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Authors

Takahashi, K
Dixon, DB
Copenhagen, DR

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Modulation of a Sustained Calcium Current by Intracellular pH in Horizontal Cells of Fish Retina

KYOH-ICHI TAKAHASHI, DONALD B. DIXON, and DAVID R. COPENHAGEN

From the Departments of Ophthalmology and Physiology, University of California, San Francisco, San Francisco, California 94143-0730

ABSTRACT A sustained high voltage–activated (HVA), nifedipine- and cadmium-sensitive calcium current and a sustained calcium action potential (AP) were recorded from horizontal cells isolated from catfish retina. pH indicator dyes showed that superfusion with NH_4Cl alkalinized these cells and that washout of NH_4Cl or superfusion with Na-acetate acidified them. HVA current was slightly enhanced during superfusion of NH_4Cl but was suppressed upon NH_4Cl washout or application of Na-acetate. When 25 mM HEPES was added to the patch pipette to increase intracellular pH buffering, the effects of NH_4Cl and Na-acetate on HVA current were reduced. These results indicated that intracellular acidification reduces HVA calcium current and alkalinization increases it. Sustained APs, recorded with high resistance, small diameter microelectrodes, were blocked by cobalt and cadmium and their magnitude varied with extracellular calcium concentration. These results provide confirmatory evidence that the HVA current is a major component of the AP and indicate that the AP can be used as a measure of how the HVA current can be modified in intact, undialyzed cells. The duration of APs was increased by superfusion with NH_4Cl and reduced by washout of NH_4Cl or superfusion with Na-acetate. The Na-acetate and NH_4Cl washout–dependent shortening of the APs was observed in the presence of intracellular BAPTA, a calcium chelator, IBMX, a phosphodiesterase inhibitor, and in Na-free or TEA-enriched saline. These findings provide supportive evidence that intracellular acidification may directly suppress the HVA calcium current in intact cells. Intracellular pH changes would thereby be expected to modulate not only the resting membrane potential of these cells in darkness, but calcium-dependent release of neurotransmitter from these cells as well. Furthermore, this acidification-dependent suppression of calcium current could serve a protective role by reducing calcium entry during retinal ischemia, which is usually thought to be accompanied by intracellular acidosis.

Address reprint requests to Dr. Don Dixon, Box 0730, Department of Ophthalmology, UCSF School of Medicine, San Francisco, CA 94143-0730.

Kyoh-Ichi Takahashi's present address is Department of Physiology, Keio University School of Medicine, 35 Shinanomachi, Shinjuku, Tokyo 160, Japan.

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INTRODUCTION

Horizontal cells in the vertebrate retina are interneurons synaptically excited by photoreceptors through glutamatergic synapses. Horizontal cells have relatively large receptive fields and mediate surround antagonism observed in the light responses of bipolar cells and cones (Baylor, Fuortes, and O'Bryan, 1971; Fuortes and Simon, 1974). The center-surround receptive field organization resulting from these antagonistic interactions is an important contrast-enhancing feature of signal processing in the visual system. The excitability and receptive field sizes of horizontal cells are modified by neuromodulators, such as dopamine, that are intrinsic to the retina (Knapp and Dowling, 1987) and by light and dark adaptation (Baldridge and Ball, 1991).

A high voltage–activated (HVA) sustained calcium current has been demonstrated in isolated horizontal cells (Tachibana, 1983; Shingai and Christensen, 1986). This current is active at the normal dark resting potential (−20 to −40 mV) for these cells in the intact retina (Yang, Tornqvist, and Dowling, 1988). Sustained APs, probably resulting from activation of an HVA calcium current, can be unmasked in horizontal cells recorded from retinas bathed with TEA (Murakami and Takahashi, 1987).

Retinal function is critically dependent on the maintenance of constant pH (Winkler, Simson, and Benner, 1977; Liebman, Mueller, and Pugh, 1984; Oakley and Wen, 1989; Donner, Hemila, Kalamkarov, Koskelainen, and Shevchenko, 1990). The level of cellular metabolism is sufficient to produce a standing pH gradient across the retina which changes with light and dark adaptation (Yamamoto, Borgula, and Steinberg, 1992). Moreover, in nervous tissue and in the retina, periods of ischemia cause significant changes in intracellular and extracellular pH (Nedergaard, Goldman, Desai, and Pulsinelli, 1991). Changes in pH can affect signal transmission in the retina. Extracellular acidification suppresses a calcium current in cones that probably regulates neurotransmitter release (Barnes and Bui, 1991), and suppresses an *N*-methyl-D-aspartate type glutamate current in horizontal cells that depolarizes these cells in darkness (Christensen and Hida, 1990). Intracellular pH regulates HVA calcium currents in *Paramecium*, myocytes, pancreatic cells, and chick dorsal root ganglion cells (Umbach, 1982; Plant, 1988; Katzka and Morad, 1989; Mironov and Lux, 1991).

In this article, by performing whole-cell voltage-clamp recordings, we investigated how intracellular pH modified the HVA current of isolated horizontal cells and, by recording membrane potentials of intact cells, we provide evidence that the sustained APs were generated by HVA current and were similarly regulated by intracellular pH.

Preliminary reports of some of these findings were presented earlier (Takahashi and Copenhagen, 1990).

MATERIALS AND METHODS

Preparation of Cells

All experimental procedures conformed to the recommendations of the NIH guide for the Use of Laboratory Animals and the ARVO Statement on the Use of Animals in Ophthalmic and Vision Research. Catfish (*Ictalurus punctatus*) were dark-adapted for 12 h, cooled, swiftly decapitated, and pithed. Enucleated eyes were hemisected and the retinas were removed.

Horizontal cells were isolated using papain and mechanical trituration following the procedures of Tachibana (1981). The cells were plated onto sterilized clean glass coverslips and kept at 10°C in L-15 media for periods ranging from 2 to 20 d. Microelectrode and patch pipette recordings were done at room temperature (20°C) in a superfusion chamber fitted to the stage of an inverted microscope (model IM35; Carl Zeiss, Inc., Thornwood, NY).

Electrophysiological Recordings

Microelectrodes were formed on a Brown-Flaming P88 Puller (Sutter Instrument Co., Novato, CA) and typically had resistances of 200–300 MΩ when filled with 3 M KCl. Patch pipettes were formed on a vertical puller (model PP-83; Narishige Scientific Instrument Laboratories, Tokyo, Japan) from 1.5-mm-diam borosilicate glass (Garner Glass Co., Claremont, CA). Tips were pulled to diameters yielding bubble numbers of 4.5–5.5 (Mittman, Flaming, Copenhagen, and

TABLE I

Experimental Solutions

	Control saline	NH_4Cl saline	$NaCH_3OO^-$ saline	Standard patch	High-buffer patch
NaCl	125 mM	105 mM	100 mM	4 mM	4 mM
KCl	2.6 mM	2.6 mM	2.6 mM	—	—
$MgCl_2$	1.0 mM	1.0 mM	1.0 mM	1 mM	1 mM
$CaCl_2$	2.5 mM*	2.5 mM*	2.5 mM*	—	—
$BaCl_2$	15 mM‡	15 mM‡	15 mM‡	2 mM	2 mM
Glucose	15 mM*	15 mM*	15 mM*	—	—
	5 mM‡	5 mM‡	5 mM‡	—	—
HEPES	10 mM	10 mM	10 mM	—	25 mM
NH_4Cl	—	20 mM	—	—	—
$NaCH_3OO^-$	—	—	25 mM	—	—
Cs gluconate	—	—	—	130 mM	105 mM
EGTA	—	—	—	10 mM	10 mM
ATP	—	—	—	1 mM	1 mM
GTP	—	—	—	0.1 mM	0.1 mM
pH	7.8	7.8	7.8	7.4	7.4

The salines used for the microelectrode experiments (*) contained 2.5 mM calcium. The salines used for the patch-clamp experiments (‡) contained 15 mM barium. The glucose concentration was reduced to compensate for the osmolarity difference.

Belgum, 1987) and having resistances of 5–8 MΩ. The access resistance during recordings was typically 20–30 MΩ and was not compensated during the experiments since the high input resistance of the cells (500–800 MΩ) made the errors due to access resistance less than a few percent. Recordings were made from the cone-driven cells, which are axon bearing in this species (DeVries and Schwartz, 1989). The cells chosen for recording had one to three short extensions emanating from the soma.

Intracellular pH Measurements of Isolated, Intact Cells

Intracellular pH was determined in individual horizontal cells using the fluorescent indicator dye 2',7'-bis-(2-carboxyethyl)-5-(and 6) carboxyfluorescein, acetoxymethyl ester (BCECF-AM; Molecular Probes, Inc., Eugene, OR) following the protocols of Paradiso, Tsien, Demarest, and Machen (1987). Cells were washed for 5 min with saline containing 5 μM BCECF-AM, and then

rinsed in control saline for 1 h to allow the fluorescent signal to reach equilibrium. A 10-μm spot at the center of individual horizontal cells was imaged by a Zonax Fluorescence/Photometer system (Carl Zeiss, Inc.). The cells were alternately illuminated by 200-ms flashes of 440- and 490-nm excitation lights. The ratio of 535-nm fluorescence induced by the two different excitation lights was used to calculate the intracellular pH. The excitation and barrier filters and the dichroic mirror were a BCECF filter set from Omega Optical Inc. (Brattleboro, VT). The measurement protocol was repeated every 5 s throughout the course of the experiment. The K^+/H^+ exchanger nigericin (10 μM; Molecular Probes, Inc.) was superfused at the end of experimental runs to calibrate the pH changes measured during the experiments. The nigericin calibrations were done in 50 mM potassium saline to depolarize the cells and thereby minimize the electrical gradient for hydrogen ions across the membrane.

Chemicals and Solutions

All chemicals were purchased from Sigma Chemical Co. (St. Louis, MO) except where noted. All solutions are listed in Table I. The pH and osmolarity of all solutions were measured before every experiment. NH_4Cl or Na-acetate was applied to the cells by switching to a superfusion saline in which either NH_4Cl or Na-acetate had been substituted for NaCl. For the high buffer patch solution, Cs-gluconate concentration was reduced when HEPES was added to maintain the appropriate osmolarity.

RESULTS

Characterization of the High Voltage–activated Current

Tachibana (1983) and others (Lasater, 1986; Shingai and Christensen, 1986) showed that retinal horizontal cells possess a high threshold, slowly inactivating calcium current (HVA current) that is activated at potentials positive to −40 to −30 mV. Fig. 1 shows this HVA current in an isolated catfish horizontal cell voltage-clamped with patch pipettes recorded in whole-cell mode. For these experiments external barium chloride (15 mM) was substituted for the normal 2.5 mM calcium chloride as it increased the sustained inward current several-fold. Barium also reduced the rate of HVA current rundown (Dixon, D. B., unpublished observation; Belles, Malecot, Hescheler, and Trautwein, 1988), which was beneficial since experiments often exceeded 30 min. Fig. 1 *A* shows the current recorded as the clamp potential was stepped to progressively more depolarized levels from the holding potential of −50 mV. The records in Fig. 1 *B* show the current evoked in 20 μM cadmium. Fig. 1 *C* shows the cadmium-sensitive current obtained by subtraction (traces in Fig. 1 *B* minus those in Fig. 1 *A*). The current–voltage (*I-V*) curve is shown in Fig. 1 *D*. These records confirm the existence of a slowly inactivating HVA current that is activated, in this cell, at potentials positive to −40 mV.

HVA currents were examined using a voltage ramp protocol. For these experiments the command voltage was ramped from −90 to +50 mV at a rate of 70 mV/s. In several cells the ramp-evoked current was compared with the plateau level of pulse-evoked currents. At the ramp rate of 70 mV/s the differences in clamp current were negligible. Fig. 2 shows the ramp current generated by a typical horizontal cell in control saline and in control saline plus 0.1 mM nifedipine. The difference curve shows the nifedipine-sensitive component of the current which peaks close to +10 mV and is activated near −30 mV. Superfusion with cadmium (20 μM) elicited

similar difference curves. The activation potentials measured with the ramps ranged between −30 and −40 mV, but the peaks were always close to +10 mV. The results from the nifedipine and cadmium experiments provide pharmacological confirmation that we were measuring a HVA calcium current. Because the onset and peak potentials of the nifedipine-isolated HVA current closely approximated the inward current measured in control saline, we took the inward current measured between −40 and +10 mV as a representation of the HVA current in subsequent experiments.

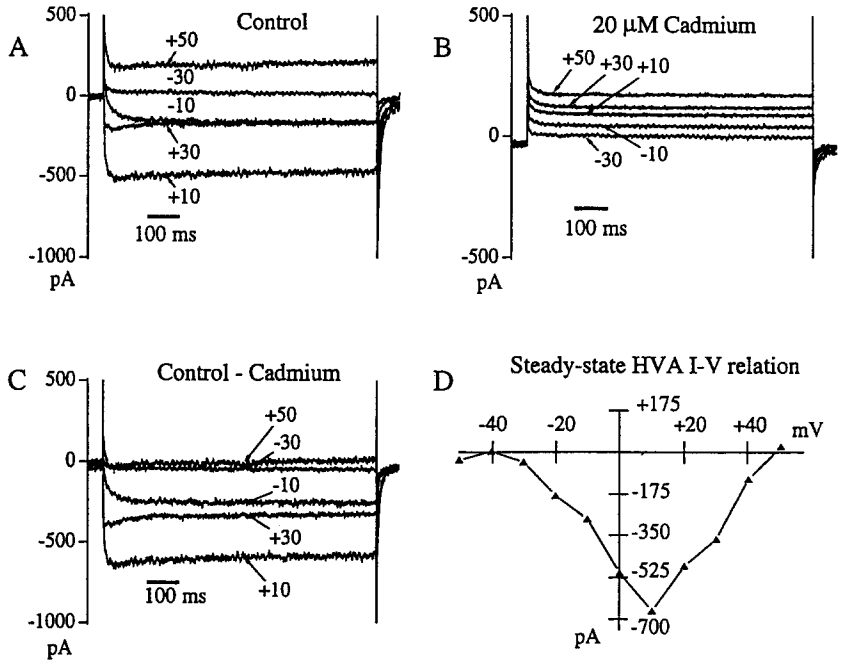

FIGURE 1. Sustained HVA currents in horizontal cells. (*A*) Horizontal cells were whole-cell voltage clamped and held at −50 mV. Pulses of 1-s duration were applied in increments of 10 mV between −40 and +50 mV. Resultant currents are shown. (*B*) The same protocol as in *A* was applied to the cell, but in the presence of 20 μM cadmium. These responses were then subtracted from those in *A* to eliminate leak currents, and displayed in *C*. The *I-V* relation for this cell, measured 500 ms into the pulse, is shown in *D*. Tracings are averages of six pulses each. Not all steps have been shown in *A–C* for clarity. The currents were recorded in 15 mM barium.

Effects of NH_4Cl and Na-Acetate Substitutions on Intracellular pH of Horizontal Cells

NH_4Cl and Na-acetate are commonly used to manipulate pH_i (Roos and Boron, 1981). An advantage of these compounds is that pH_i can be changed while holding extracellular pH (pH_e) constant. Horizontal cells were loaded with the pH indicator dye BCECF-AM and determinations were made of resting pH_i and the pH_i changes induced by NH_4Cl and Na-acetate. These measurements revealed that the mean pH_i

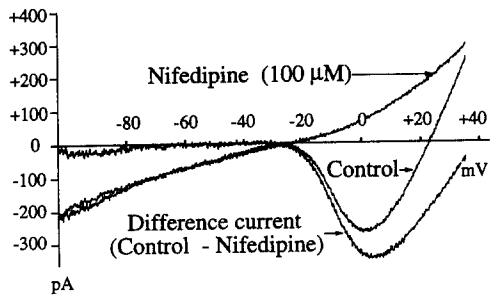

FIGURE 2. Isolation of HVA current with nifedipine. In whole-cell voltage-clamp recording mode, the voltage was ramped from −90 to +50 mV at 70 mV/s. The resultant current is shown during the voltage ramp. The nifedipine curve was performed in 0.1 mM nifedipine. The difference current is the nifedipine-sensitive current defined as an HVA calcium current. The patch pipette contained Cs-gluconate. Each trace is the average of six separate voltage ramps.

of the horizontal cells was 7.40 ± 0.06 (n = 18; pH_e 7.8) and confirmed that both NH_4Cl and Na-acetate changed the pH_i of the isolated horizontal cells.

Exposure to weak acids is known to acidify cells (Roos and Boron, 1981). Undissociated free acids penetrate cell membranes, after which they dissociate to liberate protons. Fig. 3 shows the acidification induced in a horizontal cell by a brief (2 min) exposure to Na-acetate (25 mM). At the peak, Na-acetate dropped the pH by slightly less than 0.6 U. The recovery to resting pH after washout took 2 min in this cell. The recovery varied from 2 to 10 min among different horizontal cells. Similar acidifications of catfish horizontal cells by Na-acetate were reported by DeVries and Schwartz (1989).

A 2-min exposure to 20 mM NH_4Cl typically alkalinized the horizontal cells, but immediately upon washout pH_i dropped below control levels and slowly recovered over a period of 8–15 min. The increase and subsequent decrease in pH_i result from the dissociation of NH_4^+ to NH_3 and H^+ and a higher membrane permeability to NH_3. NH_3 more easily permeates the cells and the increased cytosolic NH_3 serves as a H^+ acceptor which alkalinizes the cell's interior. Upon washout, NH_3 leaves the

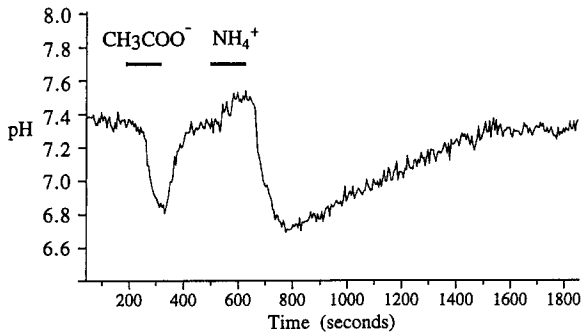

FIGURE 3. Changes in pH_i induced by NH_4Cl and Na-acetate. Horizontal cells plated on coverslips were washed briefly in BCECF-AM (5 μM) and then rinsed for ~1 h in control HEPES-buffered saline (pH_e 7.8). The ratio of fluorescence (>535 nm) induced by 440- and 490-nm excitation was calculated every 5 s. pH calibrations were done at the end of experimental runs by superfusing with nigericin (10 μM), an H^+ ionophore, and high potassium (50 mM). Changes in pH_e produced similar changes in pH_i under these conditions. In this figure, alkalinization is shown as an upward deflection. Na-acetate (25 mM) was applied and then rinsed out. After recovery to near control level, NH_4Cl (20 mM) was bath applied and then washed out.

cells rapidly, which leads to further dissociation of NH_4^+ to NH_3 and a liberation of hydrogen ions (Roos and Boron, 1981). Fig. 3 shows an example of an alkalinization and acidification produced by a 2-min exposure to NH_4Cl in an isolated horizontal cell. The alkalinization, shown here as an upward deflection, was 0.2 U above the resting pH and the rebound acidification was 0.7 U below it.

Effects of NH_4Cl and Na-Acetate on HVA Currents

Fig. 4 shows how the *I-V* curves and HVA current were affected by Na-acetate and NH_4Cl. Fig. 4 *A* illustrates the effects of NH_4Cl (20 mM) on one horizontal cell. The

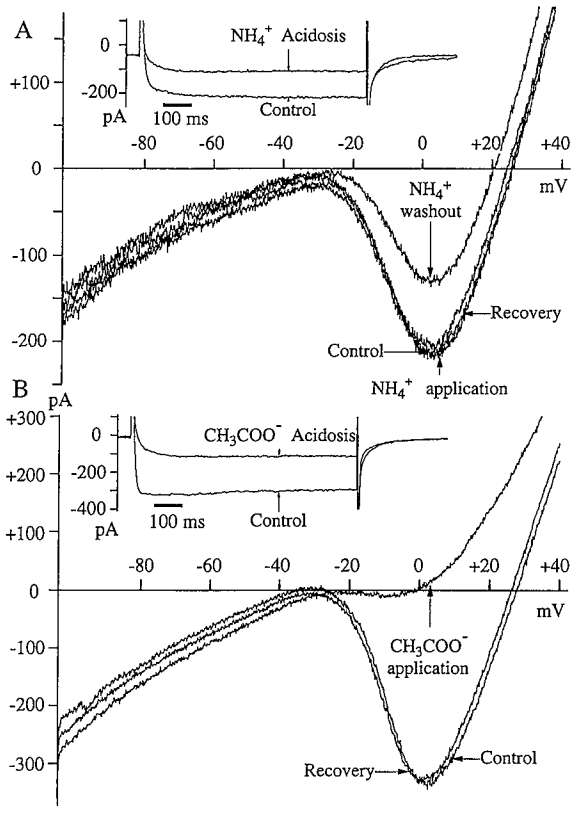

FIGURE 4. Effects of NH_4Cl and Na-acetate on the sustained HVA current. Both Na-acetate and NH_4Cl caused reversible reductions in the HVA current. (*A*) NH_4Cl application resulted in a small augmentation of the HVA current. However, upon washout the HVA current was quickly reduced to ~55% of the control magnitude. After 16 min, the HVA current returned to control levels. (*Inset*) 1-s pulses from −50 to +10 mV are shown for control and NH_4Cl washout conditions. (*B*) Sodium acetate (25 mM) strongly suppressed the HVA current. Recovery to control levels occurred in 8.5 min in this cell. (*Inset*) 1-s pulses from −50 to +10 mV before and during Na-acetate application. Command voltage was ramped from −90 to +50 mV over a 2-s period. Traces are averages of six ramps. Holding potential was −50 mV. Inset traces are averages of six pulses each.

peak current near +10 mV was slightly enhanced during NH_4Cl. Upon acidification induced by NH_4Cl washout, the current near +10 mV was reduced to ~55% of control. After 14.5 min of washout the HVA current recovered to control levels. During NH_4Cl, the peak HVA current, on average, increased by 8.8 ± 6.7% (n = 7) and upon washout it was reduced by 42.7 ± 6.7% (n = 7) below control level. The shape of the *I-V* curve was not discernibly altered in NH_4Cl, consistent with there being no change in the activation voltage of the inward HVA current. The inset in

Fig. 4 *A* shows the current elicited by a 1-s voltage-clamp pulse to +10 mV from a holding potential of −50 mV. The larger amplitude trace with the fastest time to peak shows the HVA current in control. The lower amplitude trace shows the HVA current during NH_4Cl washout. In Fig. 4 *B*, Na-acetate (25 mM) essentially eliminated the inward HVA current in this cell. The tracing marked recovery was obtained 11.5 min after washout of Na-acetate and shows that the suppression of the HVA current was reversible, as it was for NH_4Cl treatment. We found that the peak HVA current, measured near +10 mV, was reduced, on average, 86 ± 8.9% by Na-acetate (n = 5). The inset shows the inward currents generated by a 1-s voltage-clamp pulse to +10 mV from a holding potential of −50 mV in control and in Na-acetate. These results illustrated in Fig. 4, *A* and *B*, suggest that the HVA current is modulated by intracellular pH; however, there is a chance that both Na-acetate and NH_4Cl might be exerting nonspecific effects on the HVA current. This possibility was addressed in the experiments described below.

Effects of pH Buffers on the NH_4Cl and Na-Acetate Modulation of HVA Current

If hydrogen ions were regulating the HVA current, then increased intracellular pH buffering should reduce or eliminate the actions of NH_4Cl and Na-acetate. This result would make less tenable the possibility of a nonspecific action of these compounds. In whole-cell patch pipette experiments shown in Fig. 4, no pH buffer was added to the pipette solution. In Fig. 5, the patch pipette solution included 25 mM HEPES. The *I-V* curves (Fig. 5 *A*) show the currents recorded in control, during NH_4Cl application, and during washout of NH_4Cl. The peak HVA current, near +10 mV, was increased slightly by NH_4Cl. The peak HVA current, near +10 mV, was reduced slightly by the washout of NH_4Cl. Finally, the peak HVA current returned to control levels in 7 min. In all cells combined, the average decrease of HVA current during the acidification evoked by NH_4Cl washout was 10 ± 4.9% (n = 4), much less than was observed with unbuffered pipette solutions shown in Fig. 4. With 25 mM HEPES in the pipette Na-acetate produced a 25.4 ± 8.3% (n = 5) reduction in the peak of the HVA current (Fig. 5 *B*). Again, this reduction was much less than in the unbuffered patch pipette experiments (86 ± 8.9%, n = 5). These results provide strong support for the hypothesis that intracellular pH changes modulate HVA current in these horizontal cells and make it unlikely that NH_4Cl and Na-acetate could be affecting HVA current directly.

To assess how the pH-dependent effects on HVA current might affect the ionic membrane properties of more intact, less dialyzed horizontal cells, we recorded membrane potentials with small diameter microelectrodes. The advantage of these electrodes was that internal dilution of the endogenous cytoplasmic buffers would be minimized, compared with patch pipettes, so we could more accurately determine if the pH changes manifested themselves under conditions that more closely approximated the in vivo state of these cells. Below, we first characterize the HVA-dependent sustained AP, then we examine the pH dependency of the AP, and finally we test whether the pH effects could result from the actions of hydrogen ions on conductances other than the HVA calcium conductance or on cyclic nucleotide–mediated intracellular processes.

Effects of Cobalt and External Calcium on the Sustained Action Potential

Brief, depolarizing currents applied to isolated fish horizontal cells produce sustained APs that can last from several seconds to several minutes depending on the particular cell (Tachibana, 1981; Shingai and Christensen, 1986; Takahashi and Copenhagen, 1992). Fig. 6 *A*, showing the membrane potential recorded from an isolated horizontal cell, illustrates the APs. Here, a series of depolarizing currents (upward arrows) were applied in control and cobalt (5 mM) saline. The initial current pulse, in control saline, evoked an AP that was maintained at a membrane potential of −7 mV for 5 min before a brief hyperpolarizing current (downward arrow) returned the potential

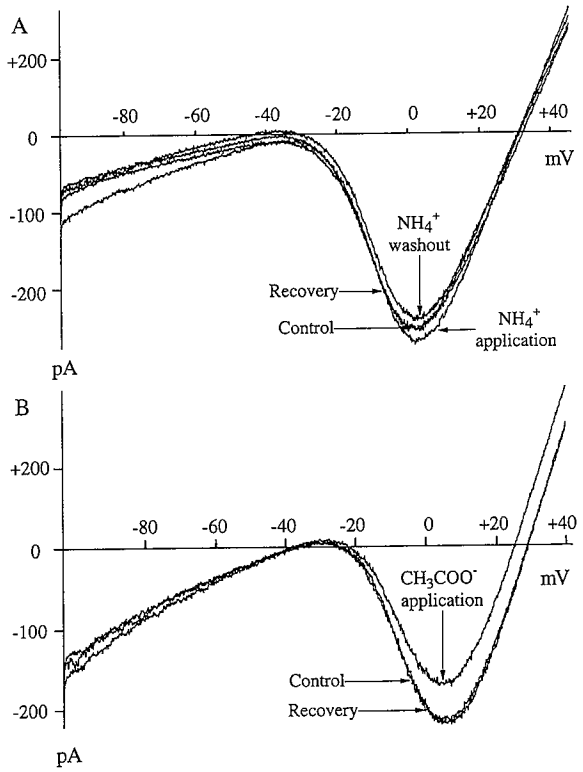

FIGURE 5. Effects of pH buffer on NH_4Cl and Na-acetate regulation of HVA currents. The effects of NH_4Cl and Na-acetate were reduced when the patch pipette solution contained 25 mM HEPES. (*A*) During NH_4Cl application, the HVA current was slightly augmented. Washout of NH_4Cl caused a small decrease in the HVA current, which recovered after 12 min. These changes represent only a small fraction of what is seen when the pipette does not contain HEPES (see Fig. 4). (*B*) Na-acetate produced only a small decrease in the HVA current in the presence of internal HEPES. The HVA current returned to control after 7 min of washout. The command voltage was ramped from −90 to +50 mV over a 2-s period. Traces are averages of six ramps. The holding potential was −50 mV.

to rest (−80 mV). A 3.5-min superfusion of cobalt was applied simultaneously with the next depolarizing current pulse. The evoked AP lasted only 1 min, reflecting the wash-in time of cobalt. Further depolarizing current pulses failed to evoke APs until 8 min after cobalt washout. Similar suppression of APs was seen in all 20 cells treated with cobalt and in all 3 cells superfused with cadmium (30 μM).

The sustained potential of APs varied with extracellular calcium concentration ($[Ca^{2+}]_o$), as shown in Fig. 6 *B*. The initial AP, evoked in 2.5 mM $[Ca^{2+}]_o$, plateaued at +10 mV. A switch to 0.5 mM $[Ca^{2+}]_o$ hyperpolarized the membrane potential by 30 mV and terminated the AP. The plateau membrane potential of a subsequent AP

evoked in 0.5 mM $[Ca^{2+}]_o$ was −15 mV. Raising $[Ca^{2+}]_o$ sequentially to 2.5, 5, and 10 mM progressively depolarized the plateau potential. Similar calcium substitutions were made on a total of eight cells. Increasing $[Ca^{2+}]_o$ 5- 10-, and 20-fold from 0.5 mM depolarized the plateau potential by 19.25 ± 3.50, 26.75 ± 4.32, and 34.75 ± 3.36 mV (mean ± SD), respectively.

The block of the APs by cobalt and cadmium and the dependence of the plateau on $[Ca^{2+}]_o$ confirm earlier conclusions that the APs in horizontal cells are generated by a sustained HVA calcium conductance (Tachibana, 1981; Lasater, 1986; Shingai and Christensen, 1986). It has been proposed by Lasater (1986) and Shingai and Christensen (1986) that at the depolarized plateau of the AP, a sustained inward

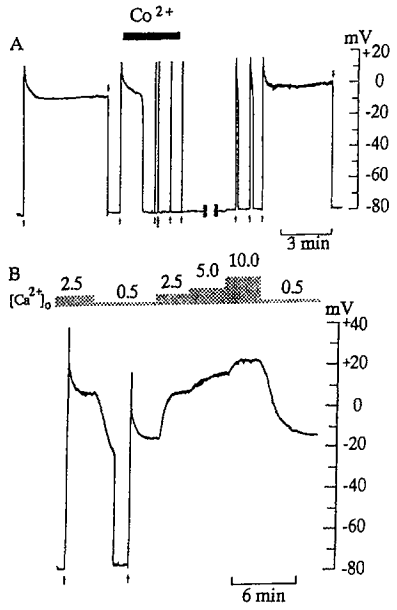

FIGURE 6. Effects of cobalt and calcium on the AP. (*A*) The first AP, recorded in control saline, lasted 5 min before a hyperpolarizing current (*downward arrow*) returned the membrane potential to rest (−80 mV). Bath application of cobalt (5 mM $CoCl_2$) terminated a second evoked AP spontaneously after < 1 min. Subsequent attempts to evoke APs failed until ~8 min after the washout of cobalt. The small upward arrows show 200-ms, 0.15-nA current pulses. The large arrow shows a 1,100-ms, +0.3-nA pulse of current. During cobalt superfusion even the larger current pulse failed to elicit an AP. An AP similar to that in control was evoked ~ 10 min after cobalt washout. Similar results were obtained using 30 μM cadmium (n = 3). The vertical bars in the membrane potential record indicates where 6 min of the record was deleted. (*B*) External calcium $[Ca^{2+}]_o$ was changed from 2.5 to 0.5 mM. The AP was terminated by this change. A subsequent AP was evoked in 0.5 mM and $[Ca^{2+}]_o$ was raised successively to 2.5, 5.0, and 10 mM.

calcium current, the HVA current, is balanced by an outward sustained potassium current. On this idea, it can be predicted when the HVA current is reduced, the outward current will hyperpolarize the membrane, bringing the potential below the activation range for the HVA current. Below this level the potential will return quickly to near the potassium equilibrium potential.

Influences of NH_4Cl and Na-Acetate on Action Potentials

Horizontal cells were briefly superfused with NH_4Cl (20 mM) or Na-acetate (25 mM). Fig. 7 *A* shows the effects of NH_4Cl on the evoked APs. In control saline, the evoked AP terminated spontaneously after ~ 1 min. During superfusion with NH_4Cl, the AP

remained at its plateau potential until after the NH_4Cl was washed out (~2 min). Subsequent attempts to evoke APs with depolarizing currents failed until 12 min after the washout of NH_4Cl. (The record is interrupted for 6 min for clarity.) Typically APs were prolonged during NH_4Cl application and were shortened or eliminated for a period of several minutes after washout. Fig. 7 *B* shows the effects of 25 mM Na-acetate on the APs. In this cell, the initial AP, evoked in control saline, lasted ~35 s. After the application of Na-acetate (2.5 min), the duration of the APs was shortened. The duration returned to pre-Na-acetate levels ~6 min after washout. In general, the APs were shortened beginning 0.5–2 min after Na-acetate was intro-

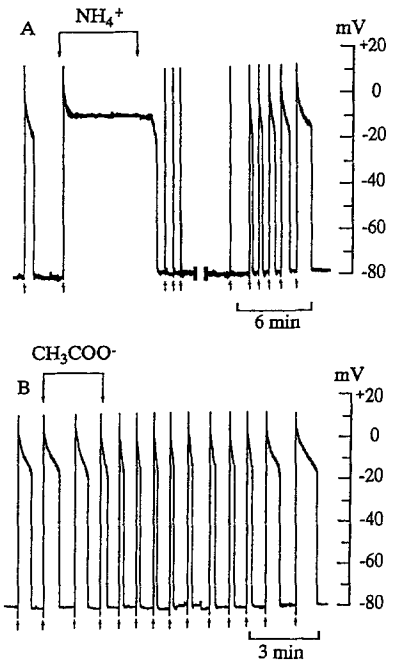

FIGURE 7. Effects of NH_4Cl and Na-acetate on the AP. (*A*) The initial evoked AP lasted ~1 min. Bath application of NH_4Cl (20 mM) prolonged the AP and washout of NH_4Cl eliminated further APs. 9 min after washout of NH_4Cl the AP returned to control. The vertical lines indicate where 6 min of the recording are not shown. (*B*) The first AP, recorded in control saline, remained in its depolarized state for 35 s before spontaneously reverting to the resting membrane potential (−80 mV). Na-acetate (25 mM) was bath applied for 2.5 min. Subsequent evoked APs were shortened to <15 s after the Na-acetate. Note that the initial peak of the AP is artifactually elevated by the current pulse. The sloping portion of the record shows the cell's membrane potential. The initial height of the AP was also reduced by Na-acetate. 6 min after Na-acetate, the AP durations and amplitude returned to control. The saline contained 2.5 mM Ca^{2+} and was adjusted to pH_e 7.8.

duced and they stayed foreshortened for a period of 2–10 min after washout. These results show that Na-acetate superfusion and NH_4Cl washout shorten the AP, consistent with an acid-induced suppression of the HVA current, and that NH_4Cl lengthens the AP, consistent with an alkaline-induced enhancement of the HVA current.

Comparisons of the patch pipette and microelectrode experiments suggest there were differences in the degree to which the AP and HVA current were altered by NH_4Cl and Na-acetate. For example, alkalinization by NH_4Cl seemed to lengthen the AP to a greater degree than the HVA current was enhanced. To investigate

whether these differences might be caused by the effects of intracellular pH on ionic conductances or cellular processes in addition to, or instead of, those on the HVA current, we performed the following experiments.

Effects of BAPTA and IBMX on pH Regulation of Action Potentials

Calcium currents in neurons can be blocked by intracellular Ca^{2+} (Brehm and Eckert, 1978; Tillotson, 1979). Tachibana (1983) has demonstrated that sustained calcium currents in goldfish horizontal cells were inactivated by intracellular calcium. To test the notion that NH_4Cl and Na-acetate might be regulating the AP by affecting intracellular calcium levels through some pH-dependent process, we determined the NH_4Cl sensitivity of APs in cells loaded with the calcium chelator, BAPTA. Fig. 8 *A*

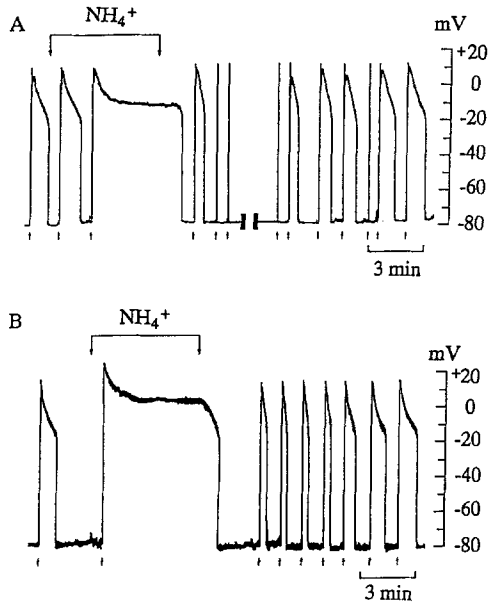

FIGURE 8. Effects of intracellular BAPTA and IBMX on pH modulation of APs. (*A*) 1 h before this recording, the cell was bathed in 70 μM BAPTA-AM for 14 min. The cell was recorded with a microelectrode containing 1 M BAPTA-tetrapotassium and 1 M KCl. The initial evoked AP lasted ~1 min and then spontaneously reverted to the resting potential. NH_4Cl (20 mM) prolonged the AP duration and washout produced a shortening and eventual elimination of the AP. 9 min after washout, the evoked AP returned control. All eight cells that were tested showed the same prolongation and shortening of the AP with NH_4Cl. The interruption in the displayed record lasted 5 min. This cell was recorded with a microelectrode containing 1 M BAPTA-tetrapotassium and 1 M KCl. (*B*) The cell was bathed with 500 μM IBMX for 15 min before the recording. The initial AP lasted ~50 s. NH_4Cl prolonged the AP; washout shortened the AP. The AP returned to control ~10 min after NH_4Cl washout. All seven cells tested in IBMX showed the prolongation and shortening of APs upon application and washout of NH_4Cl.

shows a horizontal cell that had been loaded with BAPTA by preincubating it in BAPTA-AM (70 μM) for a short time (~5 min) 1 h before the recording. In addition, the recording microelectrode was filled with 1 M BAPTA-tetrapotassium and 1 M KCl. Under these conditions an initial AP lasting 1 min was evoked in control saline. NH_4Cl (20 mM) prolonged the next evoked AP and then, after washout, reduced the duration of APs and finally eliminated subsequent ones. 9 min after washout (the record was interrupted for 5 min) the AP returned to control. APs in all eight of the cells that were tested with intracellular BAPTA showed the same prolongation and then shortening or elimination with NH_4Cl. Two of these cells were tested with BAPTA-AM only, four were tested with BAPTA-AM and BAPTA-filled microelec-

trodes, and two were tested with BAPTA-filled microelectrodes only. The above results are consistent with the idea that NH_4Cl regulation of APs is not dependent on intracellular Ca^{2+}, but by themselves they must be interpreted cautiously since there was no independent verification that calcium levels were actually buffered to low levels by BAPTA. However, the conclusion regarding an absence of a pH-dependent, calcium-induced suppression of HVA currents is strengthened by the patch-clamp experiments discussed above. In those experiments barium was substituted for external calcium. Although barium substitutes for calcium as a charge carrier, it does not usually suppress calcium currents (Ohya, Kitamura, and Kuriyama, 1988; Plant, 1988).

Increases of intracellular cAMP enhance the efficacy of kainate-type glutamate channels in fish horizontal cells (Knapp and Dowling, 1987) and cause uncoupling of gap junctions between horizontal cells (Lasater, 1987; DeVries and Schwartz, 1989). The cAMP levels are thought to be regulated in part by dopamine binding to D1 receptors, which stimulates adenylyl cyclase (Lasater, 1987). One possibility to account for the NH_4Cl dependence of the AP is that a pH-sensitive, cAMP-dependent process in these cells could be influencing the phosphorylation of the calcium channels and changing their state of activation (Belles et al., 1988). To test for a cAMP-mediated mechanism we bathed cells in IBMX, a cyclic nucleotide phosphodiesterase inhibitor that should block the hydrolysis of cAMP and cGMP. Fig. 8 *B* shows the effects of NH_4Cl on a cell preincubated in 500 μM IBMX for 15 min before and during the experiment. The prolongation of the AP during NH_4Cl treatment and the shortening and elimination of APs during washout was not eliminated by IBMX. These same findings held for two other horizontal cells bathed in 500 μM IBMX and five other cells bathed in 100 μM IBMX. In other retinal studies, comparable amounts of IBMX blocked phosphodiesterase activity in bipolar cells and photoreceptors (Ames and Barad, 1989; Nawy and Jahr, 1990). In turtle eyecups a concentration of IBMX as low as 20 μM blocked cAMP-mediated uncoupling of horizontal cells (Piccolino, Neyton, and Gerschenfeld, 1984). Therefore, the above results make it unlikely that NH_4Cl modulation of AP duration is mediated by a process that depends on phosphodiesterase activity and hence changes in cAMP or cGMP levels.

NH_4Cl Regulation of APs in TEA and Na-free Saline

Fish horizontal cells have a TEA-sensitive potassium conductance; application of TEA increases the duration of the APs in these cells (Tachibana, 1981). Outward rectifying potassium currents, which are TEA sensitive, have been shown in some preparations to be blocked by acidification (Moody, 1980). Blockage of the TEA-sensitive potassium current by acidification would be expected to prolong APs, so it would seem unlikely that acidification would suppress the APs via this mechanism. Nonetheless, we tested this possibility. Fig. 9 *A* shows that in the presence of 30 mM TEA, NH_4Cl superfusion prolonged the APs and then eliminated them during washout. *I-V* relations obtained in TEA-treated cells showed that an outward rectifier current was suppressed (data not shown) by TEA at this concentration. The pH dependence of the outward rectifying potassium was not studied further in these experiments, but it

was evident that this current did not contribute to the shortening of the AP upon intracellular acidification.

Voltage-sensitive sodium currents are known to be regulated by pH_i (for review see Bass and Moore, 1973). In addition, a sodium-hydrogen exchanger is known to regulate pH_i in many nerve cells (Hoffmann and Simonsen, 1989). Fig. 9 *B* demonstrates that NH_4Cl modulation of the APs is not likely to be due to modulation of sodium conductances or activity of sodium-hydrogen exchangers. This cell, recorded in Na-free saline, generated APs that were first prolonged and then eliminated by the application and subsequent washout of NH_4Cl. The ability to generate an AP in Na-free saline confirms the findings of Tachibana (1981) that sodium currents do not contribute to the AP. Since NH_4Cl modulated the AP in the absence of a sodium gradient, we conclude that the activity of the sodium-hydrogen exchanger is not regulating the duration of the APs.

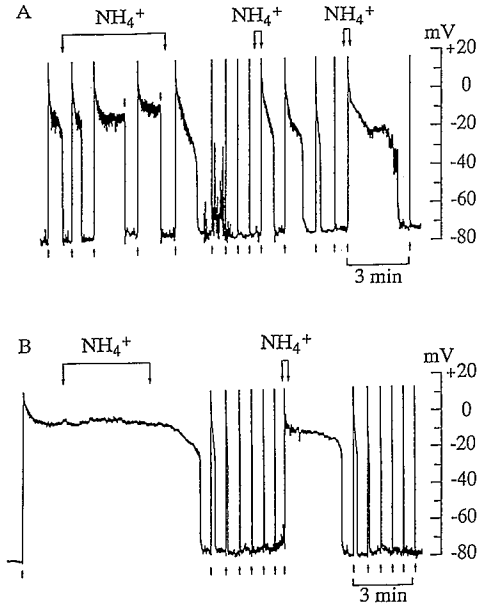

FIGURE 9. Effects of TEA and sodium-free saline on the AP. (*A*) The initial AP, recorded in TEA (30 mM) saline, remained in its depolarized state for ~40 s before reverting to rest potential. During both applications of NH_4Cl (20 mM) the second and third APs were prolonged. They were terminated by hyperpolarizing current pulses (*downward arrows*). Four attempts to evoke APs after 2 min of washout failed. Two attempts to evoke APs after a brief (20 s) reapplication of NH_4Cl resulted in sustained APs. Again after ~3 min of washout, APs could no longer be evoked. (*B*) In Na-free saline the AP remained in its depolarized state (~0 mV) during bath application of NH_4Cl (20 mM) and for a period of ~2.5 min after NH_4Cl washout. During the next 4 min after washout, subsequent evoked APs became shorter and were eventually eliminated. A brief (~30 s) reapplication of NH_4Cl prolonged the AP to 3 min. A gradual shortening and eventual elimination of the APs were observed again as NH_4Cl was washed out. Similar results were seen in all three cells tested in Na-free saline. This record was started 20 min after control saline was switched to Na-free saline. These results rule out the possibility that a pH-dependent effect on a sodium current can account for the changes in AP duration.

DISCUSSION

Sustained APs and high voltage, slowly inactivating calcium currents were shown to be altered by salines formulated to change the pH_i of the horizontal cells. Na-acetate and NH_4Cl superfusion are standard techniques that have been used to modulate

pH_i in a variety of preparations including *Paramecium*, pancreatic acinar cells, and retinal pigment epithelial cells (Umbach, 1982; Paradiso et al., 1987; Lin and Miller, 1991) and are shown to similarly alter pH_i in the isolated horizontal cells studied here (Fig. 3). Therefore, we interpret these results to indicate that alkalinization from pH_i 7.4 prolonged the AP by increasing the HVA current. Acidification from pH_i 7.4 reduced or eliminated the AP by suppressing the HVA current.

The mean resting pH_i in isolated horizontal cells was found to be 7.4. Of physiological interest is the question of whether pH_i is normally close to 7.4 where the pH regulation of APs and HVA currents are seen. There is no data on in vivo pH_i of retinal neurons in fish. Several studies at least suggest that pH_i could be $\sim$7.4. Heisler (1979) reported that skeletal and heart muscle cells from carp had a pH of 7.4. He has also shown that the pH of carp blood at 20°C is 7.85, close to the extracellular pH in the present experiments. On this basis it is likely that intracellular the pH of retinal neurons could be close to 7.4.

Effects of Intracellular Acidification on Membrane Potential of Horizontal Cells Recorded in the Intact Retina

Previously published studies provide evidence that the pH-dependent modulation of HVA current might occur in the intact retina (Negishi, Teranishi, and Kato, 1985; Takahashi and Copenhagen, 1992). Takahashi and Copenhagen (1992) showed that intracellular acidification induced by increases of CO_2 hyperpolarized horizontal cells (see Fig. 2 of Takahashi and Copenhagen, 1992). Negishi et al. (1985) showed that in eyecup preparations of fish retina, ammonia initially depolarized and then hyperpolarized horizontal cells (see Fig. 3). Furthermore, when they gassed the preparation with increased CO_2, the horizontal cells were hyperpolarized. These previous results are consistent with the notion that the resting membrane potential of horizontal cells can be influenced by changes in pH_i. In light of our data demonstrating the effects of pH_i on the HVA current, these membrane polarizations reported above in intact retina could be plausibly explained by pH-dependent modulation of the HVA current.

Regulation of Intracellular pH

Intracellular pH of fish retinal neurons could be modulated by changes in ionic concentrations, metabolism, and temperature. Many neurons maintain their intracellular pH by using sodium-hydrogen or bicarbonate-chloride exchangers (Thomas, 1977). We have preliminary data indicating that these two exchangers regulate pH of horizontal cells (Copenhagen and Takahashi, 1991). Changes in extracellular concentrations of any of the ions carried by the exchangers could lead to an intracellular acidification or alkalinization. For example, salt balance in the blood could vary with salinity of the fishes' environment, which could alter intracellular pH. Hydrogen ions produced by the hydrolysis of ATP during anaerobic metabolism could acidify horizontal cells, and other retinal neurons as well. This acidification would be exaggerated during brief ischemia (Nedergaard et al., 1991). Moreover, Heisler (1979) has shown that the pH of carp and other poikilotherms varies with temperature. As the temperature decreases, there is compensatory acidification of blood. This change could be reflected in the intracellular pH. Although there is scant evidence to

document how pH is regulated in vivo in any vertebrate retina, it is certainly conceivable that the pH of retinal cells could be altered by environmental and metabolic changes. Such changes could alter horizontal cell excitability, coupling, or the release of neurotransmitter.

pH Dependency of HVA Current and APs

The resting pH_i of the horizontal cells in culture was measured to be 7.4. NH_4Cl superfusion raised and lowered the pH_i about this level. It was not possible in these experiments to directly quantify the relation between intracellular pH and the enhancement or reduction of the HVA current. However, assuming that a single titratable site on the calcium channel was being protonated, the dissociation constant is probably not more than ±0.5 pH units from pH 7.4 (assuming pH 7.4 fell somewhere within the range of 20–80% of current reduction).

Given that the pH was reduced on the order of 0.5 pH units by Na-acetate and NH_4Cl washout (Fig. 3) and that the same treatments led to significant reductions in the HVA currents, it would not be unlikely for 50% suppression to fall between pH 7.1 and 7.4. In *Paramecium* the apparent dissociation constant for calcium current suppression by protons was at pH 6.2 (Umbach, 1982). Intracellular pH in *Paramecium* averaged 6.8. In ventricular cells from guinea pig, the calcium current was 50% blocked at pH 6.5 (pH_i 7.2; Irisawa and Sato, 1986). It would appear that this current in horizontal cells is inhibited at a lower proton concentration (higher pH) than the calcium currents in *Paramecium* or ventricular cells. However, in all cases the currents are capable of being enhanced or suppressed with changes of pH about the intrinsic pH_i of the cells.

Effects of pH_i on Retinal Function

Intracellular pH regulation of HVA current might be expected to affect center-surround organization in the retina by altering neurotransmitter release from horizontal cells and by altering their membrane potential. Lasater (1986) and Shingai and Christensen (1986) have postulated that the membrane potential of horizontal cells in the in vitro retina depends on a sustained HVA current. Inward currents generated by voltage-gated calcium channels and glutamate-gated channels are offset by a sustained outward potassium current. Any change in the sustained HVA current would alter the membrane potential of the horizontal cell.

Moreover, the flux of calcium through the sustained calcium channels and concomitant changes in intracellular calcium activity could alter neurotransmitter release from the horizontal cells. It has been reported that the release of GABA from toad horizontal cells was independent of extracellular concentrations of calcium (Schwartz, 1982). Other evidence suggests that there is a calcium-dependent component of GABA release (Ayoub and Lam, 1987) from fish horizontal cells. In fish retina only one of four subclasses of horizontal cell appears to use GABA as a neurotransmitter (Marc, Stell, Bok, and Lam, 1978; Lam, Su, Swain, Marc, Brandon, and Wu, 1979). Thus, it is possible that the other subclasses of horizontal cell might release other neurotransmitter molecules by calcium-dependent exocytosis.

Potential Protective Effects of an Acidification-induced Suppression of Calcium Influx

Calcium entry into cells has been proposed as one of the events leading to cell death during ischemia or metabolic insults to nervous tissue. Ischemic episodes are known

to be accompanied by an intracellular acidosis (Kraig, Ferreira-Filho, and Nicholson, 1983). It is ostensible, given the pH dependence of the HVA currents, that an intracellular acidification caused by ischemic acidosis could protect cells from death by reducing calcium influx.

pH Buffering in Cells: An Explanation of the Differences in Acetate Efficacy

Because acidification-induced changes in APs did not appear to result from pH effects on TEA-sensitive potassium currents, sodium currents, calcium-inactivated calcium currents, or cAMP hydrolysis, we propose that the differences in Na-acetate efficacy is due to differences in intracellular buffering between microelectrode and patch pipette recordings. During microelectrode experiments acetate tended to have weak effects on AP duration compared with NH_4Cl washout (see Fig. 7). Moreover, acetate sometimes did not acidify cells monitored with BCECF. In contrast, during patch-clamp experiments acetate was much more effective than NH_4Cl, often reducing the HVA current to near zero (see Fig. 4).

Cells commonly regulate their intracellular hydrogen ion concentration using a series of weak acid buffers that act in concert to maintain a stable pH_i. One prominent pH buffer in most cells is phosphate (Portner, 1987); however, several buffers combine to set and maintain a resting pH_i. Individual buffers provide a buffering capacity based on concentration and the difference between pH_i and the pK_a for that particular buffer. A cell's total buffering capacity is the sum of the individual buffers and can be calculated from the equation:

$$\beta = 2.3 \frac{[H^+] \times K'}{([H^+] + K')^2} \times [\text{total buffer}]$$

where β is the buffering capacity and K' is a constant representing the relative strength of the acid buffer (see Roos and Boron, 1981).

During microelectrode experiments we contend that the natural cellular buffering capacity of recorded cells remains virtually intact because the small tip diameters restrict fluid exchange. Under these conditions pH_i is 7.4, and the pK_a of the putative major intracellular buffer phosphate is 7.2. Presumably acid loading caused by acetate entry can be buffered readily with only a small change in pH_i. In the patch-clamp experiments, however, the innate cellular buffers are dialyzed and replaced by the major component of the patch solution, cesium gluconate. The pK_a for gluconate is 3.76; hence, at pH 7.4 (the patch solution pH) all the gluconate is unprotonated. As acetate enters the cell and releases its proton, gluconate's buffering capacity is very low; hence, the pH drops more than when the natural cellular buffers and buffering capacity are intact.

We made very rough estimates of the buffering capacity of isolated horizontal cells under the two different recording conditions. Data regarding the intracellular buffering capacity in fish cells are rare; however, Heisler and Neumann (1980) reported that the buffering capacity in dogfish muscle cells ranged between 36 and 52 meq/liter. Under the patch-clamp conditions, if we make the simplifying assumptions that the horizontal cell represents a closed system and that acetate has equilibrated across the membrane, then at pH_i 7.4 the intracellular buffering capacity due to gluconate (130 mM) and acetate (20 mM) is $\sim$0.2 meq/liter. Making similar assumptions during NH_4Cl treatment, the cells buffering capacity is $\sim$45 meq/liter.

This value is similar to the buffering capacity of fish muscle cells and presumably that for intact, undialyzed horizontal cells. Therefore, on this basis acetate would be expected to change pH_i much more with patch pipettes than with microelectrodes. The assumptions we make are clearly oversimplified; however, these values indicate a basis for the discrepancy noted in the efficacy of acetate during microelectrode and patch-clamp experiments.

At present we are unable to simultaneously image intracellular pH while recording electrophysiologically. We do not know whether the ~45% reduction in HVA current during NH_4Cl washout is more than enough or just sufficient to account for the effects seen on the AP duration. The fact that the AP plateau membrane potential in isolated cells can spontaneously return to negative potentials suggests that the interacting currents are delicately balanced, requiring only small changes in either calcium or potassium current to cause a return to baseline. We believe the reductions in HVA current noted during patch-clamp experiments with acetate or NH_4Cl washout are probably much more than necessary to cause the AP plateau to return to baseline.

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