Intracellular calcium and its sodium-independent regulation in voltage-clamped snail neurones

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1. We have used both Ca^{2+}-sensitive microelectrodes and fura-2 to measure the intracellular free calcium ion concentration ($[Ca^{2+}]_i$ or its negative log, pCa_i) of snail neurones voltage clamped to −50 or −60 mV. Using Ca^{2+}-sensitive microelectrodes, $[Ca^{2+}]_i$ was found to be ~174 nM and pCa_i, 6·76 ± 0·09 (mean ± S.E.M.; $n = 11$); using fura-2, $[Ca^{2+}]_i$ was ~40 nM and pCa_i, 7·44 ± 0·06 (mean ± S.E.M., $n = 10$).

2. Depolarizations (1–20 s) caused an increase in $[Ca^{2+}]_i$ which was abolished by removal of extracellular Ca^{2+}, indicating that the rise in $[Ca^{2+}]_i$ was due to Ca^{2+} influx through voltage-activated Ca^{2+} channels.

3. Caffeine (10–20 mM) caused an increase in $[Ca^{2+}]_i$ in the presence or absence of extracellular Ca^{2+}. The effects of caffeine on $[Ca^{2+}]_i$ could be prevented by ryanodine.

4. Thapsigargin, an inhibitor of the endoplasmic reticulum Ca^{2+}-ATPase, caused a small increase in resting $[Ca^{2+}]_i$ and slowed the rate of recovery from Ca^{2+} loads following 20 s depolarizations.

5. Neither replacement of extracellular sodium with *N*-methyl-D-glucamine (NMDG), nor loading the cells with intracellular sodium, had any effect on resting $[Ca^{2+}]_i$ or the rate of recovery of $[Ca^{2+}]_i$ following depolarizations.

6. The mitochondrial uncoupling agent carbonyl cyanide *m*-chlorophenylhydrazone (CCmP) caused a small gradual rise in resting $[Ca^{2+}]_i$. Removal of extracellular sodium during exposure to CCmP had no further effect on $[Ca^{2+}]_i$.

7. Intracellular orthovanadate caused an increase in resting $[Ca^{2+}]_i$ and prevented the full recovery of $[Ca^{2+}]_i$ following small Ca^{2+} loads, but removal of extracellular sodium did not cause a rise in $[Ca^{2+}]_i$. We conclude that there is no Na^+–Ca^{2+} exchanger present in the cell body of these neurones and that $[Ca^{2+}]_i$ is maintained by an ATP-dependent Ca^{2+} pump.

Intracellular Ca^{2+} is important in the regulation of cellular activity in many types of cells. Measurements using a variety of techniques have found the intracellular free calcium ion concentration ($[Ca^{2+}]_i$) generally to be in the range 20–200 nM under resting conditions. The millimolar extracellular Ca^{2+} concentration and negative resting potential provide a large electrochemical gradient for Ca^{2+} across the plasma membrane, the largest for any common inorganic ion. Even minor changes in the permeability of the membrane to Ca^{2+} can produce relatively large fluctuations in $[Ca^{2+}]_i$.

The level of $[Ca^{2+}]_i$ is determined by three types of processes: entry across the cell membrane, muffling within the cytoplasm, and extrusion of Ca^{2+}. By muffling we mean both chemical buffering and uptake by organelles. The entry of Ca^{2+} into excitable cells is predominantly through voltage-operated Ca^{2+} channels in the plasma membrane, although Ca^{2+} might enter via other mechanisms, e.g. receptor-operated channels and reverse Na^+–Ca^{2+} exchange (Baker, Blaustein, Hodgkin & Steinhardt, 1969). Once inside the cell, Ca^{2+} is bound to Ca^{2+}-binding proteins within the cytosol, or sequestered into intracellular organelles such as mitochondria, endoplasmic reticulum or synaptic vesicles. However, these intracellular stores have a finite capacity and in the long term Ca^{2+} must be extruded from the cell.

Three different transporters that extrude Ca^{2+} from cells have been identified, the Na^+–Ca^{2+} exchanger:

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Na^+–Ca^{2+}, K^+ exchanger and an ATP-dependent Ca^{2+} pump. The presence of a Na^+–Ca^{2+} exchanger in neurones was first demonstrated by Blaustein & Hodgkin (1969). They used cyanide to poison the squid giant axon and measured efflux of $^{45}Ca^{2+}$. Initially there was very little effect of cyanide on the rate of efflux, but after 1–2 h the $^{45}Ca^{2+}$ efflux increased to about ten times its original rate. Under these conditions the efflux of $^{45}Ca^{2+}$ was dependent on the presence of sodium ions (Na^+) in the extracellular medium. When extracellular sodium was replaced with lithium the rate of $^{45}Ca^{2+}$ efflux was greatly reduced. Further evidence for the Na^+–Ca^{2+} exchanger was provided by Baker *et al.* (1969). They demonstrated that the ouabain-insensitive part of Na^+ efflux from squid giant axon was abolished by removal of external Ca^{2+}. These experiments indicated that the exchanger could not only operate to extrude Ca^{2+} from the cell but was also reversible, causing an influx of Ca^{2+} coupled to Na^+ efflux.

The Na^+–Ca^{2+} exchanger has since been demonstrated in a variety of neuronal preparations including *Aplysia* neurones (Levy & Tillotson, 1988), synaptosomes from vertebrate brain (Sanchez-Armass & Blaustein, 1987), isolated *Helix pomatia* neurones (Kostyuk, Mironov, Tepikin & Belan, 1989), cultured rat sensory neurones (Benham, Evans & McBain, 1992), rat nucleus basalis (Tatsumi & Katayama, 1993) and cultured chick sensory neurones (Mirinov, Usachev & Lux, 1993). A related transporter, the Na^+–Ca^{2+}, K^+ exchanger has been identified in retinal rod outer segments (Cervetto, Lagnado, Perry, Robinson & McNaughton, 1989) and in synaptosomes (Coutinho, Carvalho & Carvalho, 1983). This exchanger similarly uses the energy from the sodium gradient for Ca^{2+} efflux but it also transports K^+ with a stoichiometry of $4Na^+$–$1Ca^{2+}$, $1K^+$.

Hence, there appear to be two Ca^{2+} transporters driven by the sodium gradient. Research into the exact role of the Na^+–Ca^{2+} exchanger in $[Ca^{2+}]_i$ regulation has been greatly hampered by the lack of a potent and specific inhibitor.

The existence of a specific ATPase that transports Ca^{2+} out of the cell was first described in erythrocytes by Schatzmann (1966). He showed that erythrocyte ghosts loaded with Ca^{2+} and ATP had a considerably faster Ca^{2+} efflux than cells treated in the same way except for the addition of ATP. This efflux occurred against a concentration gradient suggesting the presence of an ATP-powered Ca^{2+} pump. The existence of such a pump has since been demonstrated in a wide variety of cell types, including the squid giant axon (Baker & McNaughton, 1976) and brain synaptosomes (Rahamimoff & Abramovitz, 1978). Experiments on the squid giant axon (DiPolo & Beaugé, 1979) have shown that the Ca^{2+} pump has a high affinity for Ca^{2+} and is capable of regulating $[Ca^{2+}]_i$ down to physiological levels, approximately 0·18 μM. Recent experiments on snail neurones have demonstrated directly that the Ca^{2+} pump exchanges Ca^{2+} for H^+ (Schwiening, Kennedy & Thomas, 1993).

Mitochondria can accumulate $[Ca^{2+}]_i$. In isolated brain mitochondria, Rosgado-Flores & Blaustein (1987) demonstrated that under physiological conditions, when Ca^{2+} concentrations are 0·1–1 μM, mitochondria sequester very little Ca^{2+}. However, under conditions when the Ca^{2+} is much higher, mitochondria in snail neurones are capable of sequestering large quantities of Ca^{2+} (Meech & Thomas, 1980).

Ca^{2+} can also be stored by non-mitochondrial organelles, such as the endoplasmic reticulum. Ca^{2+} is thought to be released from the reticulum by a Ca^{2+}-induced Ca^{2+} release (CICR) mechanism. In 1968, Weber & Herz demonstrated that caffeine could release Ca^{2+} stored in the SR from skeletal muscle cells. The effect of caffeine on intracellular Ca^{2+} stores is now often used as an indicator for CICR, which has been widely shown to occur in neurones (Neering & McBurney, 1984).

We have found that $[Ca^{2+}]_i$ regulation in snail neurones does not require external sodium, but is driven by a vanadate-sensitive Ca^{2+} pump.

Early experiments on this subject have been briefly reported at meetings of The Physiological Society (Thomas & Kennedy, 1992; Kennedy & Thomas, 1993).

METHODS

General

All experiments were carried out on snail neurones in isolated suboesophageal ganglia. Cells were voltage clamped using two microelectrodes and held at a potential of −50 or −60 mV. $[Ca^{2+}]_i$ was measured using fura-2, and/or Ca^{2+}-sensitive microelectrodes using the experimental arrangement shown in Fig. 1. Depolarizations lasting for between 1 and 20 s were used to load the cell with Ca^{2+} and the recovery of Ca_i^{2+} from these loads was studied.

Preparation

Snails, *Helix aspersa*, were collected locally and kept aestivating until killed by rapid removal of the brain. The entire circumoesophageal ganglion was removed and placed dorsal side uppermost on a plastic bath insert. A clear perspex strip was placed over the nerves coming from the suboesophageal ganglia, and screwed down firmly to hold the ganglia in place. The connection between the right and left cerebral ganglia was cut. Small pins placed through the cerebral ganglion were used to slightly stretch the dissection and secure it to the bath insert. The thick connective tissue covering the visceral and pallial ganglia was removed and the bath insert was mounted in the experimental chamber at an angle of 45 deg. Once covered with snail Ringer solution, which was kept flowing until the preparation was discarded, the inner connective tissue was torn with a fine tungsten wire to expose the neurones. The preparation was then left for 30 min after which time damaged cells would appear swollen and opaque, whereas healthy cells looked translucent. All experiments were carried out at room temperature (18–22 °C).

Solutions

The normal snail Ringer solution contained (mM): NaCl, 80; KCl, 4; $CaCl_2$, 7; $MgCl_2$, 5; Hepes, 20; adjusted to pH 7·5 by addition of NaOH. In Na^+-free solutions NaCl and NaOH were replaced with *N*-methyl-D-glucamine (NMDG) or LiOH and adjusted to pH 7·5 using HCl. In K^+-free solutions KCl was replaced with NaCl and in solutions that contained no Na^+ or K^+ we replaced both with NMDG. In Ca^{2+}-free solutions, $CaCl_2$ was replaced with $MgCl_2$ and any calcium contamination was minimized by adding 1 mM EGTA. In experiments where vanadate (sodium orthovanadate) was used intracellularly, it was added to the voltage-clamp electrode (100 mM) and allowed to leak passively into the cell during the experiment. The stock orthovanadate solution was kept alkaline (pH ~9) to ensure that the anion was in the correct form. Caffeine (Sigma) was used at 10 or 20 mM and was dissolved directly into snail Ringer solution. Carbonyl cyanide *m*-chlorophenylhydrazone (CCmP, Sigma) and thapsigargin (Research Biochemicals Inc., Natick, MA, USA) were first dissolved in a small quantity of dimethyl sulphoxide (DMSO) which was in turn diluted at least 1000 times to give the final experimental concentration in snail Ringer solution.

Calibration solutions

The calibration solutions were made using the method of McGuigan, Luthi & Buri (1991) with minor modifications for use with snail neurones. All glassware was kept scrupulously clean and plastic ware was used wherever possible. Chemicals were from BDH, and were Aristar grade and Milli-Q water was used to minimize calcium contamination. Briefly, the solutions were prepared with ionic concentrations similar to those found in snail cytoplasm as follows (mM): K^+, 100; Na^+, 5; Mg^{2+} 1; buffered with Hepes, 10; and adjusted to pH 7·4. Calcium was buffered with BAPTA (4 mM), rather than EGTA, as it is less sensitive to pH. A 2-fold stock solution was prepared which was split into two equal portions so that both solutions contained identical concentrations of all the above compounds. To one solution 4 mM $CaCl_2$ 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 water was added to make a BAPTA solution. Solutions were then prepared by mixing the BAPTA and Ca-BAPTA solutions in different proportions. The Ca^{2+} in each solution was measured using a Ca^{2+}-sensitive macroelectrode (Philips IS561-Ca, Unicam, Cambridge, UK). The purity of BAPTA, its apparent binding constant and Ca^{2+} contamination were then calculated. Calibration solutions of pCa 6, 6·5, 7, 7·5 and 8 (where $pCa = -\log_{10}[Ca^{2+}]$) could then be prepared, taking into account the purity and apparent binding constant of BAPTA and Ca^{2+} contamination, by mixing the appropriate quantities of the BAPTA and Ca-BAPTA solutions.

Microelectrodes

Conventional. Micropipettes were pulled from filamented boro- or aluminosilicate glass tubing and backfilled with 2 M KCl for recording membrane potentials or passing current. Tips were broken, if necessary, by touching them on a pin in the bath to give resistances between 10 and 20 MΩ.

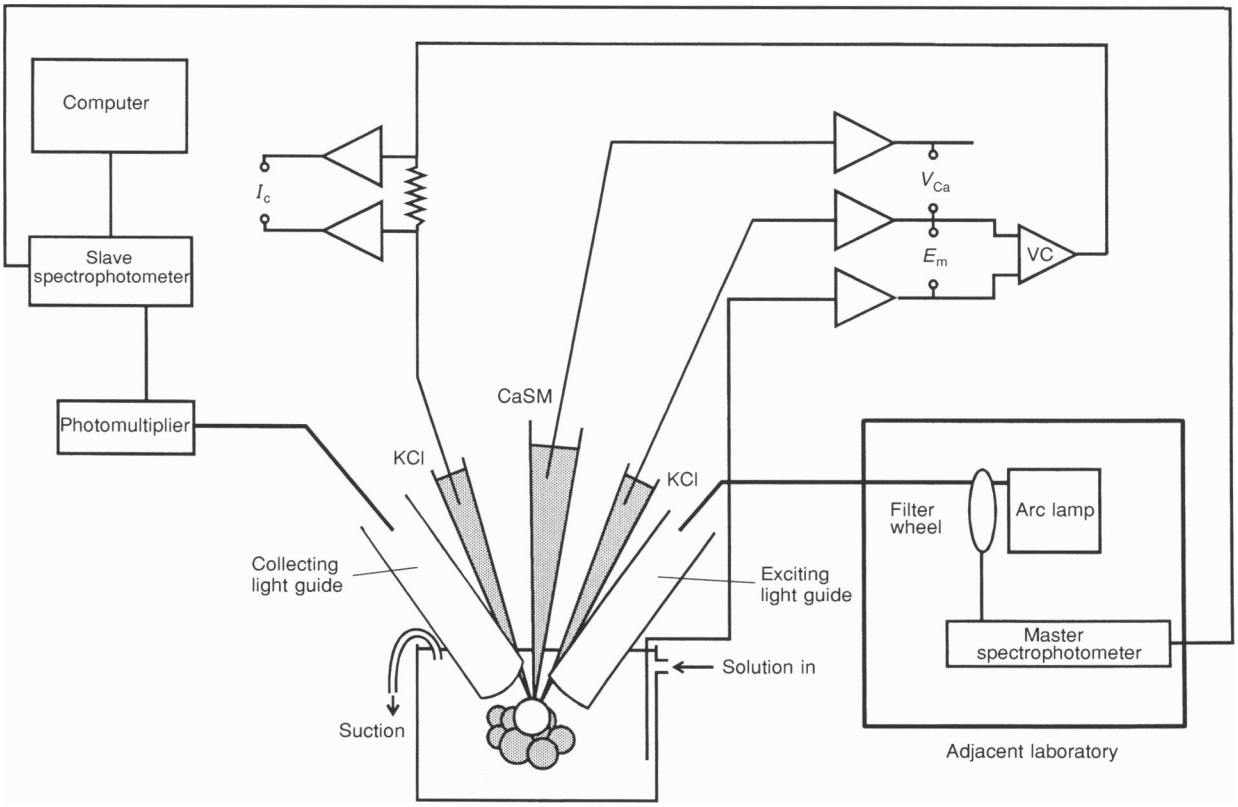

Figure 1. Diagram of the microelectrode and fluorescence apparatus

Not drawn to scale. CaSM, Ca^{2+}-sensitive microelectrode; VC, voltage clamp; I_c, clamp current; E_m, membrane potential; V_{Ca}, Ca^{2+}-sensitive microelectrode potential.

Ca^{2+} sensitive. Micropipettes were pulled in small batches from 1·5 mm diameter unfilamented aluminosilicate glass tubing. Tips were then broken to about 1·5 μm diameter and the pipettes baked in a miniature vacuum oven, volume about 20 ml, at 250 °C. After 5 min the oven was sealed and about 10 μl of bis(dimethylamino)-dimethylsilane was injected. After a further 4 min the oven was re-evacuated and allowed to cool. Selected micropipettes 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 was sucked into the tips under visual control from a small volume held in the end of a 0·2 mm diameter glass tube. The cocktail was a modification of that described by Ammann, Buhrer, Schefer, Muller & Simon (1987), and contained (mg ml^{-1}): Ca^{2+} ionophore ETH 129, 12; sodium tetrakis[3,5-bis(trifluoromethyl)phenyl]-borate, 6; 2-nitrophenyl octyl ether, 200; high molecular weight PVC, 33; and tetrahydrofuran, 749 (all from Fluka Biochemica, Buchs, Switzerland). The finished Ca^{2+}-sensitive microelectrodes were stored in air for at least an hour before use, and often required many minutes positioned in the bath before yielding a stable baseline potential. Before, or soon after insertion into a cell, air pressure of about 2 bar was applied to the back of the Ca^{2+}-sensitive microelectrodes and kept on throughout the experiment.

It usually required considerable downward movement of the Ca^{2+}-sensitive microelectrode to achieve a satisfactory insertion into a selected neurone. Often stability was improved by partial withdrawal. Once a stable potential was obtained, the penetration was regarded as satisfactory only if: (a) the current required to clamp the membrane potential at −60 mV was less than 3 nA, (b) the Ca^{2+}-sensitive microelectrode potential changed by less than 2 mV when the cell was hyperpolarized for 20 s by 30 mV, (c) $[Ca^{2+}]_i$ at least doubled when the cell was depolarized by 40 mV for 10 s, and in later experiments (d) $[Ca^{2+}]_i$ did not increase when external calcium was increased from 7 to 35 mM. Results were discarded if any electrode potential in normal snail Ringer solution changed by more than 5 mV during an experiment.

Calcium measurements with fura-2

A large (150–250 μm), well-exposed cell was chosen, before fura-2 injection, and illuminated with alternating 340, 360 and 380 nm light using a fibre optic system which has been described previously (Thomas & Schwiening, 1992). The photomultiplier voltage (supplied by the slave spectrophotometer) was increased until either the 340 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. The computer collected the total signal, and background fluorescence was subtracted during data analysis. The signals for the pen recorder and computer display were automatically corrected for background using a gain/offset module on the slave spectrophotometer.

Once background fluorescence had been recorded, the cell was impaled with a microelectrode containing 1 mM fura-2 dissolved in 100 mM KCl, with 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 which 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 which was then impaled with membrane-potential and voltage-clamp microelectrodes. Cells were considered to be healthy if: (a) the membrane potential was more negative than −40 mV when impaled with a 2 M KCl microelectrode, (b) the clamp current was less than ~4 nA when the cell was voltage clamped to −60 mV and (c) the cell was able to regulate Ca^{2+} loads following depolarizations.

Three methods of calibrating the fura-2 signal were tested: (a) an *in vivo* method using ionomycin to permeabilize the cell membrane and using Ca^{2+}-free and high-Ca^{2+} Ringer solutions to obtain maximum and minimum ratios, (b) an *in vivo* method using pressure injections of EGTA and $CaCl_2$ to produce maximum and minimum ratios and (c) calibration *in vitro* using small drops (10 μl) of calibrating solutions containing 10 μM fura-2 placed on a glass slide. The fluorescence from each drop was measured in turn by submerging the light guides in the calibration solutions. Calibration curves for fura-2 could then be plotted using the methods of Grynkiewicz, Poenie & Tsien (1985).

Following Alvarez-Leefmans, Rink & Tsien (1981) we have reported intracellular Ca^{2+} levels as the free calcium ion concentration ($[Ca^{2+}]_i$ or its negative $\log_{10}$, pCa_i) that in a calibration solution, with an ionic strength of ~0·13 M, would produce the same microelectrode potential or fura-2 ratio.

Electrical arrangement

The way in which the microelectrodes and light guides were positioned over the preparation and connected to amplifiers etc. is illustrated diagrammatically in Fig. 1. Voltages from the amplifiers were displayed on an oscilloscope and pen recorder and data were collected by a microcomputer.

Analysis of the rates of recovery

As the data were noisy, recoveries were first fitted with exponentials which were then used to calculate the rate of recovery at any given point. One exponential did not fit the data well but two exponentials did fit reasonably well. The recoveries from Ca^{2+} loads were fitted with a double exponential function in the form:

$$\text{Rate} = S_1 \exp(-t/\tau_I) + S_2 \exp(-t/\tau_{II}) + \text{offset},$$

where S_1 and S_2 are the two scalars, τ_I and τ_{II} are the two time constants in seconds and offset refers to the y-axis offset.

By normalizing the fura-2 ratio to a value of 1, and dividing the fura-2 ratio throughout the recovery by the resting fura-2 ratio at the start of the experiment, the rates of recovery at any given point in any experiment could be compared. To compare all the experimental data the rates of recovery were calculated from the equation at normalized ratios of 1·15, 1·5, 1·85, 2·2, 2·55 and 2·9. The rate was calculated at a given point by taking a small time before and after the required point and calculating the slope of the line. (This is essentially the same as drawing a tangent to the curve using the equation: $y = mx + c$, where x and y are known from the data at a given point, m is the gradient of the line and c is a constant.) The rates of recovery at each point could then be plotted against the normalized ratio.

RESULTS

Fura-2 is known to be affected by intracellular proteins, changes in ionic strength and viscosity, so ideally any calibration of the dye should be carried out *in vivo*. Attempts to calibrate the fura-2 signal in the cell by permeabilizing the cell membrane, using the ionophore ionomycin, failed. We found it impossible to clamp $[Ca^{2+}]_i$ to the level of extracellular Ca^{2+}. We believe that ionomycin was ineffective because the cells possess a powerful Ca^{2+} efflux pathway and are very large (~150 μM). We also attempted to obtain maximum and minimum ratios by pressure injection of EGTA or $CaCl_2$. Pressure injection of 1 mM EGTA gave a reproducible minimum ratio, which was similar to the *in vitro* minimum. However, pressure injections of $CaCl_2$ proved to be more difficult, mainly due to the electrodes blocking, and they rarely reached a stable maximum ratio. Using the *in vitro* method of calibration, the fluorescence ratio in several calibration solutions, containing different $[Ca^{2+}]$ values, could easily be measured and a calibration curve plotted. Such *in vitro* calibrations may be subject to some error so an *in vivo* method of calibration would be preferable.

We therefore tried to use Ca^{2+}-sensitive microelectrodes to calibrate the fura-2 ratio by measuring the resting $[Ca^{2+}]_i$, since microelectrodes were in theory easy to calibrate. At first calibrating the electrodes often proved unreliable even in known solutions, and gave very variable readings for $[Ca^{2+}]_i$. Typically when both fura-2 and microelectrodes were used in the same cell, the Ca^{2+}-sensitive microelectrode at first indicated a high $[Ca^{2+}]_i$ with no change on depolarization and only after much manipulation would record a lower $[Ca^{2+}]_i$ that increased on depolarization. Yet fura-2 in the same cell indicated a relatively low baseline $[Ca^{2+}]_i$, and large transient changes in $[Ca^{2+}]_i$ during depolarizations throughout the experiment, as illustrated in Fig. 2. Although Ca^{2+}-sensitive microelectrode performance improved with

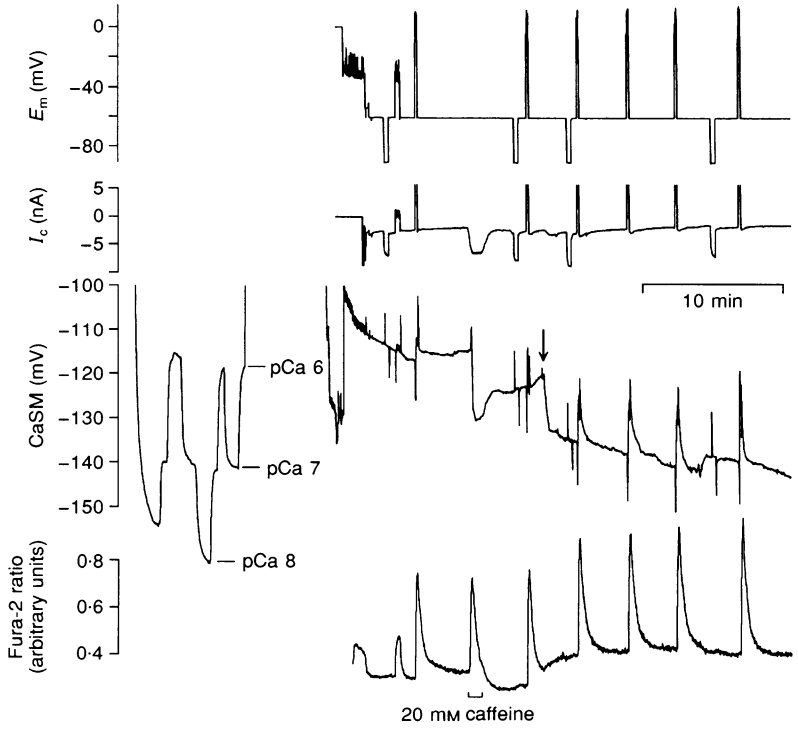

Figure 2. Measurement of $[Ca^{2+}]_i$ in a snail neurone with both a Ca^{2+}-sensitive microelectrode and the dye fura-2

Recordings are shown of membrane potential (E_m), clamp current (I_c), Ca^{2+}-sensitive microelectrode potential (CaSM), and fura-2 ratio. Before any microelectrodes were inserted the Ca^{2+}-sensitive microelectrode was calibrated by setting its potential to 0 mV in normal Ringer solution and then recording its potential in solutions of pCa 8, 7 and 6. The CaSM was inserted first, then the E_m and clamp microelectrodes. At intervals the holding potential was changed to test electrical subtraction of membrane potential from the Ca^{2+}, or the effect of opening voltage-activated Ca^{2+} channels. Caffeine was added to the superfusate for the period indicated and the Ca^{2+}-sensitive microelectrode was withdrawn slightly at the arrow. The clamp amplifier failed to keep E_m constant at −30 or 0 mV, and clamp currents greater than 5 nA were not recorded.

experience we have used Ca^{2+}-sensitive microelectrodes only to estimate an upper limit for resting $[Ca^{2+}]_i$, taking measurements only from recordings selected for minimal leakage. Due to the problems with Ca^{2+}-sensitive microelectrodes, most of the routine experiments were carried out using fura-2. In most cases we have not attempted to calibrate the fura-2 experiments, since they were used simply to study $[Ca^{2+}]_i$ recovery from depolarization-induced increases.

Normal resting $[Ca^{2+}]_i$

Ca^{2+}-sensitive microelectrode measurements. Most of the apparently high values of $[Ca^{2+}]_i$ recorded with Ca^{2+}-sensitive microelectrodes which were electrically fully inside the cell, as shown by the subtraction test, were probably due to leakage of external Ca^{2+} into the cell at the point of penetration. We found that such a leak could be detected by bathing the cell in elevated (from the normal 7 to 35 mM) external calcium. This increase usually, but not always, caused a rapid increase in the apparent $[Ca^{2+}]_i$. An example of a Ca^{2+}-sensitive microelectrode record which showed no increase in $[Ca^{2+}]_i$ with this test is shown in Fig. 3. Only eleven experiments satisfied all the criteria for electrical and chemical integrity, as listed in the Methods section. In these experiments resting pCa_i was found to be $6{\cdot}76 \pm 0{\cdot}09$ (mean ± S.E.M.; $n = 11$), equivalent to a $[Ca^{2+}]$ of ~174 nM.

Fura-2 measurements. In all experiments that met our criteria $[Ca^{2+}]_i$ was maintained at a steady level under control conditions. Having calibrated the fura-2 *in vitro*, and assuming that fura-2 behaved the same way in snail cytoplasm as in our calibration solutions, we calculated the normal resting $[Ca^{2+}]_i$. This was found to be pCa_i $7{\cdot}44 \pm 0{\cdot}06$ (mean ± S.E.M., $n = 10$) equivalent to a $[Ca^{2+}]$ of ~40 nM. This is well within the range of values measured with fura-2 in other neurones, but much lower than our Ca^{2+}-sensitive microelectode value.

Calcium entry

On depolarization to potentials around zero, Ca^{2+} enters neurones through voltage-activated Ca^{2+} channels. We used depolarizations to 0 mV for between 1 and 20 s to load the cells with Ca^{2+} and studied the recovery of $[Ca^{2+}]_i$

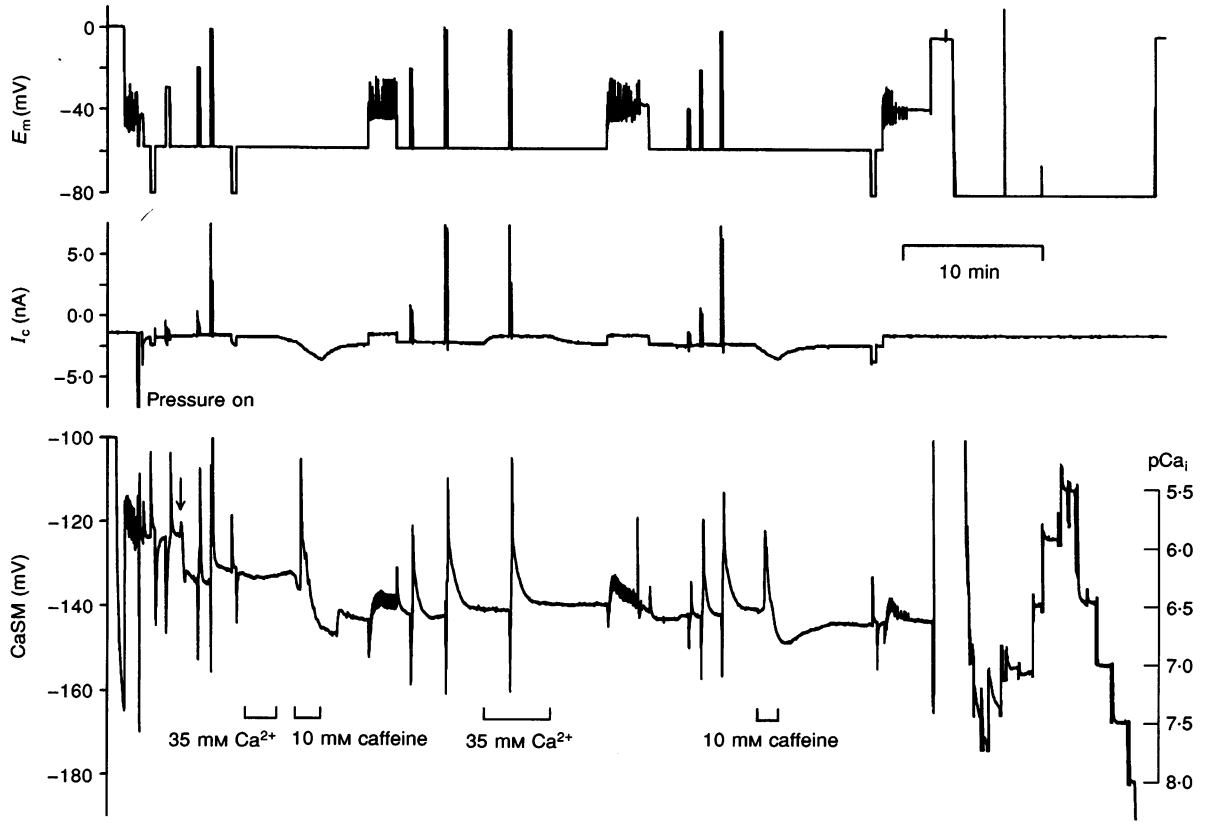

Figure 3. An example from one of eleven experiments in which the Ca^{2+}-sensitive microelectrode satisfied all criteria for a valid measurement of $[Ca^{2+}]_i$

Records of membrane potential, clamp current and Ca^{2+}-sensitive microelectrode potential. The arrow indicates the point at which pressure was applied to the back of the Ca^{2+}-sensitive microelectrode. Where indicated, external calcium was raised to 35 mM, or caffeine was applied at 10 mM. At the end the Ca^{2+}-sensitive microelectrode was calibrated in solutions of pCa 5·5 to 8, as indicated.

following such loads. The experiments illustrated in Fig. 4 show that external calcium is required for $[Ca^{2+}]_i$ to increase during a depolarization, but not when caffeine is applied.

Near the beginning of the experiment shown in Fig. 4*A* two control depolarizations were given and $[Ca^{2+}]_i$ increased. When the cell was repolarized $[Ca^{2+}]_i$ recovered to resting levels. When extracellular calcium was removed the resting level of $[Ca^{2+}]_i$ decreased, presumably due to either a decreased leak of Ca^{2+} into the cell or as a result of increased Ca^{2+} efflux. Further depolarizations failed to produce a rise in $[Ca^{2+}]_i$ as would be expected if Ca^{2+} entry through voltage-activated channels was responsible for the increase in $[Ca^{2+}]_i$ during depolarizations.

Many neurones store Ca^{2+} intracellularly. Some of the stored Ca^{2+} can be released by caffeine. The experiment in Fig. 4*B* shows that after approximately 8 min in Ca^{2+}-free Ringer solution depolarizations were unable to produce a rise in $[Ca^{2+}]_i$, but application of 20 mM caffeine produced a release of intracellularly stored Ca^{2+} even though membrane potential (E_m) was held at −60 mV.

The effects of caffeine and ryanodine

Many workers have shown that ryanodine prevents calcium or caffeine from releasing Ca^{2+} from intracellular stores. In the experiment shown in Fig. 5 we used caffeine and ryanodine to determine whether CICR was contributing to the rise in $[Ca^{2+}]_i$ during depolarizations.

Initially, a control depolarization for 10 s was given and $[Ca^{2+}]_i$ was then allowed to recover from the increase. Application of 10 mM caffeine caused a second increase in $[Ca^{2+}]_i$ presumably by releasing intracellularly stored Ca^{2+}. A depolarization in normal snail Ringer solution was then given to load the cell with Ca^{2+} and ensure that the intracellular stores contained some Ca^{2+}. This was then followed by ryanodine application (10 μM) for approximately 22 min. During this time caffeine was applied twice. The first application produced a rise in $[Ca^{2+}]_i$ which was smaller than the control application of caffeine in normal snail Ringer solution. The second application of caffeine, however, had very little effect on $[Ca^{2+}]_i$. This suggests that the caffeine-sensitive Ca^{2+} stores were no longer able to release Ca^{2+}. Subsequent depolarizations gave changes in $[Ca^{2+}]_i$ very similar to those seen with control depolarizations. This indicates that the majority of the $[Ca^{2+}]_i$ increase during depolarizations to 0 mV for 10 s is due to Ca^{2+} influx through channels rather than as a result of CICR. Similar results were obtained in a total of four experiments.

The effects of thapsigargin

A different way of investigating the possible contribution of the intracellular stores to the maintenance of resting $[Ca^{2+}]_i$ and recovery from Ca^{2+} loads is to apply thapsigargin. This is known to block the intracellular Ca^{2+}-ATPase responsible for uptake of Ca^{2+} into stores, but does not inhibit the plasma membrane ATPase (Thastrup, Cullen, Drobak, Hanley & Dawson, 1990).

In the experiment shown in Fig. 6, two control depolarizations were given and the increase in $[Ca^{2+}]_i$ was allowed to recover. Thapsigargin application then caused a small increase in resting $[Ca^{2+}]_i$. When depolarizations

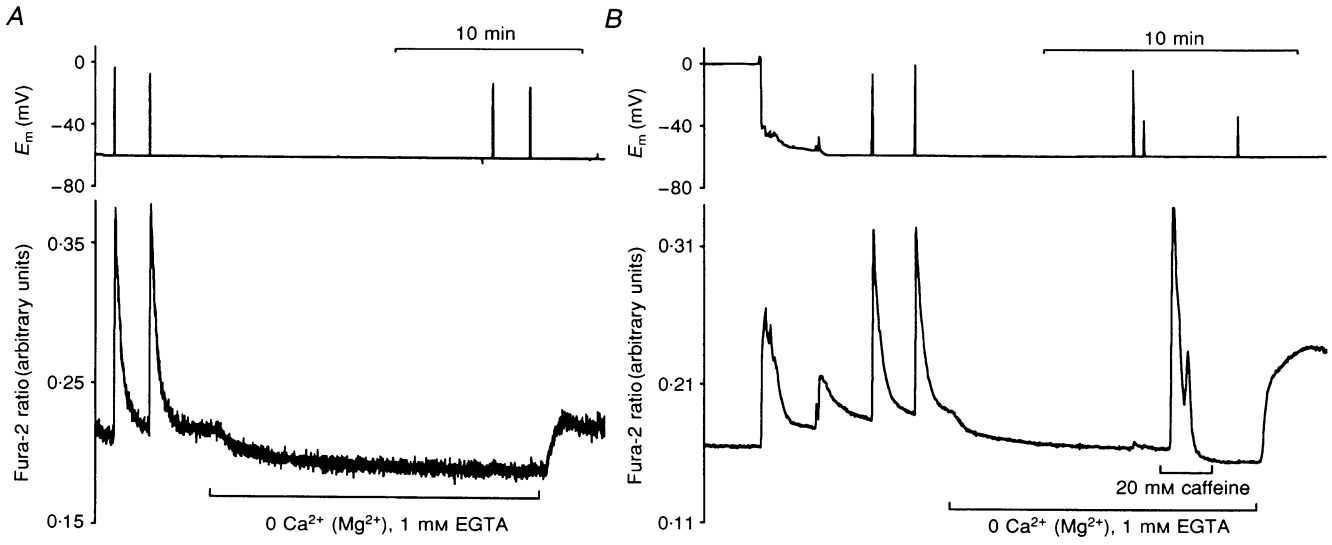

Figure 4. The effects of removing extracellular calcium on intracellular calcium during depolarization and caffeine application

In both experiments the cells were voltage clamped to −60 mV and depolarizations were held to 0 mV for 1 s. *A*, the effect of removing extracellular calcium (replaced with magnesium and minimized using EGTA) on Ca_i^{2+}. *B*, the effect of 20 mM caffeine after removing extracellular calcium (*B*).

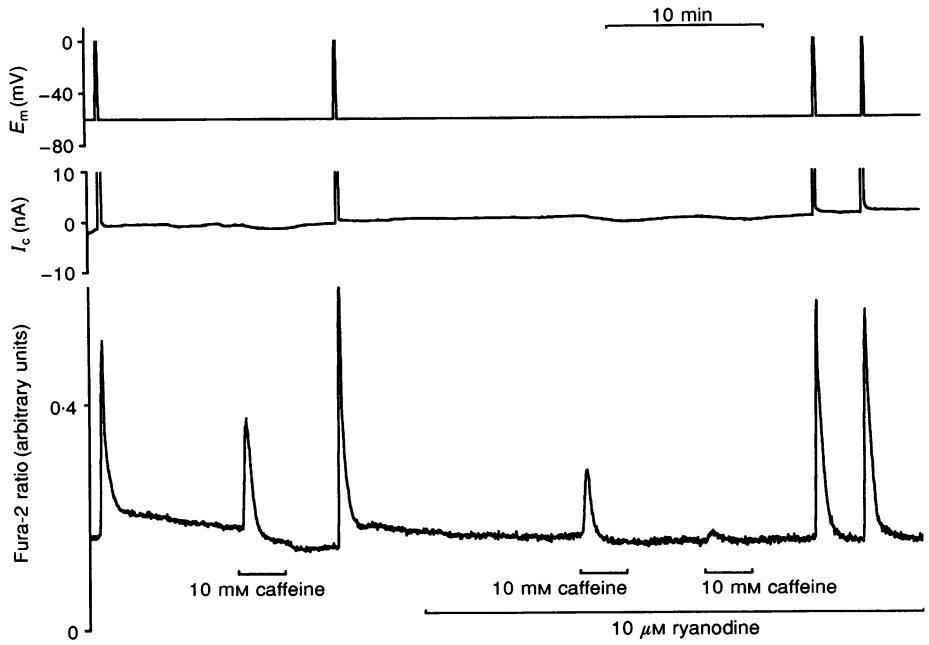

Figure 5. The effects of ryanodine on the responses to caffeine application
Cell was voltage clamped to −60 mV and depolarizations were held to 0 mV for 10 s. Both caffeine and ryanodine were incorporated into the normal snail Ringer solution.

were given following thapsigargin, $[Ca^{2+}]_i$ clearly recovered more slowly. For this experiment the rate of recovery when fitted with a single exponential was 43·5 and 44 s in control conditions and 63·5 and 63 s after treatment with thapsigargin. This suggests that the intracellular stores play only a minor role in regulating Ca^{2+} loads.

The effect of extracellular sodium removal

If Ca^{2+} is extruded by a Na^+–Ca^{2+} exchanger, then removal of extracellular sodium should prevent Ca^{2+} efflux and may even reverse the exchanger causing an increase in $[Ca^{2+}]_i$.

In preliminary experiments with external sodium removal, cells were not voltage clamped and only the

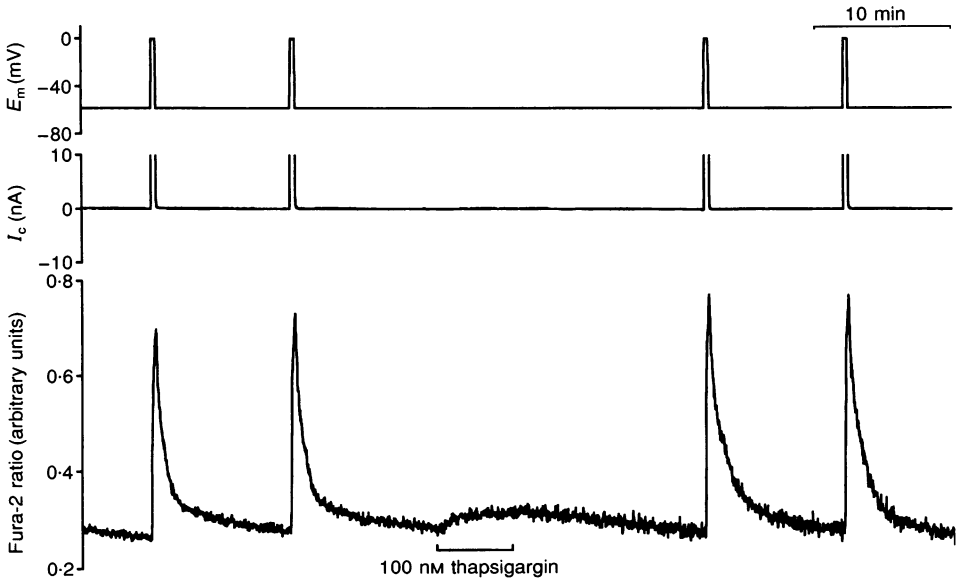

Figure 6. The effects of thapsigargin on the resting $[Ca^{2+}]_i$ and recovery from Ca^{2+} loads
The cell was voltage clamped to −60 mV and depolarized to 0 mV for 20 s. Thapsigargin in snail Ringer solution was applied to the bath through a separate solution inlet using a syringe.

membrane potential and fura-2 fluorescence were recorded as shown in Fig. 7*A*. Initially the cell had a potential of approximately −60 mV and was spontaneously firing bursts of action potentials. Each burst of action potentials caused a rise in $[Ca^{2+}]_i$ which recovered when firing stopped. Removal of extracellular sodium (replaced with NMDG) under these conditions caused the cell membrane to hyperpolarize and the cell stopped firing action potentials. $[Ca^{2+}]_i$ fell, and rapidly reached a lower steady state. When extracellular sodium was replaced, there was a short delay due to slow solution flow before the cell began to depolarize and rapidly fire action potentials. This was accompanied by a rapid rise in $[Ca^{2+}]_i$ which overshot the original resting value. After approximately 15 min the cell returned to a regular pattern of firing bursts of action potentials and $[Ca^{2+}]_i$ recovered to its original level. Subsequent removal of extracellular sodium had a similar effect. Similar results were seen in eleven other cells.

From such experiments on cells that were not voltage clamped it was difficult to distinguish between the effects of changes in membrane potential and removing extracellular sodium. In subsequent experiments, cells were voltage clamped to −60 mV to prevent changes in Ca^{2+} entry through voltage-activated Ca^{2+} channels. Near the start of the experiment in Fig. 7*B* a control depolarization to 0 mV for 10 s was given to load the cell with Ca^{2+}. $[Ca^{2+}]_i$ was allowed to recover to resting levels. When extracellular sodium was removed for 15 min there was no effect on $[Ca^{2+}]_i$. In some experiments sodium was replaced with lithium giving similar results ($n = 4$).

The Na^+–Ca^{2+} exchanger is thought to have a high capacity for transport of Ca^{2+} but a low affinity for $[Ca^{2+}]_i$. It is thus possible that it plays only a minor role in the maintenance of resting $[Ca^{2+}]_i$, but becomes much more important in regulating increases in $[Ca^{2+}]_i$. To investigate whether this was the case, depolarizations were given during the period of Na^+-free Ringer solution, as shown in Fig. 8. Control depolarizations in normal Ringer solution were given before and after the period of extracellular sodium removal. Removal of extracellular sodium had no effect on the resting $[Ca^{2+}]_i$ or the recovery from Ca^{2+} loads. Similar results were seen in the nine other cells tested.

Removal of extracellular sodium did not slow the rate of recovery of $[Ca^{2+}]_i$ as would be expected if a Na^+–Ca^{2+} exchanger was partly responsible for extruding Ca^{2+}.

The recoveries from Ca^{2+} loads were fitted with double exponentials and the rate of recovery was calculated at six fura-2 ratios. The average rate (dr/dt, measured as ratio change per second (s^{-1})), from nine experiments, was plotted against the normalized ratio as shown in Fig. 9.

In some experiments the depolarizations were shorter than in others, causing smaller rises in $[Ca^{2+}]_i$; therefore, the rate of recovery could not be calculated at all the normalized ratios. However, it is clear that there was no difference in the rate of recovery in control and Na^+-free Ringer solution at any of the fura-2 ratios.

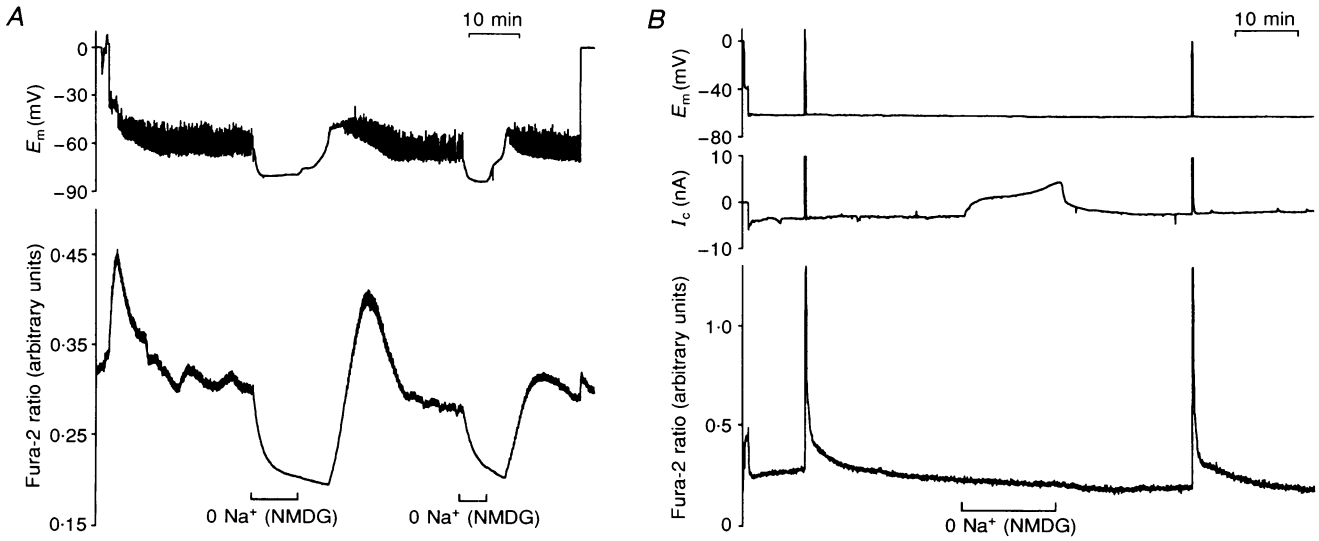

Figure 7. A comparison of the effects on $[Ca^{2+}]_i$ of removing extracellular sodium in a cell firing action potentials when compared with a cell voltage clamped to −60 mV

A, the effect of removing extracellular sodium on a cell spontaneously firing bursts of action potentials (the full height of action potentials were poorly resolved by the voltage-to-frequency conversion but is represented by the vertical lines on the E_m trace). *B*, the effect of removing extracellular sodium in a cell that had been voltage clamped to −60 mV; depolarizations near the beginning and end of the experiment were to 0 mV for 10 s.

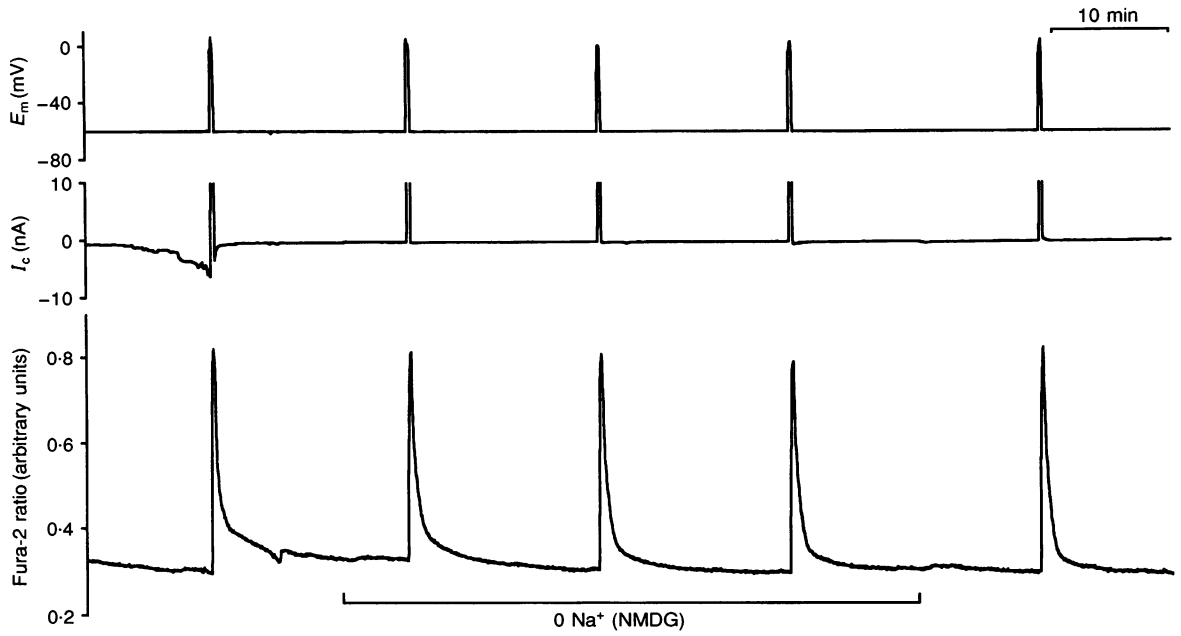

Figure 8. The effects of removing extracellular sodium (replaced with NMDG) on the resting $[Ca^{2+}]_i$ and the recovery from Ca^{2+} loads

The cell was voltage clamped to −60 mV and depolarizations held to 0 mV for 16 s were used to load the cells with Ca^{2+}.

The effects of intracellular Na^+ loading

Perhaps a Na^+–Ca^{2+} exchanger is present in these cells but was inoperative with low intracellular sodium. To check this, we first loaded the cells with sodium before removing extracellular Na^+ to reverse the gradient. Application of K^+-free Ringer solution would be expected to cause a gradual rise in intracellular sodium by blocking the Na^+–K^+ pump. This intracellular sodium loading should ensure that when extracellular sodium was removed the sodium gradient across the cell membrane was completely reversed, so increasing any Ca^{2+} influx via a Na^+–Ca^{2+} exchanger. Long depolarizations (10 s) were given to challenge the $[Ca^{2+}]_i$ regulating processes with large Ca^{2+} loads, as shown in Fig. 10.

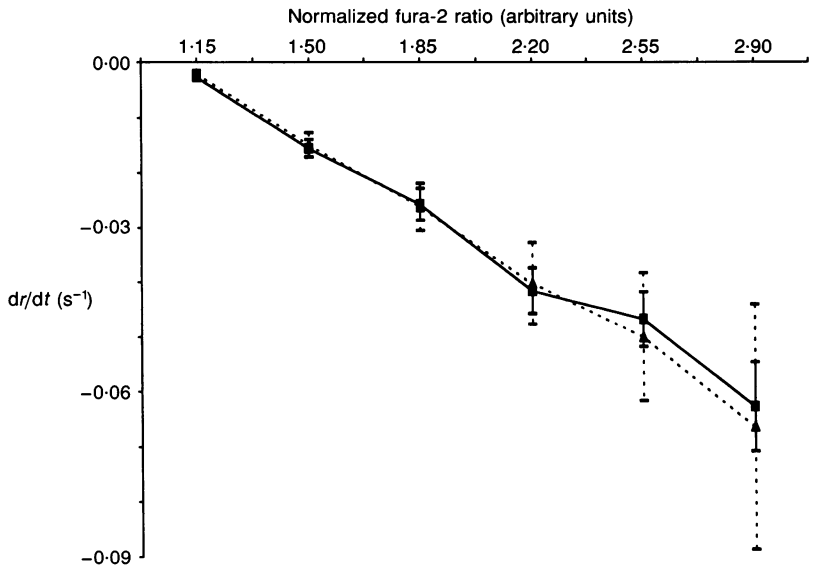

Figure 9. A plot of rate of recovery of normalized fura-2 ratio against normalized fura-2 ratio

Data pooled from 9 experiments on different cells. Continuous line and squares indicate normal Ringer solution, dashed lines and triangles indicate Na^+-free Ringer solution. Horizontal bars give standard errors of the mean.

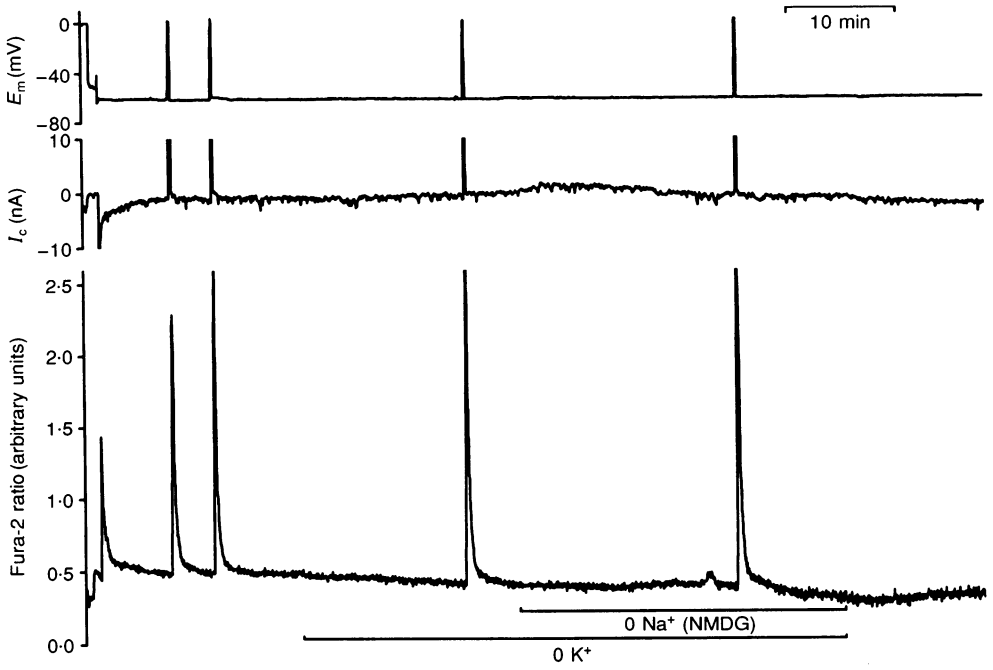

Figure 10. The effects of intracellular Na^+ loading and removal of extracellular sodium on the resting Ca_i^{2+} and recoveries from Ca^{2+} loads

The cell was voltage clamped to −60 mV and depolarizations to 0 mV for 10 s used to load the cell with Ca^{2+}. K^+-free Ringer solution was used to inhibit the Na^+ pump causing a rise in intracellular Na^+.

Initially two control depolarizations were given and $[Ca^{2+}]_i$ was allowed to recover. Removal of extracellular potassium for 20 min had little effect on the resting $[Ca^{2+}]_i$ or the recovery from depolarizations. Removing extracellular sodium for 30 min (still in the absence of extracellular potassium) had no effect on the resting $[Ca^{2+}]_i$ or the rate of recovery from Ca^{2+} loads. Similar results were seen in all four cells tested.

Analysis of the rates of recovery from Ca^{2+} loads using double exponential functions was carried out and is shown in Fig. 11. For clarity, only control and Na^+-free recoveries are shown.

There was no effect of removing extracellular sodium on the rate of recovery, even after the cell had been loaded with Na^+ to ensure a reversal of the sodium gradient.

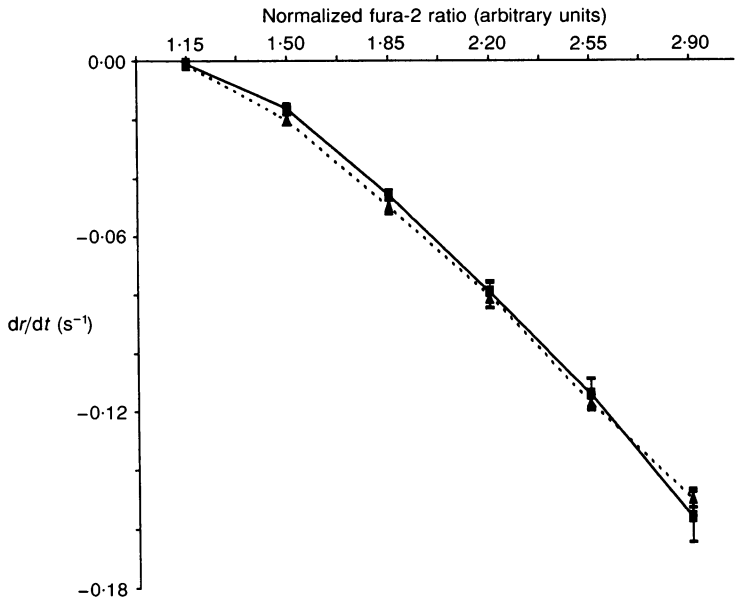

Figure 11. A plot of rate of recovery of normalized fura-2 ratio against normalized fura-2 ratio

Data pooled from 3 experiments on different cells. Continuous line and squares indicate normal Ringer solution, dashed lines and triangles indicate Na^+-free Ringer solution during intracellular Na^+ loading. Horizontal bars give standard errors of the mean.

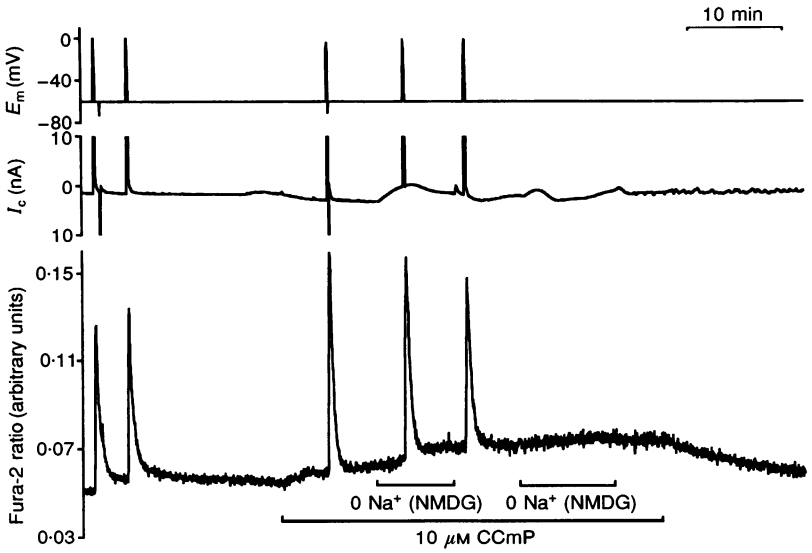

Figure 12. The effects of extracellular sodium removal on $[Ca^{2+}]_i$ during mitochondrial poisoning with CCmP

The cell was voltage clamped to −60 mV and depolarizations to 0 mV for 10 s were used to load the cell with Ca^{2+}. CCmP was incorporated into snail Ringer solution.

We also tried intracellular sodium loading by inhibition of the Na^+–K^+ pump with ouabain. This method of intracellular Na^+ loading gave essentially the same results as for intracellular Na^+ loading using K^+-free Ringer solution.

The effect of removing extracellular Na^+ during mitochondrial poisoning

We tested the effects of uncoupling the mitochondria using CCmP on the effect of reversing the Na^+ gradient in a further attempt to demonstrate the presence of a Na^+–Ca^{2+} exchanger in these cells.

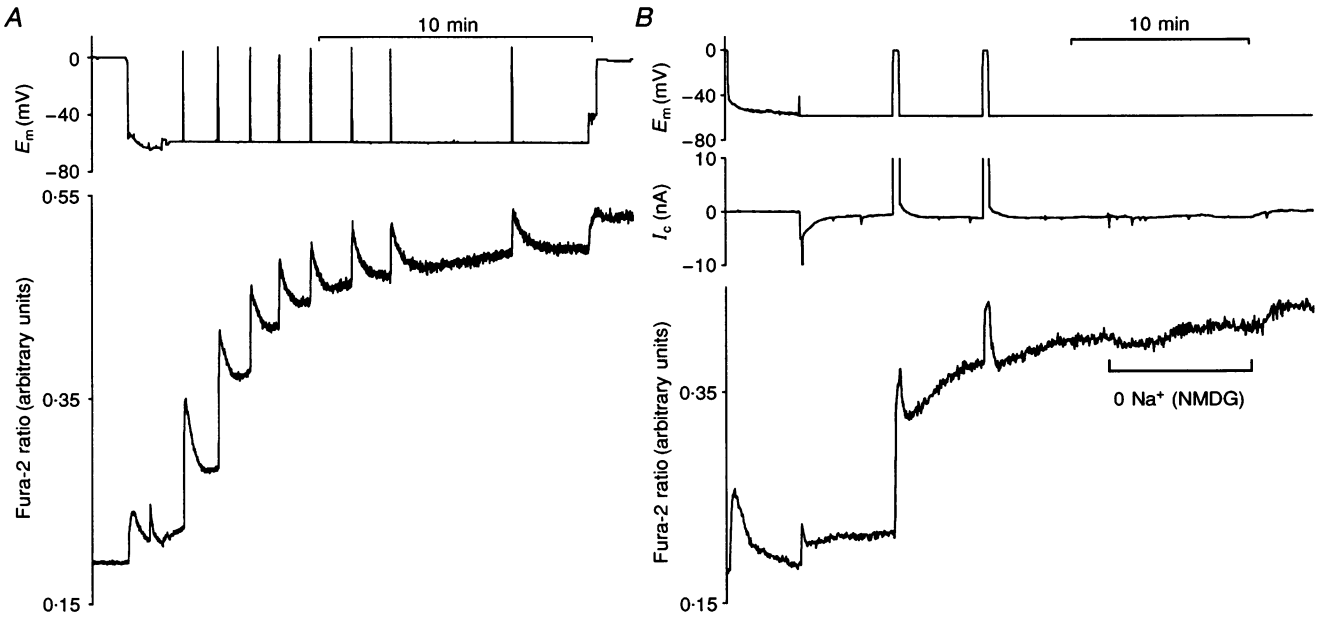

Figure 13. The effects of inhibiting ATP-dependent Ca^{2+} regulation and removal of extracellular Na^+ on Ca_i^{2+} and the recovery from Ca^{2+} loads

In both experiments the cells were voltage clamped to −60 mV. Sodium orthovanadate (100 mM) was incorporated into the voltage clamp electrode and allowed it to leak passively into the cell. *A*, the effects of intracellular vanadate on resting Ca_i^{2+} and recovery from Ca^{2+} loads are shown. Depolarizations for 1 s. *B*, the effects of removing extracellular Na^+ after inhibition of the ATP-dependent Ca^{2+} regulation by intracellular vanadate. Depolarizations for 20 s.

Near the start of the experiment shown in Fig. 12 two control Ca^{2+} loads were given and $[Ca^{2+}]_i$ was allowed to recover. During the application of CCmP there was a gradual increase in resting $[Ca^{2+}]_i$ suggesting a release of Ca^{2+} from an ATP-dependent intracellular store or inhibition of an ATP-dependent Ca^{2+} extrusion process. Nevertheless, all depolarization-induced $[Ca^{2+}]_i$ increases, in the presence or absence of extracellular sodium, recovered as quickly as the control. When CCmP was removed the $[Ca^{2+}]_i$ level declined slowly. Similar results were obtained in the two other cells tested. Three similar experiments using lithium as a sodium substitute, rather than NMDG, were carried out with similar results.

The effects of intracellular vanadate

Although in the short term intracellular Ca^{2+} stores are capable of contributing to $[Ca^{2+}]_i$ regulation, in the long term Ca^{2+} must be pumped out of the cell. We have demonstrated in a previous paper that snail neurones possess a Ca^{2+}–H^+-ATPase in the plasma membrane (Schwiening *et al.* 1993). ATP-dependent Ca^{2+} efflux has been demonstrated in a wide variety of neurones including the squid giant axon (Baker & McNaughton, 1976) and brain synaptosomes (Rahamimoff & Abramovitz, 1978).

In neurones, sodium orthovanadate is known to block many ATPases including the Ca^{2+}-ATPase but it does not inhibit the Na^+–Ca^{2+} exchanger (DiPolo, Rojas & Beaugé, 1979). We used vanadate to block the Ca^{2+}-ATPase and then removed extracellular sodium in an attempt to demonstrate the operation of the Na^+–Ca^{2+} exchanger.

Vanadate (100 mM) was incorporated into the voltage-clamp electrode and allowed to leak into the cell; therefore, no control depolarizations could be given. Depolarizations for 1 s produced small Ca^{2+} loads which did not fully recover to resting values (in contrast to experiments without vanadate where $[Ca^{2+}]_i$ always rapidly recovered). The overall $[Ca^{2+}]_i$ increased to a higher steady state as shown in Fig. 13*A*. Similar results were obtained in fourteen other cells. It was impossible to estimate the concentration that vanadate reached inside the cell. Only a small proportion of the vanadate was expected to leak into the cell as no pressure or iontophoresis was used. The amount of vanadate leaking into the cell would depend on the electrode resistance which, in these experiments, was generally found to increase from 15 to approximately 100 MΩ during the experiment. Hence, these results are consistent with vanadate inhibition of the Ca^{2+}-ATPase in the plasma membrane.

To determine if the operation of a Na^+–Ca^{2+} exchanger could be demonstrated after inhibition of the ATP-driven Ca^{2+} pump, extracellular sodium was removed following vanadate inhibition. The experiment in Fig. 13*B* shows that removal of extracellular sodium after inhibition by vanadate caused no further increase in $[Ca^{2+}]_i$. Similar results were obtained in four other cells.

DISCUSSION

Our results show that the plasma membrane Ca^{2+}-ATPase is the main mechanism responsible for $[Ca^{2+}]_i$ regulation and that it is the only mechanism for Ca^{2+} efflux. Unlike many other studies on neurones, we can find no sign of a functional Na^+–Ca^{2+} exchanger, even in poisoned cells loaded with sodium. The endoplasmic reticulum Ca^{2+}-ATPase contributes to the regulation of $[Ca^{2+}]_i$ but it is not the main mechanism by which the resting $[Ca^{2+}]_i$ is maintained.

Resting $[Ca^{2+}]_i$

Our measurements of the basal $[Ca^{2+}]_i$ in snail neurones are in reasonable agreement with previous estimates in both snail and other neurones. Our Ca^{2+}-sensitive microelectrode values, however, are higher than those measured with fura-2, even though the cells were held at the same potential and bathed in the same solutions, and the same calibration solutions were used for both methods.

Our average Ca^{2+}-sensitive microelectrode value of 174 nM (pCa_i, 6·76) is remarkably close to the 170 nM reported by Alvarez-Leefmans *et al.* (1981) in *Helix aspersa* neurones, using Ca^{2+}-sensitive microelectrodes with a different sensor. Their external calcium concentration was only 2 mM and they did not test for leakage. Other Ca^{2+}-sensitive microelectrode measurements in neurones include 130 nM in *Aplysia* neurones by Levy & Tillotson (1988) and 70 nM in rat CNS neurones by Silver & Erecinska (1990). Many more measurements have been made with indicators. With *Helix* neurones and fura-2, Kostyuk *et al.* (1989) reported a basal $[Ca^{2+}]_i$ averaging 90 nM, while Muller, Partridge & Swandulla (1993) gave resting values of 10 to 100 nM. In other neurones recent measurements of resting $[Ca^{2+}]_i$ include 136 nM in cultured rat sensory neurones (Benham *et al.* 1992), 76 nM in bullfrog sympathetic neurones (Friel & Tsien, 1992) and 100 nM in cultured rat septal neurones (Bleakman, Roback, Wainer, Miller & Harrison, 1993).

Our values are within the expected range, but do not agree with each other. We do not believe that the differences can be accounted for by increased intracellular buffering by 40–100 μM fura-2. Also, in some experiments fura-2 and Ca^{2+}-sensitive microelectrodes were used simultaneously and the Ca^{2+}-sensitive microelectrode still gave higher values for $[Ca^{2+}]_i$ than fura-2. It is unlikely that the basal cytoplasmic Ca^{2+} really was different in the two groups of neurones. Both methods are susceptible to error. The Ca^{2+}-sensitive microelectrode readings are probably too high, since it is quite possible that the Ca^{2+} at the tip of the Ca^{2+}-sensitive microelectrode was elevated by leakage from internal stores.

Calibration of fura-2 is notoriously difficult. We found the *in vitro* calibration simple to perform on a routine basis and although it may be subject to a small error we believe

it gives a reasonable estimate of resting $[Ca^{2+}]_i$. Ideally, fura-2 should be calibrated *in vivo* but this proved to be very difficult. Our original hope that Ca^{2+}-sensitive microelectrodes could be used to help calibrate the fura-2 ratio proved to be too problematical. However, if an *in vitro* calibration of fura-2 must be used, a more accurate estimation of $[Ca^{2+}]_i$ might be obtained if the calibration solutions mimicked the intracellular environment more closely, perhaps by including some protein, as fura-2 is known to be affected by many factors including intracellular proteins and changes in viscosity (Poenie, 1990).

Calcium entry

It is well established that electrically excitable membranes possess Ca^{2+} channels, and that mollusc neurones are capable of generating Ca^{2+} action potentials. It is not surprising, therefore, that changes in membrane potential and the occurrence of action potentials can influence $[Ca^{2+}]_i$.

In our experiments, depolarizations to 0 mV for between 1 and 20 s caused increases in $[Ca^{2+}]_i$ which began to recover when the cell was repolarized. Even when long depolarizations of 10–20 s were given, the Ca^{2+} continued to rise for the duration of the depolarization. This suggests that at least some of the Ca^{2+} channels were non-, or very slowly, inactivating. Mirinov & Usachev (1991) showed that during long depolarizations of between 10 and 15 s, in *Helix pomatia* neurones, $[Ca^{2+}]_i$ continued to rise and suggested that the Ca^{2+} channels were not inactivating. As expected for an influx of Ca^{2+} through voltage-activated Ca^{2+} channels during depolarizations, no increase in $[Ca^{2+}]_i$ was seen when the cell was depolarized in Ca^{2+}-free solution, as shown in Fig. 4*A*.

The effects of extracellular sodium removal

Our experiments appear to rule out any role for the Na^+–Ca^{2+} exchanger in the cell body of snail neurones. Removal of extracellular sodium had no effect on resting $[Ca^{2+}]_i$ or the rate of recovery from large Ca^{2+} loads even under conditions of intracellular Na^+ loading, vanadate inhibition of the ATP-dependent Ca^{2+} regulation and mitochondrial inhibition.

Much of the evidence for the presence of a Na^+–Ca^{2+} exchanger in neurones has come from work done on squid giant axons (Baker *et al.* 1969; Blaustein & Hodgkin, 1969) and in brain synaptosomes from the guinea-pig (Gill, Grollman & Kohn, 1981) and the rat (Nachshen, Sanchez-Armass & Weinstein, 1986).

There have been fewer studies of the role of the Na^+–Ca^{2+} exchanger in $[Ca^{2+}]_i$ regulation in neuronal cell bodies. In many cells the presence of the Na^+–Ca^{2+} exchanger has clearly been demonstrated, for example in *Aplysia* (Levy & Tillotson, 1988), cultured rat sensory neurones (Benham *et al.* 1992), rat nucleus basalis (Tatsumi & Katayama, 1993) and cultured chick sensory neurones (Mirinov *et al.* 1993). However, in these studies it is not thought to be the main mechanism for regulating $[Ca^{2+}]_i$.

Experiments by Kostyuk *et al.* (1989) have apparently demonstrated the presence of a Na^+–Ca^{2+} exchanger in snail neurones. Using isolated *Helix pomatia* neurones, they showed that extracellular sodium removal (replaced with Li^+) for a period of 5 min caused a reversible increase in $[Ca^{2+}]_i$ from 0·1 μM to between 0·2 and 0·3 μM, but this experiment was in a cell that had not been voltage clamped. During the period of sodium removal the cell initially hyperpolarized and then began to depolarize. In Fig. 7*A* we demonstrate that such changes in membrane potential change $[Ca^{2+}]_i$. Thus, it is difficult to rule out the effects of changes in membrane potential during extracellular sodium removal in the experiments of Kostyuk *et al.* (1989). In other experiments, when the cells were voltage clamped, they showed no effect of reversing the Na^+–Ca^{2+} exchanger on the rate of recovery from Ca^{2+} loads following membrane depolarizations (Kostyuk *et al.* 1989).

Only a few workers have been unable to demonstrate any effect of removing extracellular sodium on $[Ca^{2+}]_i$. However, in these studies the effects of removing extracellular sodium following metabolic poisoning were not studied. In the original experiments by Blaustein & Hodgkin (1969) the Na^+–Ca^{2+} exchanger could only be clearly demonstrated following cyanide poisoning. Thus, it is possible that in those studies where no metabolic poisoning was used the Na^+–Ca^{2+} exchanger may have been missed. These include studies on bullfrog sympathetic neurones (Nohmi & Kuba, 1984), rat dorsal root ganglion cells (Thayer & Miller, 1990) and cultured rat septal neurones (Bleakman *et al.* 1993).

In experiments on cultured dorsal root ganglion cells (Werth & Thayer, 1994), however, no effect of removing extracellular sodium, even during mitochondrial inhibition could be demonstrated. Hence, although the Na^+–Ca^{2+} exchanger is not thought to be the main mechanism for $[Ca^{2+}]_i$ regulation, it is present in many neuronal cells. Thus, our results are unusual in suggesting that the Na^+–Ca^{2+} exchanger is completely absent.

Vanadate

Vanadate is not a specific inhibitor and is known to inhibit both the plasma membrane Ca^{2+} pump and the endoplasmic reticulum ATP-dependent Ca^{2+} uptake (Bond & Huggins, 1980). From the experiment in Fig. 13*A* it is unclear exactly what proportion of the rise in $[Ca^{2+}]_i$ is due to inhibition of the plasma membrane Ca^{2+} pump. From other experiments carried out in the same laboratory (Schwiening *et al.* 1993), vanadate has been shown to block the Ca^{2+} efflux, which was detected using surface Ca^{2+}-sensitive microelectrodes. However, this does not rule out the possibility that vanadate may also be blocking intracellular uptake of $[Ca^{2+}]_i$.

It is clear from Fig. 4*B* that snail neurones have intracellular stores of Ca^{2+} which can be released by caffeine in the absence of external Ca^{2+}. It is possible, therefore, that such stores could contribute to the regulation of $[Ca^{2+}]_i$. However, thapsigargin, which specifically inhibits the endoplasmic reticulum ATPase, only had a small effect on the recovery from Ca^{2+} loads as shown in Fig. 6. It seems probable, therefore, that the effects of vanadate on the recovery from Ca^{2+} loads was primarily due to inhibition of the plasma membrane Ca^{2+} pump. Our experiments show that this Ca^{2+}–H^+ATPase is the sole mechanism available to extrude Ca^{2+} from snail neurones.

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