Effects of Injecting Calcium-Buffer Solutions on $[Ca^{2+}]_i$ in Voltage-Clamped Snail Neurons

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ABSTRACT We have investigated why fura-2 and Ca^{2+}-sensitive microelectrodes report different values for the intracellular free calcium ion concentration ($[Ca^{2+}]_i$ or its negative log, pCa_i) of snail neurons voltage-clamped to −50 or −60 mV. Both techniques were initially calibrated in vitro, using calcium calibration solutions that had ionic concentrations similar to those of snail neuron cytoplasm. Pressure injections of the same solutions at resting and elevated $[Ca^{2+}]_i$ were used to calibrate both methods in vivo. In fura-2-loaded cells these pressure injections generated changes in $[Ca^{2+}]_i$ that agreed well with those expected from the in vitro calibration. Thus, using fura-2 calibrated in vitro, the average resting $[Ca^{2+}]_i$ was found to be ~38 nM (pCa_i 7.42 ± 0.05). With Ca^{2+}-sensitive microelectrodes, the first injection of calibration solutions always caused a negative shift in the recorded microelectrode potential, as if the injection lowered $[Ca^{2+}]_i$. No such effects were seen on the fura-2 ratio. When calibrated in vivo the Ca^{2+}-sensitive microelectrode gave an average resting $[Ca^{2+}]_i$ of ~25 nM (pCa_i 7.6 ± 0.1), much lower than when calibrated in vitro. We conclude that $[Ca^{2+}]_i$ in snail neurons is ~40 nM and that Ca^{2+}-sensitive microelectrodes usually cause a leak at the point of insertion. The effects of the leak were minimized by injection of a mobile calcium buffer.

INTRODUCTION

Although the importance of intracellular calcium as a second messenger is universally acknowledged, its free ion concentration ($[Ca^{2+}]_i$) is not well established (Ross, 1993; Bassani et al., 1995). Its level is certainly very low, in the range of 10^{-7}-10^{-8} M (pCa_i 7–8). It is determined by the balance between several processes: entry of calcium across the cell membrane, intracellular release by and uptake into organelles, and efflux of calcium across the membrane.

There are now several techniques for measuring $[Ca^{2+}]_i$ that can be used in a wide variety of cell types. These include ion-sensitive microelectrodes, luminescent indicators such as aequorin, metallochromic indicators such as arsenazo III, and more recently, fluorescent indicators such as fura-2 and indo-1.

We have recently used fura-2 and Ca^{2+}-sensitive microelectrodes to measure $[Ca^{2+}]_i$ in snail neurons (Kennedy and Thomas, 1995). Using fura-2, calibrated in vitro, we found that the resting $[Ca^{2+}]_i$ was ~40 nM (pCa_i 7.4), but using Ca^{2+}-sensitive microelectrodes calibrated in vitro we found a much higher level, 170 nM (pCa_i 6.77). Our values of resting $[Ca^{2+}]_i$ were in the broad range previously reported, but they did not agree with each other.

We have therefore tried to calibrate our measurements in vivo by making pressure injections of calcium calibration solutions of known calcium concentration ($[Ca^{2+}]$). Injections of buffer solutions as a tool for estimating resting $[Ca^{2+}]_i$ were first used by Baker et al. (1971). They measured $[Ca^{2+}]_i$ in squid giant axons with aequorin. They used pressure injections of solutions composed of a mixture of Ca-EGTA and EGTA and estimated that $[Ca^{2+}]_i$ was 0.3 μM (pCa_i 6.5). More recently, Westerblad and Allen (1993) used pressure injections of $CaCl_2$, EGTA, and a 1:1 EGTA/Ca-EGTA mixture into mouse muscle to calibrate indo-1 and estimated $[Ca^{2+}]_i$ to be 26 nM (pCa_i 7.58).

The calcium calibration solutions that we injected not only contained a mixture of BAPTA/Ca-BAPTA but also concentrations of K^+, Na^+, Mg^{2+}, and H^+ chosen to mimic the intracellular ion levels of snail neurons. Our key assumption is that when a bolus of calcium calibration solution, which has essentially the same ionic composition as cytoplasm, is injected into a cell, the $[Ca^{2+}]_i$ level in the cytoplasm will change toward the calcium level in the injected fluid. If the cytoplasmic calcium level is the same as in the injected solution, no change will be seen. From our results we conclude that in snail neurons voltage-clamped to −50 or −60 mV, resting $[Ca^{2+}]_i$ is close to the 40 nM (pCa_i 7.4) previously measured by fura-2 calibrated in vitro and that the Ca^{2+}-sensitive microelectrode measurements are erroneously high. This error appears to be due to incomplete sealing of the electrode at the site of penetration. Thus, a tiny leak produces a large local elevation of Ca^{2+} at the electrode tip.

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MATERIALS AND METHODS

General

Experiments were carried out on neurons in isolated suboesophageal ganglia from the snail *Helix aspersa* as described recently (Kennedy and Thomas, 1995).

Cells were voltage-clamped to a potential of −50 or −60 mV and periodically depolarized to load the cell with Ca^{2+} through voltage-acti-

vated Ca^{2+} channels. Intracellular calcium was measured using fura-2, Ca^{2+}-sensitive microelectrodes, or both. All experiments were carried out at room temperature (18–22°C). Electrical arrangements were conventional, as described previously (Kennedy and Thomas, 1995).

Solutions

Normal snail Ringer contained (in mM): NaCl, 80; KCl, 4; $CaCl_2$, 7; $MgCl_2$, 5; HEPES, 20; adjusted to pH 7.5 with NaOH. In Ca-free solutions $CaCl_2$ was replaced with $MgCl_2$. The high-pH solution was the same as normal snail Ringer, except that it was buffered to pH 9 using AMPSO ((3-[1,1-dimethyl-2-hydroxyethyl)amino]-2-hydroxypropane-sulfonic acid). Caffeine (10 mM) was dissolved directly into the snail Ringer. All solutions were nominally bicarbonate-free.

Calibration solutions

The same calcium calibration solutions were used to calibrate both fura-2- and Ca^{2+}-sensitive microelectrodes. They were prepared using the methods of McGuigan et al. (1991) with some modifications for use with snail neurons.

Initially, a stock solution, twofold concentrated with respect to the expected intracellular ions and Ca^{2+}-buffer, was prepared with the following concentrations (mM): K^+, 200; Na^+, 10; Mg^{2+}, 2; HEPES, 20; BAPTA (1,2-bis(2-aminophenoxy)ethane-N,N,N,N-tetraacetic acid), 8. BAPTA was used because it is relatively insensitive to pH (Tsien, 1980). This stock solution was then split into two equal portions. To one portion $CaCl_2$ (4 mM) was added before making up to the final concentration with Milli-Q water to make the Ca-BAPTA solution. To the other solution only Milli-Q was added to make the BAPTA solution. The Ca-BAPTA and BAPTA solutions were then mixed in different proportions to produce a series of solutions with a range of $[Ca^{2+}]$. Each solution was adjusted to pH 7.4 by addition of HCl. A Ca^{2+}-sensitive macroelectrode (Phillips IS561-Ca; Unicam, Cambridge), which had previously been calibrated in solutions containing ionic concentrations similar to those of the calcium calibration solutions but no BAPTA and 4, 1, 0.4, and 0.2 mM $CaCl_2$, was used to measure the potential of each of the series of solutions. From these measurements the purity and apparent binding constant of BAPTA could be calculated. In this instance the purity of BAPTA was found to be 96.6% and the apparent binding constant was 6.74. Calibration solutions in the range pCa 4.5–8 were then prepared by mixing appropriate quantities of the BAPTA and Ca-BAPTA solutions, which had been calculated taking into account the purity and apparent binding constant of BAPTA.

Calcium contamination in the solutions was minimized by using Milli-Q water and Aristar grade chemicals (BDH). All glassware was kept scrupulously clean and plasticware was used wherever possible. The background contamination was always checked by measuring the potential in a solution that contained all the constituents except calcium and BAPTA, and was usually found to be <2 μM.

Before the calibration solutions were used in experiments, their $[Ca^{2+}]$ was checked using a Ca^{2+}-sensitive microelectrode. These microelectrodes were capable of giving a linear response to Ca^{2+} of ~29 mV/pCa down to ~pCa 9. In general, providing the purity of BAPTA was >95%, the theoretical pCa of the solutions was found to be accurate as determined by the Ca^{2+}-sensitive microelectrode.

For injection into cells, the calcium calibration solutions were colored by addition of fast green FCF (0.6 mg/ml) or fura-2 (10 μM, 200 μM or 1 mM).

Microelectrodes

Conventional

Micropipettes for measuring membrane potential, voltage clamping, and pressure injection of solutions were pulled from 1.5-mm filamented borosilicate glass tubing (Clark Electromedical Instruments, Reading, England) or similar aluminosilicate tubing. Microelectrodes for recording membrane potential or for passing current were backfilled with 2 M KCl. The tips of these microelectrodes were broken if necessary, by touching them on a pin in the bath, to give resistances of between 10 and 20 MΩ. In general, calcium calibration solutions were injected using a series of short (5 ms) or long (10–1000 ms), low-pressure (20 psi) injections until a change in the 360 fluorescence signal or a green spot was clearly visible.

Ca^{2+}-sensitive microelectrodes

Ca^{2+}-sensitive microelectrodes were prepared and tested as described previously (Kennedy and Thomas, 1995). Briefly, micropipettes were pulled from 1.5-mm-diameter unfilamented aluminosilicate glass tubing. If the tip diameter was less than 1.5 μm, it was broken slightly, and the pipettes were silanized in an evacuated glass tube heated to 250°C around the micropipettes (Thomas, 1994). Once the micropipettes were cool they were backfilled with a solution containing 10 mM KCl and 10 μM $CaCl_2$, and air was expelled from the tips by pressure from a syringe. Finally, a column of sensor cocktail 80–100 μm long was sucked into the tips under visual control. The cocktail was a modification of that described by Ammann et al. (1987) and contained (mg/ml, all from Fluka, Buchs, Switzerland): Ca ionophore ETH 129, 12; sodium tetrakis[3,5-bis(trifluoromethyl)phenyl]borate, 6; 2-nitrophenyloctylether, 200; high-molecular-weight PVC, 33; and tetrahydrofuran, 749. After at least an hour in air and a few minutes dipped in snail Ringer, Ca^{2+}-sensitive microelectrodes were calibrated in vitro by immersing the tip in a series of calibration solutions, usually pCa 6.5, 7, 7.5, and 8.

Calcium measurements with fura-2

Intracellular calcium was measured with fura-2 using a quartz light guide system as described previously (Kennedy and Thomas, 1995). Briefly, two 200-μm quartz light guides were positioned close to a well-exposed cell (diameter ~75–200 μm). One light guide provided the alternating 340-nm, 360-nm, and 380-nm light that was directed over the whole cell. The second light guide was used to collect the emitted fluorescence from the whole of the cell body. Before fura-2 injection the photomultiplier voltage (supplied by the slave spectrophotometer) was increased until either the 340-nm or 380-nm fluorescence signal had reached 1 V. The fluorescence at this level was recorded for 1 min to give a record of background fluorescence.

Once background fluorescence had been recorded, the cell was impaled with a microelectrode containing 1 mM fura-2 (K-salt) dissolved in 100 mM KCl (resistance ~10 MΩ). Brief applications of pressure were given to inject dye until the 360-nm signal had at least doubled. Pressure was provided by a picospritzer that was connected to the microelectrode by plastic tubing. Previous measurements in this laboratory indicate that this procedure produces an intracellular fura-2 concentration of 40–100 μM (C. J. Schwiening, unpublished observations). The fura-2 injection microelectrode was then removed to prevent any leak of fura-2 into the cell.

Calibration of fura-2

Fura-2 was calibrated in vitro after a successful experiment as follows: 8-μl drops of the calcium calibration solutions of pCa 8, 7.5, 7, 6.5, 6, 5.5, and 4.5 containing 10 μM fura-2 were placed on a clean microscope slide. Both quartz light guides were then submerged in one of these drops of calcium calibration solution and carefully positioned to maximize fluorescence. The fluorescence in response to 340, 360, and 380 nm light was then recorded, and again for each calibrating solution. The average of the 340/380 nm fluorescence ratio, from a stable part of recording for each calibration solution, was taken and plotted against the $[Ca^{2+}]$ of the solution. These data were then fitted with the least-squares fit of Eq. 1:

$$pCa = pK_{app} - \log\frac{(\text{ratio} - R_{min})}{(R_{max} - \text{ratio})}, \quad (1)$$

where pK_{app} was the apparent pK of fura-2; the ratio is of 340 nm excited fluorescence against 380 nm excited fluorescence; R_{min} is the minimum

fluorescence ratio; R_{max} is the maximum fluorescence ratio. The above equation and the derived perameters were then used to convert experimental ratio measurements into pCa_i.

An example of a typical calibration is shown in Fig. 1.

In this experiment the apparent pK of fura-2 was 5.6, R_{min} was 0.57, and R_{max} was 26.3. We found little variation in the calibrations during an experimental day. However, a small amount of variation did occur over longer periods of time. For example, over a 1-week period the values were as follows: apparent pK of fura-2 was 5.52 ± 0.05, R_{min} was 0.58 ± 0.02, and R_{max} was 25.4 ± 0.9, where all values are the mean ± SD (n = 7). Because of this variability the calibration was carried out at least once a day.

RESULTS

Resting $[Ca^{2+}]_i$ determined using fura-2 calibrated in vitro

In previous experiments (Kennedy and Thomas, 1994) we attempted and failed to calibrate the fura-2 signal in vivo. Using pressure injections of EGTA it was possible to produce a value for R_{min}, but injections of enough $CaCl_2$ to produce a value for R_{max} were impossible without destroying the cell. Permeabilizing the cell to Ca^{2+} using ionomycin did not raise $[Ca^{2+}]_i$ enough to produce a value for R_{max}, presumably because the cell was still able to regulate $[Ca^{2+}]_i$. In the work described here we carried out routine calibrations of fura-2 in vitro, as described in Materials and Methods.

For each fura-2 experiment we calculated the resting $[Ca^{2+}]_i$ by averaging the fluorescence ratio for a short duration, taken from near the beginning of the experiment when $[Ca^{2+}]_i$ had stabilized after impalement with microelectrodes. We then converted this fluorescence ratio into $[Ca^{2+}]_i$ using the in vitro calibration curve for fura-2, obtained the same day. The average resting $[Ca^{2+}]_i$ for 31 cells calculated in this way was 38 nM (pCa_i 7.42 ± 0.05; mean ± SEM, n = 31).

The effects of injecting calibration solutions

Many workers have shown that fura-2 can be affected by changes in viscosity, ionic strength, and binding to certain intracellular proteins (Konishi et al., 1988). It is quite likely, therefore, that when fura-2 is used intracellularly it behaves differently compared to how it behaves in calibration solutions. In an attempt to validate the values of resting $[Ca^{2+}]_i$ calculated using the in vitro calibration technique, we examined the effects of pressure injections of calibration solutions of known pCa (7, 7.5, and 8) on $[Ca^{2+}]_i$. These solutions were made as described in Materials and Methods; as well as ~4 mM BAPTA, they contained 10 μM fura-2, so that successful injections of these solutions could be seen by the increase in the relatively calcium-insensitive 360-nm fluorescence signal.

In the experiment in Fig. 2, resting $[Ca^{2+}]_i$ at the start of the experiment was 30 nM (pCa_i 7.52), as determined by in vitro calibration. Near the beginning of the experiment a depolarization to 0 mV for 10 s caused an increase in the ratio signal, corresponding to an increase in $[Ca^{2+}]_i$, which

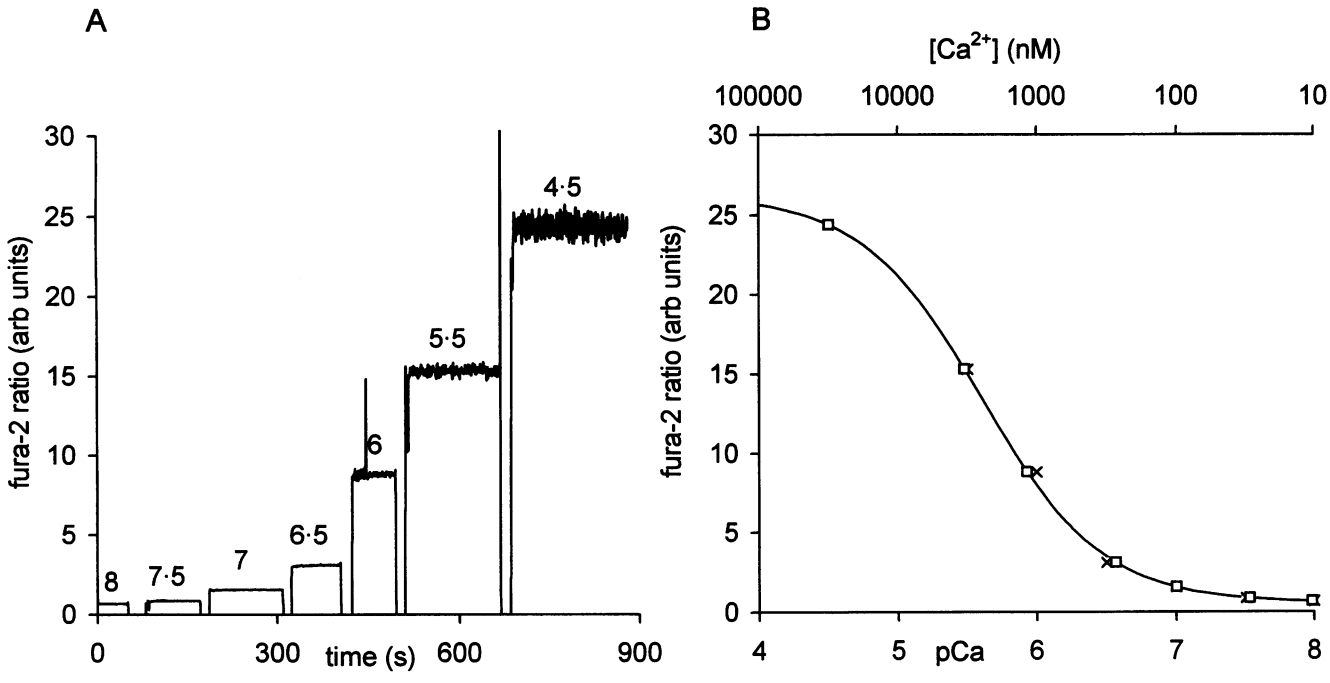

FIGURE 1 Measurements were made in vitro by submerging the light guides in 8-μl drops of calibration solutions of pCa 8, 7.5, 7, 6.5, 6, 5.5, and 4.5. The brief glitches in the fluorescence ratio in *A* occurred when the light in the experimental set-up was switched on or off. (*B*) A plot of the 340/380 fluorescence ratio against $[Ca^{2+}]$ in the calibration solutions. The two scales show $[Ca^{2+}]_i$ in nM (*top scale*) and pCa (*bottom scale*). Both the average fluorescence ratios taken from the experiment for each calibrating solution (*crosses*) and the least-squares fit of the data (*squares*) are shown. The line was calculated from the least-squares fit of the data.

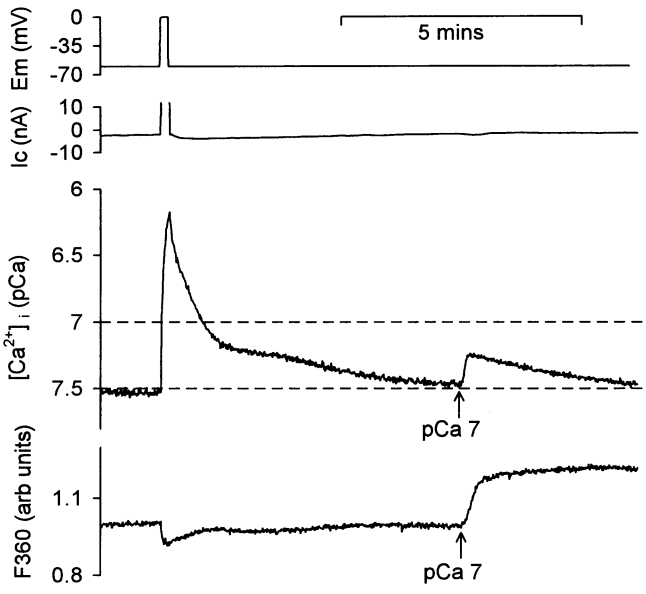

FIGURE 2 The effects on resting $[Ca^{2+}]_i$ of a 10-s depolarization and injecting a pCa 7 calibration solution. Recordings are shown of membrane potential (E_m), clamp current (I_c), and $[Ca^{2+}]_i$ derived from the fura2 ratio and plotted on a pCa scale and 360 fluorescence in arbitrary units. The cell was voltage-clamped and held at −60 mV. For clarity the clamp current has only been shown between −10 and 10 nA. A depolarization to 0 mV for 10 s was given to load the cell with Ca^{2+} to ensure that the cell could regulate $[Ca^{2+}]_i$ back to resting levels. The injection solution that contained calibration solution pCa 7 with 10 μM fura-2 was injected where indicated by the arrows. The initial decrease in the 360 signal shows that in this experiment this wavelength is not completely Ca^{2+} insensitive; the later increase in the 360 signal indicates a successful injection.

recovered to the resting level after the cell had been repolarized to −60 mV. We then made a series of short, low-pressure injections (over a period of several seconds) of calibration solution pCa 7. The increase in the 360-nm fluorescence that occurred at the time of the injection confirmed that a substantial quantity of calibration solution entered the cell. In all four experiments where pCa 7 calibration solution was injected at resting $[Ca^{2+}]_i$, there was always an increase in the ratio signal, corresponding to an increase in $[Ca^{2+}]_i$. This was as expected, if $[Ca^{2+}]_i$ really was 30 nM (pCa_i 7.52), as indicated by the in vitro calibration.

Injections of calcium calibration solution pCa 7.5 caused either an increase or a decrease in the ratio signal, corresponding to an increase or decrease in $[Ca^{2+}]_i$, consistent with the resting $[Ca^{2+}]_i$ level determined by the in vitro calibration.

In the experiment in Fig. 3 *A* the resting $[Ca^{2+}]_i$ at the start of the experiment, calculated from the in vitro calibration of fura-2, was 40 nM (pCa_i 7.4). After a depolarization to 0 mV for 10 s there was an increase in the ratio signal, corresponding to an increase in $[Ca^{2+}]_i$, which recovered to 30 nM (pCa_i 7.52). We then made a series of small-pressure injections of pCa 7.5 calibration solution, which caused only a very small increase in the ratio signal, corresponding to a small increase in $[Ca^{2+}]_i$ to 35 nM (pCa_i 7.46). As the resting $[Ca^{2+}]_i$ calculated from the in vitro calibration just before the injections was 30 nM (pCa_i 7.52), we expected little change in the ratio. The increase was indeed very small, even though it was clear from the 360-nm signal that the amount of buffer injected was substantial. This effect was seen in two other cells and suggests that the in vitro calibration of fura-2 gives a value very close to the actual resting $[Ca^{2+}]_i$.

In the experiment in Fig. 3 *B* the (in vitro calibrated) resting $[Ca^{2+}]_i$ at the start of the experiment was 56 nM (pCa_i 7.25). Near the beginning of the experiment the cell was depolarized to 0 mV for 1 s, and the increase in the ratio signal, corresponding to an increase in $[Ca^{2+}]_i$, was allowed to recover. We then made a series of pressure injections of pCa 7.5 calibration solution, which caused a decrease in the ratio signal, corresponding to a decrease in $[Ca^{2+}]_i$ as expected, if resting $[Ca^{2+}]_i$ really was close to 56 nM (pCa_i 7.25), as calculated from the in vitro calibration.

The experiment in Fig. 4 shows a representative example of the effects of injecting a calibration solution of pCa 8.

Initially the resting $[Ca^{2+}]_i$ calculated from the in vitro calibration was 79 nM (pCa_i 7.1), slightly higher than the average $[Ca^{2+}]_i$. The cell was depolarized to 0 mV for 10 s, and the increase in the ratio signal, corresponding to an increase in $[Ca^{2+}]_i$, was allowed to recover. Once $[Ca^{2+}]_i$ had returned to resting levels we made two series of small-pressure injections of pCa 8 calibration solution. Both series of injections caused a decrease in the ratio signal, corresponding to a decrease in $[Ca^{2+}]_i$, as expected from the in vitro calibration. Similar results were seen in all four cells injected with pCa 8 calibration solution.

It is clear from the experiment in Fig. 4 that $[Ca^{2+}]_i$ (as determined by the in vitro calibration) actually reached the same value as the that of the injected solution. Because the cell already contained significant levels of Ca^{2+}-buffers before our injection, $[Ca^{2+}]_i$ should have tended toward, but never actually reached, the same level as in the injected solution. This suggests that at very low $[Ca^{2+}]_i$ levels, the in vitro calibration of fura-2 may less accurately reflect the in vivo $[Ca^{2+}]_i$ level, leading to an underestimate of $[Ca^{2+}]_i$.

The effects of injecting calibration solutions at resting and raised $[Ca^{2+}]_i$ measured with fura-2

To see whether the injection effects varied in the same cell when $[Ca^{2+}]_i$ was varied we injected calibration solutions at resting $[Ca^{2+}]_i$ and when $[Ca^{2+}]_i$ had been raised by prolonged depolarization (~2 min). A representative experiment is shown in Fig. 5.

Near the beginning of this experiment the cell was depolarized to 0 mV for 1 s, and the increase in $[Ca^{2+}]_i$ was allowed to recover. The resting $[Ca^{2+}]_i$ calculated from the in vitro calibration of fura-2 after this depolarization was 63 nM (pCa_i 7.2). We then made series of small-pressure

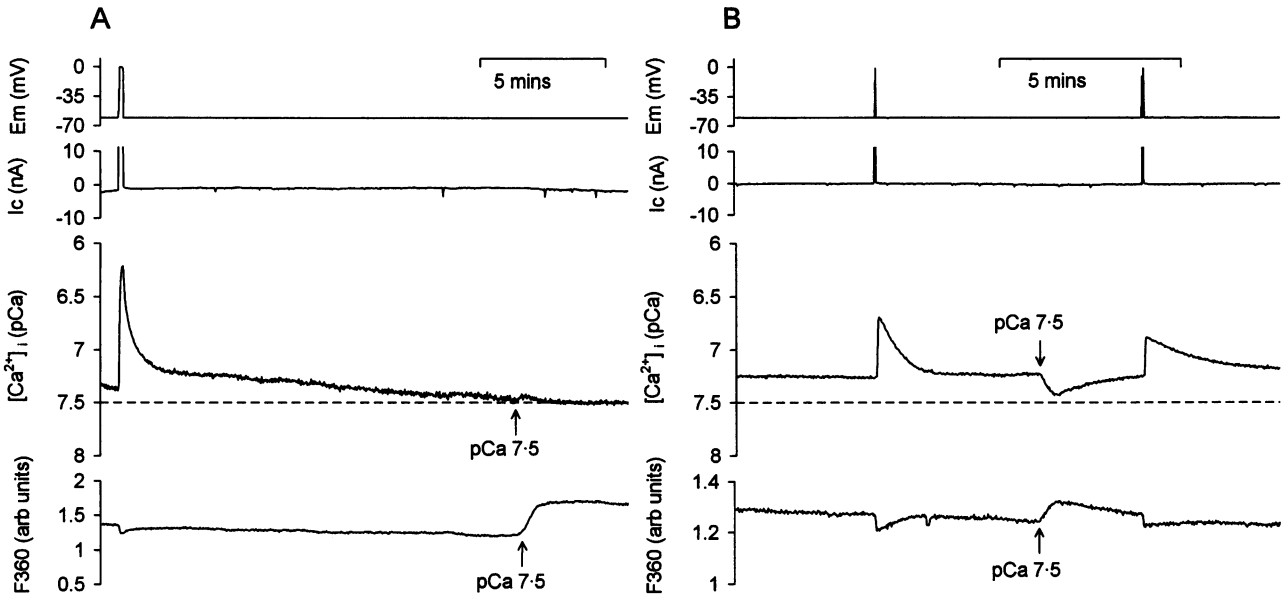

FIGURE 3 The effects on $[Ca^{2+}]_i$ of depolarizations and injecting pCa 7.5 calibration solution. For clarity the clamp current is only shown between −10 and 10 nA. Arrows indicate when pCa 7.5 calibration solution was injected. Depolarizations were for 10 s (*A*) and 1 s (*B*).

injections of pCa 7 calibration solution, which caused an increase in the ratio signal, corresponding to an increase in $[Ca^{2+}]_i$, as expected if $[Ca^{2+}]_i$ really was 63 nM (pCa_i 7.2). The cell was then depolarized and held at −30 mV. This caused an increase in the ratio signal, corresponding to an increase in $[Ca^{2+}]_i$, which stabilized at a higher level. Injections of pCa 7 calibration solution at this new increased level of $[Ca^{2+}]_i$, calculated to be 562 nM (pCa_i 6.25) using the in vitro calibration, caused a large decrease in the ratio signal, corresponding to a decrease in $[Ca^{2+}]_i$. The cell was then repolarized to −60 mV, and $[Ca^{2+}]_i$ began to recover to resting levels. Similar results were seen in four other cells.

In two experiments (results not shown) injections of pCa 6.5 calibration solution were given after $[Ca^{2+}]_i$ had been raised by prolonged depolarization to above 316 nM (pCa_i 6.5). In both cases injection of this calibration solution caused a decrease in the ratio signal, corresponding to a

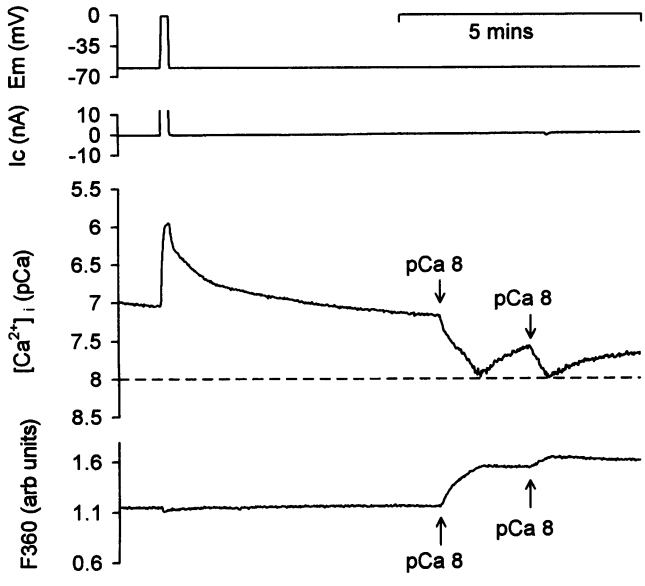

FIGURE 4 The effects on $[Ca^{2+}]_i$ of a 10-s depolarization and injecting pCa 8 calibration solution. Depolarizations for 10 s to 0 mV. For clarity clamp current is only shown between −10 and 10 nA. The arrows indicate the injection of pCa 8 calibration solution.

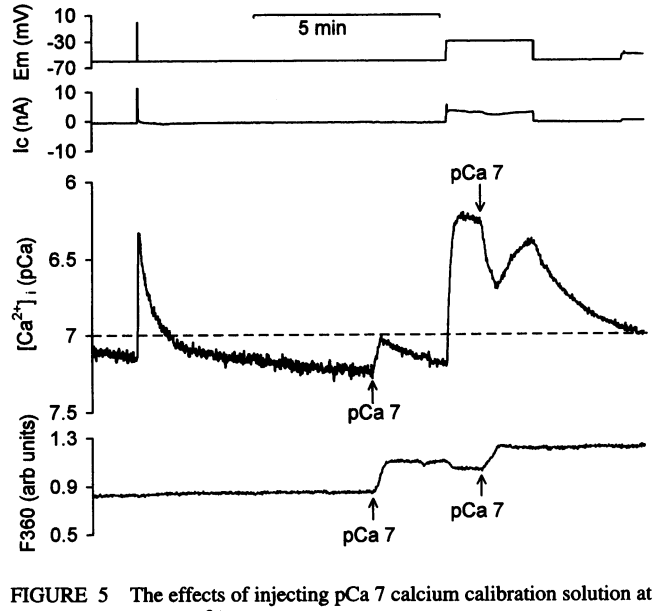

FIGURE 5 The effects of injecting pCa 7 calcium calibration solution at resting and raised $[Ca^{2+}]_i$ levels. Initial depolarization for 1 s to 0 mV. $[Ca^{2+}]_i$ was raised using a sustained (approximately 2 min) depolarization to −30 mV. Arrows indicate the injection of calcium calibration solution. For clarity, the clamp current is shown between −10 and 10 nA.

decrease in $[Ca^{2+}]_i$, as expected. In four experiments (results not shown) prolonged depolarization failed to raise $[Ca^{2+}]_i$ to more than 316 nM (pCa_i 6.5). In these experiments injections of pCa 6.5 calibration solution caused an increase in the ratio signal, corresponding to an increase in $[Ca^{2+}]_i$. Hence, the effects of injecting calibration solutions at both resting and raised $[Ca^{2+}]_i$ corresponded well with the values for $[Ca^{2+}]_i$ calculated using the in vitro calibration of fura-2.

The effects of injecting calibration solutions at resting and raised $[Ca^{2+}]_i$ measured with Ca^{2+}-sensitive microelectrodes

To investigate the previously reported discrepancy (Kennedy and Thomas, 1995) between the fura-2- and Ca^{2+}-sensitive microelectrode measurements of resting $[Ca^{2+}]_i$, we used pressure injection of calibration solutions to calibrate the microelectrode in vivo. In one set of experiments we injected different cells with one of three different calibration solutions colored with Fast Green; pCa 7, pCa 7.4, or pCa 8. We attempted to inject enough dyed solution to make a bolus inside the cell, which appeared to be at first about 10 μm in diameter. We depolarized the cell briefly at intervals to see how much the injected BAPTA had increased intracellular Ca^{2+} buffering. After the first few injections at the normal holding potential we changed $[Ca^{2+}]_i$ by depolarizing or applying high pH or caffeine. An experiment representative of the five successful ones done with pCa 7 solution is shown in Fig. 6.

In this experiment, as in most cases, the Ca^{2+}-sensitive microelectrode insertion required considerable movement of the electrode tip. Often the tip was apparently deep within the cell, as observed through the microscope, before it showed any electrical signs of entering the cell. Once in the cell, to help the Ca^{2+}-sensitive microelectrode seal in, we increased the external Ca^{2+} from 7 to 35 mM. This reduced the clamp current and often accelerated the recovery from damage during impalement. For clarity, the experiments have only been shown from where the Ca^{2+}-sensitive microelectrode potential had stabilized, just before the voltage-clamp electrode was removed and replaced with the injection electrode. In this case the KCl-filled clamp electrode was removed 28 min after the insertion of the Ca^{2+}-sensitive microelectrode (*first solid arrow*). This withdrawal caused a paradoxical dramatic negative shift in the voltage recorded by the Ca^{2+}-sensitive microelectrode (V_{Ca}). This negative shift in V_{Ca} suggests that a Ca^{2+} leak that had been raising the local $[Ca^{2+}]_i$ at the Ca^{2+}-sensitive microelectrode tip was somehow reduced, so that the local $[Ca^{2+}]_i$ had fallen severalfold. As the clamp electrode was removed, the cell membrane must have changed its position in such a way that the Ca^{2+}-sensitive microelectrode sealed in better. We then replaced the KCl electrode with one filled with the pCa 7 calibration solution (*second solid arrow*), inserted it into the cell, and switched the voltage clamp on again. We then tried several pressure injections, at increasing pressures, but saw no green spot. So we switched off the clamp, withdrew the electrode (*third solid arrow*), broke its tip slightly on a nearby pin, reinserted it (*fourth solid arrow*), and switched the clamp on again. Once $[Ca^{2+}]_i$ had stabilized we again attempted a pressure injection, at the point indicated by the first arrow (a) above the V_{Ca} record. This time green dye was clearly visible.

This first injection (a) caused an apparent brief increase in $[Ca^{2+}]_i$ (perhaps due to Ca^{2+} influx or release from

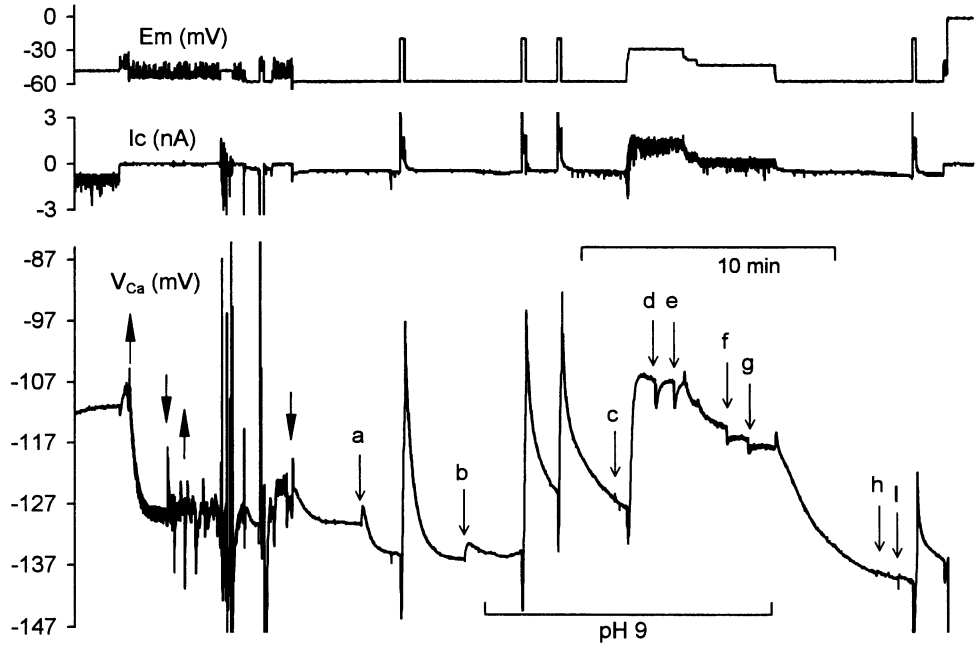

FIGURE 6 Calibrating a Ca^{2+}-sensitive microelectrode in vivo by injecting pCa 7 calibration solution. The four solid arrows above the V_{Ca} record indicate two removals and replacements of current electrode. Arrows (*a–i*) indicate pressure injections of pCa 7 calibration solution. Clamp current above 3 nA is not shown. Where indicated, superfusate had a pH of 9.

stores) followed by an undershoot to what seemed to be a new stable level (V_{Ca} = −136 mV). The reason for the undershoot following only the first injection is not clear, although it is probably due to the injected buffer somehow reducing a leak, or the effect of a leak, around the Ca^{2+}-sensitive microelectrode tip.

Next we depolarized the cell by 40 mV for 10 s. This caused a much larger change in V_{Ca} than that seen before any injections had been made (not shown). After one more injection (*arrow b*) we depolarized the cell after raising the external pH to increase $[Ca^{2+}]_i$ by inhibiting Ca^{2+} extrusion (Schwiening et al., 1993). We made five more injections (*arrows c–g*) at elevated $[Ca^{2+}]_i$ levels before returning external pH and E_m to normal. Injection c was made when V_{Ca} was −126 mV; it had no effect. Injection d was made at a V_{Ca} of −106 mV; it caused an apparent decrease in $[Ca^{2+}]_i$, as did injections e, f, and g. If the changes in V_{Ca} caused by injections b, c, and d were due simply to $[Ca^{2+}]_i$ equilibrating between the cytoplasm and the injected solution, the null effect at injection c, when V_{Ca} was −126 mV, suggests that $[Ca^{2+}]_i$ was then the same (100 nM) as in the pCa 7 solution injected.

If the relationship in the cell between V_{Ca} and pCa was the same as seen in a typical in vitro calibration curve, which gives 29 mV per pCa, we can estimate that the new stable pCa_i after the first injection (when V_{Ca} was −136 mV) was 7.0 + 10/29 = 7.4. Before that first injection the corresponding pCa_i was 7.2.

An experiment representative of three in which we injected pCa 8 calibration solution colored with Fast Green is shown in Fig. 7. Again we had to withdraw and break the pCa 8 injecting microelectrode (*solid arrows*) before we were able to make a successful pressure injection. This first injection (a) caused a large transient apparent increase in $[Ca^{2+}]_i$, followed by a sustained undershoot. Again the subsequent test depolarization caused a much larger V_{Ca} transient than when it was applied before the pCa 8 injection. Injections b, c, and d all caused small increases in $[Ca^{2+}]_i$. We then depolarized the cell for several minutes and increased external pH. Injections e, f, and g all caused apparent falls in $[Ca^{2+}]_i$. The V_{Ca} at which injections would cause no change was estimated graphically at −146 mV. The corresponding pCa_i after the first injection was 8.2.

Injection of calibration solutions with added fura-2

Coloring the injection solution with fura-2 has the advantage that the relative size can be estimated from the 360-nm fluorescence, but the disadvantage is that injections cannot be seen directly. Fig. 8 shows one of four experiments in which pCa 7.4 calibration solutions containing ~4 mM BAPTA and 0.2 mM fura-2 were injected after V_{Ca} had stabilized and the KCl current electrode had been replaced (*solid arrows*) with one that allowed injections of pCa 7.4 solution (the first trial failed).

The first successful injection (a) caused a rapid negative shift in V_{Ca}, or a fall in the apparent $[Ca^{2+}]_i$, and an increase in the response to a 40 mV depolarization. The next injection (b), about 9 min later, caused another decrease in $[Ca^{2+}]_i$, but the third (c) was made during superfusion of a Ca^{2+}-free saline, which lowered $[Ca^{2+}]_i$, and this injection caused a small increase in $[Ca^{2+}]_i$. The V_{Ca} at which there would have been no change was estimated to be −146 mV, and from this we calculate that the stable pCa_i about 5 min before the end of the experiment was 7.3. This estimate agrees well with the observation that the last injection (*arrow e*), made at this time, had no effect on either the V_{Ca}

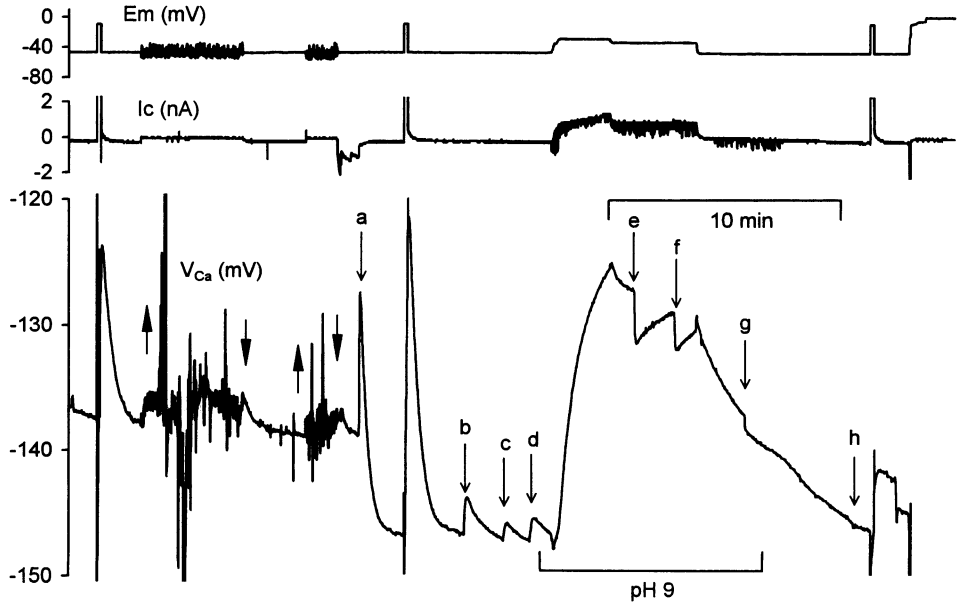

FIGURE 7 Calibrating a Ca^{2+}-sensitive microelectrode in vivo by injecting pCa 8 calibration solution. Double-ended arrows above the V_{Ca} record show where the electrode position was adjusted. Arrows (*a–h*) show where pressure injections were made.

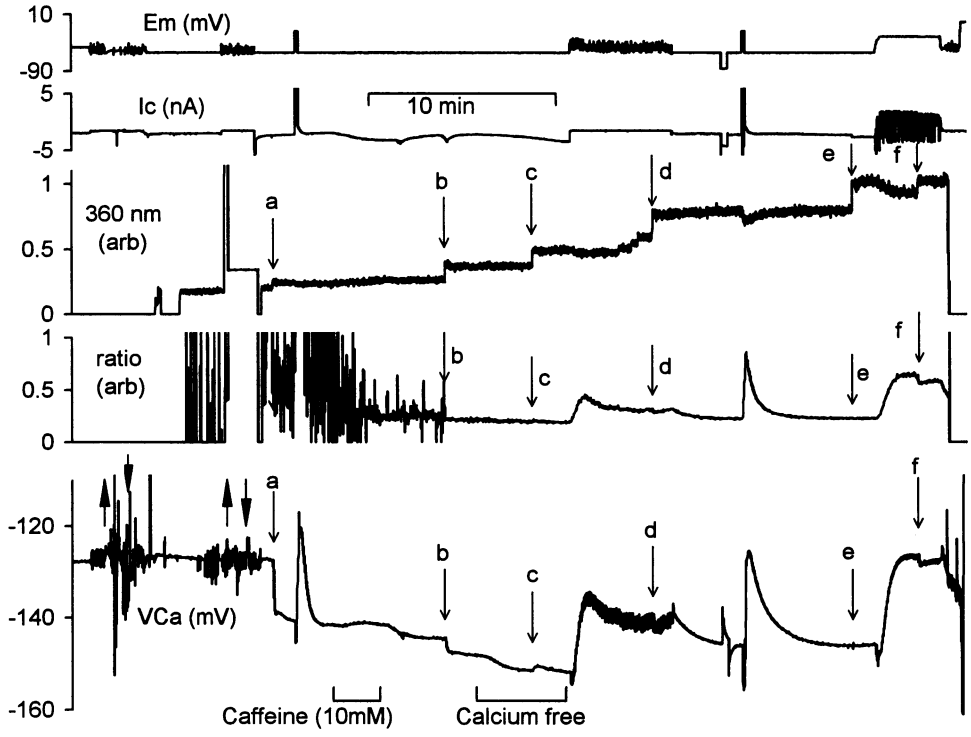

FIGURE 8 Calibration of Ca^{2+}-sensitive microelectrode by injection of a pCa 7.4 calibration solution containing fura-2. As well as E_m, I_c, and V_{Ca}, the total fluorescence at 360 nm and the 340/380 nm fluorescence ratio (both in arbitrary units) were recorded once fura-2 had been injected. Arrows (*a–f*) indicate successful injections of calibration solution.

or the fura-2 ratio, although the 360 nm record shows it was a large injection.

Average resting $[Ca^{2+}]_i$ calculated from the Ca^{2+}-sensitive microelectrode experiments

In a total of 13 experiments we were able to make repeated injections of calibration solutions, of which at least one injection after the first in each experiment caused an apparent decrease, and at least one caused an increase or no change in $[Ca^{2+}]_i$. (In several other experiments all injections caused an increase in the apparent $[Ca^{2+}]_i$, but we have not analyzed these.) For each of the 13 experiments we graphically estimated the null V_{Ca} (i.e., the V_{Ca} at which an injection would have caused no change) and assumed that at this V_{Ca} the pCa in the cell was the same as in the injected calibration solution. (We made no allowance for the progressive increase in buffering as more and more BAPTA was injected, or any variation in injection size.) Assuming that the Ca^{2+}-sensitive microelectrode generated a 29-mV change for a tenfold change in $[Ca^{2+}]_i$ as it did in vitro, we used the null value to convert the intracellular V_{Ca} values to pCa_i. The resulting stable resting intracellular pCa values measured after at least one injection are plotted (*triangles* and *crosses*) against the corresponding V_{Ca} in Fig. 9. The average such pCa_i was thus found to be 7.6 (n = 13) or 25 nM. We also plot in Fig. 9 a typical extracellular calibration curve obtained with a fresh Ca^{2+}-sensitive microelectrode.

The intracellular measurements all fall above the in vitro calibration line, suggesting that the electrode sensitivity to calcium may somehow be offset inside the cell (see Blatter and Blinks, 1991). This difference between in vivo and in vitro performance of the Ca^{2+}-sensitive microelectrodes may explain in part why our previous values for resting $[Ca^{2+}]_i$, obtained when the electrodes were calibrated after withdrawal from the cell, were 170 nM (pCa_i of 6.77) (Kennedy and Thomas, 1995).

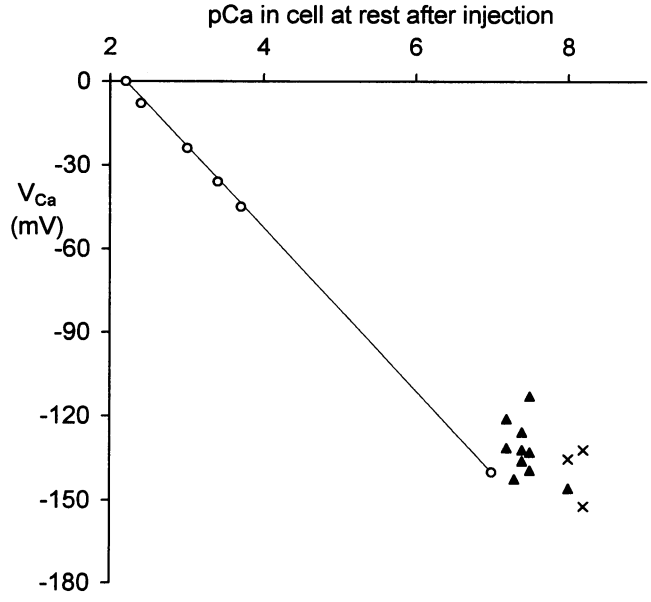

FIGURE 9 Ca^{2+}-sensitive microelectrode responses to calcium in vitro and in vivo. ○, Calibration in vitro of a freshly made Ca^{2+}-sensitive microelectrode. ▲, Resting pCa values in cells after at least one injection of pCa 7 or 7.4 calibration solution. ×, Resting pCa values in three cells after at least one injection of pCa 8 calibration solution.

DISCUSSION

Our results with both fura-2- and Ca^{2+}-sensitive microelectrodes suggest that the average $[Ca^{2+}]_i$ in the cytoplasm of snail neurons held at membrane potentials between −50 and −60 mV is about 40 nM (pCa_i 7.4). With fura-2, calibrated in vitro average $[Ca^{2+}]_i$ was 38 nM (pCa_i was 7.42 ± 0.05, mean ± SEM, $n = 31$), and with Ca^{2+}-sensitive microelectrodes calibrated in vivo, using the null point method, it was 25 nM (pCa_i 7.6 ± 0.10, mean ± SEM, $n = 13$). Pressure injections of calcium calibration solutions verified that the in vitro calibration of fura-2 was a reasonably accurate method for determining $[Ca^{2+}]_i$ in the range pCa 6.5–7.5.

Accuracy of our calibration solutions

Both methods of measuring the resting $[Ca^{2+}]_i$ are dependent on knowing the $[Ca^{2+}]$ in the calcium calibration solutions accurately. Unfortunately there is, as yet, no international standard for calcium buffer solutions with very low $[Ca^{2+}]$. Thus, calcium calibration solutions must be prepared in the laboratory. This is a complicated and time-consuming process with many opportunities for error, and the techniques vary between laboratories. Many workers prepare their solutions using recipes and equations, rather than actual measurements, to allow for the effects of temperature, ionic strength, and pH on the Ca^{2+} buffer they have chosen (Harrison and Bers, 1989). However, few take into account the actual purity of their buffer. This is likely to be <100% because of impurities, such as water, in the buffer itself, or inaccuracies in measuring out the chemical. Clearly the level of calcium in a calibration buffer is critically dependent on the amount of Ca^{2+} buffer added to the solution.

Our calibration buffer solutions were prepared using the methods of McGuigan et al. (1991), but we used BAPTA rather than EGTA to buffer our calcium, as it is relatively insensitive to pH (Tsien, 1980). The method relies on making up calibration solutions at the correct pH, ionic strength, and temperature, with $[Ca^{2+}]$ initially in the range of pCa 4–6. The potential in these solutions is measured with a Ca^{2+}-sensitive macroelectrode (previously calibrated using solutions with the same ionic concentrations but no BAPTA and relatively high calcium, pCa 2–4). Scatchard plot analysis of these measurements enables the purity and binding constant of the BAPTA to be determined (see McGuigan et al., 1991). The final calibration solutions in the range pCa 6–8 can then be prepared, taking these two extra factors into account. As the solutions already contain all the other ions and have the correct pH, no additional corrections are needed with this method. In the present case, before using the solutions in experiments they were all measured using a Ca^{2+}-sensitive microelectrode (Nernstian to pCa 9 when used in vitro) to check for errors.

How valid is the key assumption that injection does not cause influx or release of Ca^{2+}?

Our in vivo calibration techniques depend on the key assumption that the free Ca^{2+} concentration in a solution that causes no change in measured $[Ca^{2+}]_i$ when injected is the same as that in the cytoplasm. With the Ca^{2+}-sensitive microelectrode in vivo calibration, the first injection often caused a brief increase in $[Ca^{2+}]_i$ followed by a reduction. The increase may have been due to a brief release of stored Ca^{2+} or a mechanically induced increase in influx. If all injections did this to some extent, the null V_{Ca} would tend to be too low. On the other hand, pCa_i values determined by the injection of pCa 8 solution seemed to be rather high, as if leakage of BAPTA from the voltage-clamp electrode might be lowering $[Ca^{2+}]_i$. If we calculate our mean pCa_i without data for pCa 8 injection, it becomes 7.44 ± 0.07 (mean ± SEM; $n = 10$).

With fura-2 measurements, calibration solution injections only caused small increases or decreases in $[Ca^{2+}]_i$, as expected from the in vitro calibration of fura-2. Occasionally the cells were damaged during injection, presumably because of electrode movement. This resulted in a large increase in $[Ca^{2+}]_i$ and clamp current. Such results were not included in the analysis.

Validity of in vitro calibration of fura-2

We have previously tried to calibrate fura-2 in vivo by pressure injection of $CaCl_2$ or EGTA, or by permeabilizing the cell with ionomycin (Kennedy and Thomas, 1995). However, this proved to be impossible without killing the cell. We therefore calibrated our fura-2 in vitro using calcium calibration solutions that contained ionic concentrations close to those found in snail cytoplasm but no proteins or other agents to increase viscosity. Many studies have shown that fluorescent indicators such as fura-2 are affected by changes in viscosity, ionic strength, and binding to intracellular proteins (Grynkiewicz et al., 1985; Konishi et al., 1988; Poenie, 1990; Uto et al., 1991; Hollingworth et al., 1992; Westerblad and Allen, 1994). There are also reports that some fluorescent indicators may not change their dissociation constant for Ca^{2+} upon binding to intracellular proteins (Ikenouchi et al., 1991). Thus, fluorescent indicators may, in some instances, behave quite differently inside the cell compared to in vitro calibration solutions. This can lead to errors if in vitro calibrations, or the apparent dissociation constant for Ca^{2+} (K_D) from in vitro measurements, is used to estimate $[Ca^{2+}]_i$. It is perhaps surprising, therefore, that our values for resting $[Ca^{2+}]_i$ calculated from the in vitro calibration of fura-2 are in reasonable agreement with the effects of injecting calcium calibration solutions in the range of pCa 6.5–7.5. This suggests that at resting $[Ca^{2+}]_i$ levels our in vitro calibration provides a reasonably accurate value for $[Ca^{2+}]_i$. However, in experiments in which pCa 8 solution was injected, our calculated $[Ca^{2+}]_i$ actually reached the same pCa as the solution (we would

only have expected it to tend toward pCa 8), indicating that at these very low $[Ca^{2+}]_i$ levels the in vitro calibration of fura-2 gives a less accurate estimate of $[Ca^{2+}]_i$, tending to underestimate the in vivo $[Ca^{2+}]_i$ level.

As changes in viscosity and ionic strength may affect fura-2, it seems possible that injection of large amounts of buffer solutions may affect the intracellular environment. Westerblad and Allen (1993) used indo-1 to measure $[Ca^{2+}]_i$ in intact mouse muscle fibres. They calibrated the dye using pressure injections of 0.5 M EGTA for R_{min}, 10 mM $CaCl_2$ for R_{max} and a 1:1 mixture of Ca-EGTA/EGTA to estimate the K_D. They found resting $[Ca^{2+}]_i$ in their cells to be 26 nM. Baylor et al. (1994) argue that this technique will cause swelling, damage, and a change in the intracellular environment, which will affect the value of resting $[Ca^{2+}]_i$. In our experiments pressure injections did occasionally cause visible cell swelling and a large increase in voltage-clamp current, presumably due to cell damage, but these experiments were not included in our analysis. In general, the only long-lasting effect of these injections was a slowing of the rate of recovery from Ca^{2+} loads (see Fig. 3 *B*), as would be expected from the increase in intracellular buffering. The level of $[Ca^{2+}]_i$ as measured by fura-2 always returned to the same resting levels over a period of several minutes after both increases and decreases brought about by buffer injection.

The effect of BAPTA on the Ca^{2+}-sensitive microelectrode voltage

The effect of even small injections of calibrating solution on the voltage recorded by an intracellular Ca^{2+}-sensitive microelectrode in a neuron not previously loaded with BAPTA or fura-2 was very striking. The average negative shift in V_{Ca} seen after the first injection of calibration solution was 10 mV ($n = 13$). It was as if the injected buffer either reduced a Ca^{2+} leak or, more likely, reduced its effect on the Ca^{2+} level at the Ca^{2+}-sensitive microelectrode tip, because no such effects were seen with fura-2. One possible explanation is that the mobility of the injected buffer allows the Ca^{2+} leaking in around the point of insertion to diffuse away more rapidly, so that the electrode tip is less affected. The supposed leak could in some cases apparently be reduced suddenly by movements of the cell membrane, as seen in the experiment of Fig. 6, but the injection of mobile buffer always reduced the apparent $[Ca^{2+}]_i$. Neher and Augustine (1992) suggest that there may be little or no mobile Ca^{2+} buffer in a normal cell. If so, the diffusion of Ca^{2+} in a cell with fura-2 or other small buffers added may be abnormal. Presumably larger, less mobile indicators such as fura-2 coupled to dextran would avoid this distortion.

Ca^{2+}-sensitive microelectrode problems

Our present results suggest that our previous Ca^{2+}-sensitive microelectrode measurement of resting $[Ca^{2+}]_i$ was too high by at least 100 nM. There seem to be two possible reasons for this. The first is that without added mobile buffer, a small leak at the point of electrode insertion causes a large and persistent but very local increase in $[Ca^{2+}]_i$. The second may be that the Ca^{2+}-sensitive microelectrode is somehow less sensitive inside the cell than in vitro. Perhaps the intracellular milieu somehow generates about 10 mV of error. The cytoplasm may contain some component, not present in the calibration solution, to which the electrode is sensitive.

Our finding that the first injection of BAPTA caused a large fall in the apparent $[Ca^{2+}]_i$ as recorded by a well-inserted Ca^{2+}-sensitive microelectrode suggests that Ca^{2+}-sensitive microelectrodes must be used with caution for measuring calcium in any situation where calcium is likely to be increased by leakage around the point of insertion, such as intracellularly. Such leaks around the point of insertion of microelectrodes have been reported previously for sodium in crustacean muscle (Taylor and Thomas, 1984) and are very hard to eliminate. The methods we have used to calibrate in vivo may themselves permanently change the resting $[Ca^{2+}]_i$ level, but this seems unlikely. Incremental injections of extra BAPTA after the first one have little effect even when the buffering power is so high that transients are visibly slower.

Comparison with other resting $[Ca^{2+}]_i$ measurements

Many measurements of $[Ca^{2+}]_i$ have been made in a variety of different neuronal cell types. In *Helix aspersa* neurons Meech and Standen (1975) used the potential at which there was no apparent Ca^{2+} influx to estimate $[Ca^{2+}]_i$ and found it was between 30 and 80 nM. When fura-2 was used, $[Ca^{2+}]_i$ was found to be 90 nM in *Helix pomatia* neurons by Kostyuk et al. (1989), 136 nM in rat dorsal root ganglion neurons by Thayer and Miller (1990), 100 nM in bullfrog sympathetic neurons by Nohmi et al. (1992), 76 nM in bullfrog sympathetic neurons by Friel and Tsien (1992), 100 nM in cultured rat septal neurons by Bleakman et al. (1993), 47 nM in isolated rat nucleus basalis neurons by Tatsumi and Katayama (1993), 10–100 nM in *Helix* neurons by Muller et al. (1993), 100 nM in cultured rat cortical neurons by Ou-Yang et al. (1994), and 250 nM in rat isolated neurohypophyseal nerve endings by Stuenkel (1994). These and other studies using different fluorescent dyes indicate that $[Ca^{2+}]_i$ is probably within the range of 10–300 nM in neurons. It is not clear whether this large range is physiological or is an indication of inaccurate measurements.

The resting level of $[Ca^{2+}]_i$ is dependent on many experimental conditions, including the level of extracellular calcium in the bathing Ringer, the membrane potential, and whether or not the cell is voltage-clamped. Clearly, experimental conditions will vary between different studies, and these differences may, in part, explain some of the variation in the values for resting $[Ca^{2+}]_i$. In our study extracellular

calcium was 7 mM, and the neurons were voltage-clamped to between −50 and −60 mV, which should minimize the influx of Ca^{2+} through Ca^{2+} channels, perhaps leading to a relatively low $[Ca^{2+}]_i$ level.

In studies using Ca^{2+}-sensitive microelectrodes $[Ca^{2+}]_i$ was found to be 170 nM in *Helix aspersa* neurons (Alvarez-Leefmans et al., 1981), 130–180 nM in *Aplysia* neurons (Gorman et al., 1984; Levy and Tillotson, 1988), and 70 nM in rat CNS neurons (Silver and Erecinska, 1990).

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REFERENCES

Alvarez-Leefmans, F. J., T. J. Rink, and R. Y. Tsien. 1981. Free calcium ions in neurons of *Helix aspersa* measured with ion-selective microelectrodes. *J. Physiol.* 315:531–548.

Ammann, D., T. Buhrer, U. Schefer, M. Muller, and W. Simon. 1987. Intracellular neutral carrier-based Ca^{2+} microelectrode with subnanomolar detection limit. *Pflügers Archiv. Eur. J. Physiol.* 409:223–228.

Baker, P. F., A. L. Hodgkin, and E. B. Ridgeway. 1971. Depolarization and calcium entry in squid giant axons. *J. Physiol.* 218:709–755.

Bassani, J. W. M., R. A. Bassani, and D. M. Bers. 1995. Calibration of indo-1 and resting intracellular $[Ca]_i$ in intact rabbit cardiac myocytes. *Biophys. J.* 68:1453–1460.

Baylor, S. M., A. B. Harkins, and N. Kurebayashi. 1994. Response to Westerblad and Allen. *Biophys. J.* 66:927–928.

Blatter, L. A., and J. R. Blinks. 1991. Simultaneous measurement of Ca^{2+} in muscle with Ca electrodes and aequorin. *J. Gen. Physiol.* 98: 1141–1160.

Bleakman, D., J. D. Roback, B. H. Wainer, R. J. Miller, and N. L. Harrison. 1993. Calcium homeostasis in rat septal neurons in tissue culture. *Pflügers Arch. Eur. J. Physiol.* 600:257–267.

Friel, D. D., and R. W. Tsien. 1992. A caffeine- and ryanodine-sensitive Ca^{2+} store in bullfrog sympathetic neurons modulates effects of Ca^{2+} entry on $[Ca^{2+}]_i$. *J. Physiol.* 450:217–246.

Gorman, A. L. F., S. Levy, E. Nasi, and D. Tillotson. 1984. Intracellular calcium measured with calcium-sensitive micro-electrodes and Arsenazo III in voltage-clamped *Aplysia* neurons. *J. Physiol.* 353:127–142.

Grynkiewicz, G., M. Poenie, and R. Y. Tsien. 1985. A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. *J. Biol. Chem.* 260:3440–3450.

Harrison, S. M., and D. M. Bers. 1989. Correction of proton and Ca association constants of EGTA for temperature and ionic strength. *Am. J. Physiol.* 256:C1250–C1256.

Hollingworth, S., A. B. Harkins, N. Kurebayashi, M. Konishi, and S. M. Baylor. 1992. Excitation-contraction coupling in intact frog skeletal muscle fibres injected with mmolar concentrations of fura-2. *Biophys. J.* 63:224–234.

Ikenouchi, H., G. A. Peeters, and W. H. Barry. 1991. Evidence that binding of indo-1 to cardiac myocyte protein does not markedly change K_D for Ca. *Cell Calcium.* 12:415–422.

Kennedy, H. J., and R. C. Thomas. 1994. The effects of injecting calcium buffer solutions on intracellular calcium in snail neurons in isolated ganglia. *J. Physiol.* 479:11P.

Kennedy, H. J., and R. C. Thomas. 1995. Intracellular calcium and its sodium-independent regulation in voltage-clamped snail neurons. *J. Physiol.* 484:533–548.

Konishi, M., A. Olson, S. Hollingworth, and S. M. Baylor. 1988. Myoplasmic binding of fura-2 investigated by steady-state fluorescence and absorbance measurements. *Biophys. J.* 54:1089–1104.

Kostyuk, P. G., S. L. Mironov, A. V. Tepikin, and P. V. Belan. 1989. Cytoplasmic free Ca in isolated snail neurons as revealed by the fluorescent probe fura-2: mechanisms of Ca recovery after Ca load and Ca release from intracellular sites. *J. Membr. Biol.* 110:11–18.

Levy, S., and D. Tillotson. 1988. Effects of Na^+ and Ca^{2+} gradients on intracellular free Ca^{2+} in voltage-clamped *Aplysia* neurons. *Brain Res.* 474:332–342.

McGuigan, J. A. S., D. Luthi, and A. Buri. 1991. Calcium buffer solutions and how to make and use them: a do it yourself guide. *Can. J. Physiol. Pharmacol.* 69:1733–1749.

Meech, R. W., and N. B. Standen. 1975. Potassium activation in *Helix aspersa* neurons under voltage-clamp: a component mediated by calcium influx. *J. Physiol.* 249:211–239.

Muller, T. H., L. D. Partridge, and D. Swandulla. 1993. Calcium buffering in bursting *Helix* pacemaker neurons. *Pflügers Arch. Eur. J. Physiol.* 425:499–505.

Neher, E., and G. J. Augustine. 1992. Calcium gradients and buffers in bovine chromaffin cells. *J. Physiol.* 450:273–301.

Nohmi, M., S.-Y. Hua, and K. Kuba. 1992. Basal Ca^{2+} and the oscillation of Ca^{2+} in caffeine-treated bullfrog sympathetic neurons. *J. Physiol.* 350:513–528.

Orkand, R. K., and R. C. Thomas. 1995. Effects of low doses of caffeine on $[Ca^{2+}]_i$ in voltage-clamped snail *Helix aspersa* neurons. *J. Physiol.* 489:19–28.

Ou-Yang, Y., T. Kristian, P. Mellergard, and B. K. Siesjo. 1994. The influence of pH on glutamate and depolarisation-induced increases of intracellular calcium concentration in cortical neuron in primary culture. *Brain Res.* 646:65–72.

Poenie, M. 1990. Alteration of intracellular Fura-2 fluorescence by viscosity: a simple correction. *Cell Calcium.* 11:85–91.

Ross, W. N. 1993. Calcium on the level. *Biophys. J.* 64:1655–1656.

Schwiening, C. J., H. J. Kennedy, and R. C. Thomas. 1993. Calcium-hydrogen exchange by the plasma membrane Ca-ATPase of voltage-clamped snail neurons. *Proc. R. Soc. Lond.* 253:285–289.

Silver, I. A., and M. Erecinska. 1990. Intracellular and extracellular changes of $[Ca^{2+}]$ in hypoxia and ischemia in rat brain in vivo. *J. Gen. Physiol.* 95:837–866.

Stuenkel, E. L. 1994. regulation of intracellular calcium and calcium buffering properties of rat isolated neurohypophysial nerve endings. *J. Physiol.* 481:251–271.

Tatsumi, H., and Y. Katayama. 1993. Regulation of the intracellular free calcium concentration in acutely dissociated neurons from rat nucleus basalis. *J. Physiol.* 464:165–181.

Taylor, P. S., and R. C. Thomas. 1984. The effect of leakage on microelectrode measurements of intracellular sodium activity in crab muscle fibres. *J. Physiol.* 352:539–550.

Thayer, S. A., and R. J. Miller. 1990. Regulation of the intracellular free calcium concentration in single rat dorsal root ganglion neurons in vitro. *J. Physiol.* 425:85–115.

Thomas, R. C. 1994. Current construction protocol for calcium-sensitive intracellular microelectrodes. *J. Physiol.* 476:9P.

Tsien, R. Y. 1980. New calcium indicators and buffers with high selectivity against magnesium and protons: design, synthesis and properties of prototype structures. *Biochemistry.* 19:2396–2404.

Uto, A., H. Arai, and Y. Ogawa. 1991. Reassessment of fura-2 and the ratio method for determination of intracellular Ca^{2+} concentrations. *Cell Calcium.* 12:29–37.

Westerblad, H., and D. G. Allen. 1993. The influence of intracellular pH on contraction, relaxation and $[Ca^{2+}]_i$ in intact single fibres from mouse muscle. *J. Physiol.* 466:611–628.

Westerblad, H., and D. G. Allen. 1994. Methods for calibration of fluorescent calcium indicators in skeletal muscle fibres. *Biophys. J.* 66: 926–927.