Modulation of N-Type Ca\textsuperscript{2+} Channels by Intracellular pH in Chick Sensory Neurons

LASZLO KISS AND STEPHEN J. KORN
Department of Physiology and Neurobiology, University of Connecticut, Storrs, Connecticut 06269

Kiss, Laszlo and Stephen J. Korn. Modulation of N-type Ca\textsuperscript{2+} channels by intracellular pH in chick sensory neurons. J. Neurophysiol. 81: 1839–1847, 1999. Both physiological and pathological neuronal events, many of which elevate intracellular [Ca\textsuperscript{2+}]\textsubscript{i}, can produce changes in intracellular pH of between 0.15 and 0.5 U, between pH 7.4 and 6.8. N-type Ca\textsuperscript{2+} channels, which are intimately involved in exocytosis and other excitable cell processes, are sensitive to intracellular pH changes. However, the pH range over which N-type Ca\textsuperscript{2+} channels are sensitive, and the sensitivity of N-type Ca\textsuperscript{2+} channels to small changes in intracellular pH, are unknown. We studied the influence of intracellular pH changes on N-type calcium channel currents in dorsal root ganglion neurons, acutely isolated from 14-day-old chick embryos. Intracellular pH was monitored in patch-clamp recordings with the fluorescent dye, BCECF, and manipulated in both the acidic and basic direction by extracellular application of NH\textsubscript{4}\textsuperscript{+} in the presence and absence of intracellular NH\textsubscript{4}\textsuperscript{+}. Changes in intracellular pH between 6.6 and 7.5 produced a graded change in Ca\textsuperscript{2+} current magnitude with no apparent shift in activation potential. Intracellular acidification from pH 7.3 to 7.0 reversibly inhibited Ca\textsuperscript{2+} currents by 40%. Acidification from pH 7.3 to pH 6.6 reversibly inhibited Ca\textsuperscript{2+} currents by 65%. Alkalization from pH 7.3 to 7.5 potentiated Ca\textsuperscript{2+} currents by approximately 40%. Channels were sensitive to pH changes with high intracellular concentrations of the Ca\textsuperscript{2+} chelator, bis-(o-aminophenoxy)-N,N,N',N'-tetraacetic acid, which indicates that the effects of pHi did not involve a Ca\textsuperscript{2+}-dependent mechanism. These data indicate that N-type Ca\textsuperscript{2+} channel currents are extremely sensitive to small changes in pHi in the range produced by both physiological and pathological events. Furthermore, these data suggest that modulation of N-type Ca\textsuperscript{2+} channels by pHi may play an important role in physiological processes that produce small changes in pHi and a protective role in pathological mechanisms that produce larger changes in pHi.

INTRODUCTION

Both excitatory and inhibitory stimuli can acidify neurons. Elevation of intracellular Ca\textsuperscript{2+} (Ahmed and Connor 1980; Meech and Thomas 1977; Werth and Thayer 1994), application of glutamate (Canzoniero et al. 1996; Dixon et al. 1993; Hartley and Dubinsky 1993; Irwin et al. 1994; Wang et al. 1994), and application of gamma-aminobutyric acid (GABA) (Kaila et al. 1993; Luckermann et al. 1997) produce transient intracellular acidification by as much as 0.15–0.5 pH units in mammalian and nonmammalian neurons. In addition, Ca\textsuperscript{2+} influx associated with action potentials acidifies neurons by ~0.2 U (Ahmed and Connor 1980; Trapp et al. 1996), experimentally induced hypoxia acidifies neurons by as much as 0.5 U (O'Donnell and Bickler 1994), and reuptake of glutamate into rat hippocampal neurons is accompanied by intracellular acidification of ~0.2 pH units (Amato et al. 1994). Intracellular acidification is associated with changes in membrane excitability (Church 1992), neurotoxicity (Nedergaard et al. 1991), and possibly protection from pathological events such as reperfusion injury after ischemia (Bond et al. 1993; Scholz et al. 1992; Tombaugh and Sapolsky 1990; Voronov et al. 1996). However, the potential importance of small changes in intracellular pH (pHi), and the mechanisms by which small or large changes in pHi can influence physiological and/or pathological events, is poorly understood.

Both N- and L-type Ca\textsuperscript{2+} channels in mammalian and nonmammalian tissues are sensitive to changes in pHi (Kaibara and Kameyama 1988; Klockner and Isenberg 1994; Mirinov and Lux 1991; Takahashi et al. 1993; Tombaugh and Somjen 1997). In catfish horizontal cells, L-type Ca\textsuperscript{2+} channels are quite sensitive to pH changes between 6.6 and 7.6; acidification from pH 7.3 to 7.0 produced a 30–40% reduction in current amplitude, and alkalinization from pH 7.3 to 7.6 potentiated Ca\textsuperscript{2+} currents by ~40% (Dixon et al. 1993). Although not as precisely quantified, L-type Ca\textsuperscript{2+} currents from mammalian vascular smooth muscle were also sensitive to pH changes between 6 and 8.4 (Klockner and Isenberg 1994). In contrast to these results, the pHi range over which N-type Ca\textsuperscript{2+} channels are sensitive is not known.

In hippocampal neurons, N-type Ca\textsuperscript{2+} channels appear to be more sensitive than L-type Ca\textsuperscript{2+} channels to changes in pH (Tombaugh and Somjen 1997). This difference was reflected as a larger change in amplitude of N- than L-type currents on application of NH\textsubscript{4}\textsuperscript{+}. However, pHi was not measured, and pHi among cells and over time can vary by as much as 0.4–0.5 U under identical patch clamp recording conditions (unpublished data). Consequently, it is not known whether this difference reflected a difference in the pHi ranges over which these two channel types were sensitive or different channel responses to identical absolute changes in pHi.

The observations described above raise two important questions: are the small (0.15–0.5 U) changes in pHi that can be produced by a variety of physiological and pathological stimuli relevant to the physiology of N-type Ca\textsuperscript{2+} channels and is the pHi sensitivity of N-type Ca\textsuperscript{2+} channels similar to or different from that reported for L-type Ca\textsuperscript{2+} channels? To address these questions, we examined the pHi dependence of N-type Ca\textsuperscript{2+} channel currents in acutely isolated chick dorsal root ganglion neurons (DRGs), which contained a pharmacologically pure population of N-type Ca\textsuperscript{2+} channels. To obtain consistent, quantitative data regarding pH sensitivity below pH 7.3, we...
controlled pH with both intracellular and extracellular NH$_4^+$ (Grinstein et al. 1994). Our results indicate that N-type Ca$^{2+}$ channels are quite sensitive to small changes in pH between 6.6 and 7.5, and that the pH sensitivity is quantitatively similar to that reported for L-type Ca$^{2+}$ channels in catfish horizontal cells (Dixon et al. 1993). These data suggest that acidification-induced inhibition of N-type Ca$^{2+}$ channel activity may be important during both physiological and pathological events. Some of these data were presented in abstract form (Callahan et al. 1995).

**METHODS**

**Cells**

Dorsal root ganglion neurons were acutely isolated from lumbar level ganglia of 14-day white leghorn chick embryos (UCONN Poultry Farm, Storrs, CT). Cells were prepared as described previously (Polo-Parada and Korn 1997). In photometry experiments, cells were plated on polyornithine-coated glass cover slips glued to the drilled-out bottom of 35-mm culture dishes. Otherwise, cells were plated on polyornithine-coated plastic culture dishes. Cells were used in experiments 1–8 h after plating.

**Patch clamp recording**

Recordings were made with both the standard whole cell patch-clamp configuration (Hamill et al. 1981) and perforated patch technique (Korn and Horn 1989). Patch pipettes were fabricated from N51A glass (Garner Glass, Claremont, CA), coated with silicone elastomer (Sylgard 184, Dow Corning, Midland, MI) and fire-polished. Pipette resistance varied from 0.5 to 2.0 MΩ. Capacitive transients were neutralized electronically, and series resistance compensation was used at 80–90% (Dagan 3911A patch-clamp amplifier, Dagan, Minneapolis, MN, or Axopatch 1D, Axon Instruments, Foster City, CA). In whole cell recordings, series resistance ranged from 1–4 MΩ (2.3 ± 0.1, mean ± SE; n = 82). Series resistance in perforated patch experiments ranged from 8.8 to 19.8 MΩ (13.5 ± 1.2, n = 8). Membrane currents were filtered at 2 kHz (internal patch-clamp filter) and digitized at sample intervals of 100–400 μs. Unless otherwise stated, the holding potential was −80 mV, and Ca$^{2+}$ currents were evoked by a 100-ms depolarizing stimulus once every 6–10 s. Experiments were performed at room temperature (20–24°C). Data were acquired and measured with pClamp 6 (Axon Instruments).

**Photometry**

Intracellular pH was measured in cells loaded with BCECF. To load cells with dye, cells were incubated at 37°C for 10 min with 2.5 μM of the membrane permeant, BCECF-AM (Molecular Probes, Eugene OR, or Texas Fluorescence Laboratories, Austin, TX). Cells then were washed with media and used immediately. When whole cell patch-clamp experiments were combined with photometry, 10 μM of the acid form of BCECF was added to the pipette solution. This prevented the loss of intracellular BCECF during the course of a patch clamp experiment. Electrophysiological experiments were begun after the fluorescence intensity stabilized (stabilization took 1–2 min).

Cells were excited with light from a 150 W Xenon lamp (cut by 60–90% with a neutral density filter), alternately passed through a 450- or 490-nm filter (Omega Optical, Brattleboro, VT) and then through a liquid light guide with quartz-collecting lenses on either end. The light then passed through a 515-nm dichroic mirror. Emitted light passed through a 535-nm barrier filter into an A/D converter for acquisition by pClamp. In this way, electrophysiological and photometric signals were collected simultaneously.

Photometry and electrophysiology were under the control of a user-written program. The protocols used for simultaneous recording of photometry and electrophysiology data are illustrated in Figs. 5 and 6. Briefly, every trace measured a 450- and a 490-nm signal before the voltage-clamp stimulus and a 490-nm signal during and after the voltage-clamp stimulus. There was no change in 490-nm signal during the depolarizing command. Calibration of the 490/450 ratio for pH is described later. In several experiments, the order of the 450- and 490-nm excitation was alternated so that the 450 nm measurement was made during the stimulus. The results obtained were identical.

For photometry experiments on intact cells (Fig. 1), cells were exposed to excitation light for 60 ms at each wavelength with a delay of 60 ms between each excitation. A ratio measurement was collected every 5 s for ≤15 min. Cells that showed measurable photobleaching were rare and were discarded.

**BCECF calibration**

Intracellular pH was calibrated for BCECF with the nigericin method (Boyarski et al. 1988). Briefly, cells loaded with BCECF were acutely isolated from lumbar level ganglia of 14-day white leghorn chick embryos (UCONN Poultry Farm, Storrs, CT). Cells were prepared as described previously (Polo-Parada and Korn 1997). In photometry experiments, cells were plated on polyornithine-coated glass cover slips glued to the drilled-out bottom of 35-mm culture dishes. Otherwise, cells were plated on polyornithine-coated plastic culture dishes. Cells were used in experiments 1–8 h after plating.

**FIG. 1.** Calibration of BCECF. Intracellular pH was equilibrated for BCECF with the nigericin method (Boyarski et al. 1988). Briefly, cells loaded with BCECF were
exposed to high-potassium bathing solutions (which contained, in mM, 150 KCl, 15 N-methylglucamine Cl, 5 MgCl₂, 10 glucose, and 10 HEPES or MES buffer; pH 7.3, osmolality = 325 ± 5 mosm/kg) of different pH in the presence of 10 μM nigericin. In the presence of nigericin, intracellular and extracellular pH equilibrated within 1–2 min. After equilibration, emission intensity was measured after excitation at 450 and 490 nm in five cells at each pH, and the average plotted as a function of pH (Fig. 1A). The ratio was normalized according to Eq. 1 (Boyaraski et al. 1988)

\[
\frac{I_{490}}{I_{450}} = 1 + b \left[ \frac{10^{(pH-pK)}}{1 + 10^{(pH-pK)}} - \frac{10^{(pH-pK)}}{1 + 10^{(pH-pK)}} \right]
\]

where \(I_{490}\) and \(I_{450}\) are emission intensities at the two excitation wavelengths and pH is the extracellular pH. Figure 1B shows a plot of the normalized ratio as a function of pH, with fitted values of pK and b of 7.02 and 1.50, respectively.

Whereas BCECF was calibrated in ‘‘intact’’ cells, pH measurements usually were recorded in cells loaded with 10 mM HEPES and, in some cases, high concentrations of bis(o-aminophenoxo)N,N′,N″,N″-tetraacetic acid (BAPTA). With internal and external solutions set to pH 7.30, the following pH was calculated from BCECF measurements in resting patch-clamped cells: 7.30 ± 0.03 (n = 8) and 7.30 ± 0.03 (n = 14) in perforated patch experiments with and without intracellular BAPTA, respectively; 7.32 ± 0.04 (n = 6) and 7.20 ± 0.02 (n = 30) in whole cell experiments with and without intracellular NH₄⁺. Thus neither HEPES nor BAPTA appeared to influence the accuracy of the calibration.

Solutions

Recordings were made from cells plated in 35-mm Nunc tissue culture dishes containing 1.5–2.0 ml of either static or flowing bathing solution. In static bath solutions (Figs. 3 and 4), the solution bathing the cell contained (in mM): 110 NaCl, 30 tetraethylammonium (TEA)-Cl, 20 N-methylglucamine (NMG)-Cl, 2 CaCl₂ or BaCl₂, 1 MgCl₂, 20 glucose, 10 HEPES, and 0.001 tetrodotoxin, pH 7.3 (NaOH), osmolality = 320 ± 5 mosm/kg. In the standard whole cell configuration, the pipette solution usually contained (in mM): 150 CsCl, 10 EGTA-Cs, 10 HEPES, 4 MgCl₂, 4 creatine phosphate, 4 ATP-Na, and 0.2 mM GTP-Na, leupeptin and creatine kinase, pH 7.3 (CsOH), osmolality = 305 ± 5 mosm/kg. In perforated-patch recordings, the pipette solution contained (in mM): 55 CsCl, 75 Cs₂SO₄, 8 MgCl₂, and 10 HEPES, pH 7.3 (CsOH), osmolality = 285 ± 5 mosm/kg. Substitutions are listed in the figure legends. In some perforated patch experiments, cells were loaded with BAPTA. To accomplish this, 40 min before recording, cells were incubated for 30 min with media containing 100 μM BAPTA-AM (Molecular Probes) in the 37°C incubator. The media containing BAPTA was replaced with BAPTA-free media and cells incubated for an additional 10 min. This procedure completely eliminated both intracellular Ca²⁺ elevation as measured by Fura-2, and activation of Ca²⁺-dependent Cl⁻ currents, after Ca²⁺ channel activation (data not shown). Except for the solution in the tip of the electrode, the pipette solution in perforated-patch experiments contained 16 μg/ml nystatin.

Two different methods were used to manipulate intracellular pH. In some experiments, intracellular pH was alkalinized by equimolar substitution of 20 mM NH₄⁺ (Cl⁻ salt) for extracellular NMG⁺ (Figs. 3–6) (Boron and De Weer 1976). In other experiments, intracellular pH was controlled by establishing a dual NH₄⁺ equilibrium, with 20 mM intracellular NH₄⁺ and varying concentrations of extracellular NH₄⁺ (Figs. 7 and 8) (Grinstein et al. 1994). This technique is described in more detail in RESULTS section.
paired Student’s t

...presence of NH₄⁺ blocked Ca²⁺ current in 11- to 12-day-old embryos (Cox and Dunlap 1994), and removal (Recov) of NH₄⁺. Current in the presence of NH₄⁺ was recorded 22–25 s after NH₄⁺ application was begun. B: current-voltage relationship in the absence (○) and presence (●) of NH₄⁺. Intracellular solution contained 0.5 mM EGTA. Cₘ = 16.6 pF; Rₑ = 1.9 MΩ.

**Data analysis**

All curve fitting and statistics were done with SigmaPlot 2.0 for Windows (Jandel Scientific, Corte Madera, CA). Error bars in plots represent the standard error of the mean. Statistical significance was tested by unpaired Student’s t-test except in Fig. 8D, which used a paired Student’s t-test.

**RESULTS**

**Ca²⁺ channel subtype**

Although chick DRGs contain predominantly N-type Ca²⁺ channels (Cox and Dunlap 1994), they can express L-type channels depending on age and time in culture (Cox and Dunlap 1992, 1994). Because L-type Ca²⁺ channels are sensitive to changes in pH, it was important to determine whether our cell population contained a fraction of L-type Ca²⁺ channels.

We characterized the subtype of Ca²⁺ channel(s) in our cells by voltage-dependent inactivation and pharmacological sensitivity. To examine inactivation, cells were held at progressively more depolarized potentials for 10 s followed by a test stimulus to 0 mV (Fig. 2A). In all cells tested, Ca²⁺ currents completely inactivated between −10 and 0 mV, with the half-maximal inactivation at −63.7 ± 1.7 mV (Fig. 2B; n = 8).

We then tested the ω-conotoxin GVIA sensitivity and dihydropyridine sensitivity. In 14 cells tested, 10 μM conotoxin irreversibly blocked the Ca²⁺ current by 94.4 ± 2.7%. Ten of these 14 cells were blocked by 100% by 1 μM conotoxin (Fig. 2, C and D). The mean block in the other four cells was 80.6 ± 4.3%. Importantly, 100% current block was achieved in cells of all sizes (Fig. 2D). One μM conotoxin also irreversibly blocked Ca²⁺ currents by 100% in four of four cells tested. Consistent with these results, the dihydropyridine nimbodipine (1 μM) had no effect on Ca²⁺ currents in six cells tested. Thus as previously described for acutely isolated chick DRGs taken from 11- to 12-day-old embryos (Cox and Dunlap 1994), cells of all sizes contained either entirely or almost entirely ω-conotoxin GVIA-sensitive Ca²⁺ channels.

**Potentiation of N-type Ca²⁺ currents by extracellular NH₄⁺**

Application of 20 mM NH₄⁺ reversibly potentiated voltage-activated Ca²⁺ currents (Fig. 3A). There was no shift in the voltage dependence of activation and little or no change in reversal potential (Fig. 3B).

Extracellular application of NH₄⁺ is a standard technique for elevation of intracellular pH. Because NH₄⁺ permeates through K⁺ channels, however, it was possible that this increase in voltage-activated inward current reflected the addition of an inward current carried by NH₄⁺ to the Ca²⁺ current. The experiment in Fig. 4 demonstrates that this was not the case. The data in Fig. 4 were collected under identical conditions as that in Fig. 3, except that TEA was eliminated from the bath solution (equimolar replacement with Na⁺). As in Fig. 3, application of NH₄⁺ resulted in an increase in the inward current (Fig. 3A). Also evident in this figure is the presence of a slow inward tail current (Fig. 4A, arrow) during NH₄⁺ application. Immediately after removal of NH₄⁺, the slow tail current disappeared and the inward current during the depolarization was reduced slightly. This tail current was blocked by extracellular application of 30 mM TEA, consistent with the tail current representing NH₄⁺ flux through K⁺ channels. We interpret these data to mean that, in the absence of TEA, the slow tail and perhaps a fraction of the step current during NH₄⁺ application was carried by NH₄⁺. However, the complete disappearance of tail current at a time when inward step current still was potentiated markedly indicates that the dominant effect of NH₄⁺ application was potentiation of the voltage-activated Ca²⁺ current. This interpretation is supported by the plots in Fig. 4B. The top plot shows that the inward tail current was present during application of NH₄⁺ and disappeared immediately on termination of NH₄⁺ application. This is consistent with what is expected with application and removal of an ion that permeates a voltage-gated channel. In contrast, Fig. 4B, bottom, shows that the inward current during the step grew slowly on NH₄⁺ application and decayed back to the control level slowly after removal of NH₄⁺. In all other experiments, the bathing solution contained 30 mM TEA to minimize the contribution of an NH₄⁺ current through K⁺ channels to measured currents.

**FIG. 3.** Potentiation of Iᵥ by extracellular application of NH₄⁺. A: currents evoked by 100-ms depolarization to 0 mV from −80 mV, recorded before (Cont.), during (NH₄⁺), and after removal (Recov) of NH₄⁺. Current in the presence of NH₄⁺ was recorded 22–25 s after NH₄⁺ application was begun. B: current-voltage relationship in the absence (○) and presence (●) of NH₄⁺. Intracellular solution contained 0.5 mM EGTA. Cₘ = 16.6 pF; Rₑ = 1.9 MΩ.

**FIG. 4.** Potentiation of inward current is not due to addition of non-Ca²⁺ current to Iᵥ. A: currents evoked by 100-ms depolarization to −10 mV before (Cont.), during (NH₄⁺), and 5 s after termination (1st off) of NH₄⁺ application. Arrow, inward tail current in the presence of extracellular NH₄⁺ that was absent in the absence of NH₄⁺. Intracellular solution contained 0.5 mM EGTA. Cₘ = 30.5 pF; Rₑ = 1.5 MΩ. B: time course of tail current increase, measured at arrow in A. Stimuli were delivered every 5 s. On the 1st pulse after NH₄⁺ application, the tail current reached maximum magnitude. On the 1st pulse after NH₄⁺ termination, the tail current returned to control value. C: time course of Iᵥ increase. Iᵥ was measured at the peak. Iᵥ grew slowly during NH₄⁺ application and recovered slowly after termination of NH₄⁺.
appears to be due to diffusion of NH$_4^+$

techniques exhibited no rebound acidification after removal of NH$_4^+$

if pH = 7.78), and shortly after termination of NH$_4^+$

patch recording: effect of NH$_4^+$
served previously, Ca$_{2+}$
nitude. On removal of external NH$_4^+$
multaneously with the photometric output in Fig. 5

A.

of these data are presented in the following text.

stabilized at four different plateau levels, and with each incre-

tion of NH$_4^+$

by NH$_4^+$

obtained at 450 and 490 nm before, during, and after applica-

BAPTA, recorded with the perforated patch configuration.

pH-sensitive dye, BCECF. In these experiments, 20 mM NH$_4^+$

was applied externally to alkalinize the cells.

We used two approaches to examine the pH$_i$ dependence and

sensitivity of Ca$_{2+}$

channel current magnitude. In the first set of

experiments (Figs. 5 and 6), we simultaneously measured

pH$_i$ and Ca$_{2+}$
current magnitude from cells loaded with the

pH-sensitive dye, BCECF. In these experiments, 20 mM NH$_4^+$

was applied externally to alkaline the cells.

Figure 5 shows results from a cell also preloaded with

BAPTA, recorded with the perforated patch configuration.

Figure 5A illustrates digitized light intensity measurements

obtained at 450 and 490 nm before, during, and after applica-

tion of NH$_4^+$. The internal pH in these three conditions, calcu-

lated from the calibration in Fig. 1, was 7.02, 7.78, and 7.23,

respectively. Figure 5B illustrates Ca$_{2+}$
currents recorded si-

multaneously with the photometric output in Fig. 5A. As ob-

served previously, Ca$_{2+}$
currents were potentiated reversibly by

NH$_4^+$

application. The association of changes in pH$_i$ and

Ca$_{2+}$
current magnitude can be compared in Fig. 5, C and D.

On application of NH$_4^+$, intracellular pH rose from 7.0 and 7.7,

and there was a corresponding increase in Ca$_{2+}$
current magnitude. On removal of external NH$_4^+$, pH$_i$ fell and Ca$_{2+}$
current magnitude was reduced. The change in Ca$_{2+}$
current magnitude routinely lagged behind the measured change in pH$_i$

during the on-phase of NH$_4^+$

application, making precise cor-

relation of pH$_i$ and Ca$_{2+}$
current magnitude difficult (this lag

will be addressed in DISCUSSION). Note, however, that pH$_i$
stabilized at four different plateau levels, and with each incre-

ment of alkalization, which was as little as 0.2 pH units,

Ca$_{2+}$
current magnitude was increased. More detailed analysis of

data are presented in the following text.

Figure 6A illustrates simultaneous recordings of pH$_i$ (top)

and Ca$_{2+}$

channel currents (bottom) from a standard (ruptured patch) whole cell recording. As described earlier, application of

NH$_4^+$

rapidly alkalinized the cell interior to pH 7.5, and the

Ca$_{2+}$
current was potentiated. Similar to the results observed in

Fig. 5, C and D, the change in Ca$_{2+}$
current magnitude more closely paralleled the change in pH$_i$ during the off-phase of NH$_4^+$

application. Consequently, we plotted Ca$_{2+}$
current magnitude as a function of pH$_i$ measured after removal of NH$_4^+$

(Fig. 6B). The data illustrate data from the cell in Fig. 6A; ○ plot the

pH dependence of Ca$_{2+}$
currents from the cell described in Fig. 5. Data were recorded once every 6 s, so the decline from pH 7.5 to 7.3 took ~90 s. These data indicate that Ca$_{2+}$
current magnitude is responsive to pH$_i$ changes between pH 7.0 and 7.5 and that prevention of intracellular Ca$_{2+}$
elevation with BAPTA had no apparent effect on pH sensitivity (NH$_4^+$
in-

duced potentiation also was observed in 6 whole cell record-

ings that included 5 mM intracellular BAPTA; data not shown).

Of particular interest from these experiments though, is the observation that Ca$_{2+}$
currents were sensitive to very small changes between pH 7.3 and 7.5.

**Control of intracellular pH by a dual-NH$_4^+$
equilibrium technique**

Manipulation of pH$_i$ solely by extracellular NH$_4^+$
posed three problems. First, the possibility of a long-lasting influence of

NH$_4^+$

itself on Ca$_{2+}$
current magnitude could not be ruled out from these experiments. Second, as described in Figs. 4–6, the

change in Ca$_{2+}$
current magnitude lagged behind the change in pH$_i$ especially during the on-phase of NH$_4^+$
application. This effect, which may have been due at least partially to the pH$_i$
buffering effect of BCECF, precluded an accurate quantifica-

tion of pH dependence of Ca$_{2+}$
current magnitude. Third, pH$_i$
could be measured and manipulated, but not quantitatively

controlled, with this technique. To ameliorate these problems, we used a dual NH$_4^+$
equilibrium technique, in which NH$_4^+$
is included in the intracellular solution and varied in the external

**FIG. 5.** Simultaneous recording of intracellular pH and I$_{ca}$ in perforated

patch recording: effect of NH$_4^+$

Cell was preloaded with bis-(o-aminophenoxy)-N,N,N',N'-tetraacetic acid (BAPTA). A and B: traces shown illustrate

BCECF emission intensity (A) and Ca$_{2+}$
currents (B) before (pH 7.02), during (pH 7.78), and shortly after termination of NH$_4^+$
application (pH 7.23). C and D: Ca$_{2+}$
current magnitude (C) and intracellular pH (D) as a function of time. Note: whereas intact cells in our laboratory (data not shown) always display the well documented rebound acidification after removal of NH$_4^+$
(Boron and De Weer 1976), cells recorded with patch-clamp techniques exhibited no rebound acidification after removal of NH$_4^+$
. This appears to be due to diffusion of NH$_4^+$
into the pipette through the perforated or ruptured patch (unpublished results).

**FIG. 6.** I$_{ca}$ is sensitive to small changes in pH between pH 7.0 and pH 7.6. 
A: standard whole cell recording from cell loaded with BCECF. Top: voltage output from photometer showing emission intensity at 450 nm excitation and 490 nm excitation wavelength. Three traces are shown. 1 recorded before NH$_4^+$
application (pH 7.12), 1 during NH$_4^+$
application (pH 7.48), and an interme-

diate signal (pH 7.32) recorded shortly after termination of NH$_4^+$
application. **Bottom:** Ca$_{2+}$
currents evoked by depolarization to 0 mV, recorded simulta-

neously with the photometry traces shown above. C$_m$ = 14.0 pF, R$_e$ = 2.5 MΩ.

B: Ca$_{2+}$
current magnitude as a function of intracellular pH for 2 cells. ○ data from the cell shown in A. ○ data from the cell described in Fig. 5. Currents were measured at the peak. Right axis (perforated patch data) and left axis (whole cell data) are scaled identically.
solution (Fig. 7) (Grinstein et al. 1994). With NH₄⁺ in the pipette solution and no external NH₄⁺, cells are acidified by the exit of NH₃ across the cell membrane (Fig. 7A, top). With NH₄⁺ in both the internal and external solution, the internal proton concentration is determined by the relative concentrations of internal and external NH₄⁺ (Fig. 7A, bottom). In contrast to techniques that use just internal or external NH₄⁺, this allows for precise, reproducible control of pHᵢ. This is illustrated in Fig. 7B. In patch-clamp recordings that included 20 mM NH₄⁺ in the pipette solution, application of different external [NH₄⁺] resulted in rapid, controlled changes in pHᵢ and produced a significant reduction in Ca²⁺ current magnitude (not shown), so this technique was not used to alkalinate cells in the experiments below. The plot in Fig. 7C illustrates the results of NH₄⁺ application to six cells recorded in the whole cell patch-clamp configuration. Cells were held at −80 mV throughout, and no depolarizing stimuli were applied. At external [NH₄⁺] of 0, 5, and 20 mM, pHᵢ attained values of 6.6, 7.0, and 7.3, respectively.

Figure 8 illustrates the pH sensitivity of Ba²⁺ currents using the dual NH₄⁺ equilibrium technique. The intracellular solution contained 20 mM NH₄⁺. Figure 8A illustrates currents activated by depolarization to 0 mV in the presence of 20 mM external NH₄⁺ (control and recovery traces are shown) and in the presence of 5 mM NH₄⁺. The complete current-voltage relationship from the same cell (Fig. 8B) illustrates that Ba²⁺ current was reduced by acidification with no accompanying change in reversal potential or voltage sensitivity. At 0 mV, Ba²⁺ current magnitude was reduced by 40% on switching from 20 to 5 mM NH₄⁺ (Fig. 8D). Figure 8C illustrates four superimposed currents, recorded alternately, in the presence of 20 or 0 mM external NH₄⁺. Currents recorded in the presence of 0 NH₄⁺ were reduced in magnitude by 65% compared with those recorded in the presence of 20 mM NH₄⁺ (Fig. 8D). These results demonstrate that Ca²⁺ channel currents are extremely sensitive to pHᵢ changes between 7.0 and 7.3 (the physiological range) and that acidification below pH 7.0 results in further reduction in Ba²⁺ current magnitude. Ba²⁺ current responses to changes in pHᵢ were readily reversible, which suggests that acidification to values as low as pH 6.6 did not damage the Ca²⁺ channel, but rather, that changes in pHᵢ modified channel function. Finally, because NH₄⁺ always was present in these experiments, these results demonstrate that it was the change in pHᵢ, not the presence of NH₄⁺, that modulated Ca²⁺ current magnitude.

**DISCUSSION**

Ca²⁺ influx via voltage-gated Ca²⁺ channels, glutamate application, GABA application, and hypoxia all produce intracellular acidification by 0.15–0.5 U (Ahmed and Connor 1980; Canzoniero et al. 1996; Dixon et al. 1993; Hartley and Dubinsky 1993; Kaila et al. 1990, 1993; Luckermann et al. 1997; sky 1993; Kaila et al. 1990, 1993; Luckermann et al. 1997;
The main finding of our experiments is that N-type Ca\(^{2+}\) channels in hippocampal neurons (Tombaugh and Somjen 1997), resting pH\(_r\) varied and/or the pH\(_i\) values traversed on application of NH\(_4\)\(^+\) differed. Alternatively, the apparently different channel sensitivities to pH\(_i\) in hippocampal neurons indeed may reflect a difference between mammalian and nonmammalian channel sensitivity to pH\(_i\) or different mechanisms of pH sensitivity in different cell types. Resolution of this discrepancy awaits precise quantitative examination of the pH dependence of different Ca\(^{2+}\) channel subtypes from different cells.

### Time lag between pH change and Ca\(^{2+}\) current response

In all of our experiments, there was a delay between the measured pH\(_i\) change and Ca\(^{2+}\) current response (see Fig. 5 for example). This delay was not entirely due to the presence of intracellular BCECF or HEPES, both H\(^+\) buffers, because the lag occurred in the absence of these chemicals (Figs. 4 and 5).

We imagine one of three possible reasons for the delay. First, the pH\(_i\)-induced modulation may have been produced indirectly by protonation of another Ca\(^{2+}\) channel modulator rather than by a direct protonation of the Ca\(^{2+}\) channel. Second, the pH\(_i\)-induced modulation may have resulted from protonation of a site in the Ca\(^{2+}\) channel pore near the outer vestibule (Chen et al. 1996). Whereas H\(^+\)-induced modulation of this externally located site usually is studied via extracellular pH changes (cf. Chen et al. 1996; Prod’hom et al. 1989), it is possible that intracellular protons could make their way to this site on repetitive Ca\(^{2+}\) channel activation. Finally, this delay may have represented a lag in pH\(_i\) change adjacent to the membrane relative to that measured in the entire cell by BCECF. Elucidation of the mechanism of pH\(_i\)-induced modulation of Ca\(^{2+}\) currents must await future studies.

### Functional significance

The variety of physiological and pathological stimuli that alter pH\(_i\) and the observation that Ca\(^{2+}\) channel currents are quite sensitive to small changes in pH\(_i\) in the physiological range suggest that changes in pH\(_i\) may play an important role in both physiological and pathological events. Some potential roles are described here.

#### FEEDBACK INHIBITORY MECHANISM

Mitochondrial uptake of Ca\(^{2+}\) is an important Ca\(^{2+}\) removal mechanism under conditions of both moderate and high intracellular Ca\(^{2+}\) load (Herrington et al. 1996; Meech and Thomas 1980; Park et al. 1996; Thayer and Miller 1990; Wang et al. 1994). After Ca\(^{2+}\) influx, the H\(^+\) pumped out of the mitochondria during respiration can become a significant source of intracellular acidification and can produce changes in pH\(_i\) of \(\pm 0.2\) U (Werth and Thayer 1994). Thus Ca\(^{2+}\)-induced acidification after Ca\(^{2+}\) influx might form a feedback inhibitory control on Ca\(^{2+}\) channel activity. Given the prominent role of N-type Ca\(^{2+}\) channels in the secretory process in both neurons and endocrine cells, such a feedback mechanism may be particularly important in the secretion process.

#### GABA AS A POTENTIAL MODULATOR OF Ca\(^{2+}\) CHANNEL FUNCTION

Activation of GABA\(_{A}\) receptors results in changes in pH\(_i\) by flux of HCO\(_3^-\) through GABA-activated Cl\(^-\).
channels (Kaila et al. 1990, 1993; Luckermann et al. 1997; Voipio et al. 1991). Under physiological conditions, GABA can acidify the cell interior, on average, by as much as 0.25–0.4 U. Thus inhibition of cell function by GABA may be accomplished, at least in part, by acidification-induced inhibition of Ca\(^{2+}\) channel function. Interestingly, this effect may depend on the physiological state of the cell or organism because membrane potential and pCO\(_2\) will influence the magnitude and even direction of GABA-induced pH changes without influencing GABA-activated conductance changes (Luckermann et al. 1997).

**NEURONAL PROTECTION DURING ISCHEMIC CONDITIONS.** Cellular damage from ischemia and reperfusion injury after ischemia are associated with large increases in intracellular Ca\(^{2+}\) (cf. Bickler and Hansen 1998; Kristian and Siesjo 1998; Meissner and Morgan 1995; Shimazaki et al. 1998; Vornov 1998). In both neurons and cardiac tissue, maintenance of acidic pH, during recovery from ischemia protects cells from cell death (Bond et al. 1993; Scholz et al. 1992; Tombaugh and Sapolsky 1990; Vornov et al. 1996). Acidification-induced inhibition of Ca\(^{2+}\) channels, under conditions that might otherwise serve to activate Ca\(^{2+}\) channels, may be one mechanism by which cells are protected from neurotoxic damage.

In summary, although the functional significance of pH-dependent modulation of Ca\(^{2+}\) channels remains speculative, the exquisite sensitivity of N-type Ca\(^{2+}\) channels to changes in pH, between pH 6.6 and 7.5 suggests that modulation by pH may contribute to a wide array of physiological and pathological events.

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