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CYTOPLASMIC ALKALIZATION REDUCES CALCIUM BUFFERING IN MOLLUSCAN CENTRAL NEURONS

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SUMMARY

The effect of raised cytoplasmic pH (pH_i) on intracellular calcium concentration ($[Ca^{2+}]_i$) transients following calcium influx during membrane depolarization was studied in identified neurons in the abdominal ganglion of *Aplysia californica*. The pH_i was monitored with pH-sensitive microelectrodes. Sea water containing 15 mM NH_4Cl at pH 7.7 elevated pH_i about 0.35 pH units from the normal level of 7.17. These cells have an estimated buffering power of about 60 mM/pH unit. Calcium influx was elicited by depolarizing pulses under voltage clamp and $[Ca^{2+}]_i$ transients were monitored with the photoprotein aequorin or the metallochromic dye arsenazo III. Aequorin photo-emissions increased by 21–131% (mean, 48%) and arsenazo III absorbance changes accompanying depolarization increased by 9–33% (mean, 20%) after 30 min in NH_4^+, corresponding roughly to a 14% increase in $[Ca^{2+}]_i$ transients. Calcium-dependent potassium tail currents following a depolarizing pulse were somewhat slower and 4–91% (mean, 38%) larger in NH_4^+. The magnitude and time- and voltage-dependence of the membrane calcium conductance was studied using calcium tail currents following depolarizing pulses. The calcium current was unaffected by NH_4^+, so the enhanced $[Ca^{2+}]_i$ transients must reflect reduced calcium buffering at high pH_i. Either reduced cytoplasmic calcium binding or slowed active extrusion of calcium may be responsible for this effect.

INTRODUCTION

In a recent report[43], Dr. S. Smith and I showed that when *Aplysia* central neurons filled with aequorin are bathed in a solution containing 100 mM or more tetraethylammonium (TEA) ion, light emissions accompanying membrane depolarizations were increased. This indicates that depolarization evokes a larger intracellular

free calcium concentration ($[Ca^{2+}]_i$) transient in TEA than in its absence. In an effort to determine how TEA exerts this effect, I measured intracellular pH (pH_i) with pH-sensitive microelectrodes and found that externally applied commercial preparations of TEA alkalized neural cytoplasm[44]. The present study was therefore undertaken to disclose any effect of cytoplasmic alkalization on $[Ca^{2+}]_i$ transients accompanying membrane depolarization.

Not much is known about the interaction between intracellular pH and calcium. Meech and Thomas[26] and Ahmed and Connor[3] found that injection of calcium or its entry through membrane channels could acidify neural cytoplasm. Rose and Rick[32] found a similar effect in salivary gland cells. Lea and Ashley[24], Rose and Rick[32], Ahmed and Connor[3] and Rink et al.[31] found that acidification of muscle, salivary gland, molluscan neuron and *Xenopus* embryo cytoplasm with external CO_2 or H^+ injection often resulted in an increase in resting $[Ca^{2+}]_i$ levels, while Mullins and Requena[29] found that cytoplasmic alkalization of squid axon by external NH_4^+ reduced $[Ca^{2+}]_i$. In contrast, Baker and Honerjäger[6] reported that alkalization of squid giant axon with external NH_4^+ sometimes increased $[Ca^{2+}]_i$, while acidification had the opposite effect. The only published study on the effect of pH_i on $[Ca^{2+}]_i$ transients is that of Mullins and Requena[29], who found an increase in $[Ca^{2+}]_i$ transients near the periphery, but a decrease in peak $[Ca^{2+}]_i$ transients in the center of squid axons, when cytoplasm was alkalized by external NH_4^+. The influence of pH_i on $[Ca^{2+}]_i$ transients in *Aplysia* central neurons is the subject of this paper.

METHODS

Aplysia californica (200–400 g) were obtained from Pacific Biomarine (Venice, CA) and kept in a 60 gal Instant Ocean tank at 16 °C. All experiments were performed on identified neurons from the abdominal ganglion. Ganglia were usually desheathed without pronase, and small clumps of axotomized cells were pinned to a Sylgard-bottom dish in a temperature-controlled chamber. Intact ganglia were used for aequorin experiments only. Cells were usually bathed in a normal artificial sea water (NASW) consisting of (mM): 495 Na^+, 10 K^+, 10 Ca^{2+}, 50 Mg^{2+}, 620 Cl^- and 10 HEPES buffer pH 7.5. Solutions containing 0.24 mM dissolved NH_3 gas were made by adding 15 mM NH_4Cl to NASW, and adjusting the pH to 7.7. The ammonium concentration of such solutions, as measured with ammonium-specific electrodes, remains stable for months.

Procedures for voltage clamping neurons, and for filling cells with aequorin or arsenazo III and detecting signals from these calcium indicators have been described in detail elsewhere[35]. In order to obtain simultaneous information about absorbance changes at several wavelengths, an air turbine spinning chopper wheel similar in design to that described by Brinley et al.[12] and Gorman and Thomas[21] was constructed. Apparatus designed by Dr. Stephen J. Smith was used to de-multiplex the optical signal into its individual wavelength components. This device integrates each light signal during a pulse of light, corrects for dark current sampled between pulses, and measures the ratio of transmitted light to incident light as described earlier[35]. An

output corresponding to each wavelength and a differential output for any selected wavelength pair are provided.

Intracellular pH measurements were made either with arsenazo III (see below) or with pH-sensitive recessed-tip microelectrodes fabricated as described by Thomas[37]. The electrodes used in these experiments responded in 20 s with a slope of 57–58 mV/pH unit in the range pH 5–8. A signal linearly related to pH was obtained by recording differentially between the pH electrode and the voltage-sensing electrode.

In most experiments voltage, current, and pH or optical signals were recorded on magnetic tape and a chart recorder. In some experiments using arsenazo III, these signals were digitized, collected using a Processor Technology Sol 20 microcomputer, and stored on minifloppy discs and a chart recorder. In these experiments the microcomputer was also used to generate pulse sequences for the command potential of the voltage clamp. Signals stored on magnetic discs were later retrieved and displayed in analogue form on a storage oscilloscope. Finally, in experiments on rapid deactivation or 'tail' currents following voltage-clamp pulses, the currents following an equal number of pulses of equal and opposite magnitude were averaged using a Neurolog NL750 signal averager, with a time resolution of 256 bits for a 10 ms sweep duration. Individual tail currents following single pulses of either polarity were simultaneously displayed on an oscilloscope screen and photographed.

Calibration of arsenazo III

Arsenazo III was used to measure changes in $[Ca^{2+}]_i$ at different pH_is. Arsenazo III signals corresponding to changes in pH_i were also recorded. Arsenazo III is known to respond nearly linearly to changes in calcium ion concentration between 0.1 and 20 μM, and also to be sensitive to pH changes in the presence of excess Mg^{2+} [13,18,21,35,36]. We previously calibrated our sample of arsenazo III for calcium concentration changes under conditions of ionic strength, pH, and magnesium concentration appropriate for normal molluscan cytoplasm[35]. It was now necessary to extend this calibration to include the effect of pH on the arsenazo III response to a given change in calcium concentration, and also to measure the response of arsenazo III to a change in pH in the absence of significant free calcium.

The published spectrum of arsenazo III absorbance changes accompanying changes in $[Ca^{2+}]$ and pH in the presence of excess Mg^{2+} [13,18,21,23] suggests that changes in these ions can best be discriminated by observing the wavelength pair 660 and 630 nm. For changes in $[Ca^{2+}]$, the absorbance change at 660 nm is about double the change at 630 nm, while the reverse is true for changes in pH. I have therefore measured the absorbance change at these wavelengths and at the isosbestic point for $[Ca^{2+}]$ and pH changes (577 nm), in solutions resembling cytoplasm with different buffered calcium and pH levels. Arsenazo III was dissolved at 250 μM (the same as the final concentration after injection into neurons) in a solution of 0.4 M KCl, 50 mM HEPES to buffer pH, and 50 mM DPTA (1,3-diaminopropan-2-ol-tetraacetic acid, Sigma) to buffer $[Ca^{2+}]$ to either nominally 0 or 10 μM, as described previously[35]. These levels are comparable to the resting $[Ca^{2+}]_i$ and the peak submembrane $[Ca^{2+}]_i$ following a membrane depolarization[18,35]. The pH of the mixture was adjusted with

NaOH to either 7.1 or 7.4. These levels are similar to the resting pH_i and the pH_i after alkalization by external NH_4^+ (see Results). Absorbance measurements of these solutions were made with 1 mm pathlength cuvetttes in a Cary 14 spectrophotometer. The results are presented in Table I.

Note that the absorbance change at 660 nm, divided by the isosbestic absorbance, corresponding to a $[Ca^{2+}]$ increase of 10 μM, *decreased* from 0.139 to 0.114 when the pH was raised from 7.1 to 7.4. This result at first seemed paradoxical, because if H^+ competes with Ca^{2+} for binding sites on arsenazo III[14,27] one would expect to see a larger absorbance change, due to a larger apparent affinity for calcium binding at high pH[36]. However, raising pH increases the absorbance at 660 nm in the absence of Ca^{2+}, probably because the hydrogen ion is replaced by magnesium on an arsenazo III binding site in high pH in excess Mg^{2+} (cf. ref. 23). Therefore, the absorbance at 660 nm of the 'uncomplexed' arsenazo III species is closer to that of the Ca-arsenazo III complex at high pH, and consequently the change in absorbance on raising $[Ca^{2+}]$ is 18% less. This result is consistent with other reports that the sensitivity of arsenazo III to changes in $[Ca^{2+}]$ is diminished at alkaline pH[14], especially in excess magnesium[23]. These effects are even more pronounced at 630 nm. Consequently, the absorbance change for an increase in $[Ca^{2+}]$ using the wavelength pair 660–630 nm is very slightly larger (about 6%) in going from pH 7.1 to pH 7.4.

Table I also shows the expected result that for changes in $[Ca^{2+}]$, the absorbance change at 660 nm is 2–3 times that at 630 nm, while for changes in pH, the normalized absorbance change at 660 nm ($\Delta(A_{660}/A_{577}) = 0.070$ for zero calcium) is about half the change at 630 nm ($\Delta(A_{630}/A_{577}) = 0.123$). That the same distinction in the spectral response of arsenazo III holds for calcium and pH changes in vivo is illustrated in Fig. 1.

This figure confirms that small cytoplasmic pH changes induced by exposure to NH_4^+ are not accompanied by measurable changes in $[Ca^{2+}]_i$, although small changes would go undetected. Moreover, the influx of calcium during depolarization is not accompanied by detectable changes in pH_i, since the spectrum of arsenazo III absorbance changes matches that of a calcium concentration change. From the work of Ahmed and Connor[3], pH_i would be expected to change by less than 0.01 units for the calcium influxes occurring in the present experiments.

TABLE I

Absorbance of arsenazo III solutions with different $[Ca^{2+}]$ and pH

Abbreviations: A_n, absorbance at wavelength n; $\Delta(A_n/A_{577})$, normalized absorbance change at n nm; $\Delta(A_{660-630}/A_{577})$, normalized differential absorbance change at the wavelength pair 660 and 630 nm. Arsenazo III concentration: 250 μM. Path length: 1 mm.

pH	$[Ca^{2+}]$ (μM)	A_{577}	A_{630}	A_{660}	$\Delta\frac{A_{630}}{A_{577}}$	$\Delta\frac{A_{660}}{A_{577}}$	$\Delta\frac{A_{660-630}}{A_{577}}$
7.1	0	0.896	0.373	0.174			
7.1	10	0.900	0.435	0.300	0.067	0.139	0.0722
7.4	0	0.907	0.490	0.240			
7.4	10	0.923	0.532	0.350	0.037	0.114	0.0767

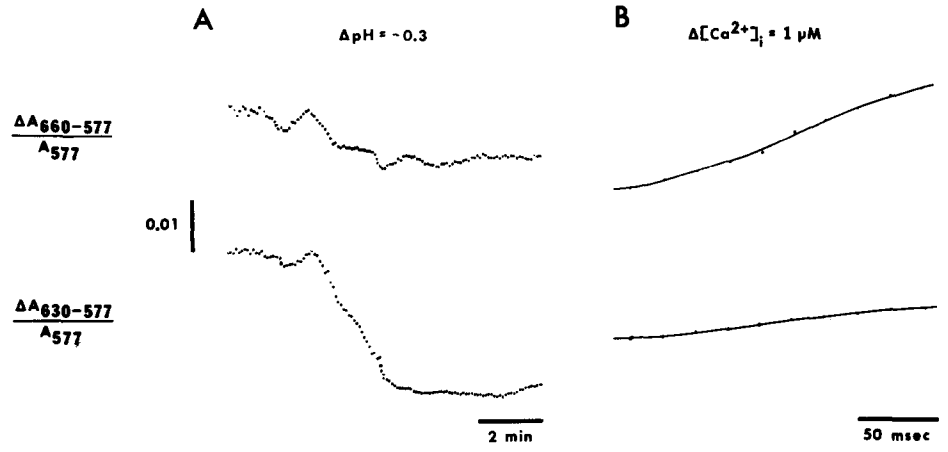

Fig. 1. Absorbance changes in a neuron filled with 0.29 mM arsenazo III, in response to changes in cytoplasmic pH and calcium concentration. A: reduction in pH_i induced by washing out a solution of normal artificial sea water containing 15 mM NH_4Cl and buffered to pH 7.7. The absorbance change at 630 nm is about triple that at 660 nm. B: increase in $[Ca^{2+}]_i$ during a 200 ms depolarizating pulse to +20 mV in normal sea water. The absorbance change at 660 nm is about 2.4 times that at 630 nm. Cell R_{15}. Temperature 16 °C. Holding potential —40 mV in all experiments. The approximate changes in pH and $[Ca^{2+}]_i$ shown in the headings are estimated from the peak normalized differential absorbance changes ($\Delta A_{660-630}/A_{577}$) and the data of Table I, assuming near-zero calcium in A and pH_i of 7.1 in B.

Table I also shows that arsenazo III is sensitive to a change in pH from 7.1 to 7.4 in zero-calcium. Such a change in pH is accompanied in vitro by a decrease in the normalized differential absorbance of the wavelength pair 660–630 nm ($\Delta(A_{660-630}/A_{577})$) of 0.052. These data are inadequate to reveal the relation between ΔpH and ΔA. Thus, the pH-dependent arsenazo III absorbance change can only be referred to this particular pH change, and no extrapolation to other pH levels or changes is warranted. The table does, however, provide calcium calibration factors appropriate for two pH levels, 7.1 and 7.4, and these values are used to convert absorbance changes to calcium concentration changes at these pH levels. Conversion at other pH levels by interpolation or extrapolation would only be approximate.

RESULTS

Effect of NH_4^+ on cytoplasmic pH

Cytoplasmic pH was raised by exposing neurons to the permeant weak base NH_3. In a solution of artificial sea water with 15 mM NH_4Cl buffered at pH 7.7, 0.24 mM of the ammonium will be in the form of NH_3. Ammonia rapidly penetrates cell membranes, and at equilibrium the cytoplasmic NH_3 concentration should also be 0.24 mM. The pH of the cytoplasm will change to a final value of pH_i^f from its initial level pH_i° by an amount ΔpH_i depending on the buffering power, β, of the cytoplasm. β is defined as the concentration of the proton donor NH_4^+ needed to change cytoplasmic pH by one unit[10]. Substituting this definition into the Henderson–Hassel-

balch equation yields the following expression for a change in cytoplasmic pH:

$$\Delta pH_i = pK_a - pH_i^o + \log_{10} \frac{[NH_3]}{\beta \Delta \text{ pH}} \quad (1)$$

where pK_a is the negative logarithm of the dissociation constant of NH_4^+ in sea water and $pK_a = 9.5$[10]. In four experiments, neurons were exposed to 15 mM NH_4Cl at pH 7.7, and pH_i was monitored with an intracellular hydrogen-selective microelectrode or by recording absorbance changes at 630, 660 and 577 nm in cells filled with arsenazo III. pH-sensitive microelectrodes report the absolute value of pH as well as its change, so that Eqn. 1 can be solved explicitly for β. In seven cells, the average pH_i° in normal artificial sea water was 7.17 (range 7.0–7.3). In four of these cells treated with NH_4Cl, pH_i rose slowly for 20–30 min to 0.35 units (range 0.2–0.5 units) above the initial level. On removing the ammonium, pH_i recovered to near or even below its original level. Such an experiment is illustrated in Fig. 2. In four other experiments, the effect of NH_4^+ on arsenazo III absorbance was observed. In all cases, the absorbance at 630 nm increased more then the absorbance at 660 nm, and $\Delta A_{660-630}/A_{577}$ was about —0.050 (range —0.021 to —0.093). These results correspond to the observed in vitro changes in arsenazo III absorbance when pH is increased from 7.1 to 7.4, and so are consistent with such a change in pH_i.

Using the values $pH_i = 7.1$ and $\Delta pH = 0.35$, a buffering power of $\beta = 86$ mM/pH unit may be calculated from Eqn. 1. In other experiments, exposure to 5% CO_2 sea water equilibrated with 29 mM HCO_3^- at pH 7.4 led to a drop in pH_i of about 0.015 unit, measured with a hydrogen-sensitive microelectrode. This corresponds to an apparent buffering power of about 68 mM/pH unit. This is substantially higher than the values calculated for snail cytoplasm[37] and squid axoplasm[10]. This difference in buffering power may be more apparent than real, however. In snail and squid, NH_4Cl and CO_2 treatment altered cytoplasmic pH more rapidly than in *Aplysia*, and the initial change was followed by a slow recovery due to the membrane permeability to NH_4^+ and to the action of active transport of protons or hydrogen buffers. Moreover,

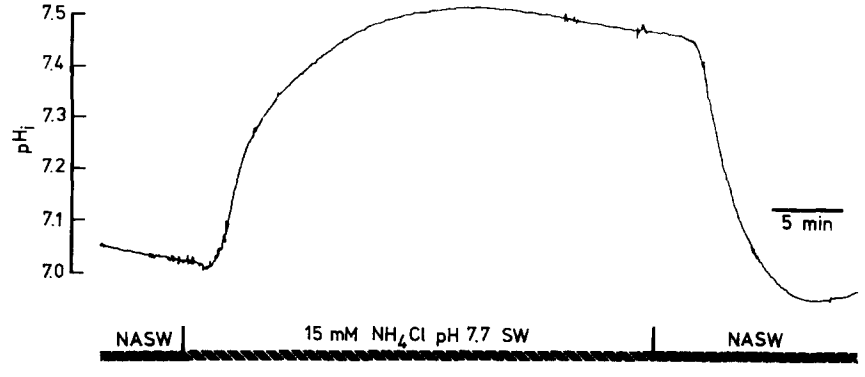

Fig. 2. Effect of sea water containing 15 mM NH_4Cl buffered to pH 7.7 (15 mM NH_4Cl pH 7.7 SW) on cytoplasmic pH. pH_i was monitored with a pH-sensitive microelectrode. After 25 min the cell was washed in normal artificial sea water (NASW). The neuron (cell L_{11}) was voltage clamped at —40 mV. Temperature 15 °C.

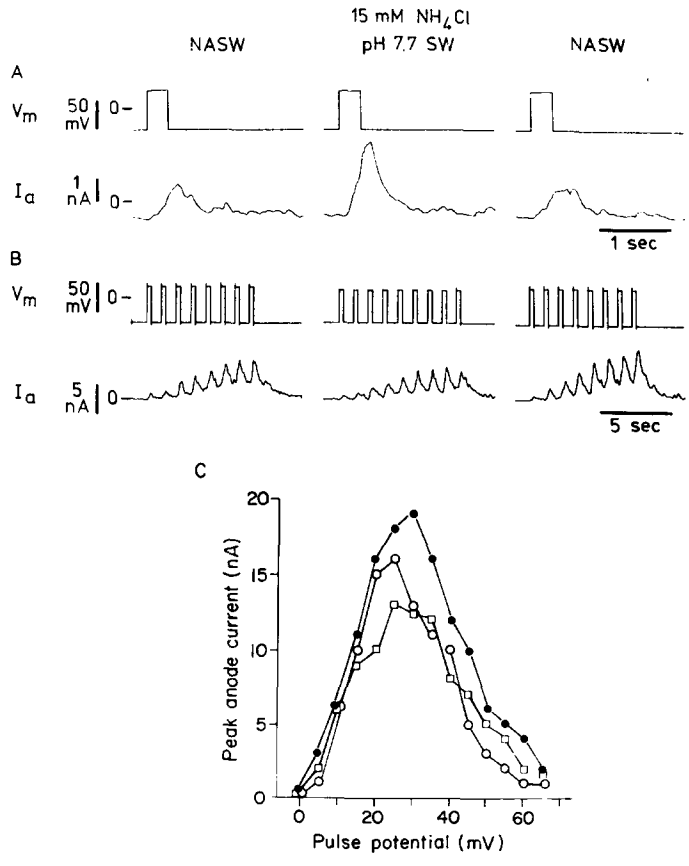

Fig. 3. A: effect of 15 mM NH_4Cl pH 7.7 sea water on aequorin photo-emissions (I_a) in response to a 0.3 s pulse to +30 mV (V_m). Cell R_2. Temperature 15 °C. Records were obtained 20–30 min after changing solutions in the order shown from left to right. B: facilitating aequorin photo-emissions in the same sequence of solutions to 0.3 s pulses of different magnitudes, adjusted to elicit similar initial aequorin responses. From left to right, the pulses depolarize the membrane to the following potentials: +20 mV, +14 mV, +20 mV. Cell R_2. Temperature 15 °C. C: voltage-dependence of effect of raising cytoplasmic pH on aequorin photoresponses to depolarizing pulses. ○, responses in normal sea water; ●, after 30 min in 15 mM NH_4Cl pH 7.7 sea water; □, 30 min after return to normal sea water. Cell L_{11}. Temperature 15 °C. Pulse duration 0.2 s.

in snail and squid removal of NH_4^+ or CO_2 was followed by an overshoot of pH_i to beyond the resting level. Such an overshoot was not consistently observed in *Aplysia*. The slower changes in pH_i observed in *Aplysia* may reflect a lower permeability to NH_3 or a slow equilibration of the bathing medium with the subglial extracellular space, but in any case they do not allow for a quasi-equilibrium to be established before the effects of active pumps and NH_4^+ permeability come into play. Thus Eqn. 1 will be inadequate to describe the ΔpH expected, and the actual cytoplasmic buffering power will be overestimated by its use. Nevertheless, the value of β (86) obtained from Eqn. 1 provides a useful means of characterizing the overall response of the cell to a weak base.

A better estimate of cytoplasmic buffering power might be obtained by assuming that the late acidification often seen in NH_4^+ (Fig. 2) reflects recovery processes. These may be back-extrapolated to estimate the pH_i change that would have occurred in the absence of such effects. This procedure results in an average ΔpH_i estimate of 0.5 units, or a β of about 60 mM/pH unit.

Effect of cytoplasmic pH on aequorin signals

The effect of cytoplasmic alkalization on intracellular calcium transients following calcium influx through voltage-dependent membrane channels was assessed by 3 measures: the size of photo-emissions from neurons injected with aequorin, the size of absorbance changes from neurons filled with arsenazo III, and the size of calcium-dependent potassium currents. In six neurons injected with about 25 μM aequorin, a 30 min exposure to 15 mM NH_4Cl pH 7.7 sea water increased calcium-evoked photo-emissions by 21–131% (mean 48%). This enhancement of aequorin responses was reversed in 5 of these 6 experiments on washing out the ammonium. Recordings from a typical experiment illustrating this effect are reproduced in Fig. 3A.

The effect of NH_4^+ on aequorin emissions is not dependent on the amplitude or duration of the depolarization used to trigger calcium influx (Fig. 3C). Thus changing cytoplasmic pH does not influence the voltage-dependence of calcium influx, but only the magnitude of the resulting calcium transient for any size depolarization. Note that the calcium–aequorin photo-reaction is virtually independent of pH in the range of pH_i variation of these experiments[28,33].

Another effect often observed is that the larger photo-emissions obtained in NH_4^+ facilitate slightly less to trains of pulses. This remained true even when the amplitude of the depolarizing pulse was reduced so that the first response was comparable to what it had been in normal artificial sea water. Fig. 3B illustrates such an experiment. In normal sea water, the sixth 0.3 s pulse to +20 mV at 1 Hz elicited a response that was 400% greater than the first. In 15 mM NH_4Cl pH 7.7 sea water, the first response to a pulse to +20 mV was now twice as large as before cytoplasmic alkalization, but the sixth response was only 350% greater than the first. When the peak pulse potential was reduced to +14 mV to compensate the effect of pH_i on the size of the first aequorin photo-emission, the sixth response was still only 350% greater than the first. This small effect of NH_4^+ on aequorin-response facilitation, like the effect on the magnitude of aequorin photo-emissions, was reversible.

Effect of raising cytoplasmic pH on arsenazo III signals

Aequorin is sensitive in a very non-linear fashion to calcium concentration changes in the micromolar region, responding to the 2.5th power of $[Ca^{2+}]^2$. Aequorin thus reports preferentially the changes in local high calcium concentration near the plasma membrane during a depolarization, and changes in aequorin signals might reflect only different distributions of the same amount of free $[Ca^{2+}]_i$[35]. To confirm the apparent effect of pH_i on free $[Ca^{2+}]_i$ transients accompanying depolarizations, 4 neurons were filled with about 250 μM arsenazo III and exposed to 15 mM NH_4Cl pH 7.7 sea water. When pH_i had presumably risen an average of 0.35 units (see above),

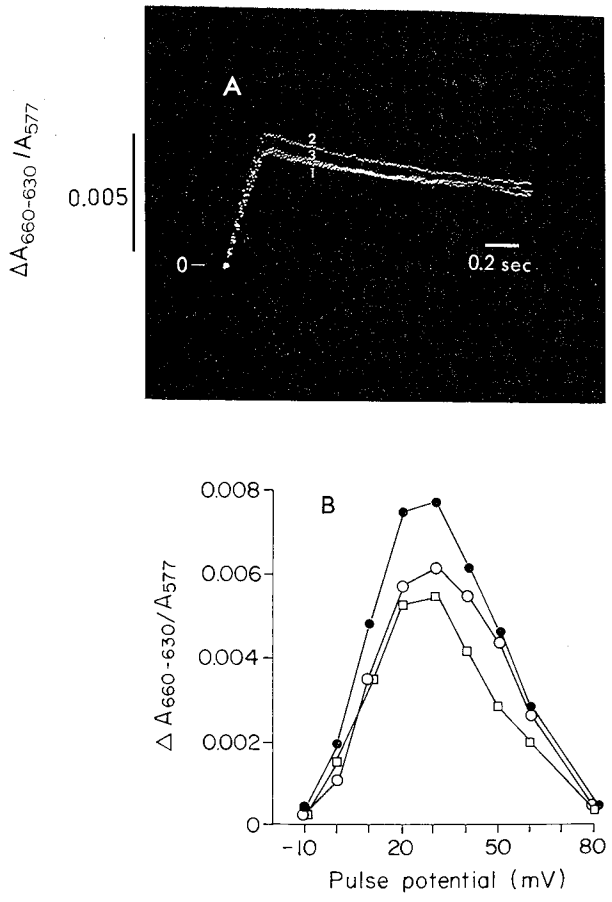

Fig. 4. Effect of cytoplasmic alkalization on arsenazo III absorbance changes in response to 0.2 s pulses to +20 mV. A: normalized differential absorbance changes in normal sea water (1), 30 min after changing to 15 mM NH_4Cl pH 7.7 sea water (2), and 30 min after a normal sea water rinse (3). B: dependence of peak absorbance changes on potential during 0.2 s depolarizating pulses in normal sea water (○), after 30 min in 15 mM NH_4Cl pH 7.7 sea water (●), and 30 min after return to normal sea water (□). In A, cell R_{15} was injected with about 300 μM arsenazo III as estimated from the absorbance at 577 nm, and $\triangle A_{660-630}/A_{577}$ of 0.005 corresponds roughly to an average $[Ca^{2+}]_i$ change of 590 nM in normal sea water, and 560 nM in NH_4Cl sea water. In B, an unidentified 250 μm diameter cell near R_{15} was filled with 250 μM arsenazo III, and $\Delta A_{660-630}/A_{577}$ of 0.005 corresponds to a $\Delta[Ca^{2+}]_i$ of about 690 mM in normal sea water and 650 nM in NH_4Cl sea water. Temperature 16 °C.

arsenazo III absorbance signals reporting a calcium concentration change during a 0.2 s depolarizing pulse were increased on average by 20% (range 9–33%). In all experiments, the absorbance change at 660 nm was 2–3 times that at 630 nm, indicating that calcium influx changed $[Ca^{2+}]_i$ with little change in pH_i. This effect was completely reversible on returning to normal sea water (Fig. 4A).

The effect of elevated pH_i on $[Ca^{2+}]_i$ transients was independent of the amplitude of the depolarizing pulse (Fig. 4B). The change in absorbance signals at the

wavelength pair 660–630 nm is greater than can be accounted for by the 6% change in the sensitivity of arsenazo III to $[Ca^{2+}]_i$ transients due to the measured pH_i change (see Methods). The results therefore suggest a real increase in free $[Ca^{2+}]_i$ transients (about 14% above normal on average) accompanying depolarization when cytoplasm is alkalized by NH_4^+. This conclusion is further supported by the records of 660 nm absorbance changes. These often increased slightly in NH_4^+. Such a change is in the opposite direction of the effect of pH on the calcium sensitivity of arsenazo III at this wavelength (see Table I).

Effect of raising cytoplasmic pH on calcium-dependent potassium current

Molluscan neurons provide a natural indicator of submembrane calcium concentration in the form of calcium-activated potassium current, $I_{K(Ca)}$[25]. Since the above chemical indicator results both suggest an increased $[Ca^{2+}]_i$ transient accompanying depolarization in elevated pH_i, similar effects on $I_{K(Ca)}$ are expected. The $I_{K(Ca)}$ component of potassium current can be most easily measured in isolation as a slow outward tail current several hundred msec following a depolarizing pulse[34,35, 38,39] at a holding potential positive to the reversial potential for $I_{K(Ca)}$. Such tail currents are illustrated in Fig. 5. When measured 200 ms after the end of a pulse, this current increased an average of 38% (range 4–91%, 8 experiments) after 30 min in 15 mM NH_4Cl pH 7.7 sea water. Furthermore, its decay rate was slowed an average of 29% (range 4–65%). The effects were partially or completely reversible after a 30 min normal sea water rinse (mean recovery to within 14% of the amplitude).

Interpretation of these observations of $I_{K(Ca)}$ is subject to the limitation that the reversal potential of $I_{K(Ca)}$ was not measured under the various experimental conditions. Increased potassium current with elevated pH_i should accentuate external

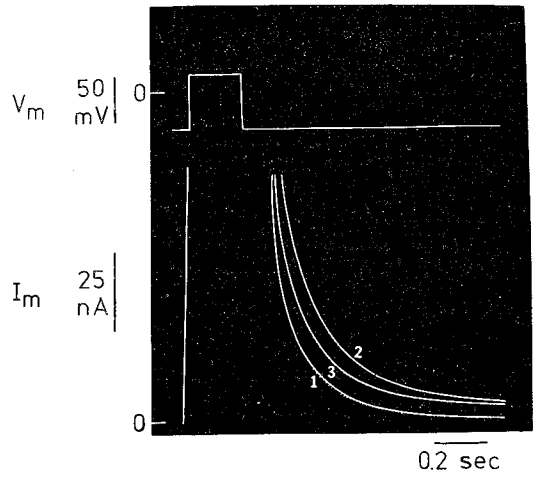

Fig. 5. Effect of raising cytoplasmic pH on calcium-activated potassium current, $I_{K(Ca)}$. I_m records $I_{K(Ca)}$ deactivation tails following a 0.2 s pulse to +20 mV (V_m) in normal sea water (1), after 30 min in 15 mM NH_4Cl pH 7.7 sea water (2), and after a 30 min normal sea water rinse (3). Cell R_{15}. Temperature 16 °C.

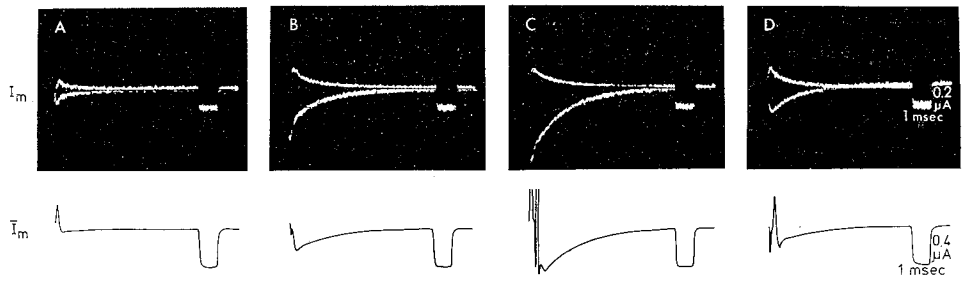

Fig. 6. Calcium deactivation tail currents following depolarizing pulses. In the top row is membrane current (I_m) following single pulses of equal magnitude and opposite polarity from a hold potential of —40 mV. In the bottom row, 8 responses of each polarity are averaged. Pulse potentials and duration are —15 mV and —65 mV, 70 ms in A (± 25 mV pulses), +15 mV and —95 mV, 70 ms in B (± 55 mV pulses), +45 mV and —125 mV, 70 ms in C (± 85 mV pulses), and +15 mV and —95 mV, 300 ms in D (± 55 mV pulses). Cell L_3. Temperature 7 °C.

potassium accumulation and reduce outward potassium tail currents. The enhanced tail currents are thus in the opposite direction from the likely effect of changes in potassium equilibrium potential. The results suggest that elevated pH_i increases the calcium-activated potassium permeability. This appears to reflect the effect of pH_i on $[Ca^{2+}]_i$ transients monitored with aequorin and arsenazo III, although a direct effect on the potassium channels cannot be excluded.

Effect of raising cytoplasmic pH on calcium current

The results so far point to an effect of cytoplasmic alkalization on the magnitude of $[Ca^{2+}]_i$ transients during membrane depolarization. These effects may be due to an influence of pH_i on calcium influx through voltage-dependent calcium channels or an interaction between pH_i and intracellular calcium buffering. To distinguish between these possibilities, membrane calcium currents were recorded under conditions of altered pH_i. Calcium currents were isolated non-pharmacologically by measuring the rapidly deactivating inward calcium tail current following depolarizing pulses[1,17,35]. These brief inward currents, occurring just after the repolarizing step which terminates a depolarizing pulse, reflect the current flowing through calcium channels that were opened by the depolarization. The current vanishes exponentially with a time-constant of 2 ms at 7 °C. (see Fig. 6), as the calcium channels close rapidly, or deactivate, at the hold potential.

The sodium current inactivates completely during a long depolarization[1], so sodium currents will not contribute to the tail current after such pulses. If the potential following a depolarizing pulse is set to —40 mV, there will be a small component of potassium deactivation current. But at 7 °C, this component decays with a time constant of about 35 ms[34] and so contributes little to the early changes in membrane current following a repolarizing step. The linear capacitance current comprises a much larger part of the early membrane current following a potential step. This component may be estimated from an equal and opposite potential step following a hyperpolarization, and may be subtracted from the current after a depolarizing step using an averaging computer. Fig. 6 shows how net inward calcium tail currents after

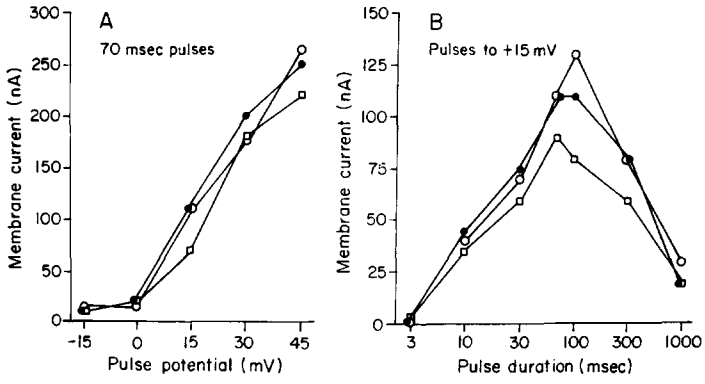

Fig. 7. Amplitudes of calcium tail currents following depolarizing pulses after 30 min in normal sea water (○), 15 mM NH_4Cl pH 7.7 sea water (●), and after returning to normal sea water (□). The current was measured from averages of 8 responses 1 ms following the repolarizing step, after subtracting capacitance current as in Fig. 6. Pulse potential in A refers to the absolute value of membrane potential during the pulse. Cell L_2. Temperature 7 °C.

depolarizing pulses are extracted from the currents which include capacitance current following positive and negative potential steps. Using these procedures, the net calcium tail current, measured 1 ms following the repolarizing step, is plotted as a function of pulse amplitude and duration in Fig. 7.

In four experiments, the effect of 15 mM NH_4Cl pH 7.7 sea water on calcium current tails was recorded. These currents were generally unaffected for any pulse duration or amplitude (Fig. 7). The average change in calcium current 1 ms after a 70 ms pulse to +20 mV was +2% (±8% S.E.). These results indicate that increasing pH_i from 7.1 to 7.4 does not influence the voltage-dependent calcium influx through the membrane, at least not sufficiently to account for the increased depolarization-induced $[Ca^{2+}]_i$ transients. Hence the latter suggest a reduction in intracellular calcium buffering at elevated pH_i.

DISCUSSION

External NH_4Cl has no effect on the time course or amplitude of calcium influx through membrane channels. The NH_3 in equilibrium with NH_4Cl permeates the membrane and its internal protonation raises pH_i, and at the same time there is an increase in $[Ca^{2+}]_i$ transients accompanying an unaltered calcium influx. It is natural to suppose that NH_3 operates exclusively through its demonstrated elevation of cytoplasmic pH, but this remains an untested assumption. Further experiments would be needed to eliminate the possibility of a direct action of NH_4^+.

The unaltered calcium currents may appear surprising, since altered calcium buffering ought to change $[Ca^{2+}]_i$, and hence the driving force for calcium current. Elevated $[Ca^{2+}]_i$ might also be expected to alter calcium inactivation[40]. However, net calcium current is apparently not very sensitive to small changes in $[Ca^{2+}]_i$[4].

Mechanism of change in internal calcium buffering

Elevated pH_i could affect $[Ca^{2+}]_i$ transients by several means. Calcium ions entering a neuron are immediately partitioned into two compartments, one rapidly bound to sites in cytoplasm and a small fraction remaining free[7,35]. Calcium is probably removed from cytoplasm by a surface membrane pump[30]. There is also some ATP-dependent uptake of calcium into organelles, but this is so slow and so insensitive to physiological levels of $[Ca^{2+}]_i$ as to be negligible in response to the small calcium influx accompanying a depolarizing pulse[11]. Thus, changes in uptake into organelles such as mitochondria are not likely to significantly affect brief $[Ca^{2+}]_i$ transients. Moreover, elevating pH_i should stimulate proton pumping by mitochondria, enhance calcium uptake, and so strengthen the mitochondrial contribution to calcium buffering[15,16], not reduce it as observed in these experiments.

The two remaining possibilities are that increasing pH_i could reduce either cytoplasmic binding of calcium or membrane extrusion systems for calcium removal. It is noteworthy that calcium binding to calmodulin isolated from pig brain does have a pH optimum, reported to be about 7.4[42]. It is perhaps relevant that calcium pumping into sarcoplasmic reticulum is pH-dependent, with a pH optimum between 6.0 and 7.0 for calcium levels of 0.1–1.0 μM[19].

From the present results it is not possible to decide whether cytoplasmic alkalization primarily affects calcium binding or calcium extrusion. Reduction of either process would enhance aequorin and arsenazo III signals of $[Ca^{2+}]_i$ transients following an unaltered calcium influx. However, the magnitude of $[Ca^{2+}]_i$ transients is much more sensitive to small changes in internal binding than to small changes in extrusion rate. Reduced calcium binding has the additional merit of speeding inward calcium diffusion from the submembrane space, because calcium diffusion in cytoplasm is retarded by binding[9]. Reduced binding would reduce the level of residual calcium accumulating underneath the membrane following an influx, and so result in reduced aequorin response facilitation, which is caused by submembrane calcium accumulation and the non-linearity of aequorin's calcium sensitivity[35]. On the other hand, a reduced rate of calcium extrusion has the merit of accounting for the slower calcium-dependent potassium tail currents observed at elevated pH_i. However, the decay of calcium-indicator responses should also be retarded by a slow pump, but this was not consistently observed. Neither mechanism alone can explain both the reduced aequorin response facilitation and the slowed calcium tail currents. Perhaps the most parsimonius explanation of the results, which can be advanced only very tentatively at this point, is to suppose that alkalization reduces cytoplasmic binding and also directly slows the kinetics of calcium-dependent potassium channels.

The results reported here may be compared to those of Mullins and Requena[29]. They found that NH_4^+ lowered the resting level of $[Ca^{2+}]_i$, apparently by *increasing* cyanide-insensitive, hence non-mitochondrial, cytoplasmic calcium buffering in squid axon. Nevertheless, when aequorin photo-emissions from the core of the axon were screened by phenol red injection, the photo-emissions from the submembrane region accompanying electrical activity were increased on elevating pH_i. Mullins and Requena interpreted this as a consequence of reduced submembrane mitochondrial

buffering at elevated pH_i, due to the lowered resting $[Ca^{2+}]_i$ level and the consequent de-priming of mitochondrial buffering of $[Ca^{2+}]_i$ transients.

The aequorin signals accompanying brief depolarizations in *Aplysia* neurons decline with a time constant of about 0.5 s, compared to a decay time constant of 1–20 min in squid axon following suprathreshold electrical stimulation at 120 s^{-1} for 1–3 min. Apparently the squid axons are heavily calcium-loaded by this stimulation and the slow decline of the aequorin response probably reflects removal of $[Ca^{2+}]_i$, while in *Aplysia* it reflects diffusion of $[Ca^{2+}]_i$ away from the membrane[35]. Thus the squid experiments were performed under conditions that would maximize the role of mitochondrial buffering, which turns on only sluggishly and at relatively high calcium levels, but then continues to work at lower $[Ca^{2+}]_i$. By lowering resting $[Ca^{2+}]_i$, mitochondrial buffers are switched off, and may fail to activate quickly enough to regulate submembrane $[Ca^{2+}]_i$ transients accompanying electrical activity, so these transients increase. In the present experiments on *Aplysia* neurons, smaller calcium loads and briefer $[Ca^{2+}]_i$ transients should fail to activate even primed mitochondrial buffering to any significant extent, so the proposal of Mullins and Requena[29] to account for increased $[Ca^{2+}]_i$ transients may not be appropriate for the present results. Nevertheless, the similarity in effect in the two preparations, namely a reduced buffering of $[Ca^{2+}]_i$ transients at high pH_i, is remarkable.

Significance of changes in $[Ca^{2+}]_i$ buffering

Changes in cytoplasmic calcium binding or active extrusion can influence $[Ca^{2+}]_i$ transients accompanying neuronal activity. Such $[Ca^{2+}]_i$ transients have been implicated in a variety of cellular processes, in particular neurosecretion[22], photoreceptor transduction[5] and adaptation[13,20], and control of potassium permeability[25]. In the present experiments, alterations in calcium buffering were manifested as changes in calcium-dependent potassium current. Such currents appear to play a critical role in the regulation of neuronal activity, particularly in repetitively firing[41] and endogenously bursting[8] neurons. Thus changes in cytoplasmic pH might regulate a number of essential neural processes by modulating intracellular calcium concentration.

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