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Cytoplasmic Free Ca in Isolated Snail Neurons as Revealed by Fluorescent Probe Fura-2: Mechanisms of Ca Recovery after Ca Load and Ca Release from Intracellular Stores

P.G. Kostyuk, S.L. Mironov, A.V. Tepikin, P.V. Belan
A.A. Bogomoletz Institute of Physiology, Kiev, 252601, USSR

Summary. Using the fluorescent probe fura-2, we measured the cytoplasmic concentration of free Ca^{2+} ($[Ca]_i$) and its changes in isolated, nonidentified neurons of the snail *Helix pomatia*. $[Ca]_i$ increased during membrane depolarization due to opening of Ca channels in the surface membrane. When the membrane potential returned to the resting level, $[Ca]_i$ recovered monoexponentially, with the time constant ranging from 10 to 30 sec. The rate of recovery remained unchanged after treatments that interferred with the normal functioning of both Ca/Na exchange and Ca-ATPase in the surface membrane or mitochondria. $[Ca]_i$ recovery slowed down upon cooling according to $Q_{10} = 2.3$ and after intracellular injection of vanadate. The data obtained suggest that the rate of $[Ca]_i$ recovery after membrane depolarization is mainly determined by Ca pump of intracellular stores (presumably by the endoplasmic reticulum). Ca release from these stores could be induced in the presence of millimolar caffeine or theophylline in the external medium when $[Ca]_i$ increased up to a certain threshold level. This depolarization-induced Ca load triggered further transient increase in $[Ca]_i$, which was accompanied by membrane hyperpolarization due to the development of Ca-activated potassium conductance. 1 mM procaine or tetracaine, but not lidocaine, inhibited this Ca-induced Ca release. In some cases stable oscillations of $[Ca]_i$ were observed. They could be induced by producing a steady Ca influx by membrane depolarization.

Key Words snail neurons · fluorescence measurements · cytoplasmic Ca · $[Ca]_i$ recovery · Ca-induced Ca release

Introduction

Intracellular homeostasis of Ca plays an important role in cell functioning (*cf.* Rasmussen & Barrett, 1984). The understanding of the main mechanisms of Ca-dependent intracellular reactions needs precise information about the cytoplasmic Ca level ($[Ca]_i$) and the mechanisms of its regulation. In the present paper we described the application of the novel fluorescent probe fura-2 for $[Ca]_i$ measurements in isolated snail neurons and revealed some mechanisms responsible for the recovery of $[Ca]_i$ after Ca load and $[Ca]_i$ increase due to its release from intracellular stores.

Materials and Methods

Neurons of the snail *Helix pomatia* were isolated by procedures not differing significantly from those developed in our laboratory (*cf.* Kostyuk & Krishtal, 1977). Cells were loaded with fura-2 at room temperature for 30–40 min by adding 0.1% stock solution of fura-2/AM (Calbiochem) in DMSO (final concentration 5 μM) to cell suspension in a normal Ringer solution, containing 100 mM NaCl, 20 mM Tris-HCl (pH 7.4), 10 mM $CaCl_2$, 5 mM $MgCl_2$, 5 mM KCl. NaN_3-containing solution was obtained replacing 10 mM NaCl by corresponding salt. In Na-free solution all NaCl was substituted by Tris-HCl. In studies on the temperature dependence of $[Ca]_i$ recovery, correspondingly warmed or cooled solutions were continuously passed through the chamber. Final temperature was monitored by a contact thermometer placed in the immediate vicinity of the examined cell.

The optical part of the experimental set-up was based on a fluorescence microscope Fluoval-2 (Karl Zeiss, Jena), which was inverted. We monitored the fluorescence of the whole cell isolated from the image by a diaphragm, which for an objective ×40, NA 0.95 corresponded to a round object with a diameter of about 50 μm. For selective excitation of fura-2 we used two interference filters centered at 362 and 387 nm, respectively (70% transmission, 3 nm bandwidth). For isolation of fura-2 fluorescence a combination of an interference filter centered at 505 nm (70% transmission, 10 nm bandwidth) and a cut-off filter centered at 489 nm was used. To increase the sensitivity of our recording system, we developed a photon-counting technique according to Horowitz and Hill (1980). Data were stored and processed by a minicomputer. Cell stimulation, membrane potential recording and electrophoretic injection were performed by using the routine microelectrode methods described by Purves (1981). Stimulation and recording microelectrodes were filled with 2.5 M KCl and had a resistance ranging from 5 to 10 MΩ. For electrophoretic injection the electrodes were filled with 100 mM Na-cAMP or Na-vanadate, respectively.

Neurons loaded with fura-2 formed from its ester (fura-2/AM) were diffusively stained with no evidence of fura-2 compartmentalization as described by Almers and Neher (1985). We attribute this to relatively low temperature of dye loading. This

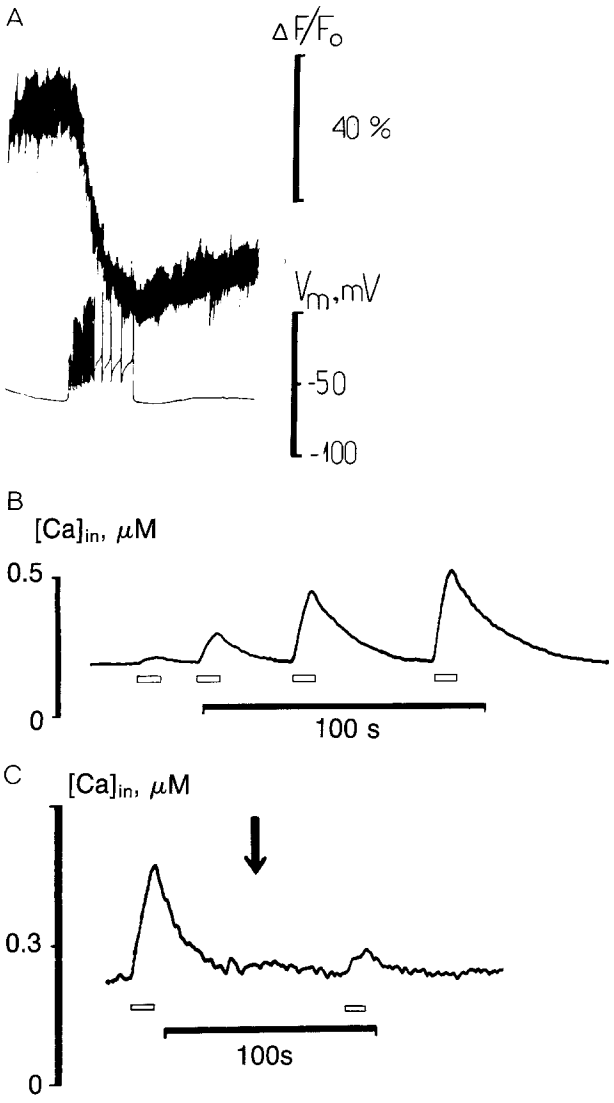

Fig. 1. Depolarization induced changes in $[Ca]_i$. (*A*) Fura-2 fluorescence excited at 387 nm (upper part) in response to a burst of action potentials (lower part) elicited in response to electric stimulus. (*B*) $[Ca]_i$ changes due to depolarization of the membrane potential to −25, −10, +5 and 20 mV, respectively. (*C*) $[Ca]_i$ responses to depolarization to 0 mV (its duration is shown by empty bar) in the normal Ringer (left) and low-Ca (0.5 mM) solutions (right)

finding is in accord with data obtained by Meldolesi et al. (J. Meldolesi, *personal communication*). They observed that fura-2 loaded in esterified form at 37°C tends to concentrate in subcellular organelles, whereas when loaded at room temperature it is evenly distributed in the cytoplasm. In some experiments (*not shown*) we directly injected fura-2 (free acid) into snail neurons, but we did not find any difference between measured parameters using these two procedures of dye loading.

$[Ca]_i$ estimates from fluorescence measurements were obtained using either single- or dual-wavelength methods. In the former case we measured only the fluorescence (F) at 387 nm, which is close to the excitation maximum of Ca-free fura-2. The necessary reference point F_{min} for each cell was obtained at the end of the experiment, applying high-frequency hyperpolarizing pulses of large amplitude to kill the cell. Such a procedure both zeroed the membrane potential and produced a sharp decrease in the fluorescence excited at 387 nm due to the Ca load in excess of fura-2 trapped inside the cell. At the same time, the fluorescence excited at 362 nm (close to the isobestic form of fura-2) was nearly constant, indicating the absence of fura-2 leakage out of the cell. From general considerations about the equilibrium between Ca-bound and Ca-free forms of fura-2 (*see*, e.g., Grynkiewicz, Poenie & Tsien, 1985) a simple formula follows

$$[Ca]_i = K_d(R - f)/(f - 1) \quad (1)$$

where $R = F_{max}/F_{min}$, $f = F/F_{min}$. R and K_d were estimated directly for our set-up in separate experiments that monitored the fluorescence of small droplets containing 10 μM fura-2 in the solution of a given free [Ca].

In a dual-wavelength method we measured the fluorescence excited both at 387 and at 362 nm and calculated free [Ca] from the ratio of these values according to the formula (2) given by Grynkiewicz et al. (1985).

Results

Figure 1*A* illustrates the ability of fura-2 to monitor changes in $[Ca]_i$ caused by electric discharge. A burst of action potentials induced a marked decrease in fura-2 fluorescence excited at 387 nm. It corresponded to a $[Ca]_i$ increase from 0.1 to 1 μM. In general, $[Ca]_i$ increased on membrane depolarization, whereas membrane hyperpolarization produced no changes in $[Ca]_i$. The depolarization-induced increase in $[Ca]_i$ was observed only for membrane potential shifts more positive than −25 mV (Fig. 1*B*), which is equal to the threshold of calcium channel activation in snail neurons (Kostyuk & Krishtal, 1977). In low Ca solutions $[Ca]_i$ transients were considerably smaller (Fig. 1*C*). All these data obviously indicate that the observed depolarization-induced increase in $[Ca]_i$ is due to Ca influx through the well-known voltage-dependent calcium channels.

Rhythmic stimulation also increased $[Ca]_i$, bringing it to a new steady level. Figure 2*A* shows that both the transient period and new steady level depended on the pulse frequency. After the end of stimulation $[Ca]_i$ always decayed to the resting level. In most cases this recovery could be well described by a single exponent with the time constant being constant for the same cell but ranging from 8 to 30 sec for different cells around the mean value of $\tau = 20 \pm 5$ sec ($n = 27$). $[Ca]_i$ recovery slowed down when the temperature was lowered (Fig. 2*B*). The mean value of Q_{10} was 2.3 ± 0.2 for temperatures ranging from 13 to 28°C.

The basal level of $[Ca]_i$ could be changed by various external treatments. Thus, in Na-free solu-

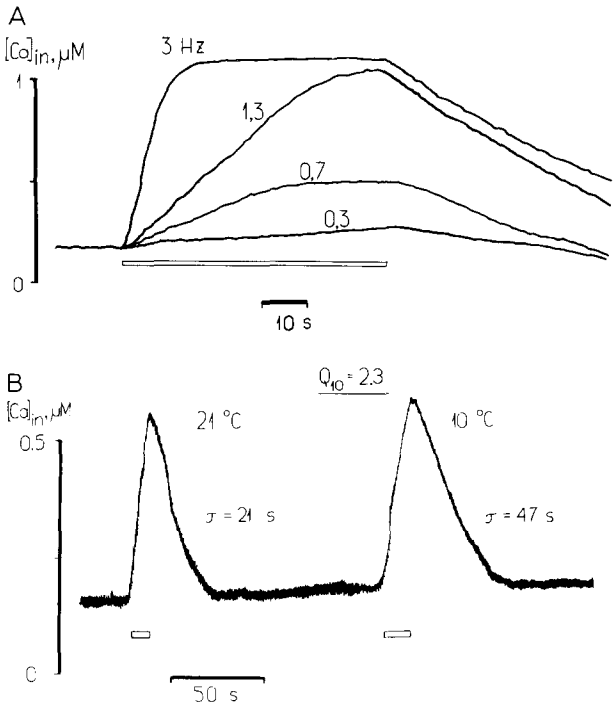

Fig. 2. Recovery of $[Ca]_i$ after depolarization-induced Ca load. (*A*) Rhythmic stimulation. The cell was depolarized to 0 mV by a series of 100-msec pulses of different frequency, indicated near each curve. The total duration of stimulation is shown by an empty bar. (*B*) Temperature dependence of $[Ca]_i$ recovery

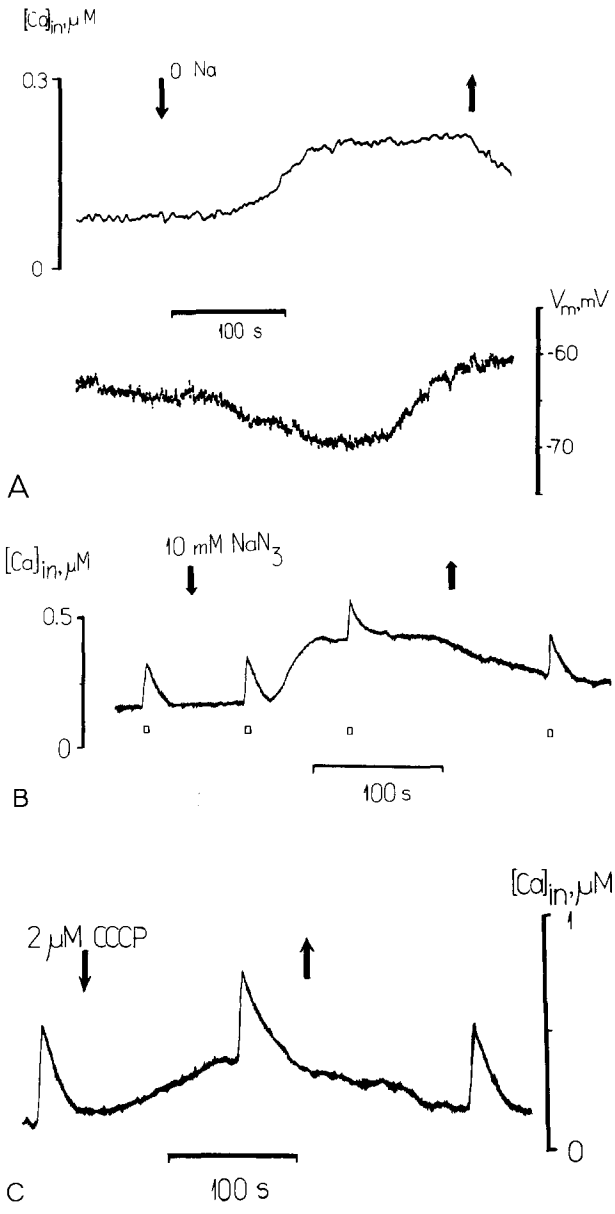

Fig. 3. Changes in basal $[Ca]_i$ level in Na-free solution (*A*) and after the application of 1 mM sodium azide (*B*) or 2 μM CCCP (*C*). In the lower part of (*A*) are shown the changes in the membrane potential

tion $[Ca]_i$ slowly rose from 0.1 to 0.2–0.3 μM (Fig. 3*A*). The effect was reversible and accompanied by membrane hyperpolarization. Mitochondrial poisons (10 mM sodium azide or 2 μM carbonyl cyanide-phenylhydrazone (CCCP)) also increased $[Ca]_i$ to about 0.3 μM (Fig. 3*B* and *C*). However, only in the latter case was the rate of $[Ca]_i$ recovery slightly increased. This is in accord with data of Barish and Thompson (1983), who observed similar effects of CCCP on intracellular Ca measured as corresponding changes in Ca-activated potassium conductance. Neither 1 mM La nor vanadate, which may affect Ca transport in the surface membrane via Ca/Na exchange or Ca pump, did not change the rate of $[Ca]_i$ recovery (*see also* Discussion).

Intracellular injection of cAMP or vanadate (Fig. 4) both increased $[Ca]_i$. Intracellular vanadate also slowed down the rate of recovery of $[Ca]_i$. Interestingly, this effect could be almost completely abolished by additional Ca load. Presumably, Ca binding to vanadate anions efficiently lowered their intracellular buffer capacity which existed before vanadate injection.

Figure 5*A* shows that in a caffeine-containing solution membrane depolarization triggered a large subsequent transient increase in $[Ca]_i$. This effect developed in an "all or none" fashion as $[Ca]_i$ reached a threshold value of 0.4 ± 0.05 μM ($n = 25$). If the initial resting potential was more positive than −50 mV, then this depolarization-induced increase in $[Ca]_i$ was followed by a marked membrane hyperpoplarization (Fig. 5*B*). Similar results were obtained with 5 mM theophylline (*not shown*). In this case, however, the threshold value of $[Ca]_i = 0.6 \pm 0.05$ μM ($n = 17$) was somewhat larger than for caffeine, whereas the maximum amplitudes of $[Ca]_i$

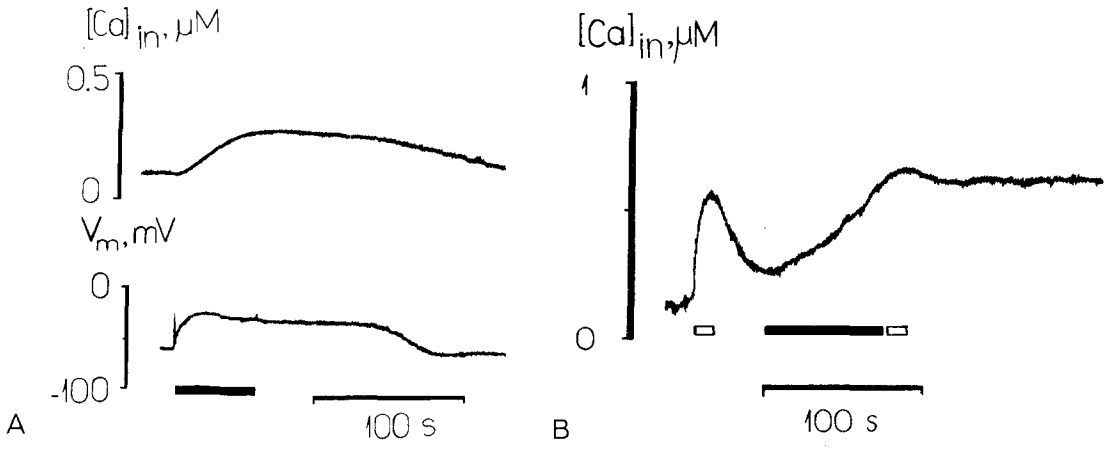

Fig. 4. $[Ca]_i$ changes after intracellular injection of cAMP (*A*) and vanadate (*B*). The duration of injection is shown by filled bars, the depolarization to 0 mV is indicated by empty bars

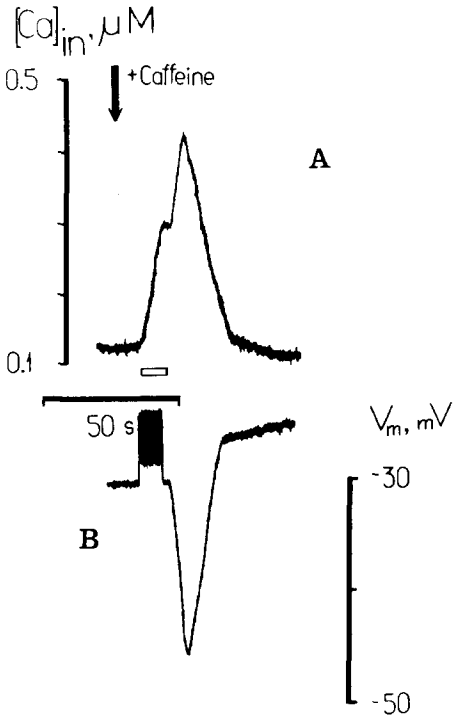

Fig. 5. Transient changes in $[Ca]_i$ (*A*) and in the membrane potential (*B*) evoked by depolarization-induced Ca load in the presence of 5 mM caffeine in the extracellular solution. The duration of the depolarizing shift to 0 mV is shown by empty bar

transients were about the same and equal to 0.9 ± 0.1 and 1.0 ± 0.1 μM, respectively.

The peak amplitude of this $[Ca]_i$ response was independent of extracellular Ca in the range from 0.5 to 20 mM, and the effect was observed for caffeine concentrations larger than 2 mM. Further increases of caffeine concentration did not change the threshold and the peak $[Ca]_i$. The time-course of $[Ca]_i$ recovery after such transient was essentially the same as that observed in the case of depolarization-induced increase in $[Ca]_i$.

Since local anesthetics inhibit Ca release from the sarcoplasmic reticulum of contractile cells, we tried them on snail neurons. Their inhibition potency appeared to be distinctly different. Thus, 1 mM lidocaine did not affect the caffeine-induced transient increase in $[Ca]_i$, whereas procaine or tetracaine in the same or even lower concentrations gradually inhibited the response by increasing its threshold (Fig. 6).

In some cases after the change of normal Ringer solution to a caffeine- or theophylline-containing solution, the cell began to generate oscillations of $[Ca]_i$ (Fig. 7*A*), which were extremely stable and could persist for several hours. Following the predictions of the corresponding mathematical model (Mironov & Tepikin, 1988), we succeeded in initiating them by producing Ca influx through the surface membrane by its steady depolarization. Figure 7*B* shows the dependence of the amplitude and frequency of these $[Ca]_i$ oscillations on membrane depolarization, i.e., the Ca influx.

Discussion

The resting value of $[Ca]_i$ in neurons of *Helix pomatia*, estimated in this work using the fluorescent probe fura-2, ranged from 0.07–0.15 μM (70% of cells studied) and corresponded to the mean value 0.09 ± 0.07 μM (n = 38). This is close to the values 0.16 and 0.24 μM obtained in neurons of *Helix aspersa* (Alvarez-Leefmans, Rink & Tsien, 1981) and *Aplysia* (Gorman et al., 1984) using Ca-sensitive microelectrodes.

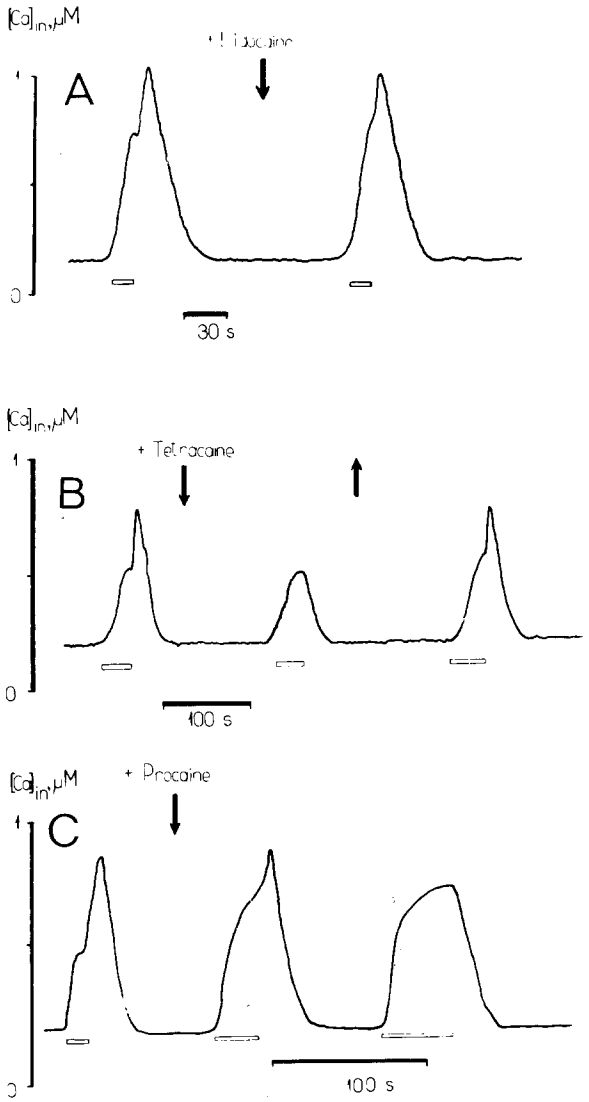

Fig. 6. Inhibition of $[Ca]_i$ transient observed in the presence of 5 mM caffeine by 1 mM lidocaine (*A*), tetracaine (*B*) or procaine (*C*). Arrows indicate the addition of the corresponding anesthetics to the bathing solution. The duration of depolarization to 0 mV is indicated by empty bars

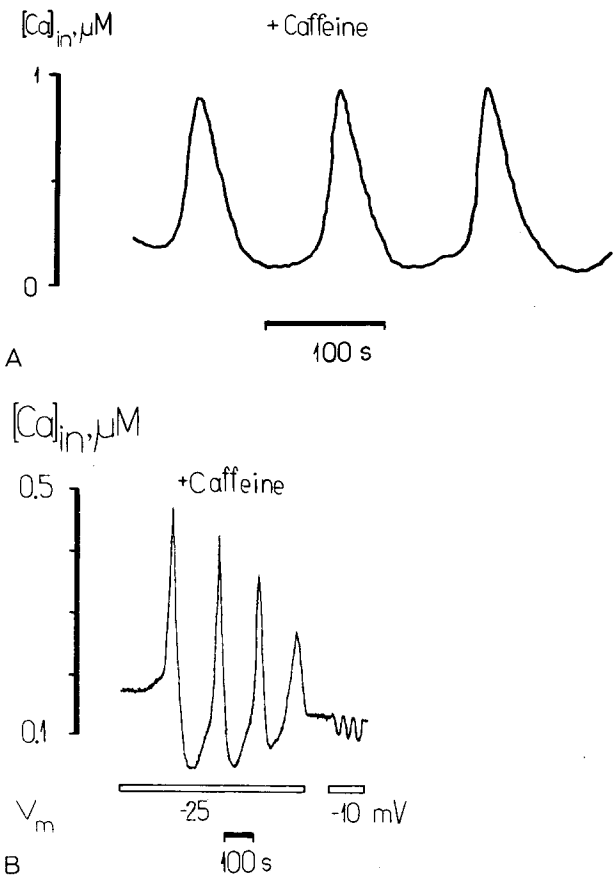

Fig. 7. Cyclic changes in $[Ca]_i$ observed in the presence of caffeine. (*A*) Spontaneous oscillations initiated by replacement of normal Ringer solution by caffeine-containing medium. (*B*) Initiation of oscillations in caffeine-containing medium during steady membrane depolarization to potentials indicated in the figure

$[Ca]_i$ increased upon membrane depolarization. These changes correlated well with the voltage dependence of calcium conductance in the somatic membrane of these neurons. When membrane potential returned to the resting value, $[Ca]_i$ recovered to the initial level. In general, several mechanisms may contribute to the whole process of $[Ca]_i$ recovery: (i) Ca binding by cytoplasmic proteins and other ligands, representing fast Ca buffers; (ii) Ca redistribution in the cytoplasm by simple diffusion; Ca extrusion through the cell surface membrane via (iii) Ca/Na exchange or (iv) Ca pump; Ca uptake within the cell by (v) mitochondria or by (vi) endoplasmic reticulum (ER). At present the exact quantitation of each mechanism is obviously impossible, because no specific pharmacological or physical tool exists to isolate it. Nevertheless, we feel that our data are sufficient to estimate a relative contribution of each mechanism listed in the process of $[Ca]_i$ recovery in snail neurones.

(1) Known on- and off-rate constants for Ca-binding proteins and for Ca chelators $k_{on} = 10^7$–10^8 M^{-1} sec^{-1} and $k_{off} = 10$–10^3 sec^{-1} (Levine & Williams, 1982) give an upper bound $\tau < 0.1$ sec for a fast Ca binding. Therefore, at present this process cannot be time resolved. In general, it should only scale Ca transients, bringing them from the mM to the μM range.

(2) Although Ca redistribution via diffusion is by no means important, especially for large cells, it can be safely excluded on the basis of observed temperature dependence of $[Ca]_i$ recovery. Measured $Q_{10} = 2.3$ is much larger than that for simple diffusion.

(3) Ca-transport systems in the surface membrane do not participate significantly in the process of $[Ca]_i$ recovery. In Na-free solutions Ca ions enter snail neurons via Ca/Na exchange (Fig. 3*A*). However, although Ca cannot exit the cell by this mechanism at $[Na]_o = 0$, the rate of $[Ca]_i$ recovery remained unchanged.

(4) La efficiently blocks not only Ca/Na exchange (Jacob, Lieberman & Liu, 1987, and references therein), but also suppresses the Ca pump (*see*, e.g., Gill & Chueh, 1985). However, La failed to change the process of $[Ca]_i$ recovery.

(5) Sodium azide and CCCP did distort the system of Ca uptake in mitochondria reflected as a long-term increase in the basal $[Ca]_i$ level. However, the rate of $[Ca]_i$ recovery was only marginally changed. It seems to indicate the absence of any significant contribution of mitochondrial Ca uptake to the process of $[Ca]_i$ recovery.

(6) Therefore, at present the data available inevitably lead to a conclusion that the largest contribution to Ca uptake in snail neurons is given by the Ca pump of endoplasmic reticulum. Although this suggestion seems to be obtained by simple rejection of all other known mechanisms of Ca removal from the cytoplasm, some direct data also favor it. First, the intracellular injection of vanadate (a well-known inhibitor of different ATPases; *cf.* Schatzmann, 1982) substantially increased both the basal level of $[Ca]_i$ and slowed down the process of its recovery. Second, the temperature dependence of $[Ca]_i$ recovery resembled that observed in biochemical studies of different Ca pumps. Q_{10} values cover the range from 2.1 to 2.4 (cited according to Schatzmann, 1985). Interestingly, Miledi, Parker and Zhu (1982), studying [Ca] transients in frog muscle fibers, estimated $Q_{10} = 2.4$ for the decay phase of Arsenazo III response. This temperature coefficient is close to that measured in snail neurons and Ca pumps.

However, for muscle fibers the time constant of $[Ca]_i$ recovery usually comprises the range from 50 to 500 msec, independent of the type of Ca-sensitive probe used (*compare*, e.g., Miledi et al. (1982), who used arsenazo III, and Timmermann and Ashley (1986), who used fura-2). The τ's for muscle cells are almost by two orders of magnitude higher than that observed for neurons. In the present work $[Ca]_i$ recovered according to a single exponent with the time constant varied from 10 to 30 sec. These values are in the range of those measured for other molluscan neurones using a metallochromic indicator dye Arsenazo III. Thus, for *Archidoris monteryensis* the time-course of $[Ca]_i$ recovery corresponded to $\tau \approx 7$ sec (Ahmed & Connor, 1979), for *Aplysia californica* τ ranged form 5 to 20 sec (Gorman & Thomas, 1978; Gorman et al., 1984). In these studies $[Ca]_i$ recovery was described as multi-exponential. This can be due to a larger size of neurons used in cited works (200–300 μm) compared to ours (ca. 50 μm). Since the effective time constant for the diffusion process $\tau' \approx x^2/D$ (where x is a linear dimension, D is the diffusion coefficient) is square-dependent on cell diameter, possibly the role of diffusion processes increases for larger cells, producing strongly nonexponential $[Ca]_i$ recovery.

The same arguments may account for the difference between τ's for nerve and muscle cells. It seems unlikely that the turnover rate or concentration of Ca pumps in muscle and nerve cells would be so strikingly different. The whole process of $[Ca]_i$ recovery is a complex one and is strongly modulated by Ca diffusion, Ca binding to cytoplasmic buffers and to Ca-sensitive dyes, which all may act as Ca sinks. At present all these processes are difficult to separate.

Obviously, our suggestion about the dominant contribution of the Ca pump to the process of $[Ca]_i$ recovery does not exclude the participation of other systems of Ca removal from the cytoplasm. Na/Ca exchange and Ca pump in the surface membrane must extrude excess Ca from the cell, otherwise the cell will be overloaded. However, according to our estimates these processes should work on a much longer time scale (e.g., minutes) than that studied in the present work. Undoubtedly, mitochondria also contribute to $[Ca]_i$ regulation, but their effectiveness is probably confined to the micromolar range of $[Ca]_i$ and extremely large Ca loads, as is constantly suggested in literature.

We tried to release stored Ca from ER by applying methylxanthines–caffeine and theophylline, which are traditionally used as inductors of Ca release from the sarcoplasmic reticulum (SR) of different contractile cells. The data indicating that in our case we also deal with the Ca release from ER are listed below.

(1) Lowering of $[Ca]_o$ to 0.5 mM did not affect the transient $[Ca]_i$ response. This can be compared with depolarization-induced Ca entry measured at different $[Ca]_o$ (Fig. 1*B*). Unfortunately, we could not remove all the Ca from the external solution, because observed $[Ca]_i$ transient could be induced only after preliminary loading of the cell with Ca.

(2) No depolarization of the surface membrane corresponds to the development of $[Ca]_i$ transient. Judging from the amplitude and the time course of this transient, one can conclude that if Ca is coming from the external medium, the corresponding inward current should be large enough to depolarize the membrane at least by 10–20 mV. Instead, we constantly observed membrane hyperpolarization, if the initial resting potential was larger than −50

mV, and no changes in membrane potential for more negative resting potentials. It is quite natural to suggest that the participation of Ca-activated potassium conductance produces the corresponding outward current whose ability to hyperpolarize the membrane is determined by the extent of the membrane potential shift from the equilibrium potassium potential.

(3) Besides their action on the SR, both caffeine and theophylline were also reported to inhibit the adenosine receptors in the surface membrane as well as the soluble cAMP-phosphodiesterase (*cf.* Fredholm, 1985). It is unclear how the first mechanism could lead to the transient effect we observed; however, the second one may account for this, because the increase in cAMP level was shown to raise $[Ca]_i$ (Hockberger & Connor, 1984; this work, Fig. 4*A*). It should be mentioned that in caffeine-containing solution $[Ca]_i$ did not change without additional Ca load, but direct intracellular cAMP injection led to a sustained increase in $[Ca]_i$ (Fig. 4*A*), which was distinctly different from that observed in the presence of caffeine.

All these arguments indicate that with the help of caffeine we were able to reveal a novel route of $[Ca]_i$ increase in nerve cells—a Ca-induced Ca release from ER, which closely resembles a well-known mechanism in contractile cells. The similarity of caffeine action on nerve and muscle cells is evident also from the inhibition of observed $[Ca]_i$ transient by local anesthetics. Their blocking potency lidocaine $\ll$ procaine $\lesssim$ tetracaine coincides with their efficacy to inhibit the contractions of guinea-pig ureter smooth muscle (Burdyga & Magura, 1986). In our case local anesthetics exert their blocking effect as a gradual increase in $[Ca]_i$ threshold. The impossibility of reaching it in the presence of anesthetics by loading the cell with Ca entering the cytoplasm via calcium channels of the surface membrane leads, finally, to a complete disappearance of Ca-induced Ca release (Fig. 6*B* and *C*).

Our data fit the suggestion (Martonosi, 1984; Palade, 1987) that caffeine facilitation of Ca-induced Ca release is due to an increase in the affinity of Ca-release channels to Ca. The intrinsic affinity of these channels in muscle cells should be higher than in nerve cells. Because of a lower threshold, a Ca-induced Ca release in SR can be observed in the absence of caffeine after relatively small Ca load, e.g., due to membrane depolarization. Caffeine decreased the threshold well below $[Ca]_i$ resting level, leading to massive spontaneous Ca release from SR. Postulated difference in Ca-affinity between Ca-release mechanisms in ER and SR may be caused by various factors, e.g., by some intracellular metabolites. In this context it is important that Ca release from SR can be activated by adenine nucleotides (Meissner, Darling & Eveleth, 1986). Therefore, discussed differences in transient Ca release from ER and SR could simply reflect the different metabolic activity of nerve and muscle cells.

Some contractile cells generate also cyclic and spontaneous contractions due to periodic release and uptake of Ca by SR (Fabiato, 1983). Recently we proposed a general mathematical model describing these effects (Mironov & Tepikin, 1988) which could explain many phenomena observed for heart cells and now for nerve cells. One of the predictions of this model is that $[Ca]_i$ oscillations should occur only in the case of constant Ca flow into the cell. We did observe this effect (Fig. 7*B*), and it is quite important that the amplitude and the frequency of oscillations depended inversely on the amplitude of Ca influx as predicted by the model.

Although these periodic changes in $[Ca]_i$ were obtained in somewhat nonphysiological conditions (namely, in the presence of caffeine), it is quite probable that some intracellular messenger might mediate the effect. Possible candidates are cAMP and inositolphosphates. The first compound produced a long-term increase in the membrane permeability to Ca, while the others may facilitate Ca release from ER. An obvious functional implication of $[Ca]_i$ oscillations should be connected with the generation of slow waves of membrane potential intrinsic for bursting neurons (*see*, e.g., Gorman & Thomas, 1978; Meech, 1979).

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