Fig. 7*B,D*). However, intracellular alkalinization by 15 mM NH_4Cl had no significant effect (Fig. 7*C,D*).

Potentiation of NMDA-induced currents by intracellular acidification

To determine directly the effect of intracellular acidification on the NMDA receptor, the soma of isolated spinal neurons was whole-cell voltage-clamped at -60 mV by using perforated patch. Local perfusion of NMDA with another micropipette (intrapipette concentration, 150 μM), as indicated in the right panel of Figure 8*A*, induced an inward current in the soma. Intracellular acidification by the substitution of all NaCl of Ringer's solution with either sodium acetate (Fig. 8*A*) or sodium propionate (Fig. 8*B*) enhanced the NMDA-induced currents ($134.6 \pm 6.4\%$ and $162.6 \pm 14.0\%$ of the control, respectively; $n = 7$ for each). On the other hand, cytosolic alkalinization with NH_4Cl did not significantly affect the NMDA-induced currents ($96.1 \pm 4.8\%$ of the control, $n = 6$) (Fig. 8*C*). However, changes of external pH from 7.6 to 8.6 or 6.6 enhanced and inhibited the NMDA-induced currents, respectively [$149.4 \pm 8.5\%$ ($n = 6$) and $37.1 \pm 6.6\%$ ($n = 4$) of the control, respectively].

DISCUSSION

Many studies have revealed that second messengers such as Ca^{2+}, cyclic nucleotides, and G-proteins modulate voltage-gated ion channels and ligand-gated receptor channels. Here we show that NMDA receptor-mediated synaptic potentiation, increase of intracellular Ca^{2+} concentration, and NMDA-induced currents are significantly influenced by the changes of either external or intracellular pH. We have observed that external acidification inhibited but intracellular acidification enhanced NMDA responses. On the other hand, external alkalinization potentiated the NMDA action, whereas intracellular alkalinization had no significant effect. The modulation of NMDA responses by both external and internal pH changes might mediate some of the physiological and pathological actions of glutamate.

Our results are different from those reported by Tang et al. (1990), who showed that NMDA-activated current was rather insensitive to the change in internal pH. The intracellular pH was altered in their study by filling the pipette with an internal solution of different values of pH. The pH of intracellular fluid may be different from that of patch pipette as a result of the buffering capacity of cytoplasm and improper diffusion of patch pipette solution. We here acidified intracellular fluid by using a weak organic acid substitution. It has long been known that weak acids can readily cross cell membranes in their uncharged forms and then dissociate, thereby acidifying the cell interior (Karuri et
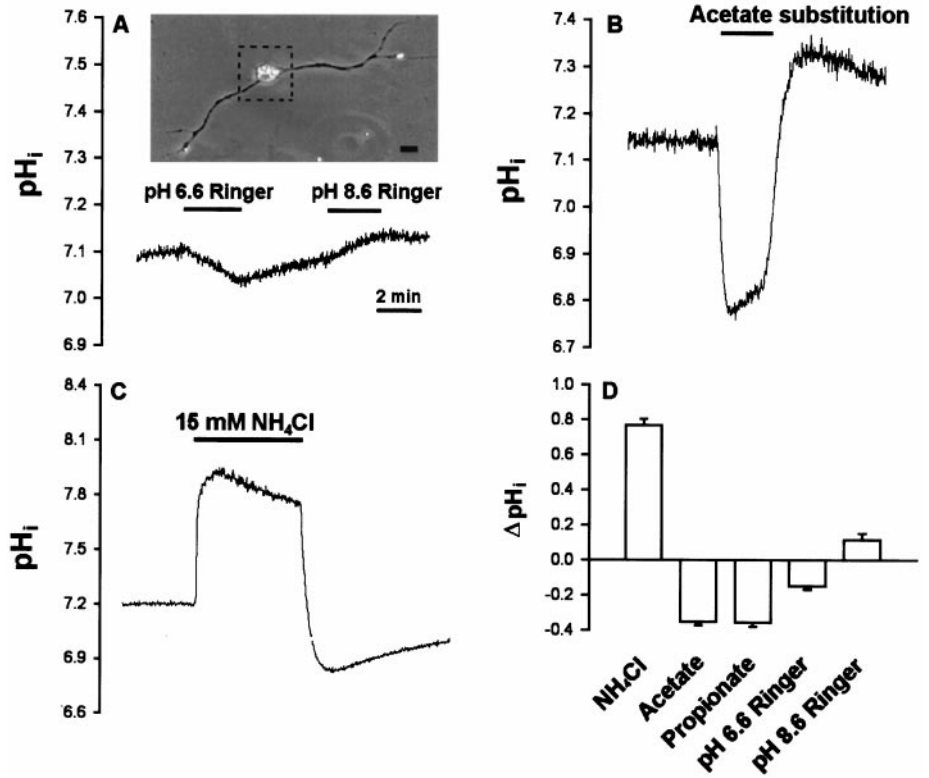
Figure 3. Cytosolic pH changes induced by organic acids and NH_4Cl in neuronal soma. One-day-old *Xenopus* cultures were loaded with BCECF-AM, and ratio fluorometric measurements of intracellular pH were made on single soma. *A*, *Top panel*: the area of single soma for the measurement of pH_i was outlined by the *dotted box*. Scale bar, 20 μm. *Bottom panel*: change of external pH slightly affected the intracellular pH. *B*, Replacement of all NaCl in Ringer's solution with sodium acetate resulted in a marked cytosolic acidification. *C*, Addition of NH_4Cl to the bath caused cytosolic alkalinization. *D*, Summarized effect on cytosolic pH by the changes of external pH or by the addition of organic acids or NH_4Cl. Data are presented as mean ± SEM ($n = 4$–8).
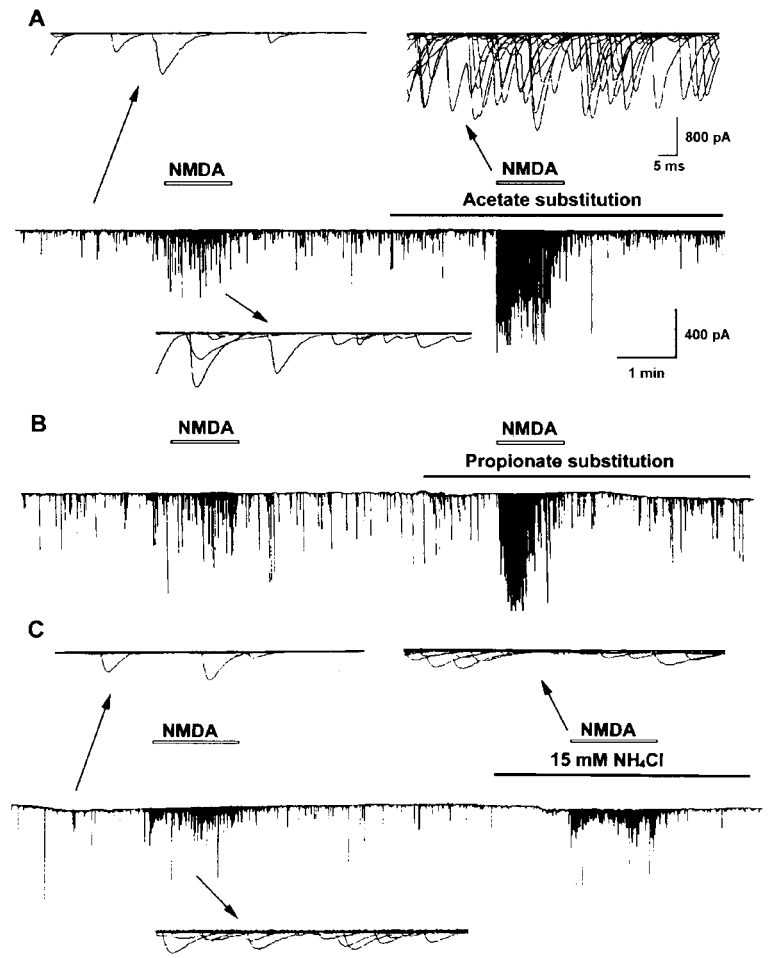

Figure 4. Effect of intracellular pH changes on the SSC-increasing action of NMDA. Shown is a continuous tracing of SSCs recorded from an innervated muscle cell in 1-d-old *Xenopus laevis* culture. The myocyte was voltage-clamped at a potential of −60 mV. Downward deflections are SSCs (filtered at 150 Hz). Perfusion of NMDA at synaptic region with another micropipette increased SSC frequency. NMDA responses were potentiated by cytosolic acidification with organic acid substitution and not significantly affected by cytosolic alkalinization with NH_4Cl. Samples of superimposed SSCs in 1 sec before and after NMDA treatment are shown at higher time resolution.
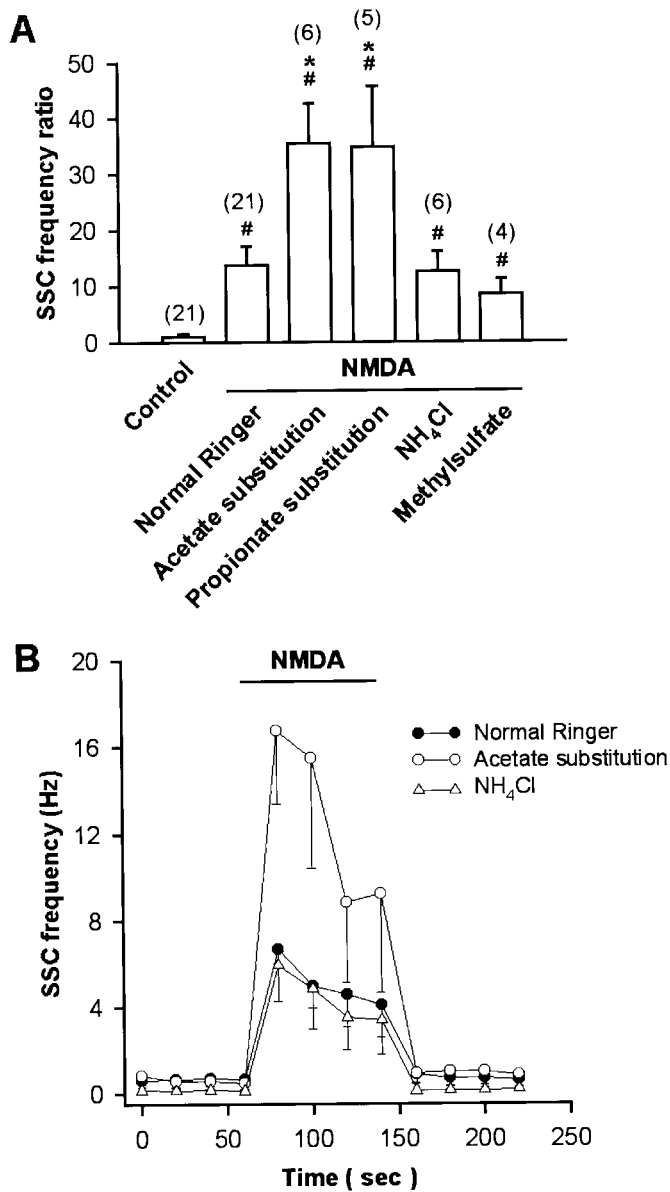
A

B

Figure 5. Summary of the effects of intracellular pH changes on the SSC-increasing action of NMDA. Note that NMDA increased SSC frequency in normal Ringer's solution, and intracellular acidification with either acetate or propionate potentiated NMDA responses (*A*). *B*, Time course-response curves of the NMDA action on the cytosolic pH changes. Data are presented as mean ± SEM (*n*). #*p* < 0.05 compared with control. **p < 0.05 compared with NMDA in normal Ringer's solution.

al., 1993). Once inside the cell, the pH_i is well above pK_a, leading to the dissociation of the protonated form and intracellular acidification. Therefore, the anions of weak acids such as acetate (pK_a 4.75) or propionate (pK_a 4.87) caused larger internal acidification ($pH_i \doteq 6.8$), and the anions of strong acids such as methylsulfate ($pK_a < 1.0$) induced little pH_i change of the cells (Chesler, 1990).

The potentiation of NMDA action was in parallel with the levels of cytosolic acidification by these organic acids. We found no effect on NMDA response by methylsulfate substitution, ruling out the likelihood that the Cl^- substitution itself was affecting NMDA receptor. Modulation of the NMDA-activated current by external pH changes resulted primarily from changes in the number of channel openings. The single-channel conductance of
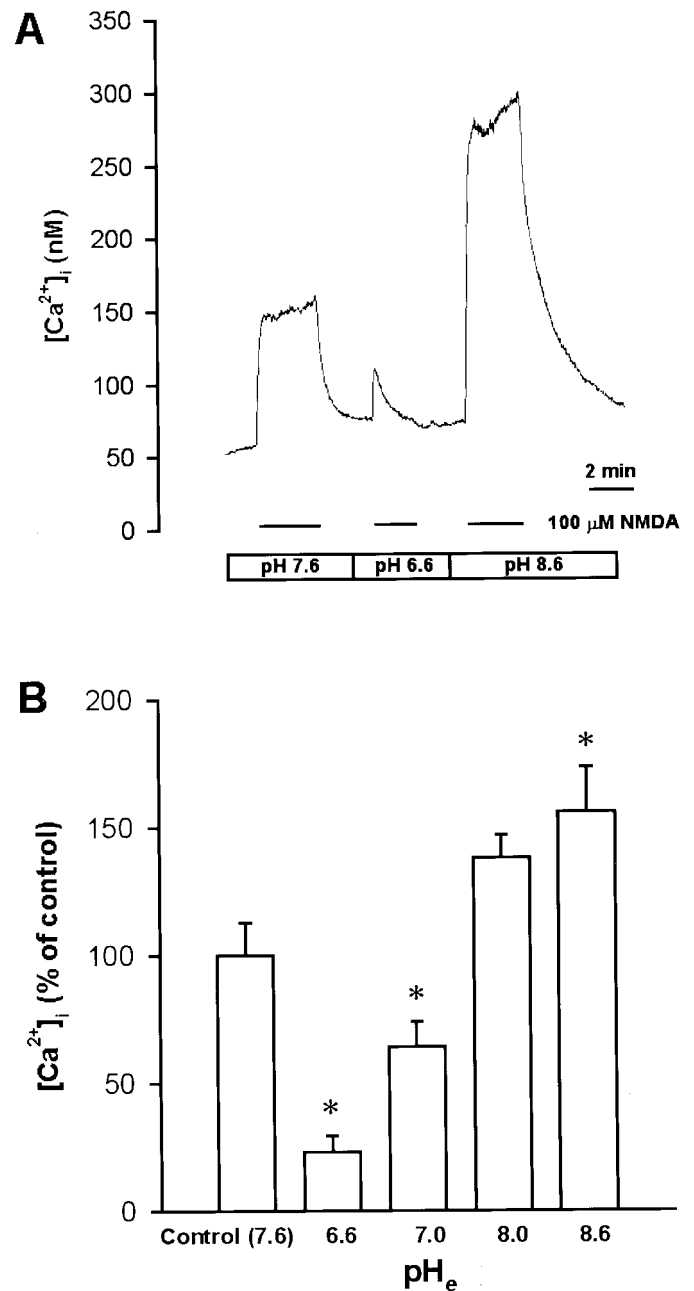
A

B

Figure 6. Effect of external pH changes on the $[Ca^{2+}]i$-increasing action of NMDA. *A*, *Xenopus* nerve–muscle cultures were loaded with fura-2 AM, and ratio fluorometric measurement of intracellular Ca^{2+} was made on a single soma. Superfusion with NMDA increased $[Ca^{2+}]i$, and this response was potentiated in pH 8.6 Ringer's solution and antagonized in pH 6.6 Ringer's solution. *B*, Summary of the effect of external pH changes on the $[Ca^{2+}]i$-increasing action of NMDA. Data are presented as mean ± SEM (*n* = 4). **p < 0.05 compared with control.

NMDA receptor was not significantly affected (Tang et al., 1990; Vyklicky et al., 1990; Traynelis and Cull-Candy, 1991). We here confirmed previous reports that external alkalinization enhances and external acidosis inhibits NMDA receptor activation (Tang et al., 1990; Vyklicky et al., 1990; Traynelis and Cull-Candy, 1991; Takahashi and Copenhagen, 1996). A lowering of pH_e from 7.6 to 6.6 inhibits the functional activation of NMDA receptors, whereas a rise of pH_e from 7.6 to 8.6 significantly enhanced NMDA responses. However, we provide additional evidence that
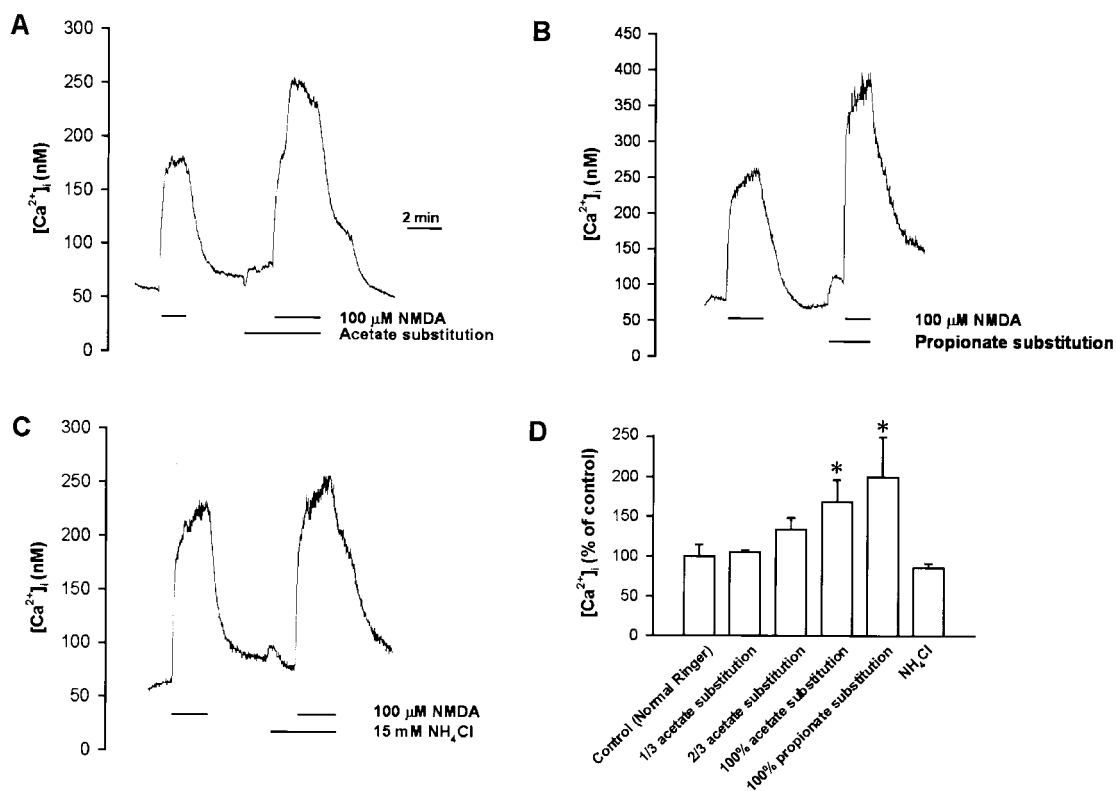
Figure 7. Effect of intracellular pH changes on the $[Ca^{2+}]i$-increasing action of NMDA. *Xenopus* nerve–muscle cultures were loaded with fura 2-AM, and ratio fluorometric measurement of intracellular Ca^{2+} was made on a single soma. Superfusion with NMDA increased $[Ca^{2+}]i$, and this response was enhanced by cytosolic acidification with either acetate (*A*) or propionate (*B*) but not affected by cytosolic alkalinization with NH_4Cl (*C*). *D*, Summary of the effects of intracellular pH changes on the $[Ca^{2+}]i$-increasing action of NMDA. Data are presented as mean $\pm$ SEM (*n* = 3–22). *p < 0.05 compared with control.
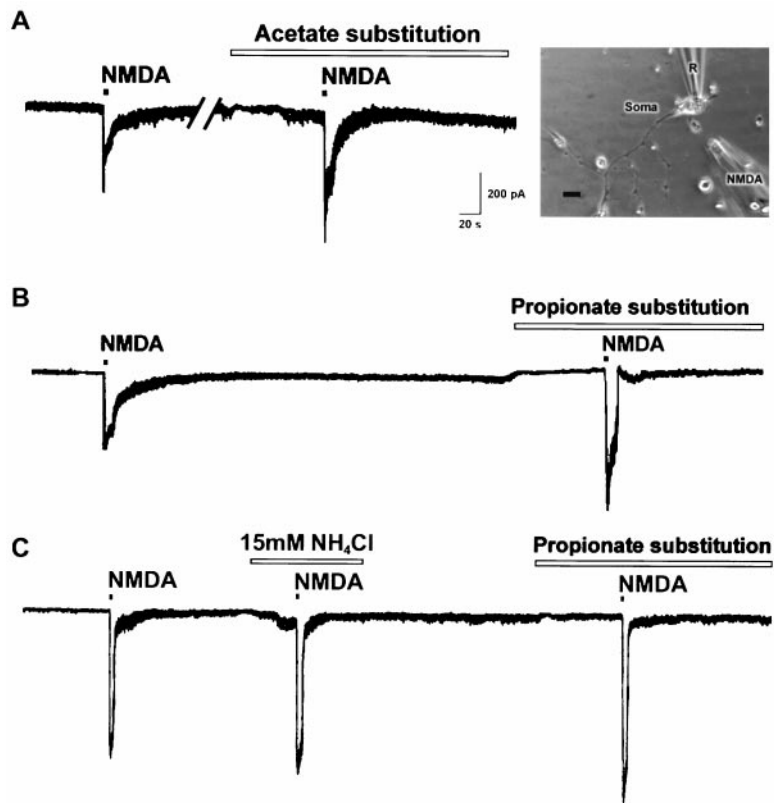

Figure 8. Potentiation of NMDA-induced current by intracellular acidification. *A*, The soma of an isolated neuron was whole-cell voltage-clamped at −60 mV. Local perfusion with NMDA from a glass micropipette (*NMDA*) as indicated in the *right panel* induced an inward current. NMDA-induced current was enhanced by intracellular acidification with either acetate (*A*) or propionate (*B*) but not affected by cytosolic alkalinization (*C*). Scale bar, 10 μm. *R*, Patch pipette.

the function of the NMDA receptor can also be regulated by the changes of intracellular pH. The potentiation of SSC and an increase in intracellular free Ca^{2+} by NMDA are regulated in parallel. We found previously that a larger decrease of pH_i (~1.4–1.6 pH units) is required for SSC frequency-increasing effect by weak organic acid substitution in pH_e 6.6 Ringer's solution (Chen et al., 1998). In the current study, the intracellular fluid revealed an acidific shift in pH of only 0.2–0.4 units by weak organic acid substitution in normal Ringer's solution. Therefore, the potentiation effect on SSC frequency can be fully accounted for by changes in the NMDA-induced $[Ca^{2+}]_i$ increase. Furthermore, direct evidence is shown in Figure 8 that NMDA-induced inward currents were increased in the presence of acetate or propionate. On the other hand, intracellular alkalinization had no significant effect on these NMDA responses. Because the NMDA receptor is activated in the absence of glycine, the modulation of intracellular H^+ appears not to be related to the glycine recognition site of the NMDA receptor. How the internal H^+ modulates the gating of the NMDA channel needs further investigation. Although pH_i was reduced by 0.1 units after the pH_e was lowered from 7.6 to 6.6, no potentiation of NMDA responses was observed, probably because of the larger inhibitory effect on NMDA receptors by external acidification and the insufficient intracellular acidification.

Changes in the concentration of H^+ have been clearly shown to serve important biological roles by regulating the properties of protein macromolecules. Accumulating evidence suggests that extracellular and intracellular pH can change significantly under not only pathological but also physiological circumstances. The local pH shifts could be large in synaptic cleft and subsynaptic regions under these conditions. Repetitive neuronal activity may lower intracellular pH through several mechanisms. These include the metabolic production of CO_2 and lactic acid, the release of protons from internal sites in response to elevation of free intracellular Ca^{2+} concentration, and the net entry of acid through ligand- or voltage-gated channels (Chesler and Kaila, 1992). Brief exposure of hippocampal neurons to NMDA results in a concentration-dependent pH_i reduction (0.2–0.4 pH units) (Irwin et al., 1994). Acute focal electrical stimulation evoked an intracellular acidosis of approximately pH 6.8 in cortical tissue (Yaksh and Anderson, 1987). Changes in pH_i and pH_e have been known to modify several types of voltage-gated ion channels (Takahashi and Copenhagen, 1996). Modulation of excitatory transmission by pH shifts may be especially pertinent to forms of long-term potentiation (LTP) that have been linked to NMDA receptor activation (Blanton et al., 1989). Experimentally, LTP is often induced by trains of high frequency stimulation. Both the intracellular and external pH shifts may thus influence the relationship between LTP induction and stimulus frequency. Pathologically, hypoxia and glucose depletion in hippocampal slices have also been shown to result in a decrease in pH_i within 5 min of exposure (Chesler, 1990). Furthermore, brain ischemia has been reported to lower brain pH_e and pH_i (Nedergaard et al., 1991; Fujiwara et al., 1992). Recent evidence suggests that mild extracellular acidosis protects central neurons from injury induced by oxygen and glucose deprivation (Giffard et al., 1990; Tombaugh and Sapolsky, 1990). Extracellular acidosis prevents the activation of NMDA receptors, which play a major role in mediating ischemic neuronal injury. By contrast, extracellular alkalosis can induce epileptiform activity, which is blocked by NMDA antagonists in mammalian brain slices (Aram and Lodge, 1987). Our results showed that intracellular acidic shift in pH of

only 0.2–0.4 units enhanced NMDA responses. Therefore, intracellular acidosis may enhance NMDA responses in some physiological conditions and NMDA neurotoxicity in certain pathological diseases. The proton sensitivity of NMDA receptors could influence synaptic plasticity and seizure generation and may be relevant for glutamate-induced neurotoxicity during ischemia. These results add another level of complexity to the regulation of NMDA synaptic events by endogenous factors. Intracellular H^+, acting as a second messenger, may influence the neuronal excitability via modulation of NMDA channel activity.

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