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

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Kiss, Laszlo and Stephen J. Korn. Modulation of N-type Ca\(^{2+}\) 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\(^{2+}\)], can produce changes in intracellular pH of between 0.15 and 0.5 U, between pH 7.4 and 6.8. N-type Ca\(^{2+}\) 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\(^{2+}\) channels are sensitive, and the sensitivity of N-type Ca\(^{2+}\) 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\(_4\)\(^+\) in the presence and absence of intracellular NH\(_4\)\(^+\). Changes in intracellular pH between 6.6 and 7.5 produced a graded change in Ca\(^{2+}\) current magnitude with no apparent shift in activation potential. Intracellular acidification from pH 7.3 to 7.0 reversibly inhibited Ca\(^{2+}\) currents by 40%. Acidification from pH 7.3 to pH 6.6 reversibly inhibited Ca\(^{2+}\) currents by 65%. Alkalization from pH 7.3 to 7.5 potentiated Ca\(^{2+}\) currents by approximately 40%. Channels were sensitive to pH changes with high intracellular concentrations of the Ca\(^{2+}\) chelator, bis-(o-aminophenoxy)-N,N,N’,N’-tetraacetic acid, which indicates that the effects of pH did not involve a Ca\(^{2+}\)-dependent mechanism. These data indicate that N-type Ca\(^{2+}\) channel currents are extremely sensitive to small changes in pH in the range produced by both physiological and pathological events. Furthermore, these data suggest that modulation of N-type Ca\(^{2+}\) channels by pH may play an important role in physiological processes that produce small changes in pH, and a protective role in pathological mechanisms that produce larger changes in pH.

INTRODUCTION

Both excitatory and inhibitory stimuli can acidify neurons. Elevation of intracellular Ca\(^{2+}\) (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\(^{2+}\) influx associated with action potentials activates 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; Vornov 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\(^{2+}\) channels in mammalian and nonmammalian tissues are sensitive to changes in pHi (Kaila 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\(^{2+}\) channels are quite sensitive to pHi changes between 6.6 and 7.6; acidification from pH 7.3 to 7.0 produced a 30–40% reduction in current amplitude, and alkalization from pH 7.3 to 7.6 potentiated Ca\(^{2+}\) currents by ~40% (Dixon et al. 1993). Although not as precisely quantified, L-type Ca\(^{2+}\) channels from mammalian vascular smooth muscle were also sensitive to pHi changes between 6 and 8.4 (Klockner and Isenberg 1994). In contrast to these results, the pHi range over which N-type Ca\(^{2+}\) channels are sensitive is not known.

In hippocampal neurons, N-type Ca\(^{2+}\) channels appear to be more sensitive than L-type Ca\(^{2+}\) channels to changes in pHi (Tombaugh and Somjen 1997). This difference was reflected as a larger change in amplitude of N- than L-type currents on application of NH\(_4\)\(^+\). 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\(^{2+}\) channels and is the pHi sensitivity of N-type Ca\(^{2+}\) channels similar to or different from that reported for L-type Ca\(^{2+}\) channels? To address these questions, we examined the pHi dependence of N-type Ca\(^{2+}\) channel currents in acutely isolated chick dorsal root ganglion neurons (DRGs), which contained a pharmacologically pure population of N-type Ca\(^{2+}\) 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$_i$ 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 ms. 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 dicroic mirror. Emitted light passed through a 535-nm barrier filter through an aperture to a photometer. The aperture was set to be as close to the diameter of the cell as possible. The analog voltage output from the photometer was routed 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...
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 (Boyarski et al. 1988)

\[
\frac{I_{490}}{I_{450}} = 1 + b \left[ \frac{10^{\pH - pK1} - 10^{1/pK2}}{1 + 10^{\pH - pK1}} \right]
\]

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-aminophenoxy)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 cells was changed by manually lowering a large-bore pipette that contained the desired test solution near the culture dishes containing 1.5–2.0 ml of either static or flowing 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.

**FIG. 2.** Voltage-dependent inactivation and block of Ca²⁺ currents by ω-CgTx GVIA. A: cells were held at a series of holding potentials (values shown) between −110 and 0 mV. After 10 s at the holding potential, cells were depolarized to 0 mV for 100 ms. Last current shown (−80 mV) was evoked after the holding potential was returned to −80 mV from 0 mV, to demonstrate that full recovery of the Ca²⁺ current would occur. Currents were recorded in the absence of extracellular Na⁺ (replaced by TEA). Cm = 18 pF; Rm = 2.2 MΩ. B: voltage dependence of inactivation. Data points are averages obtained from 8 cells tested as in A. —, best fit to the data points of the Boltzmann function, \(V_{1/2} = l/[1 + \exp(-(V - V_{1/2})/\sigma)]\), where \(V_{1/2}\) is the voltage at which currents are 50% inactivated and \(\sigma\) is the slope factor. C: ω-CgTx currents before (Cont.) and after application of 10 μM ω-CgTx GVIA. Block by ω-CgTx was always completely irreversible. Cm = 27 pF; Rm = 2.0 MΩ. D: results of ω-CgTx application to 14 cells, which ranged in size from 8 to 39 pF.
paired Student's t

consistent with these results, the dihydropyridine nimodipine
activated Ca2+

potentiation of N-type Ca2+

toxin GVIA-sensitive Ca2+
of all sizes contained either entirely or almost entirely
from 11- to 12-day-old embryos (Cox and Dunlap 1994), cells
as previously described for acutely isolated chick DRGs taken

fig. 2 D

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; R = 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

calcium 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 sen-
titive to changes in pHi, 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 sensi-
tivity. 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 dihy-
dropyridine 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 10 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 nimodipine
(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-cono-
toxin 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+ applica-
cation. Immediately after removal of NH4+, the slow tail cur-
rent disappeared and the inward current during the depolari-
zation 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 dis-
pappearance 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 consist-
ent 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 mea-
sured currents.

fig. 4

potentiation of inward current is not due to addition of non-Ca2+
current to ICa. 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; R = 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 ICa increase. ICa was measured at the peak. ICa grew slowly during NH4+ application and recovered slowly after termination of NH4+.
and Ca²⁺ channel currents (bottom) from a standard (ruptured patch) whole cell recording. As described earlier, application of NH₄⁺ rapidly alkalinized the cell interior to pH 7.5, and the Ca²⁺ current was potentiated. Similar to the results observed in Fig. 5, C and D, the change in Ca²⁺ current magnitude more closely paralleled the change in pH_i during the off-phase of NH₄⁺ application. Consequently, we plotted Ca²⁺ current magnitude as a function of pH_i measured after removal of NH₄⁺ (Fig. 6B). ○ illustrate data from the cell in Fig. 6A; □ plot the pH dependence of Ca²⁺ 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 Ca²⁺ current magnitude is responsive to pH changes between pH 7.0 and 7.5 and that prevention of intracellular Ca²⁺ elevation with BAPTA had no apparent effect on pH sensitivity (NH₄⁺-induced potentiation also was observed in 6 whole cell recordings that included 5 mM intracellular BAPTA; data not shown). Of particular interest from these experiments though, is the observation that Ca²⁺ currents were sensitive to very small changes between pH 7.3 and 7.5.

Control of intracellular pH by a dual-NH₄⁺ equilibrium technique

Manipulation of pH_i solely by extracellular NH₄⁺ posed three problems. First, the possibility of a long-lasting influence of NH₄⁺ itself on Ca²⁺ current magnitude could not be ruled out from these experiments. Second, as described in Figs. 4–6, the change in Ca²⁺ current magnitude lagged behind the change in pH_i, especially during the on-phase of NH₄⁺ application. This effect, which may have been due at least partially to the pH buffering effect of BCECF, precluded an accurate quantification of pH dependence of Ca²⁺ 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 NH₄⁺ equilibrium technique, in which NH₄⁺ is included in the intracellular solution and varied in the external

pH dependence of change in Ca²⁺ current magnitude

We used two approaches to examine the pH dependence and sensitivity of Ca²⁺ channel current magnitude. In the first set of experiments (Figs. 5 and 6), we simultaneously measured pH_i and Ca²⁺ current magnitude from cells loaded with the pH-sensitive dye, BCECF. In these experiments, 20 mM NH₄⁺ was applied externally to alkalinize 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 application of NH₄⁺. The internal pH in these three conditions, calculated from the calibration in Fig. 1, was 7.02, 7.78, and 7.23, respectively. Figure 5B illustrates Ca²⁺ currents recorded simultaneously with the photometric output in Fig. 5A. As observed previously, Ca²⁺ currents were potentiated reversibly by NH₄⁺ application. The association of changes in pH_i and Ca²⁺ current magnitude can be compared in Fig. 5, C and D. On application of NH₄⁺, intracellular pH rose from 7.0 and 7.7, and there was a corresponding increase in Ca²⁺ current magnitude. On removal of external NH₄⁺, pH_i fell and Ca²⁺ current magnitude was reduced. The change in Ca²⁺ current magnitude routinely lagged behind the measured change in pH_i during the on-phase of NH₄⁺ application, making precise correlation of pH_i and Ca²⁺ current magnitude difficult (this lag will be addressed in Discussion). Note, however, that pH_i stabilized at four different plateau levels, and with each increment of alkalinization, which was as little as 0.2 pH units, Ca²⁺ current magnitude was increased. More detailed analysis of these data are presented in the following text.

Figure 6A illustrates simultaneous recordings of pH_i (top) and Ca²⁺ channel currents (bottom) from a standard (ruptured patch) whole cell recording. As described earlier, application of NH₄⁺ rapidly alkalinized the cell interior to pH 7.5, and the Ca²⁺ current was potentiated. Similar to the results observed in Fig. 5, C and D, the change in Ca²⁺ current magnitude more closely paralleled the change in pH_i during the off-phase of NH₄⁺ application. Consequently, we plotted Ca²⁺ current magnitude as a function of pH_i measured after removal of NH₄⁺ (Fig. 6B). ○ illustrate data from the cell in Fig. 6A; □ plot the pH dependence of Ca²⁺ 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 Ca²⁺ current magnitude is responsive to pH changes between pH 7.0 and 7.5 and that prevention of intracellular Ca²⁺ elevation with BAPTA had no apparent effect on pH sensitivity (NH₄⁺-induced potentiation also was observed in 6 whole cell recordings that included 5 mM intracellular BAPTA; data not shown). Of particular interest from these experiments though, is the observation that Ca²⁺ currents were sensitive to very small changes between pH 7.3 and 7.5.

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Manipulation of pH_i solely by extracellular NH₄⁺ posed three problems. First, the possibility of a long-lasting influence of NH₄⁺ itself on Ca²⁺ current magnitude could not be ruled out from these experiments. Second, as described in Figs. 4–6, the change in Ca²⁺ current magnitude lagged behind the change in pH_i, especially during the on-phase of NH₄⁺ application. This effect, which may have been due at least partially to the pH buffering effect of BCECF, precluded an accurate quantification of pH dependence of Ca²⁺ 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 NH₄⁺ equilibrium technique, in which NH₄⁺ is included in the intracellular solution and varied in the external
solution (Fig. 7) (Grinstein et al. 1994). With NH$_4^+$ in the pipette solution and no external NH$_4^+$, cells are acidified by the exit of NH$_3$ across the cell membrane (Fig. 7A, top). With NH$_4^+$ in both the internal and external solution, the internal proton concentration is determined by the relative concentrations of internal and external NH$_4^+$ (Fig. 7A, bottom). In contrast to techniques that use just internal or external NH$_4^+$, this allows for precise, reproducible control of pH$_i$. This is illustrated in Fig. 7B. In patch-clamp recordings that included 20 mM NH$_4^+$ in the pipette solution, application of different external [NH$_4^+$] resulted in rapid, controlled changes in pH$_i$ (Fig. 7B). Note that application of 40 mM NH$_4^+$ did not produce the expected alkalization of pH$_i$ and produced a significant reduction in Ca$^{2+}$ 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 NH$_4^+$ 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 [NH$_4^+$] of 0, 5, and 20 mM, pH$_i$ attained values of 6.6, 7.0, and 7.3, respectively.

Figure 8 illustrates the pH sensitivity of Ba$^{2+}$ currents using the dual NH$_4^+$ equilibrium technique. The intracellular solution contained 20 mM NH$_4^+$. Figure 8A illustrates currents activated by depolarization to 0 mV in the presence of 20 mM external NH$_4^+$ (control and recovery traces are shown) and in the presence of 5 mM NH$_4^+$ The complete current-voltage relationship from the same cell (Fig. 8B) illustrates that Ba$^{2+}$ current was reduced by acidification with no accompanying change in reversal potential or voltage sensitivity. At 0 mV, Ba$^{2+}$ current magnitude was reduced by 40% on switching from 20 to 5 mM NH$_4^+$ (Fig. 8D). Figure 8C illustrates four superimposed currents, recorded alternately, in the presence of 20 or 0 mM external NH$_4^+$. Currents recorded in the presence of 0 NH$_4^+$ were reduced in magnitude by 65% compared with those recorded in the presence of 20 mM NH$_4^+$ (Fig. 8D). These results demonstrate that Ca$^{2+}$ channel currents are extremely sensitive to pH$_i$ changes between 7.0 and 7.3 (the physiological range) and that acidification below pH 7.0 results in further reduction in Ba$^{2+}$ current magnitude. Ba$^{2+}$ current responses to changes in pH$_i$ were readily reversible, which suggests that acidification to values as low as pH 6.6 did not damage the Ca$^{2+}$ channel, but rather, that changes in pH$_i$, modified channel function. Finally, because NH$_4^+$ always was present in these experiments, these results demonstrate that it was the change in pH$_i$, not the presence of NH$_4^+$, that modulated Ca$^{2+}$ current magnitude.

**DISCUSSION**

Ca$^{2+}$ influx via voltage-gated Ca$^{2+}$ 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;
Comparison of pH sensitivity of L- and N-type Ca\(^{2+}\) channels

The pH\(_{i}\)-sensitivity of N-type Ca\(^{2+}\) channels in chick DRGs is nearly identical to that of L-type Ca\(^{2+}\) channels in catfish horizontal cells (Dixon et al. 1993). Both L-type Ca\(^{2+}\) currents (Dixon et al. 1993) and N-type Ca\(^{2+}\) currents (Fig. 8) were reduced by \(\sim 40–45\%\) when pH\(_{i}\) was reduced from pH 7.3 to 7.0 and potentiated by 40–45% with alkalinization from pH 7.3 to 7.5 (Fig. 6). In rat hippocampal neurons, N-type Ca\(^{2+}\) channel currents appeared to be more sensitive to application of NH\(_{4}\)\(^{+}\) than L-type Ca\(^{2+}\) channel currents (Tombaugh and Somjen 1997). In these studies, however, pH\(_{i}\) was not measured. In whole cell patch-clamp experiments, with the pipette solution buffered to pH 7.3 with 10 mM HEPES, pH\(_{i}\) can vary between 6.9 and 7.3 from cell to cell and over time (unpublished data). Consequently, it is possible that under the different conditions necessary to isolate N- and L-type currents in hippocampal neurons (Tombaugh and Somjen 1997), resting pH\(_{i}\) 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 is likely 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 \(\sim 0.2\) 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.
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 pH\(_i\) will influence the magnitude and even direction of GABA-induced pH\(_i\) changes without influencing GABA-activated conductance changes (Luckermann et al. 1997).

**Neuonal Protection during Ischemic Conditions.** Celluar damage from ischemia and reperfusion injury after ischemia are associated with large increases in intracellular Ca\(^{2+}\) (cf. Bickler and Hansen 1998; Kristian and Siesjö 1988; Meissner and Morgan 1995; Shimazaki et al. 1998; Vornov 1998). In both neurons and cardiac tissue, maintenance of acidic pH\(_i\), 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\(_i\)-dependent modulation of Ca\(^{2+}\) channels remains speculative, the exquisite sensitivity of N-type Ca\(^{2+}\) channels to changes in pH\(_i\), between pH 6.6 and 7.5 suggests that modulation by pH\(_i\) may contribute to a wide array of physiological and pathological events.

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