*Journal of Physiology* (1989), **415**, *pp.* 143–158
*With 7 text-figures*
*Printed in Great Britain*



# VOLTAGE-GATED AND AGONIST-MEDIATED RISES IN INTRACELLULAR $Ca^{2+}$ IN RAT CLONAL PITUITARY CELLS ($GH_3$) HELD UNDER VOLTAGE CLAMP

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(*Received 19 September 1988*)

SUMMARY

1. Intracellular free calcium ($Ca_i^{2+}$) was estimated in single $GH_3$ cells by dual wavelength emission spectrofluorimetry using the $Ca^{2+}$ indicator dye Indo-1, while cells were held under voltage clamp using patch clamp techniques.

2. Depolarization of cells evoked a transient rise in $Ca_i^{2+}$ that increased with increasing duration of depolarization to a peak at about 10 s.

3. Calcium transients showed a bell-shaped dependence on the amplitude of the depolarizing pulse. They were abolished in the absence of extracellular calcium and by application of 10 $\mu$M-nifedipine.

4. Thyrotrophin-releasing hormone (TRH) evoked a transient rise in $Ca_i^{2+}$ that was followed by a more sustained period of elevated $Ca_i^{2+}$ in some cells. The transient phase of the response but not the sustained phase was seen in the absence of extracellular calcium.

5. $Ca_i^{2+}$ transients evoked by depolarization were not affected by pre-release of internal $Ca^{2+}$ stores with TRH.

6. The results demonstrate that voltage-gated $Ca^{2+}$ entry and $Ca^{2+}$ store release can each elevate cytoplasmic free calcium in $GH_3$ cells and may both be important for stimulus–secretion coupling. Non-voltage-gated $Ca^{2+}$ entry is not a major source of $Ca^{2+}$ under these conditions.

## INTRODUCTION

$GH_3$ cells, prolactin-secreting cell lines from rat anterior pituitary gland have been widely studied as a convenient model of stimulus–secretion coupling (for recent reviews see Gershengorn, 1986; Ozawa & Sand, 1986; Mason, Rawlings, Cobbett, Sikdar, Zorec, Akerman, Benham, Berridge, Cheek & Moreton, 1988). TRH-stimulated prolactin secretion by $GH_3$ cells is a $Ca^{2+}$-dependent process (Thorner, Hackett, Murad & Macleod, 1980) and involves a biphasic $Ca^{2+}$ signal. An initial $Ca^{2+}$ transient, not dependent on extracellular $Ca^{2+}$ is followed by a sustained plateau of $Ca^{2+}$ that is partly blocked by antagonists of voltage-gated $Ca^{2+}$ channels (Albert & Tashijan, 1984; Gershengorn & Thaw, 1985). The membrane potential changes during this response are an initial hyperpolarization followed by a sustained depolarization and burst of action potential firing (Ozawa, 1981).

During action potential discharge, extracellular $Ca^{2+}$ enters $GH_3$ cells through voltage-gated $Ca^{2+}$ channels (Kidokoro, 1975). $GH_3$ cells have two types of voltage-gated $Ca^{2+}$ channels (Hagiwara & Ohmori, 1982; Armstrong & Matteson, 1985), which could both serve as pathways for $Ca^{2+}$ entry during the second phase of the secretagogue response. Further support for this is provided by recent evidence using Fura-2 as the $Ca^{2+}$ indicator that a rise in $Ca_i^{2+}$ in response to single action potential discharge occurs under certain conditions in $GH_3$ cells (Schlegel, Winiger, Mollard, Vacher, Wuarin, Zahnd, Wollheim & Dufy, 1987).

The initial $Ca^{2+}$ transient is temporally associated with a rise in cytoplasmic inositol trisphosphate ($IP_3$) levels evoked by activation of phosphoinositidase C (Rebecchi & Gershengorn, 1983) and $IP_3$ injection can mimic the initial transient events following agonist stimulation (Dufy, Jaken & Barker, 1987). It is not known whether any membrane potential changes are required for this response. Protein kinase C activation which is also a consequence of phosphoinositidase C activation has a delayed inhibitory effect on $Ca_i^{2+}$ (Drummond, 1985) and this may be due to inhibition of voltage-gated $Ca^{2+}$ currents (Marchetti & Brown, 1988). These results, taken together, have led to the widely accepted hypothesis that the initial $Ca^{2+}$ transient is due to intracellular store release while the second phase of the response is partly due to voltage-gated $Ca^{2+}$ entry.

In addition to voltage-gated $Ca^{2+}$ entry there is some evidence that another agonist-stimulated pathway for $Ca^{2+}$ entry exists which might explain the incomplete block of the second phase of the $Ca_i^{2+}$ response by $Ca^{2+}$ antagonists. A non-voltage-dependent, $Ca^{2+}$-permeable conductance activated by secretagogues has recently been described in gonadotrophs which would be expected to contribute to agonist-mediated $Ca^{2+}$ influx into the cell (Mason & Waring, 1985).

The combination of microspectrofluorimetry and patch clamp techniques (Almers & Neher, 1985) allows measurement of $Ca^{2+}$ levels in single $GH_3$ cells under voltage clamp and hence characterization of each of the various mechanisms for the elevation of $Ca_i^{2+}$. This paper describes studies to assess the contribution of these processes to the elevation of $Ca_i^{2+}$ in $GH_3$ cells. A preliminary account of some of this work has been published as an abstract (Benham, 1988).

## METHODS

$GH_3$ cells were plated onto glass No. 1·5 cover slips and grown in Dulbecco's modified Eagle's medium supplemented with 16% horse serum, 2·5% fetal calf serum and 50 U/ml penicillin, 0·05 mg/ml streptomycin. Cells were used 3–8 days after plating. Cover-slips were sealed with silicon grease over a circular hole (diameter 12 mm) in a stainless-steel plate making a recording chamber with the cover-slip forming the base.

Cells were bathed in an extracellular solution containing (mM): 130 NaCl, 5 KCl, 10 glucose, 1 $CaCl_2$, 1·2 $MgCl_2$, 10 HEPES, buffered to pH 7·3 with NaOH. The pipette-filling (intracellular) solution was made up in Milli-Q water (Millipore) and in most experiments contained (mM): 125 KCl, 2 $Na_2ATP$, 4 $MgCl_2$, 10 HEPES, 0·025–0·1 Indo-1 (potassium salt), buffered to pH 7·2 with NaOH. No other $Ca^{2+}$ buffers were added to the pipette solution. The slightly hypotonic solution was found to help maintain stable whole-cell recording.

Voltage clamp and membrane current recording were made with standard patch clamp techniques (Hamill, Marty, Neher, Sakmann & Sigworth, 1981) using a List EPC-7 patch clamp

amplifier. Voltage steps were produced by a step command generator (University of Newcastle) triggered by a Digitimer. Membrane potential and current signals were passed through a modified Sony PCM 701 digital audio processor (Lamb, 1985) and stored on video tape.

*Fluorescence measurements*

$Ca^{2+}$ was estimated from Indo-1 fluorescence by the ratio method using single wavelength excitation and dual emission (Grynkiewicz, Poenie & Tsien, 1985; Cobbold & Rink, 1987). The optical system was developed around a Zeiss IM-35 microscope equipped for epifluorescence. Single cells loaded with Indo-1 from the patch pipette were centred under a window created by a pinhole placed in the emission beam. Light collection was thus limited to an area just greater than the dimensions of an average cell. Excitation was provided at 360 nm by a Zeiss XBO 75 Xenon arc lamp and emitted light after splitting by a dichroic mirror was detected by two parallel photomultipliers at 405 and 480 nm (10 nm bandwidth) in photon counting mode.

Subsequent signal processing was performed by a Thorn EMI Photon Detection System interfaced with an HP Vectra personal computer. Simultaneous counts at the two wavelengths were sampled at 2–20 Hz and stored on disc for subsequent analysis. A crude ratio was calculated on-line and displayed on a Lectromed chart recorder with the membrane current signal allowing the experiment to be monitored and the two signals to be synchronized. All $Ca_i^{2+}$ records have been smoothed by taking a three-point moving average of the $Ca_i^{2+}$ values. Means values are expressed as mean $\pm$ S.E.M. and time constants were fitted by eye to semilogarithmic plots of the data.

*Estimation of $Ca_i^{2+}$*

Background fluorescence contributed by cell autofluorescence and Indo-1 in the pipette was measured at both wavelengths in the cell attached mode. On break-through to whole-cell mode, loading could be monitored from the emitted light which reached equilibrium at 3–5 min after break-through with counts of 10–50 times background (0·1 mM-Indo-1) and recording was started at this point. Background values measured before break-through were then subtracted from each count in whole-cell mode. These corrected values were used to give the 405/480 ratio ($R$) at each time point. This was initially used to estimate $Ca_i^{2+}$ according to the relationship:

$$Ca_i^{2+} = K(F_o/F_s)\,(R-R_{min})/(R_{max}-R),$$

where $R_{min}$ and $R_{max}$ are the ratios obtained in the absence of $Ca^{2+}$ and at saturating $Ca^{2+}$, $K$ is the effective dissociation constant, $F_0$ is the 480 signal in the absence of $Ca^{2+}$, and $F_s$ the 480 signal in the presence of saturating $Ca^{2+}$ (Grynkiewicz *et al.* 1985). $R_{min}$ and $R_{max}$ were determined *in vitro* by sandwiching small drops of pipette solution with 10 mM-EGTA or 10 mM-$Ca^{2+}$ added between two cover-slips and measuring the ratio.

However, during experiments it became clear that the behaviour of Indo-1 inside cells was altered as it was possible to get $R$ values greater than $R_{max}$ determined *in vitro*. So, calibration values were determined for Indo-1 within cells, a similar approach to the one that Almers & Neher (1985) used for Fura-2 calibration in mast cells. $R_{min}$ was determined in cells by loading cells with pipette solution containing 20 mM-EGTA and taking the 405/480 ratio (after background subtraction) when it had stabilized as $R_{min}$. $R_{max}$ was determined at the end of each experiment by hyperpolarizing the cell to $-200$ mV (Fig. 1). This caused membrane break-down and a large increase in $Ca^{2+}$ permeability without resulting in significant dye leakage. $Ca_i^{2+}$ initially rose rapidly and then more slowly for several minutes before stabilizing. This slow increase could be due to slow saturation of high-capacity, low-affinity $Ca^{2+}$ buffers within the cell. $R_{max}$ was taken as the 405/480 ratio when the ratio had stabilized which normally ocurred within 5 min of hyperpolarizing the cell. As this measurement could be attempted in every experimental cell it provided a check for stability of recording conditions. These values were subject to change if filters were changed or as the characteristics of the lamp changed slowly during its lifetime. If $R_{max}$ values changed significantly from one day to the next than recalibration of $R_{min}$ was made. On four representative days when this was done $R_{min}$ values were $0{\cdot}04 \pm 0{\cdot}01$ *in vitro* and $0{\cdot}08 \pm 0{\cdot}01$ *in vivo* and for $R_{max}$ were $0{\cdot}61 \pm 0{\cdot}04$ and $0{\cdot}71 \pm 0{\cdot}05$ *in vitro* and *in vivo* respectively. This difference between *in vitro* and *in vivo* $R_{min}$ and $R_{max}$ values would give a 65% overestimate of resting $Ca_i^{2+}$ for a typical 405/480 ratio obtained in an unstimulated cell.

As the behaviour of Indo-1 cytoplasm is not identical to that in saline solution this raises a question over the validity of using the published value for the dissociation constant for Indo-1. So an intracellular value for the quantity $K^* = K(F_o/F_s)$ was also determined according to Almers & Neher (1985). The 405/480 ratio was determined in cells loaded with pipette solution containing 6 mM-Ca–EGTA and 3 mM-free EGTA. Assuming an apparent dissociation constant for EGTA of 151 nM at pH 7·2, then this solution had a buffered free $Ca^{2+}$ concentration of 309 nM. The ratio was taken when filling was complete, at which time (about 5 min after break-through) depolarization to 0 mV for 5 s failed to change the ratio, suggesting that $Ca_i^{2+}$ was adequately buffered by the Ca–EGTA. For example, a mean value for $K^*$ of 952 nM was derived from four cells on a day when $R_{max}$ was 0·85 and $R_{min}$ was 0·08. As it was not possible to obtain $F_o$ and $F_s$ values in the same cell a true value for the dissociation constant of Indo-1 *in vivo* could not be derived. $F_o/F_s$ values obtained at the same time *in vitro* gave a value for $K$ of 213 nM (the value obtained by Grynkiewicsz *et al.* (1985) is 250 nM at 37 °C and at similar ionic strength).

*Reliability of estimated $Ca_i^{2+}$ values*

The attempts described here to obtain accurate calibration of the fluorescence ratio signals illustrate how quite large errors can be introduced by relatively small changes in $R_{max}$ and $R_{min}$ values. Any error in $K^*$ will directly translate into the same magnitude error in estimated $Ca_i^{2+}$. Thus, for example, 10% errors in $R_{max}$ and $K^*$ would give an error of 40% in $Ca_i^{2+}$ for values around 350 nM. The maximum error in individual cells is probably about 50%. Independent evidence suggests that larger errors are unlikely. Experiments on vascular smooth muscle cells show good agreement between $Ca_i^{2+}$ estimated from contractile activity and the activity of $Ca^{2+}$-activated $K^+$ currents when compared to that derived from Indo-1 measurements on the same cells (C. D. Benham, unpublished observations).

Although the ratio technique may not be the most accurate for absolute estimations of $Ca_i^{2+}$, it is highly sensitive for detecting changes in intracellular $Ca^{2+}$ in the same cell. It is this particular strength that is exploited in the experiments described here.

*Indo-1 acetoxymethyl ester (AM)-loaded cells*

Some recordings were made from unclamped cells that were loaded with the cell penetrant acetoxymethyl (AM) ester. Cells on cover-slips were loaded for 40 min at room temperature in bathing solution containing 5 μM-Indo-1 AM. The cells were then washed and left for a further 20 min for ester hydrolysis to go to completion. After this time cells displayed resting ratios and total counts similar to those under voltage clamp with 480 nm and 405 nm counts at 10- to 20-fold higher than background values obtained from unloaded cells. Also, depolarization with high-$K^+$ solution resulted in ratios approaching $R_{max}$, suggesting that most of the indo-AM had been hydrolysed to the $Ca^{2+}$-sensitive form. It was assumed that the hydrolysed Indo-1 behaved in a similar way to that in the Indo-1 potassium salt-filled cells for calibration purposes.

*Chemicals*

Intracellular solutions were made with Gold label-grade salts (Aldrich) where possible and Milli-Q water. These precautions to minimize $Ca^{2+}$ contamination resulted in pipette solutions containing 50–100 nM-free $Ca^{2+}$ when Indo-1 (0·1 mM) was the only $Ca^{2+}$ buffer. Intracellular solutions were stored in plastic containers frozen in aliquots and Indo-1 was added from a 1 mM stock solution each day. Indo-1 potassium salt and AM were from Molecular Probes, sodium ATP was from Boehringer, TRH was from Sigma, and culture media were from Gibco.

## RESULTS

### *Resting $Ca_i^{2+}$*

Estimated cytoplasmic free $Ca^{2+}$ ($Ca_i^{2+}$) was 128+7 nM in twenty-four cells held under voltage clamp at −70 mV. $Ca_i^{2+}$ did not increase or decrease significantly during diffusion of Indo-1 from the patch pipette into the cell, which reached an equilibrium at 3–5 min after break-through into whole-cell configuration. The degree

of loading varied with the access resistance and rate of filling but no systematic effect of this variation was seen on $Ca_i^{2+}$. This suggests that the $Ca^{2+}$-buffering capacity of the Indo-1 did not significantly affect the buffering capacity of the cell. The ability of cells to recover from a $Ca^{2+}$ load was also little affected by perfusing cells for short periods as compared with Indo-AM-loaded cells (see Fig. 5). Some deterioration in the buffering capacity of cells was seen after extended perfusion periods and heavy $Ca^{2+}$ loading. This resulted in incomplete recovery of $Ca_i^{2+}$ after $Ca^{2+}$ loads. The trace shown in Fig. 1 started more than 15 min after break-through to whole-cell mode. The steadily rising basal $Ca_i^{2+}$ was often seen in cells after this time. For this reason data was usually obtained between 5 and 15 min after break-through to whole-cell mode. Cell fluorescence on visual inspection was found to be even, without bright punctate spots or noticeably dim areas, consistent with an even cytoplasmic distribution.

*Voltage-gated $Ca^{2+}$ transients*

Depolarization of cells held under voltage clamp evoked outward $K^+$ currents which dominated the whole-cell current recorded so that net outward currents were always seen (Figs 2, 3, 5, 6 and 7). In other experiments reported elsewhere (Benham, 1988), using CsCl-based pipette solution instead of KCl to minimize $K^+$ currents, it was possible to record voltage-gated $Ca^{2+}$ currents during, and $Ca^{2+}$-activated $Cl^-$ currents after, depolarizing voltage jumps.

Depolarization of cells of 0 mV caused a transient rise in $Ca_i^{2+}$. The amplitude of the $Ca^{2+}$ transient increased with the duration of depolarization, reaching a maximum of about 500 nM after 5–10 s. Figure 1*A* shows the reciprocal changes in counts at 480 and 405 nm and estimated $Ca_i^{2+}$ derived from these values in one cell. $Ca_i^{2+}$ rose rapidly in the first 500 ms and then more slowly as it approached steady state, in which influx was balanced by efflux and intracellular buffering mechanisms. For short depolarizations (100–200 ms), sampling at 10 Hz showed that the $Ca_i^{2+}$ signal continued to rise for two 100 ms sampling points following repolarization. This could be explained by diffusion of $Ca^{2+}$ into the cell from a local high concentration under the cell membrane into the bulk cytoplasm. As this occurred, more even distribution of $Ca^{2+}$ might result in a larger integrated Indo-1 signal. $Ca^{2+}$ influx during the tail $Ca^{2+}$ current might also contribute to this continued rise (Barcenas-Ruiz & Weir, 1987).

The mean peak change in $Ca_i^{2+}$ for four cells (Fig. 1*B*) showed an approximately hyperbolic dependence on the length of depolarizing pulse. This was in contrast to the response of rat cardiac myocytes where $Ca_i^{2+}$ showed bell-shaped voltage dependence, but reached a peak after less than 0·2 s and then declined during continued depolarization. In cardiac myocytes a component of this initial peak appeared to be due to intracellular $Ca^{2+}$ store release (Callewaert, Cleeman & Morad, 1988).

The amplitude of the voltage-gated transient was also dependent on the size of the depolarizing step. Figure 2*A* shows a record for one cell which was depolarized to increasingly positive potentials for 200 ms every 20 s. The relationship between the mean increase in $Ca_i^{2+}$ and potential is plotted for four similar experiments in Fig. 2*B*. $Ca_i^{2+}$ first started to rise at about $-50$ mV, the increase reached a peak at around

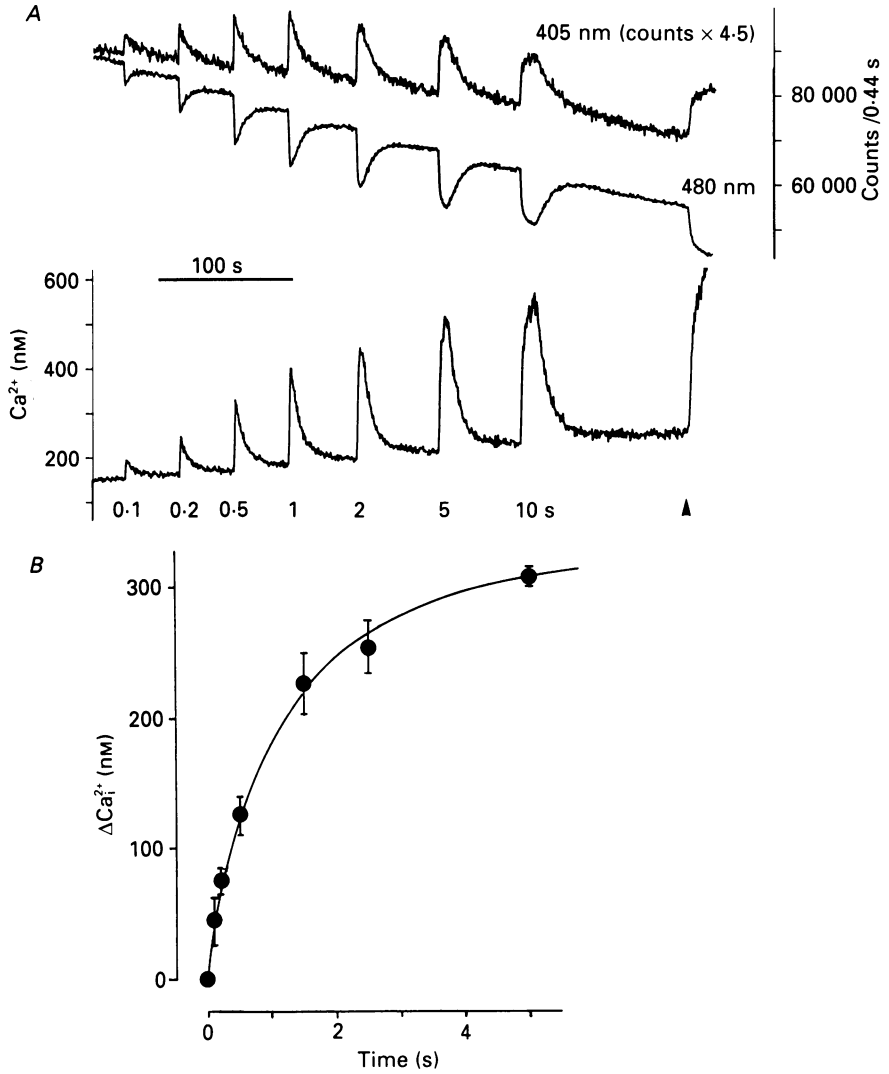


Fig. 1. Relationship between length of depolarization and amplitude of rise in $Ca_i^{2+}$. *A*, fluorescence signal at 405 nm (scaled up by a factor of 4·5 for clarity) and 480 nm (upper pair of traces) and estimated $Ca_i^{2+}$ (lower trace) from a single cell depolarized to 0 mV from −70 mV for the periods indicated below the $Ca_i^{2+}$ transients. At the arrow the cell was hyperpolarized to −200 mV to permeabilize the cell and allow estimation of $R_{max}$. At the end of the trace $R$ was 0·40 and continued to increase more slowly before stabilizing after 4 min at a value of 0·67 which was taken as $R_{max}$. *B*, peak increases in $Ca_i^{2+}$ values plotted against duration of depolarization to 0 mV from −70 mV. Mean of four cells. Error bars are S.E.M.

0 mV and then declined to a negligible level at +60 mV. This bell-shaped relationship suggests that the rise in $Ca_i^{2+}$ was dependent on $Ca^{2+}$ entry through voltage-gated $Ca^{2+}$ channels. The associated membrane current recordings showed that outward $K^+$ currents increased with depolarization which swamped the inward $Ca^{2+}$ current. The rise in $Ca_i^{2+}$ in response to these short depolarizations was completely blocked by

10 $\mu$M-nifedipine (three experiments) indicating that $Ca^{2+}$ entry was mainly through dihydropyridine-sensitive channels. The rise in $Ca_i^{2+}$ was also abolished by removal of extracellular $Ca^{2+}$ (Fig. 7*A*)

Recovery of $Ca_i^{2+}$ to resting levels on repolarization was slow, taking several seconds, and displayed complex kinetics, reflecting the variety of mechanisms that

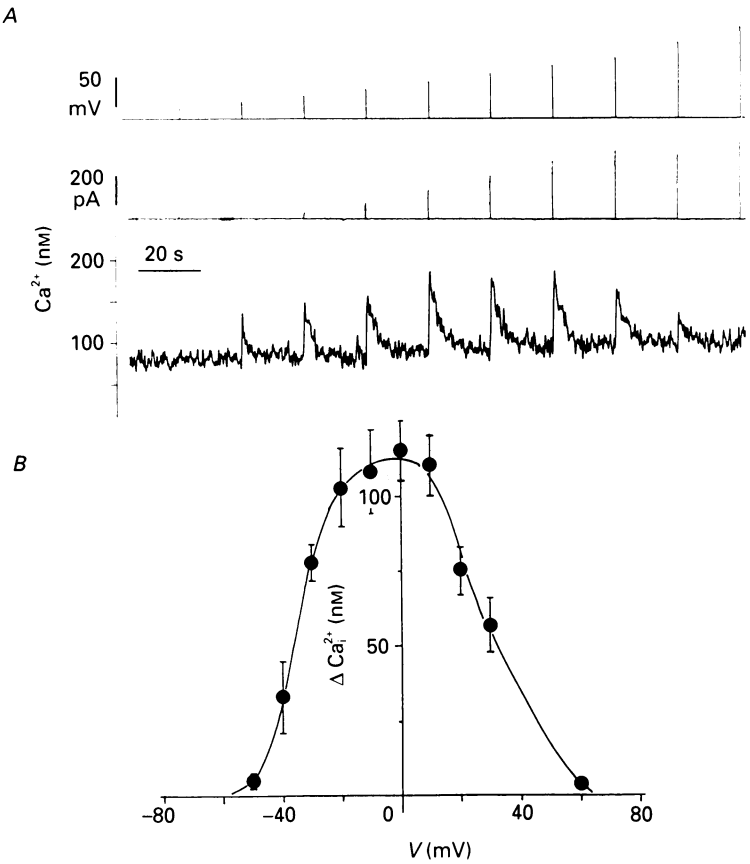


Fig. 2. Voltage dependence of $Ca_i^{2+}$ transients. *A*, membrane potential (upper) membrane current (middle) and estimated $Ca_i^{2+}$ (lower) traces from a single cell. Note net membrane currents to the first two depolarizing voltage jumps were very small. *B*, peak increases in $Ca_i^{2+}$ plotted against test potential. Means for four cells held at $-70$ mV and depolarized for 200 ms every 20 s.

might be expected to remove free $Ca^{2+}$ from the cytoplasm. Recovery from large increases in $Ca_i^{2+}$ during prolonged depolarizations was sometimes not complete, leading to a slowly rising baseline (Fig. 1*A*). However, for short depolarizations of 100 ms to a test potential of 0 mV most of the recovery phase could be fitted to a single exponential with a time constant of $3{\cdot}4 \pm 0{\cdot}5$ s ($n = 9$). The peak $Ca_i^{2+}$ attained in these responses was $337 \pm 38$ nM.

The slowness of the recovery phase suggested that trains of voltage jumps of higher frequency than about 0·25 Hz would lead to overlap and summation of $Ca^{2+}$ transients. Depolarizing voltage jumps were applied in trains to see if this occurred.

When the stimulus frequency was 0·5 Hz, this was sufficiently fast to superimpose successive $Ca^{2+}$ transients and some summation was seen. In Fig. 3 the fourth transient in the train reached a peak elevation in $Ca_i^{2+}$ that was double that in response to the first depolarization in the train. Further transients after the fourth

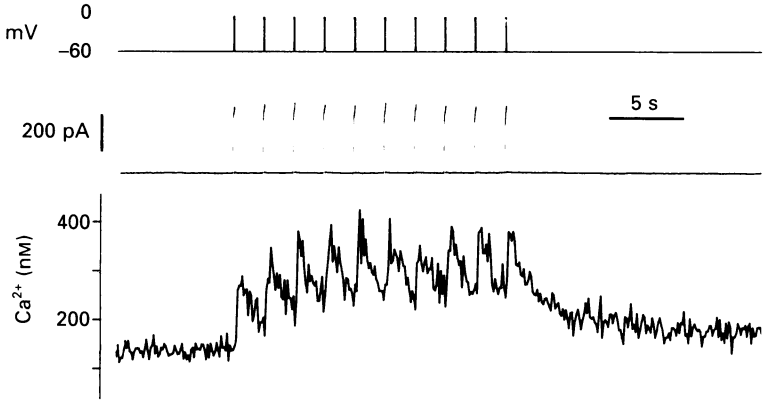


Fig. 3. Trains of depolarizing voltage steps evoke $Ca_i^{2+}$ transients that summate. Cell was held at −60 mV and depolarized to 0 mV for 200 ms at 0·5 Hz. Traces as in Fig. 2*A*.

failed to elevate $Ca_i^{2+}$ above this plateau level in all three cells where this stimulus protocol was used. This level was not the maximal rise for the cell as it could be exceeded by increasing the frequency of stimulation or by continuous depolarization of the cell. Thus each frequency of stimulation appeared to specify a particular peak $Ca_i^{2+}$.

*$Ca^{2+}$ transients in unclamped cells*

Fluorescence signals were also collected from Indo-AM-loaded cells that were not held under voltage clamp. Resting $Ca_i^{2+}$ in eleven cells was 175±14 nM. Some cells showed spontaneous oscillations in $Ca_i^{2+}$ with similar kinetics to those seen in voltage-clamped cells in response to depolarizing voltage steps. An example of this behaviour is shown in Fig. 4. When 60 mM-$K^+$-containing solution was applied to the cell this evoked a sustained rise in $Ca_i^{2+}$. The spontaneous oscillations were presumably due to action potentials occurring in the unclamped cells (Schlegel *et al.* 1987). It is interesting that in unclamped, spontaneously active cells such as this example there was little summation of transients even though $Ca_i^{2+}$ did not recover completely between transients. This was due to a decrease in the amplitude of the $Ca^{2+}$ transients so that, although they arose from a higher initial $Ca_i^{2+}$, the peaks of subsequent transients did not exceed the height of the first two. This may have been due to a reduced $Ca^{2+}$ influx during successive action potentials either because the $Ca^{2+}$ current became partly inactivated or because $K^+$ currents shortened the duration of the action potential.

The spontaneous oscillations had very similar time courses to the transients evoked by depolarizations that elicited a similar peak rise in $Ca_i^{2+}$ (Fig. 5). The similarity between these spontaneous transients and those recorded from cells under voltage clamp indicates that internal perfusion of the cell does not seriously compromise normal cell calcium buffering in response to this type of $Ca^{2+}$ load.

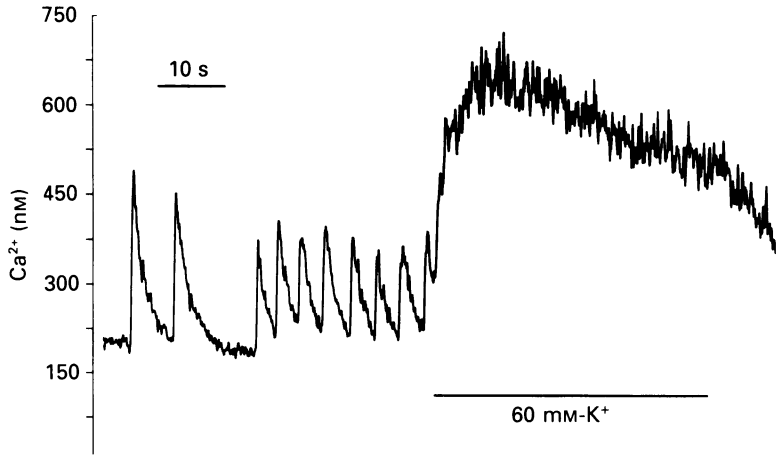


Fig. 4. Spontaneous $Ca_i^{2+}$ transients in a single unclamped cell loaded with Indo-1 AM. External $K^+$ was increased for the duration of the bar by equimolar substitution of KCl for NaCl.

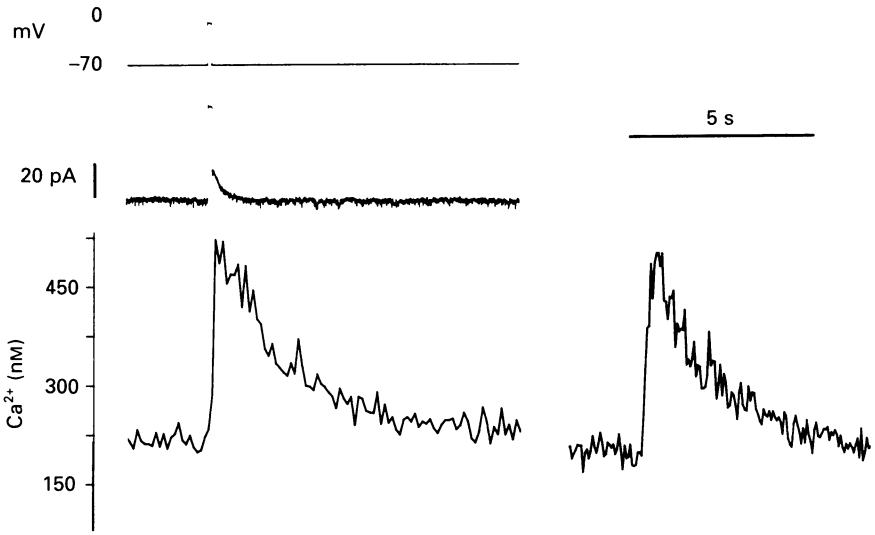


Fig. 5. Comparison of voltage-gated $Ca_i^{2+}$ transient in a perfused cell held at −70 mV and depolarized to 0 mV for 100 ms (left) with spontaneous $Ca_i^{2+}$ transient in another unclamped cell displayed on the same time scale (right). The sampling rates for the fluorescence measurements were 10 Hz for the perfused cell and 20 Hz for the unclamped cell.

*Effect of thyrotrophin-releasing hormone (TRH)*

Thyrotrophin-releasing hormone (TRH) has been shown to elevate $Ca_i^{2+}$ in suspensions of cells (Gerschengorn & Thaw, 1985), and this is thought to be mediated by a combination of intracellular store release and $Ca^{2+}$ entry through voltage-gated

$Ca^{2+}$ channels. Figure 6 shows the effect of bath application of $10^{-7}$ M-TRH, to a cell held at −70 mV which was also periodically stimulated by voltage jumps to 0 mV for 500 ms. TRH triggered a brisk rise in $Ca_i^{2+}$ which reached a peak 9 s after the onset of the response. In nine cells $Ca_i^{2+}$ rose from 116 ± 10 to 353 ± 28 nM at the peak

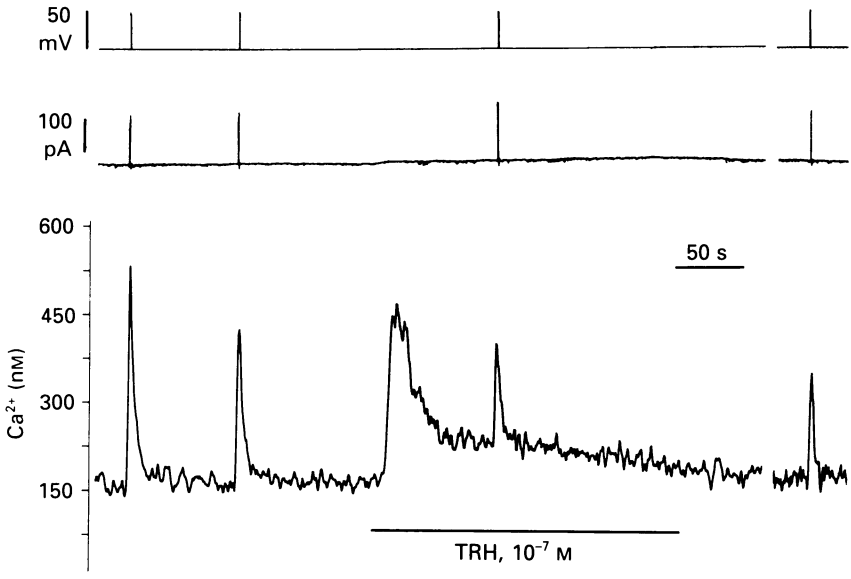


Fig. 6. TRH-evoked and voltage-gated $Ca_i^{2+}$ transients in the same cell. TRH was applied in the bathing solution for the duration of the horizontal bar. Holding potential, −60 mV.

of the response. $Ca_i^{2+}$ then declined after about 50 s to a plateau level of 156 ± 15 nM. In the response illustrated, a small outward current was seen coincident with the $Ca^{2+}$ peak, while in other cells a small inward current was associated with the response. This may reflect a varying balance in the contributions of $Ca^{2+}$-activated $K^+$ and $Cl^-$ current from cell to cell at this holding potential. Thus the TRH-mediated transient rise in $Ca_i^{2+}$ occurs in the absence of any change in membrane potential.

Voltage-gated $Ca^{2+}$ transients were stimulated in this cell before and during the TRH response. Voltage-gated responses in this cell were of approximately the same amplitude as the TRH response and declined on repetition. The response seen during the plateau phase reached a similar peak to that before and after wash-out. This suggests that the TRH-sensitive pool of $Ca^{2+}$ is independent of the response to voltage-gated jumps and $Ca^{2+}$ entry. If a voltage step was evoked at the peak of the TRH response it also evoked a near-normal response, also consistent with a lack of involvement of the TRH-sensitive store in the voltage-gated transients.

Lower concentrations of TRH ($10^{-9}$–$10^{-7}$ M) also evoked a transient rise in $Ca_i^{2+}$ followed by recovery of $Ca_i^{2+}$ to close to the basal level. No repetitive oscillations of any sort were seen in these cells under voltage clamp in response to low concentrations of TRH. Following application of $10^{-9}$ M-TRH and wash-out it was possible to record a second response to TRH if a 10-fold higher concentration was used although this

second response was of smaller amplitude. When a high concentration of $10^{-7}$ M-TRH was applied first and then the cell was washed it was not possible to elicit a second response in the same cell in five out of five attempts even after 10 min washing. Presumably intracellular mediators of the response to TRH were being exhausted.

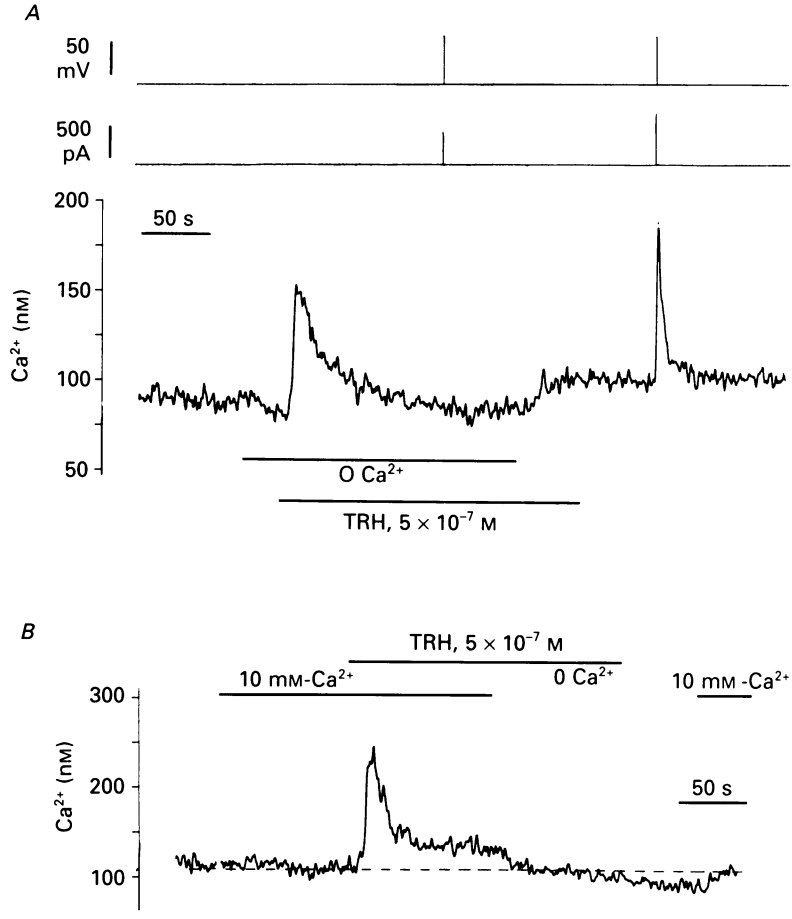


Fig. 7. Extracellular $Ca^{2+}$ dependence of TRH response. *A*, TRH application in the absence of extracellular $Ca^{2+}$. Note voltage-gated transient cannot be evoked in the absence of external $Ca^{2+}$ but is restored on restoring $Ca_o^{2+}$. *B*, removal of $Ca_o^{2+}$ during plateau phase of TRH response restores $Ca_i^{2+}$ to basal level (indicated by dashed line).

The transient phase of the TRH response could be evoked in the absence of extracellular $Ca^{2+}$. Figure 7*A* shows the effect of applying TRH to a cell bathed in nominally $Ca^{2+}$-free solution (no $Ca^{2+}$ chelator added). $Ca_i^{2+}$ rose normally but then decayed back to the basal level (three cells). The absence of extracellular $Ca^{2+}$ was tested by applying a voltage step to 0 mV which failed to evoke a rise in $Ca_i^{2+}$. Restoring $Ca_o^{2+}$ to 1 mM in the presence of TRH caused a rise in $Ca_i^{2+}$. This suggested that the plateau phase of the response might be dependent on extracellular $Ca^{2+}$ entry. If this was the case, then increasing external $Ca^{2+}$ should enhance the plateau

phase. When $Ca^{2+}$ was elevated to 10 mM (Fig. 7*B*), then a significant plateau was seen and this plateau level declined when extracellular $Ca^{2+}$ was removed as expected if TRH-stimulated $Ca^{2+}$ influx was responsible for this elevation, but the plateau level was little different from that seen in normal 1 mM-$Ca_o^{2+}$ (Figs 6 and 7). Moreover, $Ca_i^{2+}$ continued to fall to below the control resting level after TRH wash-out. This result suggests that the dependence of the plateau on extracellular $Ca^{2+}$ could be due to enhanced $Ca^{2+}$ efflux in the absence of external $Ca^{2+}$ resulting in more efficient extrusion of the $Ca^{2+}$ load delivered in the first part of the TRH response.

If an agonist-stimulated $Ca^{2+}$ entry pathway was involved in the maintenance of the plateau $Ca_i^{2+}$ level and was voltage independent then $Ca_i^{2+}$ would be dependent on the electrochemial $Ca^{2+}$ driving force. As an alternative to raising extracellular $Ca_o^{2+}$ this possibility was tested by applying hyperpolarizing voltage jumps of 10 s duration to cells before and during TRH application (Penner, Matthews & Neher, 1988). Hyperpolarizations from $-60$ to $-100$ mV had no effect on resting $Ca_i^{2+}$ or on the transient and plateau phases of the TRH response (three cells). Increasing the magnitude of hyperpolarization to $-140$ mV to give a driving force change of 80 mV started to cause non-specific break-down of membrane resistance and a rise in $Ca_i^{2+}$ both in the presence and absence of TRH. Changing the driving force by holding at potentials positive to $-60$ mV was precluded by the presence of voltage-gated $Ca^{2+}$ channels. So these restricted manipulations were unable to show clear evidence for a significant agonist-gated $Ca^{2+}$ influx through non-voltage-gated channels.

## DISCUSSION

The results demonstrate that agonist-stimulted release of intracellular stores and voltage-gated $Ca^{2+}$ channels are the two most important pathways for elevating $Ca_i^{2+}$ in these cells when stimulated by TRH. A possible contribution from agonist-gated $Ca^{2+}$ channels (Mason & Waring, 1985) was insignificant under these conditions.

Recording of agonist-evoked responses involving intracellular mediators has been difficult to achieve in some internally perfused cells due to the phenomenon of 'wash-out' of cytoplasmic constituents (Marty & Neher, 1983; Fernandez, Neher & Gomperts, 1984). Agonist responses tend to be lost after several minutes in whole-cell recording mode and multiple responses are difficult to achieve. This problem can be circumvented by limited diffusional exchange either by reducing the pipette tip diameter (Yakel, Trussel & Jackson, 1987) or by permeabilizing the cell-attached patch rather than rupturing it (Lindau & Fernandez, 1986) allowing complete dose–response curves to be generated in single cells (Horn & Marty, 1988).

In the experiments reported here the need to fill the cells with Indo-1 meant that the cells were relatively well perfused. Although it was not possible to evoke multiple responses, single responses were recorded between 5 and 10 min after break-through into whole-cell mode. Other authors have shown that TRH-induced hyperpolarizations in $GH_3$ cells were almost completely attenuated after 10 min in whole-cell recording mode (Dufy, MacDermott & Barker 1986). In intact cell populations maximal TRH concentrations evoked increases in $Ca_i^{2+}$ from resting levels of 150 nM to an initial peak of 500 nM (Gerschengorn & Thaw, 1985). Initial peaks of around 500 nM were also reported by Drummond (1985) again using Quin-2 as $Ca^{2+}$

indicator. In our perfused cells under voltage clamp the peak response was 353 nM in nine cells. In the unclamped cells some of the rise in $Ca_i^{2+}$ might be due to $Ca^{2+}$ entry through voltage-gated $Ca^{2+}$ channels in which case a fairer comparison would be with unclamped cells in $Ca^{2+}$-free conditions when the initial peak was reduced to between 350 and 400 nM (Gershengorn & Thaw, 1985). This suggests that attenuation of the $Ca^{2+}$-release phase of TRH responses was not a major problem in these experiments. Experiments using a similar single agonist dose application in parotid cells, showed that carbachol-induced release of $Ca^{2+}$ from internal stores was not impaired in perfused cells loaded with Fura-2 salt when compared with Fura-AM-loaded intact cells (Gray, 1988). These experiments in which $Ca_i^{2+}$ was measured as well as membrane currents may have been more successful in reducing run-down because care was taken to minimize disturbance of $Ca_i^{2+}$ buffering. Many experiments have been performed with millimolar or higher concentrations of EGTA in the pipette solution (Dufy *et al.* 1986) which might both attenuate any $Ca^{2+}$ release signal and also reduce the activity of $Ca^{2+}$-dependent enzymes by reducing resting $Ca^{2+}$ (Gray, 1988). Taken together, the evidence suggests that the perfused cell system can usefully be used to study complex agonist-mediated responses whilst noting that components of the response may be attenuated or lost. Comparison of $Ca_i^{2+}$ transients to voltage-gated $Ca^{2+}$ entry in perfused and intact cells suggested that perfusion did not seriously affect $Ca^{2+}$ buffering of this type of $Ca^{2+}$ load (Fig. 5).

Application of TRH in $Ca^{2+}$-free solution evoked an initial transient rise in $Ca_i^{2+}$ in cells under voltage clamp. Thus this intracellular $Ca^{2+}$ mobilization was independent of any change in membrane potential. Although maximal concentrations of TRH were normally applied, lower concentrations of TRH also gave responses with similar characteristics in normal solution. There was no evidence of any oscillatory release of internal $Ca^{2+}$ in these cells. This is in contrast to the behaviour of many non-excitable cells on sustained stimulation (Woods, Cuthbertson & Cobbold, 1986; Jacob, Merrit, Hallam & Rink, 1988; Gray, 1988). Clearly there is some diversity in the control mechanisms regulating $IP_3$-mediated $Ca^{2+}$ store release and this may not require an oscillatory control when an alternative oscillatory source such as voltage-gated $Ca^{2+}$ entry is available to the cells.

A second plateau phase of elevated $Ca_i^{2+}$ was seen in response to TRH that was not seen in the absence of extracellular $Ca^{2+}$, providing some evidence for a voltage-independent agonist-gated $Ca^{2+}$ entry. However, this response was small, and was not increased by elevating extracellular $Ca^{2+}$ or by increasing the electrical driving force by hyperpolarization. It is possible that perfusion of the cells removes a soluble link in the receptor channel coupling or that the TRH response in these cells differs from that in gonadotrophs where a $Ca^{2+}$-permeable channel has been identified that is activated by gonadotrophin-releasing hormone (Mason & Waring, 1985). Whether or not there is a small contribution from agonist-gated entry, most of the second phase of elevated $Ca_i^{2+}$ in unclamped cells can be explained by $Ca^{2+}$ influx through voltage-gated channels during increased rates of action potential discharge. This period of membrane hyperexcitability might be entirely due to depression of $K^+$ current (Dubinsky & Oxford, 1985) rather than activation of an inward current. Incomplete block of this component by organic $Ca^{2+}$ antagonists could be explained by their weak potency in polarized $GH_3$ cells (Cohen & McCarthy, 1987).

Several observations suggest that the major source of $Ca^{2+}$ for the depolarization-evoked transients is extracellular and that $Ca^{2+}$-induced $Ca^{2+}$ release is not important in these cells. This is in contrast to the situation in cardiac myocytes (Barcenas-Ruiz & Weir, 1987; Callewaert *et al.* 1988) and gut smooth muscle cells (Becker, Walsh, Singer & Fay, 1988). In $GH_3$ cells $Ca_i^{2+}$ increases with the length of depolarization up to a peak after several seconds, consistent with sustained $Ca^{2+}$ entry supplying $Ca^{2+}$. There was no evidence of an early peak in $Ca_i^{2+}$ due to internal store release. Repolarization from positive potentials where the voltage-gated $Ca^{2+}$ channels were activated results in a rapidly decaying $Ca^{2+}$ tail current which is sufficient to trigger store release in cardiac myocytes (Barcenas-Ruiz & Weir, 1987). No $Ca_i^{2+}$ transients were seen associated with repolarizations in these cells. Finally, the $Ca_i^{2+}$ transients in response to depolarization were not markedly affected by prior discharge of $Ca^{2+}$ stores by TRH in contrast to the effect of ryanodine in cardiac cells (Callewaert *et al.* 1988).

Comparison of $Ca_i^{2+}$ transients stimulated by 100 ms depolarization with spontaneous transients in unclamped cells indicated that single action potentials were associated with significant rises in $Ca_i^{2+}$, confirming the observations of Schlegel *et al.* (1987). The decay of these transients was slow, of the order of several seconds, allowing summation of $Ca_i^{2+}$ transients if the stimulus frequency was greater than 0·5 Hz. At 37 °C the rate of recovery would be more rapid but assuming a $Q_{10}$ of 3 only shortens the time constant of recovery to about 1 s. This might still allow trains of action potentials to create a sustained elevation in $Ca_i^{2+}$. Most of the recovery phase could be fitted by a single exponential although some cells showed small components with a much slower time course. This suggests that one component of buffering is rate determining for efflux of $Ca^{2+}$. This seemed to be an endogenous buffer as the time constant of decay showed no correlation with the degree of Indo-1 loading. The dissociation rate constant for $Ca^{2+}$ binding to Indo-1 ($130\ s^{-1}$; Jackson, Timmerman & Ashley, 1987) is much faster than the decay seen here and hence the rate of $Ca^{2+}$ dissociation from the dye would not limit the observed response.

In conclusion, depolarization and $Ca^{2+}$ entry appear not to have any significant interaction with internal store release in $GH_3$ cells. $Ca^{2+}$ entry and $Ca^{2+}$ store release act in parallel in these secretory cells and thus provide two more independent systems for $Ca^{2+}$ signalling within the cell.

I thank Dr J. Merritt and Ms K. Moore for help with cell culture, Dr R. Jacob for advice on fluorescence measurement and Drs J. Art and R. McBurney who developed the experimental set-up.

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