

# The Effects of HCl and CaCl<sub>2</sub> Injections on Intracellular Calcium and pH in Voltage-clamped Snail (*Helix aspersa*) Neurons

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**ABSTRACT** To investigate the mechanisms by which low intracellular pH influences calcium signaling, I have injected HCl, and in some experiments CaCl<sub>2</sub>, into snail neurons while recording intracellular pH (pH<sub>i</sub>) and calcium concentration ([Ca<sup>2+</sup>]<sub>i</sub>) with ion-sensitive microelectrodes. Unlike fluorescent indicators, these do not increase buffering. Slow injections of HCl (changing pH<sub>i</sub> by 0.1–0.2 pH units min<sup>-1</sup>) first decreased [Ca<sup>2+</sup>]<sub>i</sub> while pH<sub>i</sub> was still close to normal, but then increased [Ca<sup>2+</sup>]<sub>i</sub> when pH<sub>i</sub> fell below 6.8–7. As pH<sub>i</sub> recovered after such an injection, [Ca<sup>2+</sup>]<sub>i</sub> started to fall but then increased transiently before returning to its preinjection level. Both the acid-induced decrease and the recovery-induced increase in [Ca<sup>2+</sup>]<sub>i</sub> were abolished by cyclopiazonic acid, which empties calcium stores. Caffeine with or without ryanodine lowered [Ca<sup>2+</sup>]<sub>i</sub> and converted the acid-induced fall in [Ca<sup>2+</sup>]<sub>i</sub> to an increase. Injection of ortho-vanadate increased steady-state [Ca<sup>2+</sup>]<sub>i</sub> and its response to acidification, which was again blocked by CPA. The normal initial response to 10 mM caffeine, a transient increase in [Ca<sup>2+</sup>]<sub>i</sub>, did not occur with pH<sub>i</sub> below 7.1. When HCl was injected during a series of short CaCl<sub>2</sub> injections, the [Ca<sup>2+</sup>]<sub>i</sub> transients (recorded as changes in the potential (V<sub>Ca</sub>) of the Ca<sup>2+</sup>-sensitive microelectrode), were reduced by only 20% for a 1 pH unit acidification, as was the rate of recovery after each injection. Calcium transients induced by brief depolarizations, however, were reduced by 60% by a similar acidification. These results suggest that low pH<sub>i</sub> has little effect on the plasma membrane calcium pump (PMCA) but important effects on the calcium stores, including blocking their response to caffeine. Acidosis inhibits spontaneous calcium release via the RYR, and leads to increased store content which is unloaded when pH<sub>i</sub> returns to normal. Spontaneous release is enhanced by the rise in [Ca<sup>2+</sup>]<sub>i</sub> caused by inhibiting the PMCA.

**KEY WORDS:** pH • endoplasmic reticulum • ryanodine • caffeine • Ca<sup>2+</sup> pump

## INTRODUCTION

It is a truth universally acknowledged that all intracellular reactions are sensitive to pH, and that inside nerve cells pH is well buffered and regulated. Yet local pH can change during acidosis, ischemia, or anoxia. It is also possible that intracellular pH (pH<sub>i</sub>) will be lowered by a period of hyperactivity since Ca<sup>2+</sup> ions entering the cell are extruded in exchange for H<sup>+</sup> by the plasma membrane Ca-ATPase (PMCA)\* (Ahmed and Connor, 1980; Schwiening et al., 1993; Trapp et al., 1996). Changes in pH<sub>i</sub> can also result from bicarbonate flux through inhibitory postsynaptic channels (Ballanyi and Kaila, 1998).

Such changes in pH<sub>i</sub> are likely to have important modulatory effects due to the high pH sensitivity of cellular proteins such as enzymes, ion channels, and ion transporters (Busa, 1986; Deitmer and Rose, 1996; Tombaugh, 1998). In particular, these pH changes may have significant effects on calcium regulation in nerve

cells (Benham et al., 1992). Although changes in pH<sub>i</sub> have been associated with changes in intracellular calcium levels ([Ca<sup>2+</sup>]<sub>i</sub>) in a number of cell types, the mechanisms underlying them remain unclear. Increases in [Ca<sup>2+</sup>]<sub>i</sub> occur as a consequence of electrical activity that may allow calcium influx through voltage-gated channels, and that in turn may trigger calcium release from intracellular stores (Neering and McBurney, 1984; Friel and Tsien, 1992; Orkand and Thomas, 1995). These increases in [Ca<sup>2+</sup>]<sub>i</sub> can be modulated by several different processes. These are: the rate of extrusion from the cell via the PMCA, the rate of uptake into intracellular stores via the sarco-endoplasmic reticulum Ca ATPase (SERCA), intracellular calcium buffering, and mitochondrial uptake.

Previous work on the effects of pH<sub>i</sub> changes on resting [Ca<sup>2+</sup>]<sub>i</sub> in nerve cells has shown that relatively large experimentally imposed shifts in pH<sub>i</sub> (0.3–0.9 pH unit) can modulate neuronal calcium regulation (Dickens et al., 1989; Ouyang et al., 1994; Willoughby et al., 2001). In these studies, pH<sub>i</sub> was changed by superfusing weak acids and bases, which may well change pH inside organelles as well as in the cytoplasm. I have, therefore, now investigated the effects of the strong acid HCl, the iontophoretic injection of which should decrease pH<sub>i</sub> without directly changing pH in other compartments. I

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\*Abbreviations used in this paper: CPA, cyclopiazonic acid; pH<sub>i</sub>, intracellular pH; PMCA, plasma membrane calcium ATPase; SERCA, sarco-plasmic/endoplasmic reticulum calcium ATPase.

have explored the effects of short periods of low  $pH_i$  on resting  $[Ca^{2+}]_i$  and on calcium transients generated by caffeine by direct injection of  $CaCl_2$  or by depolarization. In contrast to previous work I have simultaneously measured both  $pH_i$  and  $[Ca^{2+}]_i$  with ion-sensitive microelectrodes that do not increase intracellular buffering.

I have found that small acidifications (0.1–0.2 pH units) caused a decrease in resting  $[Ca^{2+}]_i$ , which appeared to be due to a reduction in spontaneous release from the stores via ryanodine receptor channels. Larger intracellular acidosis caused an increase in  $[Ca^{2+}]_i$  and had little effect on depolarization-induced calcium transients, but did block release from the stores by caffeine. During  $pH_i$  recovery from large acid injections there was an additional increase in  $[Ca^{2+}]_i$  that appeared to be due to release from overloaded stores. Some preliminary data on the effect of low  $pH_i$  on responses to caffeine have been published as an abstract (Thomas, 2002).

## MATERIALS AND METHODS

### General

All the experiments were done at room temperature (18–24°C) on snail neurons 0.2–0.25 mm in diameter. I voltage clamped a suitable cell at a holding potential of –50 or –60 mV using two microelectrodes. I measured  $[Ca^{2+}]_i$  and  $pH_i$  using ion-sensitive microelectrodes and induced changes in  $pH_i$  by injecting HCl. As far as possible the tips of the microelectrodes were placed equidistantly from each other in the top part of the cell, with the calcium-sensitive microelectrode inserted first at the top center of the cell. This electrode often needed manipulation up and down to obtain a stable potential. Other microelectrodes were inserted at the corners of a square or pentagon centered on the calcium-sensitive one, with tips 50–75  $\mu$ m apart.

### Solutions

The subesophageal ganglia preparation was continuously superfused with one of up to seven different solutions held in bottles mounted on top of a Faraday cage. Flow was controlled by solenoid-operated valves near each bottle. From each valve a silica tube led to a manifold built into the experimental bath. The gravity-driven flow rate was 1.0–1.2 ml/min, and bath volume  $\sim$ 0.1 ml. The normal snail Ringer contained (mM): 80 NaCl, 4 KCl, 7  $CaCl_2$ , 5  $MgCl_2$ , 20 HEPES, titrated with NaOH to pH 7.5. Low pH (6.5) Ringer was the same but buffered with 20 mM PIPES. Low calcium Ringer was made as normal except that  $CaCl_2$  was omitted,  $MgCl_2$  was increased to 12 mM, and 1 mM EGTA was added. Caffeine was added to normal Ringer to a concentration of 10, 20, or 50 mM. Bicarbonate Ringer was made by adding 5 mM  $NaHCO_3$  to normal Ringer just before use, the bottle being covered to reduce loss of  $CO_2$ . Cyclopiazonic acid (CPA) was kept frozen as a 50 mM stock solution in dimethyl sulphoxide and diluted in snail Ringer on the day of use. Ryanodine was dissolved directly in 20 mM caffeine Ringer immediately before use. All these chemicals were from Sigma-Aldrich.

### Calcium-sensitive Microelectrodes

These were made by a method refined from those described previously (Thomas, 1994, 2001). Every few days I pulled 8–10 long,

sharp micropipettes from unfilamented aluminosilicate glass (1.2 mm diameter) and broke the tips on a glass rod to outer diameters of 1.5–2  $\mu$ m. I left them in air for  $\sim$ 30 min, then stacked them horizontally on a metal tray that I put into a borosilicate glass tube (12 mm internal diameter). One end of the tube was connected via a tap to a vacuum pump, the other sealed with a rubber cap. The tube was then evacuated for 30 s, the tap closed, and 3  $\mu$ l bis(dimethylamino)dimethylsilane injected through the cap. A heating current, set to generate a temperature of  $\sim$ 450°C around the pipette tips, was then passed through a wire coil round the middle of the tube for 10 min. The tube was then briefly reevacuated, sealed, and left to cool.

Functional microelectrodes were prepared as follows on the day of the experiment. First I backfilled three or four silanized micropipettes with a solution of 10 mM KCl and 0.01 mM  $CaCl_2$ , using a 10 ml syringe and rubber tube to force air out of the tips. I then sucked a short column (150–250  $\mu$ m) of sensor cocktail into the tip of each micropipette. The cocktail was a modification of that described by Ammann et al. (1987) with the composition (mg/ml): 12 Ca ionophore ETH 129, 6 sodium tetrakis[3,5-bis(trifluoromethyl)phenyl]borate, 200 2-Nitrophenyloctylether, 34 high molecular weight PVC, and 748 Tetrahydrofuran (all from Fluka). Filled microelectrodes were left to stabilize for at least 1 h before use, and had useful lives of only 12 h or so. During the experiment I applied  $\sim$ 100 kPa of air pressure to the back of the  $Ca^{2+}$ -sensitive microelectrode, since I have found that this maximizes its response. Before use each microelectrode was tested: the bath was superfused with Ca-free Ringer's solution (1 mM EGTA,  $pCa > 8$ ), and any electrode showing a potential change of less than 150 mV was discarded. The potentials recorded from the electrodes during the experiments were displayed as  $V_{Ca}$ , and I have not converted them either to  $pCa$  or nM free  $[Ca^{2+}]_i$ . Calibration of  $Ca^{2+}$ -sensitive microelectrodes is difficult because, in my hands, their responses change when they are withdrawn from a cell at the end of an experiment. Previous work suggests that their responses are essentially linear down to  $pCa 8$  (10 nM), with a potential change of 28 mV per decade. For a detailed account see Kennedy and Thomas (1996). Experimental data were discarded if the  $V_{Ca}$  outside the cell had changed by  $>7$  mV at the end of the experiment.

### pH-sensitive Microelectrodes

The same method was used as above except that the micropipettes were made from borosilicate glass and the tips broken to  $\sim$ 1  $\mu$ m diameter before silanization. The backfill solution was pH 6.5 Ringer, and the pH sensor Hydrogen ionophore II, cocktail A (Fluka), sucked up to give a 200–500  $\mu$ m column. They were calibrated by superfusing the preparation before and after the experiment with pH 6.5 Ringer. If the potential change in response to this solution was  $<58$  mV, or if the potential in normal Ringer changed during the experiment by  $>5$  mV, the result was discarded.

### Other Microelectrodes

Microelectrodes for recording membrane potential (filled with 2 M KCl), and for passing clamp current (filled with 1 M KCl or CsCl) were pulled from filamented borosilicate glass (1.5 mm diameter), and broken on a pin in the bath to a resistance of 7–15 M $\Omega$ . For injecting HCl, such micropipettes were unbroken, filled with 1 M HCl, and had resistances of 40–60 M $\Omega$ . For injecting  $CaCl_2$ , micropipettes were filled with 0.1 M  $CaCl_2$  and broken to a resistance of  $\sim$ 10 M $\Omega$ ; for injecting ortho-vanadate, they were filled with 100 mM Na o-vanadate (BDH), titrated to pH 9.5, and similarly broken. If the potential of the membrane potential microelectrode in normal Ringer changed by more than 5 mV during the experiment the result was discarded.

To check that the effects of HCl injection were not related to the  $\text{Cl}^-$  ions injected, and that the  $\text{Cs}^+$  ions in the clamp electrode had no effect on calcium transients, I tested the effects of large  $\text{CsCl}$  injections on  $[\text{Ca}^{2+}]_i$  and on the  $\text{Ca}^{2+}$  transients induced by caffeine application (unpublished data). Injections that, based on earlier experiments with  $\text{NaCl}$  injections (Thomas, 1972), I estimate would have increased intracellular  $\text{Cs}^+$  and  $\text{Cl}^-$  concentrations inside the cell by 30–50 mM, had no obvious effect on  $[\text{Ca}^{2+}]_i$  or the response to caffeine. The clamp current required to depolarize the membrane was, however, reduced by two-thirds, since  $\text{Cs}^+$  ions block  $\text{K}^+$  channels.

### Snail Brain Preparation

Common garden snails, *Helix aspersa*, were collected from the wild by local gardeners and kept in the laboratory where they estivated spontaneously. I isolated the subesophageal ganglia as described by Kennedy and Thomas (1995), mounted them on a plastic bath insert, and superfused them with normal snail Ringer. I then tore the inner connective sheath to expose the neurons and inserted pins to ensure that some of the large neurons at the back of the visceral and right pallial ganglia were accessible and well supported. Up to six microelectrodes were arranged over the preparation, five mounted on simple manual micromanipulators (Prior), but the  $\text{Ca}^{2+}$ -sensitive microelectrode held on a Luigs and Neumann (Ratingen) motorized manipulator that could be controlled from outside the Faraday cage.

### Data Collection and Analysis

Potentials from the microelectrodes were led to preamplifiers in the cage. Outputs from these and the clamp amplifier were filtered (8-pole Bessel) and recorded at 5 Hz on a PC via a CED interface and data collection program (Cambridge Electronic Design). For more details of most of the apparatus see Kennedy and Thomas (1995). Rates of recovery were calculated by fitting a single exponential to the data. Means are reported  $\pm$  SEM. Significance was tested using the *t* test for two samples assuming unequal variances.

## RESULTS

### The Effect of HCl Injection on Resting Intracellular Calcium

In preliminary experiments I found that injecting sufficient HCl to lower  $\text{pH}_i$  by more than one unit for more than a few minutes led to irreversible changes in the electrical properties of the neuron. Thus I sought to minimize the time at such low  $\text{pH}_i$  values. Fig. 1 A shows the effects of one period of acidification by HCl injection and of the subsequent bicarbonate-assisted  $\text{pH}_i$  recovery on the resting  $[\text{Ca}^{2+}]_i$  level in a neuron clamped at a potential of  $-60$  mV. During the initial part of the experiment, as the  $\text{Ca}^{2+}$ -sensitive microelectrode sealed into the cell and  $V_{\text{Ca}}$  decreased to a value below (i.e., more negative than)  $-120$  mV, four brief depolarizations were imposed to allow calcium entry from outside the cell. ( $V_{\text{Ca}}$  values of  $-100$ ,  $-120$ , and  $-140$  mV correspond to  $[\text{Ca}^{2+}]_i$  levels of  $\sim 1.7$   $\mu\text{M}$ , 300 nM, and 60 nM, respectively, assuming that the properties of my  $\text{Ca}^{2+}$ -sensitive microelectrodes have changed little from those used by Kennedy and Thomas (1996). The HCl injection electrode was then inserted and a 20

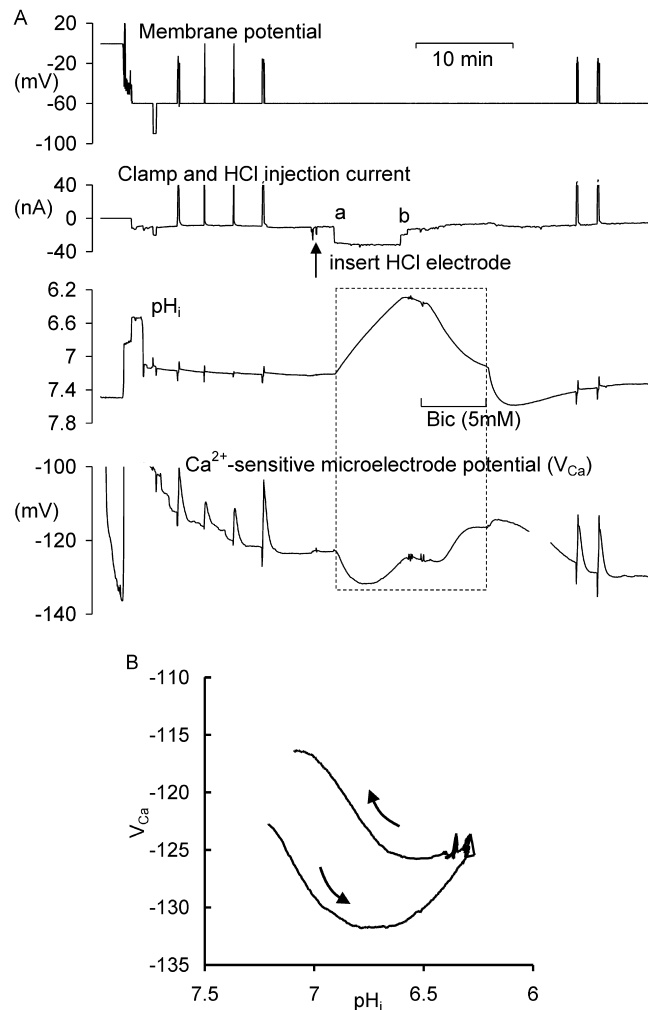


FIGURE 1. The effect of HCl injection on  $\text{pH}_i$  and steady-state  $[\text{Ca}^{2+}]_i$  in a voltage-clamped snail neuron. (A) Record of membrane potential, clamp and HCl injection current,  $\text{pH}_i$  and  $V_{\text{Ca}}$ , during a short experiment.  $V_{\text{Ca}}$  values of  $-100$ ,  $-120$ , and  $-140$  mV correspond, respectively, to  $[\text{Ca}^{2+}]_i$  values of 1.7  $\mu\text{M}$ , 300 nM, and 60 nM. The  $\text{Ca}^{2+}$ -sensitive microelectrode was inserted first, then the membrane potential and clamp electrodes (both filled with KCl, the latter pushed against the cell membrane before being zapped in by switching on the clamp) and then the  $\text{pH}$ -sensitive microelectrode. The membrane potential was clamped at  $-60$  mV except for one brief hyperpolarization and six brief depolarizations. After the first four depolarizations the HCl electrode was inserted (arrow) and used to lower  $\text{pH}_i$  to  $\sim 6.3$ . The injection current, shown as a downward deflection between a and b on the current trace, was then switched off and  $\text{pH}_i$  allowed to start recovering. 3 min after the injection current was switched off a solution with 5 mM bicarbonate was superfused (shown as bar under  $\text{pH}_i$  record) for 7 min to accelerate  $\text{pH}_i$  recovery. (B) Graph of  $V_{\text{Ca}}$  versus  $\text{pH}_i$  for the period (shown as within dashed box on Fig. 1 A) from the start of the HCl injection to the end of the bicarbonate superfusion. Arrows show direction in which time passed.

nA current passed through it for 410 s. The resulting injection of  $\text{H}^+$  and  $\text{Cl}^-$  ions lowered  $\text{pH}_i$  from 7.2 to 6.3. Within a few seconds of the start of the injection  $[\text{Ca}^{2+}]_i$  began to decrease, reaching a minimum as  $\text{pH}_i$

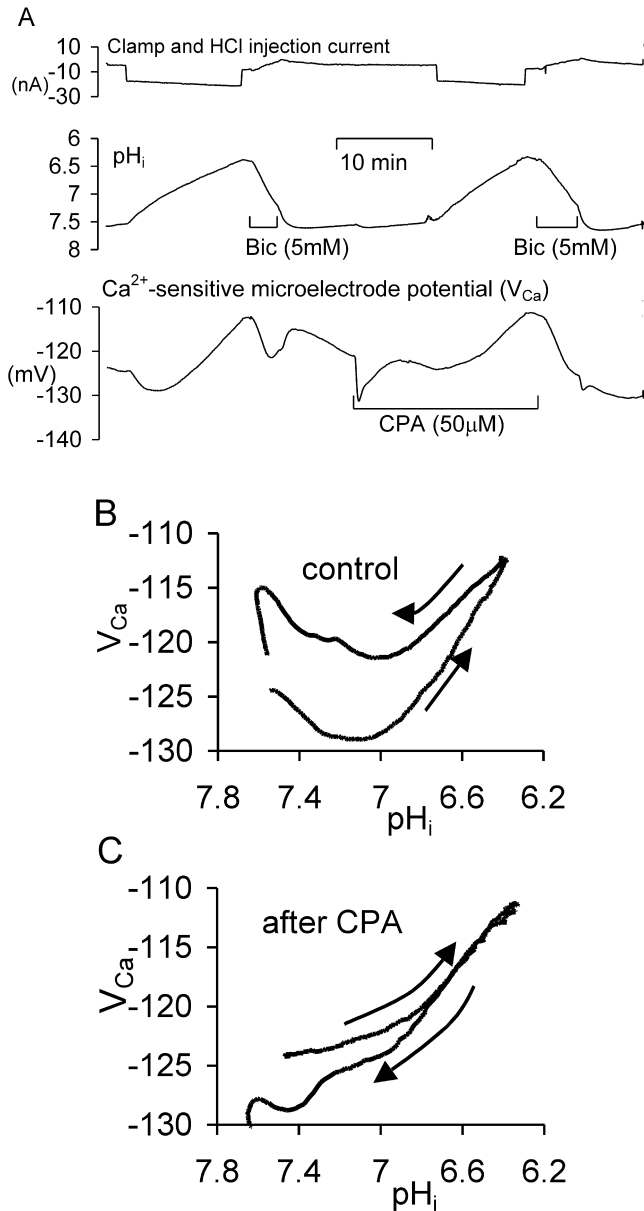


FIGURE 2. The effect of cyclopiazonic acid on the  $V_{Ca}$  response to acid injection. (A) Experiment showing two periods of acid injection (downward deflections of the current trace) before and after the start of application of 50  $\mu$ M CPA. The membrane potential was held at  $-60$  mV throughout the period displayed. (B) Plots of  $V_{Ca}$  versus  $pH_i$  for the first period during which acid was injected and then  $pH_i$  allowed to recover to the point where CPA was added. Arrows show direction of time. (C) Graph of  $V_{Ca}$  versus  $pH_i$  for the second period, after the start of CPA application, during which acid was injected and  $pH_i$  recovered. Passage of time again shown by arrows.

reached 6.75 ( $V_{Ca}$  fell from  $-123$  to  $-132$  mV). As the injection continued,  $[Ca^{2+}]_i$  began to increase, reaching almost the preinjection level by the time the injection current was switched off as  $pH_i$  reached 6.3. As  $pH_i$  then was allowed to recover from the injection, assisted by the added bicarbonate,  $[Ca^{2+}]_i$  at first changed lit-

tle, then increased, reaching a peak during an additional  $pH_i$  increase just after bicarbonate was removed. (This additional  $pH_i$  increase was caused by intracellular bicarbonate ions taking up intracellular  $H^+$  ions and leaving the cell as  $CO_2$ .) Fig. 1 B shows the relationship between  $V_{Ca}$  and  $pH_i$  during the part of the experiment outlined by the box in Fig. 1 A. In a total of 13 similar tests of the effect of HCl injection on the resting calcium level there was, during the acidification, always an initial decrease in  $[Ca^{2+}]_i$ , with an average change in  $V_{Ca}$  of  $4.7 \pm 0.8$  mV, followed by an increase as  $pH_i$  continued to fall during each injection. During the  $pH_i$  recovery there was always a transient increase in  $[Ca^{2+}]_i$ , although often on a changing baseline that made it hard to quantify. A second example of these effects of HCl injections and the subsequent  $pH_i$  recovery is shown in the first part of Fig. 2 A. In this experiment  $V_{Ca}$  first decreased by 4 mV then increased to a value 11 mV above the preinjection level as  $pH_i$  reached 6.4. In the 13 similar tests of the effect of large HCl injections, the average increase in  $V_{Ca}$  by the end of each injection was 9 mV, with  $V_{Ca}$  changes ranging from a 6 mV decrease to a 25 mV increase. The average minimum  $pH_i$  reached in these experiments was 6.5.

Willoughby et al. (2001) have shown that a  $pH_i$  increase (induced by a weak base) caused a rise in  $[Ca^{2+}]_i$  in snail neurons via release from the stores. I therefore investigated the effect on the  $pH_i$  recovery-induced transient  $[Ca^{2+}]_i$  increase of blocking the store-loading pump with cyclopiazonic acid (CPA). Fig. 2 A shows an experiment much like that of Fig. 1 A, except that after  $pH_i$  had recovered from the first HCl injection I superfused the preparation with Ringer to which CPA had been added shortly before. Many previous studies (e.g., Seidler et al., 1989; Orkand and Thomas, 1995) have shown that CPA blocks the SERCA pump. This leads to a slow emptying of the stores, since they seem to be inherently leaky. (In the experiment shown in Fig. 2 A, CPA application caused an unexpected, immediate, but transient fall in  $[Ca^{2+}]_i$ . In a total of seven similar experiments in which CPA was applied after a period of acid injection the transient fall in  $V_{Ca}$  ranged from 1 to 10 mV.)

After the transient fall in  $[Ca^{2+}]_i$ , CPA caused the expected slow rise in calcium as the stores emptied, followed by recovery as the PMCA restored the normal resting level. At this point the HCl injection was repeated, lowering  $pH_i$  from 7.4 to 6.35 over 12 min. This time there was no fall in  $[Ca^{2+}]_i$  before the rise. In a total of seven similar experiments in which HCl was injected before and after CPA, the average fall in  $V_{Ca}$  before CPA was  $7.7 \pm 2.4$  mV. After CPA, HCl injection failed to decrease  $[Ca^{2+}]_i$  in any of the experiments. Fig. 2 B shows the control relationship between  $V_{Ca}$  and  $pH_i$  from the first acid injection and recovery, whereas

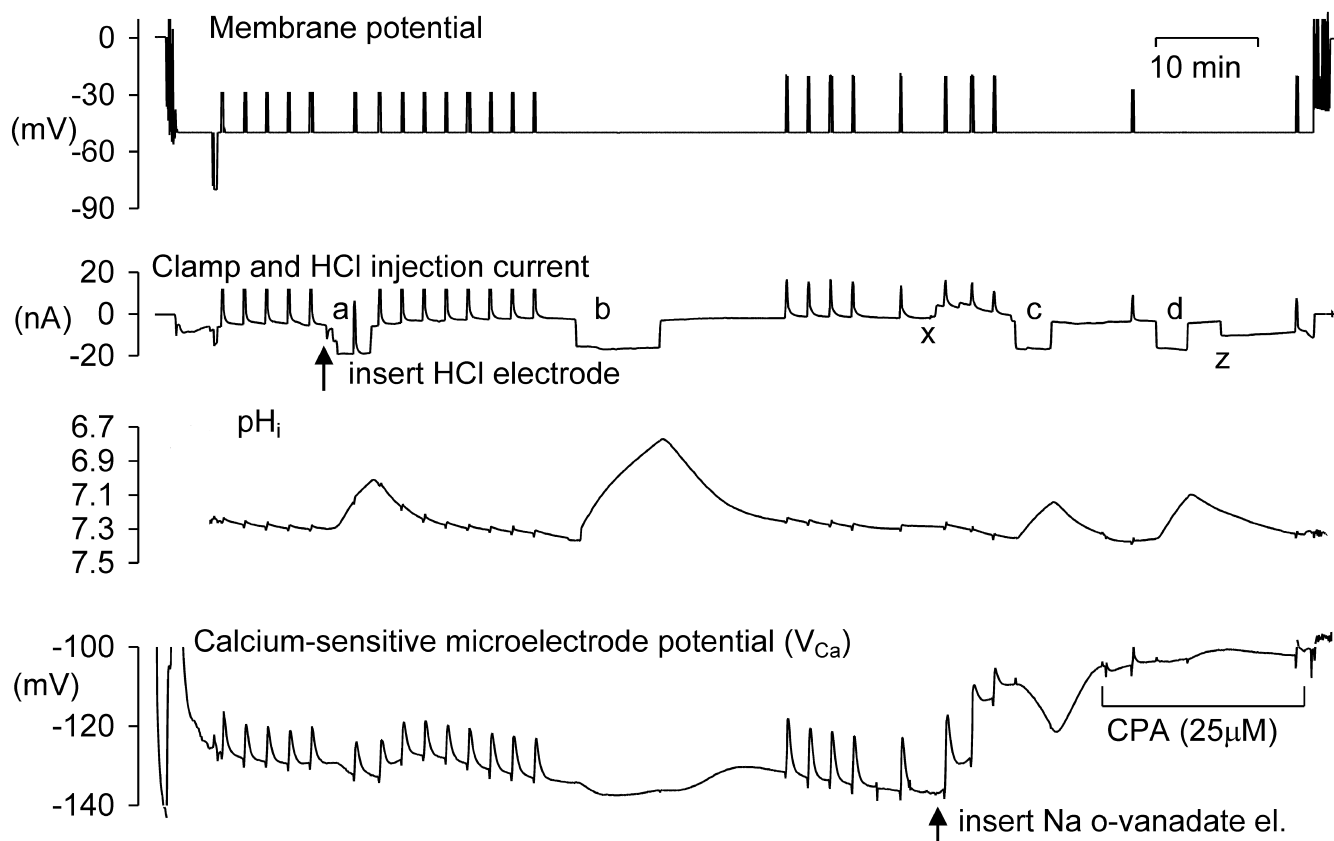


FIGURE 3. The effect of Ca-ATPase inhibitors on the response of  $V_{Ca}$  to acid injections. Records of membrane potential, clamp, and injection current,  $pH_i$  and  $V_{Ca}$ , during a complete experiment. The membrane potential was clamped at  $-50$  mV except for one brief hyperpolarization and many brief depolarizations to test homeostatic mechanisms. After the first five depolarizations the HCl electrode was inserted and current passed through it to lower  $pH_i$  four times, as shown by the downward current deflections (a–d). When  $pH_i$  had recovered from the second HCl injection, o-vanadate was injected between points x and z to inhibit the PMCA. In this experiment  $pH_i$  was allowed to recover after each injection without bicarbonate superfusion.

Fig. 2 C shows the relationship after CPA. Inhibition of the SERCA pump clearly removed most of the hysteresis in the  $V_{Ca}/pH_i$  relationship.

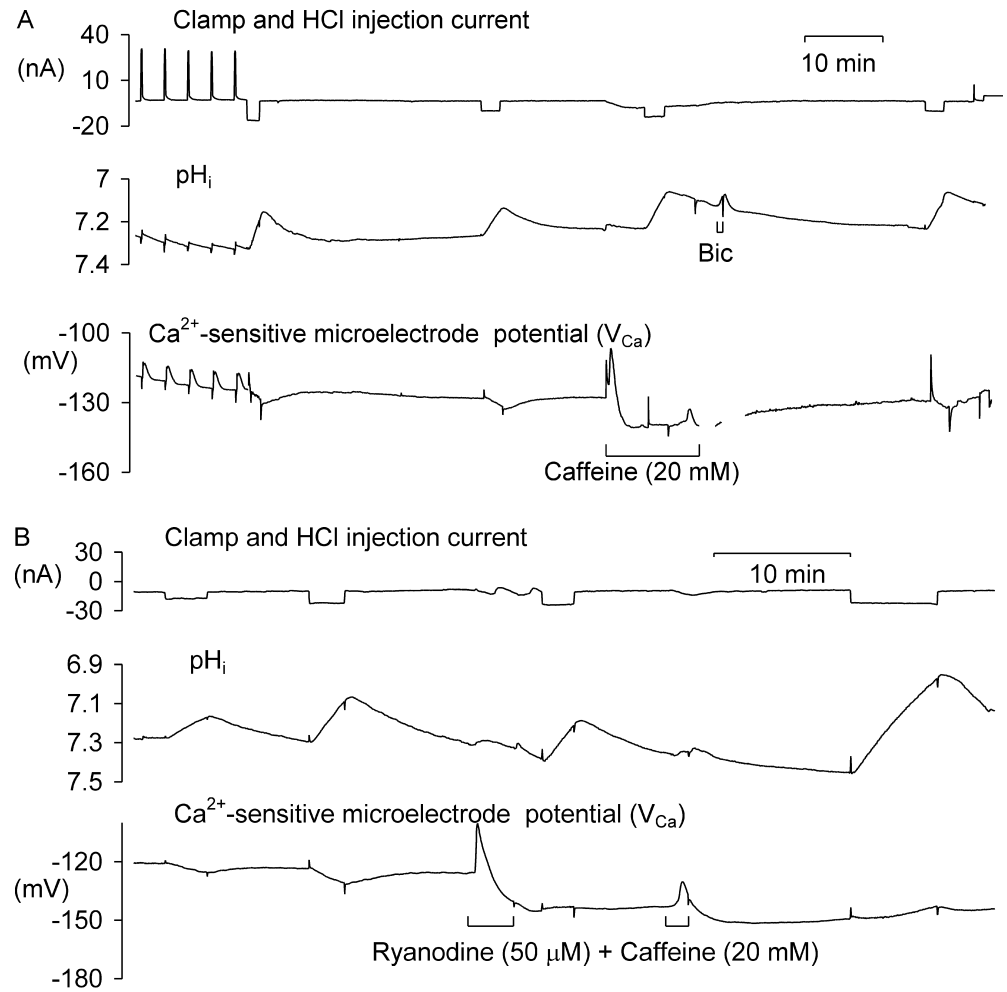
Next I investigated the possible roles of the PMCA and the steady-state calcium level on the response to acidification. I injected ortho-vanadate, a potent ATPase inhibitor (Cantley et al., 1977), which should block the PMCA, the sodium pump and possibly also the SERCA pump. Fig. 3 shows one of three similar experiments in which o-vanadate was injected iontophoretically, in this case after two periods of HCl injection. The first injection lowered  $pH_i$  from 7.3 to 7.0, and  $V_{Ca}$  from  $-129$  to  $-133$  mV. The second, larger, injection lowered  $pH_i$  from 7.35 to 6.8, and  $V_{Ca}$  from  $-134$  mV to  $-137$  mV. Once  $pH_i$  and  $V_{Ca}$  had stabilized after recovering from the second injection, a microelectrode filled with Na-o-vanadate was inserted and a 5 nA current passed to inject o-vanadate ions. The resulting inhibition of the PMCA caused  $[Ca^{2+}]_i$  to start increasing immediately. Three depolarizations imposed an extra calcium load. When  $V_{Ca}$  had reached  $-110$

mV (from  $-137$  mV, equivalent to almost a tenfold increase in  $[Ca^{2+}]_i$ ), I injected HCl for 210 s, lowering  $pH_i$  from 7.35 to 7.15. This time  $V_{Ca}$  fell rapidly to  $-121$  mV, and rose again as rapidly when the HCl injection was stopped. After superfusion with CPA, the acid injection was repeated. It had no immediate effect on intracellular calcium, although as  $pH_i$  recovered there was a small increase. These effects of vanadate and CPA were seen in a total of three similar experiments: vanadate injection to block the PMCA and raise  $[Ca^{2+}]_i$  made the  $V_{Ca}$  response to HCl injection larger, whereas CPA, which inhibits the SERCA pump, abolished it.

#### *The Effect of Caffeine and Ryanodine on the Response to HCl Injection*

Caffeine-sensitive calcium stores seem to be universal in nerve cells, although their function remains unclear (Bootman et al., 2001). Calcium is released physiologically from the stores via the RYR calcium-release channels by a local increase in  $[Ca^{2+}]_i$  in the process known as calcium-induced calcium release (CICR). High levels

FIGURE 4. The effect of caffeine alone and with ryanodine on the response of  $V_{Ca}$  to HCl injection. (A) The cell was clamped at  $-60$  mV except at the start of the section shown, when five depolarizations to  $-20$  mV for 10 s were applied. Then HCl was injected for two short periods, and when  $pH_i$  had recovered, caffeine was superfused for 12 min. During caffeine superfusion a third HCl injection was made, and near the end of the experiment, a fourth. The two missing parts of the  $V_{Ca}$  trace after caffeine washout are due to removal of noise from the clamp electrode. (B) Another cell, which was clamped at  $-60$  mV throughout. After two acid injections, a freshly-made solution of ryanodine and caffeine was superfused for 3 min. Then another HCl injection was made. A second application of ryanodine and caffeine showed that the first had not been completely effective. A second postryanodine injection again caused no decrease in  $[Ca^{2+}]_i$ .



of caffeine lower the threshold for this process so that it occurs at normal levels of  $[Ca^{2+}]_i$ , leading to store emptying. The subsequent undershoot in  $[Ca^{2+}]_i$  when caffeine is removed has been ascribed to local depletion of calcium by the hyperactive SERCA pump (Collins and Thomas, 2001). Since the RYR calcium-release channels of the sarcoplasmic reticulum of muscle cells are known to be inhibited by low pH (Meissner and Henderson, 1987; Rousseau and Pinkos, 1990; Meissner, 1994), I have tested the effect of caffeine and ryanodine on the  $V_{Ca}$  response to small acid injections. Fig. 4 A shows an experiment in which caffeine was applied after two short HCl injections, both of which caused a decrease in  $[Ca^{2+}]_i$ . Superfusion with 20 mM caffeine then caused a large transient increase in  $[Ca^{2+}]_i$  as CICR was induced, followed by a prolonged undershoot. Then, 5 min after beginning the caffeine application, during the undershoot, I made a third HCl injection. This caused no change in  $[Ca^{2+}]_i$ , although it was followed by a small release of residual calcium just before I ended the caffeine superfusion. A fourth HCl injection made 22 min later caused a small decrease in  $[Ca^{2+}]_i$ . Similar results were obtained in two other ex-

periments in which HCl was injected during a long exposure to 20 mM caffeine.

While caffeine opens RYR and thus empties the stores, ryanodine blocks the RYR. Unfortunately, it rapidly blocks only open RYR in snail neurons, so I applied it together with 20 mM caffeine to maximize its effectiveness. Fig. 4 B shows an example result. After two periods of HCl injection, each of which caused a fall in  $[Ca^{2+}]_i$ , the preparation was superfused with ryanodine and caffeine for 210 s. This caused a large transient increase in  $[Ca^{2+}]_i$  followed by a long-lasting undershoot. Both of the two subsequent HCl injections caused an increase in  $[Ca^{2+}]_i$ . In a total of six similar experiments, before ryanodine the mean decrease in  $V_{Ca}$  caused by a 0.1 pH unit acidification was  $-1.5 \pm 0.7$  mV. After ryanodine, the same acidification caused a mean increase in  $V_{Ca}$  of  $0.9 \pm 0.4$  mV. Ryanodine and caffeine thus converted the effect of small HCl injections from a decrease in  $[Ca^{2+}]_i$  to no change or an increase.

The application of ryanodine and caffeine together led to a prolonged decrease in steady-state  $V_{Ca}$  that was maintained long after the return to normal snail Ringer, unlike with the application of caffeine alone.

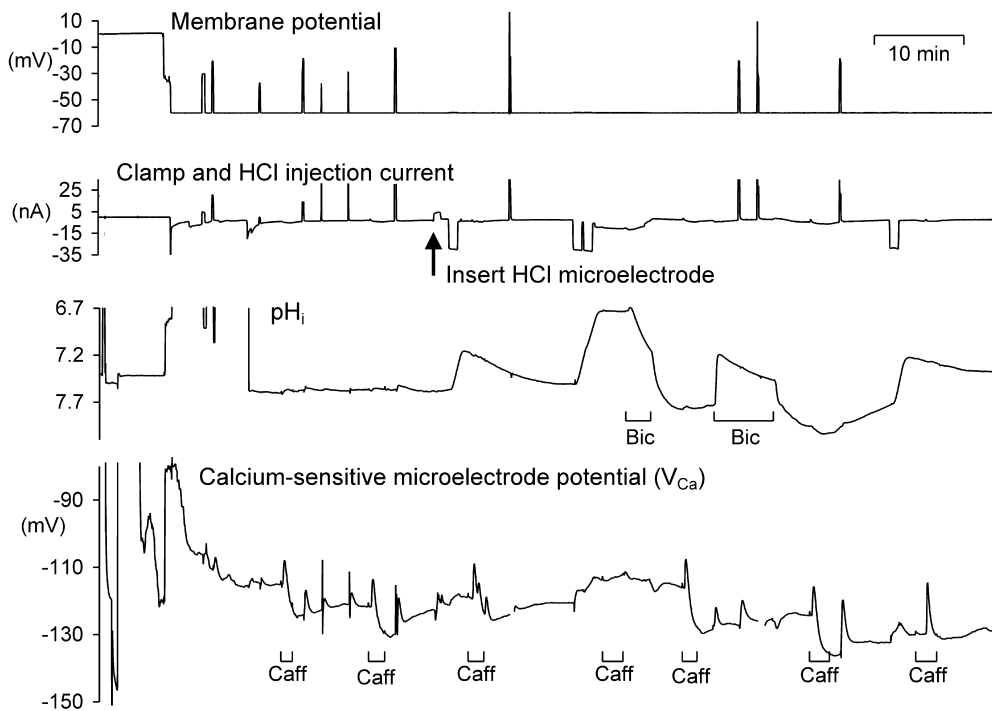


FIGURE 5. The effect of low  $pH_i$  on the response of  $V_{Ca}$  to the application of 10 mM caffeine. The first half of a long experiment is shown, including the period during which the calcium-sensitive microelectrode was pushed right through the cell and then withdrawn back into the cell. After two applications of 10 mM caffeine, an HCl microelectrode was inserted through which currents were passed to decrease  $pH_i$ , shown as downward deflections on the current record. Caffeine was applied as shown by the bars below the  $V_{Ca}$  record, and bicarbonate (5 mM) was applied for two periods as indicated.

The average  $V_{Ca}$  before ryanodine application was  $-132 \pm 3$  mV, while 10 min after removing ryanodine it was  $-145 \pm 2$  mV. This is equivalent to a decrease of  $[Ca^{2+}]_i$  from 122 to 42 nM.

#### The Effect of HCl Injection on the Response to Caffeine

At normal  $pH_i$  values 10 mM caffeine always causes a large calcium release from snail neurone calcium stores within 1 min as long as the stores have had time (4–6 min) to refill after any previous caffeine application. To test the effects of low  $pH_i$  on the effectiveness of caffeine in initiating CICR, I have observed the responses to 10 mM caffeine, applied for 2 min, as shown in Fig. 5. Seven applications of caffeine were made at different  $pH_i$  levels as changed by HCl injection or bicarbonate superfusion. At  $pH_i$  levels below seven there was no clear response to caffeine, while at higher  $pH_i$  levels caffeine consistently caused a transient increase in calcium.

Fig. 6 shows results from a total of 25 caffeine applications from three experiments in which  $pH_i$  was manipulated as in Fig. 5. As shown in Fig. 6 A all the caffeine applications made when  $pH_i$  was above 7.1 produced a fall in  $V_{Ca}$  of at least 6 mV; but the six application made when  $pH_i$  was lower than 7.1 caused a change in  $V_{Ca}$  of, at most, less than 3 mV. Fig. 6 B shows the values of  $V_{Ca}$  and  $pH_i$  at which the caffeine was applied. Although low  $pH_i$  did tend to increase the steady-state calcium level, this cannot explain the failure of the caffeine response. When caffeine was applied at 50 mM (unpublished data) it caused a large release ( $\Delta V_{Ca}$  over 10 mV) at  $pH_i$  values down to 6.75, but only a small release at lower values.

#### The Effects of Acid Injections on Calcium Transients.

Since the calcium pump (PMCA) that extrudes  $Ca^{2+}$  ions from the cell is known to carry  $H^+$  ions into the cell, it seems probable that it would be inhibited by low  $pH_i$ , as it is by high extracellular pH (Benham et al., 1992; Schwiening et al., 1993). I therefore examined the effects of HCl injection on calcium transients. I increased  $[Ca^{2+}]_i$  by two methods: by direct iontophoretic injection of  $Ca^{2+}$  (and  $Cl^-$ ) ions or by brief depolarizations. The number of ions injected for a given charge, using the same procedure, has been shown not to change in vitro when  $[Ca^{2+}]_i$  was varied over the normal  $[Ca^{2+}]_i$  range of 10–100 nM (Schwiening and Thomas, 1996). The effect of varying pH in vitro on  $Ca^{2+}$  iontophoresis has not been studied, but there is no reason to believe it would be sensitive to  $pH_i$ , at least in the range used here. The voltage-sensitive calcium channels are, however, likely to be inhibited by low  $pH_i$ , so that calcium entry will decrease as HCl is injected (see Tombaugh and Somjen, 1998). Fig. 7 shows an experiment in which I tested the effect of low  $pH_i$  on both types of calcium transient. Calcium injections were made with currents of 10 or 20 nA for 10 s. The depolarizations were by 40 mV for 10 s. After inserting the HCl electrode I passed an  $H^+$  injection current which was increased as necessary to lower pH by  $\sim 1$  U over a period of 18 min;  $Ca^{2+}$  ions were injected at  $\sim 2$  min intervals. Once the  $pH_i$  was below 6.5 I switched off the acid injection and superfused the preparation for a short time with bicarbonate Ringer to allow the cell to extrude the injected acid more rapidly. After allowing some time for  $pH_i$  and  $V_{Ca}$  to stabi-

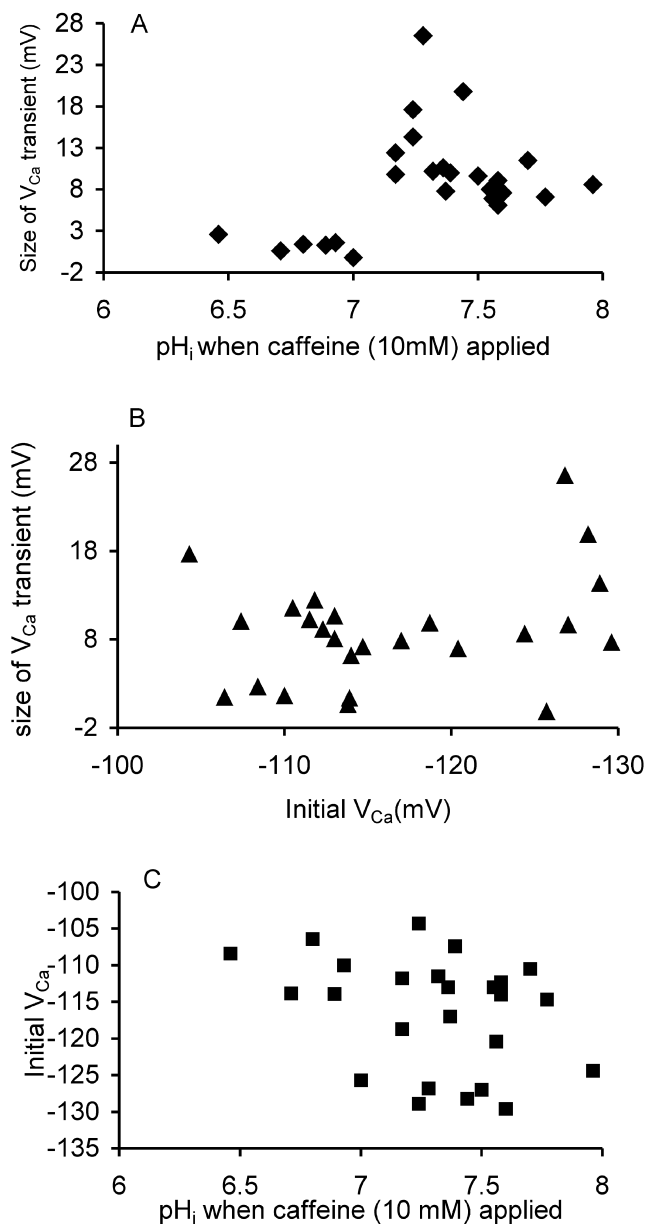


FIGURE 6. Caffeine-induced calcium release at different pH<sub>i</sub>. (A) The size of the transient change in V<sub>Ca</sub> occurring within 60 s of the start of 25 applications of 10 mM caffeine onto three different cells is plotted against the pH<sub>i</sub> at the time of application. (B) The size of the transient change in V<sub>Ca</sub> plotted against the V<sub>Ca</sub> at the time of application. (C) The initial V<sub>Ca</sub> and pH<sub>i</sub> when the caffeine applications were made. Data from three experiments in which caffeine was applied a total of 25 times.

lize, I began a series of 10 s depolarizations, and repeated the acid injection protocol. The depolarizations caused rather smaller changes in V<sub>Ca</sub> than the injections, although the pH<sub>i</sub> changes, presumably generated by the PMCA, associated with the calcium extrusions at normal pH<sub>i</sub> were similar: 0.009 for the injections and 0.015 for the depolarizations. (The scale used in Fig. 7 makes this hard to detect, but I made the comparison by displaying

the original data at high gain.) This suggests that the calcium load generated by the depolarizations was larger than that generated by the injections, assuming in both cases that the complete calcium load was extruded by the PMCA. The location of the Ca<sup>2+</sup>-sensitive microelectrode some distance from the cell membrane presumably accounts for the smaller recorded size of the depolarization-induced transients.

The size of the V<sub>Ca</sub> transients caused by the injections presumably reflects a combination of local buffering and diffusion from the injection site to the measurement point. The large acidification appears to have had little overall effect. On the other hand, the transients caused by depolarizations were greatly reduced by acidification. Fig. 8 shows another experiment in which transients resulting from injections and depolarizations were compared. In this case the depolarizations were to 0 mV, which as pH<sub>i</sub> fell caused the opening of outward H<sup>+</sup> (Thomas and Meech, 1982) as well as Ca<sup>2+</sup> channels. Thus, pH<sub>i</sub> tended to increase briefly during the depolarizations at low pH<sub>i</sub>. As before, the V<sub>Ca</sub> transients generated by Ca<sup>2+</sup> influx from outside the cell were more sensitive to low pH<sub>i</sub> than were those arising from the iontophoretic injections, although both seemed more sensitive to low pH<sub>i</sub> here than in the experiment shown in Fig. 7. The first pH<sub>i</sub> decrease, from 7.28 to 6.24, reduced the V<sub>Ca</sub> transient generated by CaCl<sub>2</sub> injection by almost 50%, from 15.7 to 8 mV. The second pH<sub>i</sub> decrease, from 7.24 to 6.49, reduced the V<sub>Ca</sub> transient generated by depolarization by 90%, from 17.3 to 1.8 mV depolarization.

Data on normalized V<sub>Ca</sub> transients from five experiments are collected in Fig. 9. The series of transients recorded just before and during each period of HCl injection were normalized to the largest response of that series, then collected in 0.2 pH unit bins. The injection-induced calcium transients were only ~20% smaller at pH 6.5 than in the physiological range of 7.2 to 7.4, although only the point at pH<sub>i</sub> 6.4 was significantly different (*P* < 0.05) from that at pH<sub>i</sub> 7.4. On the other hand, the depolarization-induced transients were reduced by ~60% by the same acidification. Only the points at pH 6.8 and 6.6 were significantly different from that at pH 7.3, that at pH 6.6 at the 0.0003 level.

Since calcium extrusion by the PMCA involves H<sup>+</sup> transport into the cell, low pH<sub>i</sub> will increase the work required to extrude Ca<sup>2+</sup> ions, and may slow the rate of recovery after a calcium injection. Fig. 10 shows the recovery time constants averaged in 0.2 pH unit bins for 25 injection-induced V<sub>Ca</sub> transients. Acidification did not slow the rates of recovery; none of the low pH<sub>i</sub> points were significantly different from that at pH<sub>i</sub> 7.45.

#### DISCUSSION

In this study I have shown that changes in pH<sub>i</sub> induced by HCl injection can have marked effects on neuronal



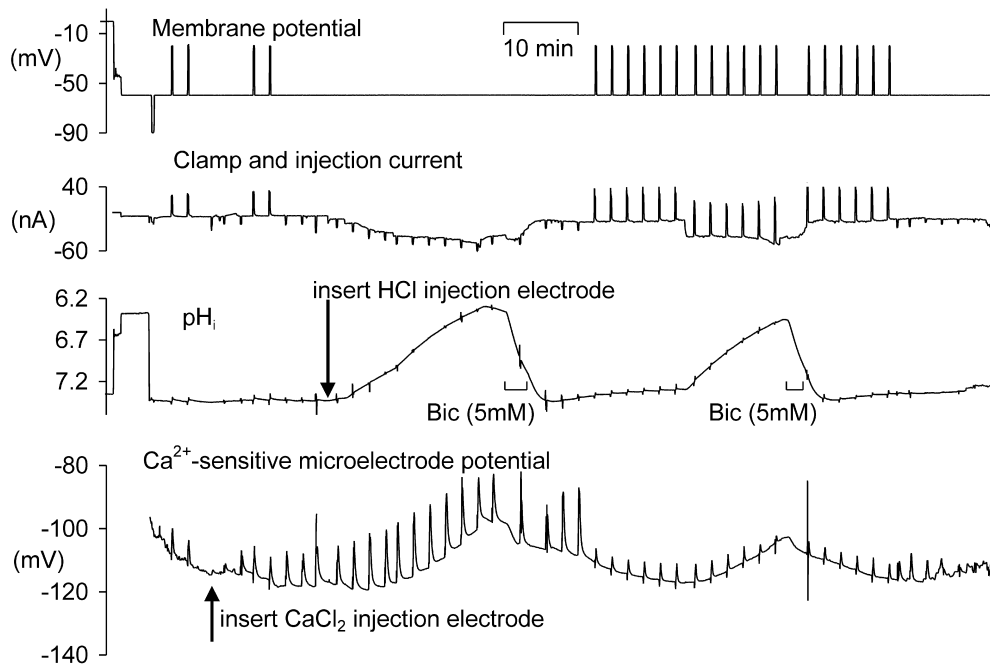


FIGURE 7. The effect of HCl injection on  $pH_i$  and on  $V_{Ca}$  transients generated either by injection of  $CaCl_2$  or by depolarizations to  $-20$  mV for 10 s. The membrane potential was clamped at  $-60$  mV except for brief depolarizations. After the first two depolarizations the  $CaCl_2$  electrode was inserted and used to make several injections. Finally the HCl electrode was inserted and used to reduce  $pH_i$ . Downward offsets on the current trace indicate the periods during which  $Ca^{2+}$  or  $H^+$  and  $Cl^-$  ions were injected iontophoretically, and upward deflections indicate depolarizations. After the two periods when  $pH_i$  was reduced by HCl injection, recovery was assisted by superfusion of Ringer containing 5 mM added bicarbonate. The large vertical excursions of the pH and calcium traces were caused by electrical pickup by the amplifier inputs.

$[Ca^{2+}]_i$ . When resting calcium was recorded while  $pH_i$  was lowered,  $[Ca^{2+}]_i$  initially fell with modest acidifications but then rose as  $pH_i$  was further reduced. When  $pH_i$  was allowed to recover,  $[Ca^{2+}]_i$  at first also began to

recover (if it had been increased above baseline by the acidification), but then transiently increased again. Both the acid-induced fall in  $[Ca^{2+}]_i$  and the recovery-induced increase were abolished by CPA. Ortho-vana-

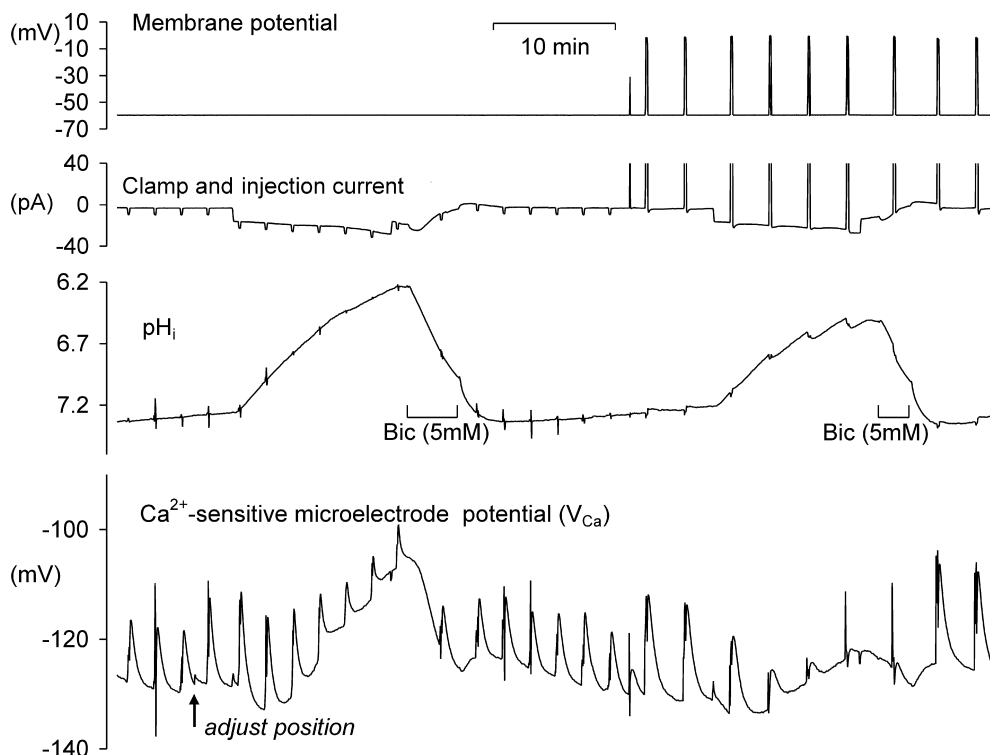


FIGURE 8. The effect of HCl injection on  $pH_i$  and on  $V_{Ca}$  transients generated either by injection (downward deflections on the current record) of  $CaCl_2$  or by depolarizations to 0 mV for 10 s (upward deflections on the current record). After the first three injections, the position of the calcium-sensitive microelectrode was adjusted; this increased the recorded  $V_{Ca}$  transients. After the two periods when  $pH_i$  was reduced by HCl injection, recovery was assisted by superfusion of Ringer containing 5 mM added  $NaHCO_3$ . The vertical excursions of the pH and calcium traces were caused by electrical pickup by the amplifier inputs.

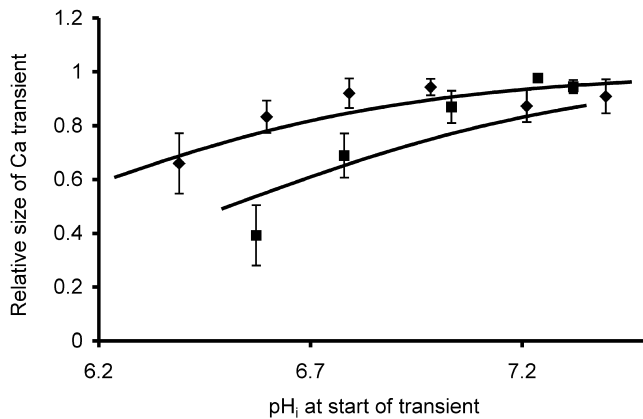


FIGURE 9. Effect of low  $pH_i$  on the size of the transient increases in  $V_{Ca}$  induced by either iontophoretic injections ( $\blacklozenge$ ) or 10 s depolarizations ( $\blacksquare$ ). The calcium transients from each period of acid injection were normalized relative to the largest transient in that series, then collected in 0.2 pH unit bins (the pH at the start of each injection). The mean transient size and SEM for each bin was plotted against the average starting  $pH_i$  for the transients in each bin. Data from five different experiments, with a total of 26 injections and 25 depolarizations. Lines are least squares best fits to the original normalized data of the following relationship:  $pH_{st} = pK + \log(\Delta Ca/1 - \Delta Ca)$ , where  $pH_{st}$  is the  $pH_i$  at the start of a  $V_{Ca}$  transient,  $pK$  the pH when  $\Delta Ca = 0.5$ , and  $\Delta Ca$  the relative size of the calcium transient. The  $pK$ s giving the two lines were 6.05 for the injections and 6.51 for the depolarizations.

date increased the steady-state  $[Ca^{2+}]_i$  and magnified the acid-induced fall  $[Ca^{2+}]_i$ . The decrease in  $[Ca^{2+}]_i$  caused by small HCl injections was abolished by caffeine and ryanodine. Calcium release from stores by 10 mM caffeine did not occur when the  $pH_i$  was below 7.1. While injections sufficient to lower  $pH_i$  by one unit had little effect on calcium transients generated by  $CaCl_2$  injection, such acidifications greatly reduced transients caused by opening voltage-gated calcium channels.

#### *The Effect of HCl Injection on Resting $[Ca^{2+}]_i$*

Resting  $[Ca^{2+}]_i$  in snail neurons is influenced by several processes. These are (a) influx from outside the cell, mainly through channels, (b) extrusion from the cell by the PMCA (Kennedy and Thomas [1995] showed that snail neurons have no Na/Ca exchanger), (c) uptake into stores by the SERCA, (d) release from the stores via the RYR and inositol 1,4,5-trisphosphate channels, and (e) uptake and release by mitochondria. Since there is no evidence for any mitochondrial involvement at the low levels of  $[Ca^{2+}]_i$  in a cell clamped at  $-50$  or  $-60$  mV I will not discuss this further.

My results show that modest reductions in  $pH_i$  caused by HCl injection consistently reduced resting  $[Ca^{2+}]_i$ . This effect of acidification is similar to that reported by Willoughby et al. (2001). They used propionate superfusion to lower  $pH_i$  by 0.2–0.3 pH units and recorded a

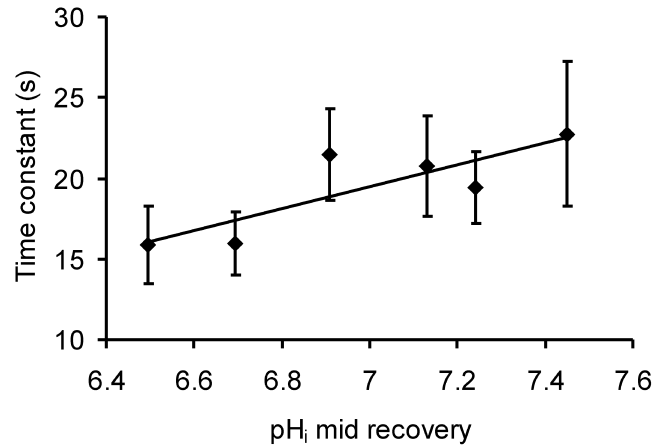


FIGURE 10. The effect of low  $pH_i$  on the rate of recovery of  $[Ca^{2+}]_i$  (expressed as  $V_{Ca}$ ) for calcium injections from three experiments, those of Figs. 6 and 7 and another. Transients were grouped into 0.2 pH unit bins ( $pH_i$  at the midpoint of the recovery), and averaged with respect to both  $pH_i$  and time constant. The bars show the standard errors of the mean time constants.

small fall in  $[Ca^{2+}]_i$  using both fura-2 and a  $Ca^{2+}$ -sensitive microelectrode. They did not investigate the effects of SERCA inhibitors on the response to low  $pH_i$ , although they showed that CPA blocked a large fraction of the rise in  $[Ca^{2+}]_i$  seen with  $pH_i$  increases induced with weak bases. My findings that CPA blocked both the fall in  $[Ca^{2+}]_i$  induced by modest acidifications and the rise in  $[Ca^{2+}]_i$  induced by the  $pH_i$  recovery after acidification show that both phenomena reflect changes in the net calcium uptake or release of calcium by the calcium stores.

What effect of  $pH_i$  on the stores caused the fall in  $[Ca^{2+}]_i$  seen with acidification? Calcium uptake into the stores is driven by the SERCA pump, whereas spontaneous release is believed to occur through RYR channels. Thus, a fall in  $[Ca^{2+}]_i$  may result from either stimulation of the pump or inhibition of a continuous release process. In the experiments in which I saw clear falls in  $[Ca^{2+}]_i$  with HCl injection the membrane potential was clamped near the resting potential, so that  $[Ca^{2+}]_i$  was presumably well below the normal threshold for calcium-induced calcium release. Perhaps, nevertheless, continuous release of calcium from the stores was occurring, until inhibited by the fall in  $pH_i$ . The finding that caffeine and ryanodine block, indeed reverse, the fall in  $[Ca^{2+}]_i$  caused by acid injection supports this suggestion. There is also considerable evidence from other preparations that low  $pH_i$  inhibits release by the stores. In intact cardiac myocytes both uptake and release of  $Ca^{2+}$  were inhibited by acidosis (Hulme and Orchard, 1998) and the frequency of spontaneous calcium sparks, caused by release from the stores, was reduced by low  $pH_i$  (Balnave and

Vaughan-Jones, 2000). There is also clear evidence that calcium release by isolated sarcoplasmic reticulum RYR receptors is  $\text{pH}_i$  sensitive (Meissner and Henderson, 1987; Rousseau and Pinkos, 1990; Meissner, 1994), although recent work with RYR receptors from rabbit skeletal muscle incorporated in lipid bilayers suggests low  $\text{pH}$  on the cytoplasmic side may be less inhibitory than reported previously (Laver et al., 2000).

Alternatively or additionally, the reduction in  $[\text{Ca}^{2+}]_i$  might indicate that low  $\text{pH}_i$  was stimulating the SERCA pump. However, there is no evidence for this in other preparations. On the contrary, a recent study of the effect of acidification on sarcoplasmic reticulum Ca-ATPase activity found that it was  $\sim 60\%$  less at  $\text{pH}$  6.5 than 7.4. (Stienen et al., 1999). Thus, the processes controlling both calcium store uptake and release are inhibited by acidosis in muscle.

Together, the inhibitory effects of caffeine, ryanodine, and CPA show that the initial effects of HCl injection on  $[\text{Ca}^{2+}]_i$  must be due to inhibition of release via RYR. As  $\text{pH}_i$  is reduced calcium release is inhibited, causing  $[\text{Ca}^{2+}]_i$  to fall. Further acidification, and/or the passage of time, caused  $[\text{Ca}^{2+}]_i$  to rise, often to levels higher than the baseline. This increase was not blocked by CPA, suggesting that it was due to low  $\text{pH}$  effects on either the PMCA or mitochondria. It was unlikely to be due to an effect on the plasma membrane calcium channels, since they are inhibited by low  $\text{pH}_i$ .

The long-term fall in  $[\text{Ca}^{2+}]_i$  produced by ryanodine, as well as the effects of small HCl injections, lead to the conclusion that resting  $[\text{Ca}^{2+}]_i$ , at least as recorded by a  $\text{Ca}^{2+}$ -sensitive microelectrode, may be supplemented by a continuous leak from the stores. It is difficult to understand how such a steady leak could contribute in the long term to resting  $[\text{Ca}^{2+}]_i$ , unless the release and uptake sites are in different parts of the endoplasmic reticulum. If they are colocalized, as usually assumed, and the rate of uptake depends on the store content, there is presumably a balance at rest between local release and uptake. Reducing release by HCl injection would increase the store content and thus reduce uptake. Any reduction in  $[\text{Ca}^{2+}]_i$  would be only transient. Perhaps, as suggested by Verkhatsky and Petersen (2002) the ER acts as a conduit, in this case carrying calcium from the plasma membrane into the cell interior. There would then be a cytoplasmic gradient from the release sites to the plasma membrane. Blocking release would lead to a long-term fall in resting  $[\text{Ca}^{2+}]_i$  in the cell interior.

The transient decrease in  $[\text{Ca}^{2+}]_i$  sometimes seen on the application of CPA, as in Fig. 2 A, has not been observed before. In my experiments the calcium stores were often likely to have been overloaded when CPA was applied. Perhaps the sudden blockage of the SERCA pump also briefly reduces the leakage by some unknown mechanism. If the SERCA pump is electro-

genic, there might be a change in the potential across the ER membrane.

Further evidence for a continuous resting leak is that inhibition of the SERCA pump by CPA does lead to store emptying, which has always been presumed to occur via leakage. On the other hand, CPA has not been reported to cause a significant long-term reduction in  $[\text{Ca}^{2+}]_i$  as might be expected when leakage ceases when the stores are exhausted, while ryanodine and caffeine do cause such a reduction. In nonexcitable cells, of course, emptying the stores greatly increases calcium influx from outside the cell via store-operated calcium entry. Perhaps at least a modest increase occurs in neurons too when the stores are emptied by CPA, sufficient to offset the effects on  $[\text{Ca}^{2+}]_i$  when there is no further leakage from stores. The stores are not emptied by ryanodine, so the same increase in influx would not occur.

If the extent of the fall in  $[\text{Ca}^{2+}]_i$  with acidification reflects a fall in the rate of leakage from the stores, then my results with *o*-vanadate suggest that blocking the PMCA and thus raising  $[\text{Ca}^{2+}]_i$  greatly increases leakage from the stores. Indeed this is further evidence that CICR operates at near-normal  $[\text{Ca}^{2+}]_i$  levels. Alternative mechanisms that could explain both the large fall in  $[\text{Ca}^{2+}]_i$  with acidification and the blocking effect of CPA are difficult to conceive.

#### *The Effect of the Postacidification $\text{pH}_i$ Recovery on Resting $[\text{Ca}^{2+}]_i$*

The transient rise in  $[\text{Ca}^{2+}]_i$ , seen when  $\text{pH}_i$  was allowed to recover from HCl injection, is similar to that reported recently by Willoughby et al. (2001) for a  $\text{pH}_i$  increase from its resting level induced by application of a weak base. Thus, it seems that a relative  $\text{pH}_i$  increase magnifies spontaneous calcium release. The stores in my experiments behave as if the period in low  $\text{pH}_i$  has overloaded them, suggesting that the uptake pump was much less inhibited by low  $\text{pH}_i$  than was the release process.

The  $\text{pH}_i$  sensitivity of  $\text{Ca}^{2+}$  release via RYR implies that  $\text{pH}_i$  shifts seen during normal neuronal activity might influence  $[\text{Ca}^{2+}]_i$  also (Chesler and Kaila, 1992). A study of rat hippocampal neurons showed a train of action potentials (for 1 min at 0.5 Hz) was accompanied by a fall in  $\text{pH}_i$  of 0.18 pH unit (Trapp et al., 1996). Similarly, in *Helix* neurons a burst of depolarizations to 0 mV (50 times at 5 Hz) produced an acidification of 0.06 pH unit (Willoughby et al., 1999). In both studies  $\text{pH}_i$  returned rapidly to resting values. Such activity-induced acidifications will lead to a build-up of calcium in the stores, so that, as  $\text{pH}_i$  recovers, the stores will be hypersensitive to a subsequent influx of calcium. This could be a mechanism contributing to long-term changes in neuronal properties.

Of all the processes I have tested which are involved in influencing or controlling  $[Ca^{2+}]_i$ , the RYR calcium release channels seem to be in practice the most sensitive to  $pH_i$ . Modest acidifications inhibit release much more than they inhibit the SERCA pump, so acidified stores increase their calcium content. Conversely, alkalizations or simple recovery of normal  $pH_i$  from acidifications sensitize the stores and enhance CICR.

#### *Inhibition by Low $pH_i$ of the Response to Caffeine*

It is well established that the calcium stores in a wide variety of preparations are sensitive to  $pH_i$  (see Willoughby et al., 2001) but complete inhibition of the response to caffeine by low  $pH_i$  has not been reported before. It is unlikely that the stores were empty, or that access of caffeine was restricted in any way by the low  $pH_i$ . Although the calcium release channel is inhibited by low pH in various sarcoplasmic vesicle preparations, the inhibition seen at  $pH_i$  7.0 in the recent careful study of Laver et al. (2000) was <20%.

#### *Lack of Effect of Acidification on Injection-induced Calcium Transients*

The repeated iontophoretic injections of  $CaCl_2$  caused transient increases in  $[Ca^{2+}]_i$  at the tip of the  $Ca^{2+}$ -sensitive microelectrode that was some tens of micrometers distant from the injection site. Assuming that calcium stores were so well-loaded that they played no significant role in shaping the transients, the recovery after the end of the injections presumably reflects  $Ca^{2+}$  extrusion by the PMCA (Schwiening et al., 1993). The modest slowing of the rate of  $V_{Ca}$  recovery caused by large HCl injections correlates well with the relatively low sensitivity of the PMCA of rat sensory and other neurons to high extracellular pH (Benham et al., 1992). Presumably the PMCA has a very low affinity for intracellular  $H^+$  ions. Similarly, the size of the injection-induced  $V_{Ca}$  transients was little reduced by low  $pH_i$ , presumably because calcium buffering is not very sensitive to  $pH_i$ . Much of the size reduction with acidification may be due to the rise in  $[Ca^{2+}]_i$  that accompanied the HCl injection at low  $pH_i$  levels. Schwiening and Thomas (1996) found that the calcium transients (recorded with fura-2) generated by iontophoretic injection were reduced while  $[Ca^{2+}]_i$  was increased by changing the clamp potential. The greater sensitivity of the depolarization-induced calcium transients found here was presumably due to the pH sensitivity of the calcium channels. The identity of the channels likely to operate in snail neurons is unknown, but all such channels are likely to be inhibited by low  $pH_i$  (Tombaugh and Somjen, 1998).

In summary, the mechanism by which modest intracellular acidosis is most likely to influence  $[Ca^{2+}]_i$  in nerve cells is by inhibiting spontaneous or calcium-

induced calcium release from the endoplasmic reticulum. All the other processes which raise or lower  $[Ca^{2+}]_i$  in the cytoplasm appear to be far less sensitive to changes in pH.

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