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Mechanism of spontaneous intracellular calcium fluctuations in single GH_4C_1 rat pituitary cells

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Individual unstimulated GH_4C_1 cells exhibited spontaneous dynamic fluctuations in cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_i$). Either chelation of extracellular Ca^{2+} with EGTA or treatment with nifedipine inhibited spontaneous $[Ca^{2+}]_i$ fluctuations, indicating that the $[Ca^{2+}]_i$ profile was dependent on the entry of extracellular Ca^{2+} via voltage-operated Ca^{2+} channels (VOCC). Spontaneous $[Ca^{2+}]_i$ fluctuations did not resume immediately after exposure of EGTA-pretreated cells to extracellular Ca^{2+}, supporting the hypothesis that the complex $[Ca^{2+}]_i$ profiles observed in unstimulated cells required filling of an intracellular Ca^{2+} pool. BAY K 8644 elicited large rapid oscillations in $[Ca^{2+}]_i$. After chelation of extracellular Ca^{2+}, however, re-addition of Ca^{2+} plus BAY K 8644 did not result in $[Ca^{2+}]_i$ oscillations. The intracellular Ca^{2+} pool necessary for BAY K-induced oscillations was not the same Ins(1,4,5)P_3-sensitive pool stimulated by thyrotropin-releasing hormone (TRH), because the TRH-stimulated Ins(1,4,5)P_3-induced $[Ca^{2+}]_i$ spike and the BAY K 8644-induced oscillations were differentially sensitive to chelation of extracellular Ca^{2+} and thapsigargin. Caffeine caused an increase in $[Ca^{2+}]_i$ fluctuations in quiescent cells, supporting a role for Ca^{2+}-induced Ca^{2+} release (CICR) in the generation of spontaneous $[Ca^{2+}]_i$ fluctuations. In conclusion, the complex spontaneous changes in $[Ca^{2+}]_i$ observed in single GH_4C_1 cells depend on both the influx of extracellular Ca^{2+} through VOCC and the action of an intracellular Ca^{2+} pool that increases $[Ca^{2+}]_i$ through a CICR-like mechanism.

INTRODUCTION

Regulated change in cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_i$) is an important intracellular signal transduction mechanism responsible for the regulation of many aspects of cellular biochemistry, including gene expression [1,2], protein synthesis [3], stimulus–secretion coupling [4,5] and stimulus–contraction coupling [6]. Time-resolved measurements of $[Ca^{2+}]_i$ in individual cells have shown, however, that in some cell types the relatively constant $[Ca^{2+}]_i$ baseline observed in populations of cells actually represents the sum of dynamic fluctuating spontaneous $[Ca^{2+}]_i$ profiles of individual cells. The clonal rat pituitary cell strain GH_4C_1 [7,8] is one such cell type [9].

Although the $[Ca^{2+}]_i$ response to pharmacological agents has been extensively studied in populations of GH_4C_1 cells and a related clone, GH_3 [10–13], much less is known about the $[Ca^{2+}]_i$ response in individual cells [9,14–16]. In particular, the mechanisms that underlie the generation of spontaneous $[Ca^{2+}]_i$ fluctuations in these secretory cells remain to be elucidated. It has been reported that individual GH_3 cells exhibit spontaneous action potentials which cause a transient increase in $[Ca^{2+}]_i$ due to influx of extracellular Ca^{2+} through voltage-operated Ca^{2+} channels (VOCC) [4,9,15]. The possible role of intracellular Ca^{2+} stores in the maintenance of the spontaneous $[Ca^{2+}]_i$ profile has not been studied, however.

In the present investigation, we have examined the mechanism by which spontaneous fluctuations in $[Ca^{2+}]_i$ are generated in individual GH_4C_1 cells. Our results demonstrate that spontaneous $[Ca^{2+}]_i$ oscillations require both the entry of extracellular Ca^{2+} through VOCC and the release of Ca^{2+} from an intracellular pool. This intracellular pool is distinct from the Ins(1,4,5)P_3-sensitive pool responsible for the thyrotropin-releasing-hormone (TRH)-induced transient $[Ca^{2+}]_i$ spike, and is regulated through a Ca^{2+}-induced-Ca^{2+}-release (CICR) mechanism.

MATERIALS AND METHODS

Materials

BAY K 8644 and nifedipine were from Calbiochem (La Jolla, CA, U.S.A.). TRH was from Peninsula Labs (Belmont, CA, U.S.A.). Thapsigargin was from LC Services Corp. (Woburn, MA, U.S.A.). All other compounds were of reagent grade.

Cell culture

GH_4C_1 cells were grown at 37 °C in Ham's F10 medium (GIBCO, Grand Island, NY, U.S.A.) containing 15% horse serum and 2.5% fetal-bovine serum in a humidified air/CO_2 (19:1) environment. At 48 h before each experiment cells were suspended with Viokase (A. H. Robbins, Richmond, VA, U.S.A.) and plated on glass coverslips coated with Cell-Tak (Collaborative Research, Bedford, MA, U.S.A.). In control experiments it was determined that Cell-Tak did not affect $[Ca^{2+}]_i$ fluctuations in unstimulated cells or cellular $[Ca^{2+}]_i$ responses to pharmacological agents (results not shown).

Calcium measurements

Cells were rinsed with Hanks' balanced salts solution (HBSS; concns. in mM: NaCl 118.0, KCl 4.6, $CaCl_2$ 1.0, D-glucose 10.0, Hepes 20.0) and loaded with 9 μM Fluo-3 AM (Molecular Probes, Eugene, OR, U.S.A.) in HBSS containing 0.04% Pluronic F-127 (Molecular Probes) for 45 min at room tem-

Abbreviations used: $[Ca^{2+}]_i$, cytosolic free Ca^{2+} concn.; VOCC, voltage-operated Ca^{2+} channels; TRH, thyrotropin-releasing hormone; CICR, Ca^{2+}-induced Ca^{2+} release; HBSS, Hanks' balanced salts solution.

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perature. Cells were then rinsed with HBSS and remained in HBSS until use.

Pharmacological agents were administered with a Pasteur pipette to remove the buffer bathing the cells and to replace it with buffer containing the agent(s) of interest. The act of removing and replacing the buffer surrounding the cells did not result in a significant change in Fluo-3 fluorescence. Unless otherwise indicated, all data were collected by using cells in HBSS containing 1 mM $CaCl_2$ (Ca/HBSS). Ca^{2+}-free experiments were conducted in HBSS without $CaCl_2$ containing 0.5 mM EGTA (EGTA/HBSS). All experiments were performed at room temperature.

$[Ca^{2+}]_i$ was measured in single cells by using an ACAS 570 interactive laser cytometer (Meridian Instruments, Okemos, MI, U.S.A.) [17–19]. The coverslip on which the cells had been plated was placed in a tissue chamber (Biophysica Technologies, Baltimore, MD, U.S.A.), which was then mounted on the stage of an Olympus IMT-2 inverted fluorescence microscope. The coverslip was scanned as the computer-controlled microscope stage moved through a prescribed *x–y* pattern over the fixed excitation laser beam. Fluo-3 fluorescence was excited by using the 488 nm line of an argon laser. Fluorescence emission was passed through a 515 nm long-pass filter and monitored with a photomultiplier tube.

Time-resolved changes in $[Ca^{2+}]_i$ over an entire GH_4C_1 cell were monitored by using the image-scan mode of the ACAS 570. During an image scan, the stage was moved so as to scan a rectangular field covering the area of a single cell or a small group of cells. From a series of such experiments, it was determined that $[Ca^{2+}]_i$ fluctuations in GH_4C_1 cells occurred over the entire cell, without identifiable peaks of fluorescence intensity associated with any particular area of the cell such as the nucleus (results not shown). Individual cell images were scanned with 1.5 μm spatial resolution and 4–7 s temporal resolution. Greater time resolution was gained by using the line-scan mode of the ACAS 570. In this mode the stage was moved along the *x*-axis only. Measurements were therefore taken as a linear trace across the same portion of the cell for the entire experiment. GH_4C_1 cells had a rounded morphology and, for an experiment using the line-scan mode, the stage was aligned so that the laser beam scanned through the equator of the cell. As the cell passed over the laser beam, fluorescence measurements were taken at 0.6 μm intervals; the fluorescence value recorded for each point was the mean of 8 sequential readings. Since each reading was taken in approx. 4 μs, the total time required for a line scan of a single cell was approx. 17 ms. The relative fluorescence value for each time point represented the integral of the fluorescence measurements recorded across the cell divided by the length of the scan. All data reported here were obtained by using the line-scan mode. Fluorescence measurements were repeated at 300 ms intervals unless otherwise noted.

All calcium measurements reported here were made with the indicator dye Fluo-3, because this dye is relatively resistant to photobleaching. The major disadvantage of using Fluo-3 is that relative fluorescence data cannot be readily calibrated to yield absolute Ca^{2+} concentrations [20,21]. Data were therefore expressed as relative fluorescence values. Although we found that the fluorescence of an individual Fluo-3-loaded cell varied with the amount of dye loaded, in experiments on > 500 cells, baseline fluorescence counts were found not to correlate with cellular $[Ca^{2+}]_i$ responses to the various pharmacological treatments. Further, many of the experiments reported here were repeated with the calcium indicator Indo-1 (Molecular Probes), with similar results (not shown). Indo-1 fluorescence was monitored simultaneously at two emission wavelengths, including a $[Ca^{2+}]_i$-sensitive fluorescence at 410 nm and a $[Ca^{2+}]_i$-insensitive fluorescence at 485 nm. The 410/485 nm ratio of fluorescence intensities was independent of cell thickness, dye leakage and variability in dye loading of individual cells. Since Indo-1 and Fluo-3 gave similar $[Ca^{2+}]_i$ profiles in unstimulated and treated cells, the Fluo-3 fluorescence profiles in the present paper appeared to represent faithfully $[Ca^{2+}]_i$ fluctuations in single GH_4C_1 cells rather than dye artifacts or variations in dye loading.

RESULTS

Spontaneous $[Ca^{2+}]_i$ profiles in unstimulated GH_4C_1 cells

GH_4C_1 cells exhibited spontaneous fluctuations in $[Ca^{2+}]_i$ (Figures 1a, 1b and 1c). These complex patterns were composed of three basic elements: spikes, oscillations and quiet periods. A spike was defined as a high-amplitude calcium transient. A period of oscillation was defined as a series of two or more spikes. A quiet period was defined as the absence of either spikes or oscillations. Figure 2 illustrates that an unstimulated cell could switch spontaneously among these three $[Ca^{2+}]_i$ dynamics. The first 50 s of this $[Ca^{2+}]_i$ profile is expanded to demonstrate that the individual spikes within a period of oscillations were similar in amplitude, duration (1–2 s), and frequency. Individual $[Ca^{2+}]_i$ spikes observed were asymmetric, in that a rapid increase in $[Ca^{2+}]_i$ was followed by a more gradual decrease in $[Ca^{2+}]_i$. Analysis of the spontaneous $[Ca^{2+}]_i$ profiles of 458 cells for a period of 1–10 min each (average observation time 3.55 min per cell) indicated that individual GH_4C_1 cells were, on average, spiking or oscillating 48 % of the time and quiet 52 % of the time.

Spontaneous changes in $[Ca^{2+}]_i$ required the influx of extracellular Ca^{2+} through L-type VOCC

The extracellular Ca^{2+} requirement for the maintenance of spontaneous $[Ca^{2+}]_i$ fluctuations is shown in Figure 3. Extracellular Ca^{2+} was removed by replacing the Ca/HBSS buffer with EGTA/HBSS. Removal of extracellular Ca^{2+} caused a gradual cessation of $[Ca^{2+}]_i$ oscillations and the appearance of a quiet $[Ca^{2+}]_i$ profile. The $[Ca^{2+}]_i$ oscillations did not return immediately, but rather several minutes after replacing Ca^{2+} in the buffer (Figure 3). Ca/HBSS was replaced shortly after the cessation of $[Ca^{2+}]_i$ fluctuations in the experiment shown in Figure 3. In similar experiments in which cells were exposed to EGTA/HBSS for longer periods of time (up to 15 min) after the fluctuations ended, no spontaneous $[Ca^{2+}]_i$ fluctuations were observed before extracellular Ca^{2+} was restored by returning cells to Ca/HBSS (results not shown). Further, it appeared that the length of exposure to EGTA/HBSS was correlated directly with the length of the lag period between replacement of Ca/HBSS and return of $[Ca^{2+}]_i$ oscillations. These findings suggest that intracellular Ca^{2+} pools were involved in the return of $[Ca^{2+}]_i$ oscillations, since, if Ca^{2+} influx across the plasma membrane were solely responsible for the unstimulated fluctuations in $[Ca^{2+}]_i$, then the oscillatory pattern would have been expected to return within seconds after re-introduction of extracellular Ca^{2+}.

To eliminate the possibility that $[Ca^{2+}]_i$ oscillations did not return more rapidly because residual EGTA chelated some portion of the re-introduced Ca^{2+}, control experiments using KCl-induced depolarization were performed (Figure 4). In populations of GH_4C_1 cells, KCl (35–50 mM) strongly depolarizes the plasma membrane and causes a rapid influx of extracellular Ca^{2+} through VOCC [22–24]. Depolarization of individual GH_4C_1 cells by treatment with KCl (35 mM) in Ca/HBSS resulted in a rapid and sustained increase in $[Ca^{2+}]_i$ (Figure 4a). The $[Ca^{2+}]_i$ plateau phase persisted as long as the high-KCl buffer was

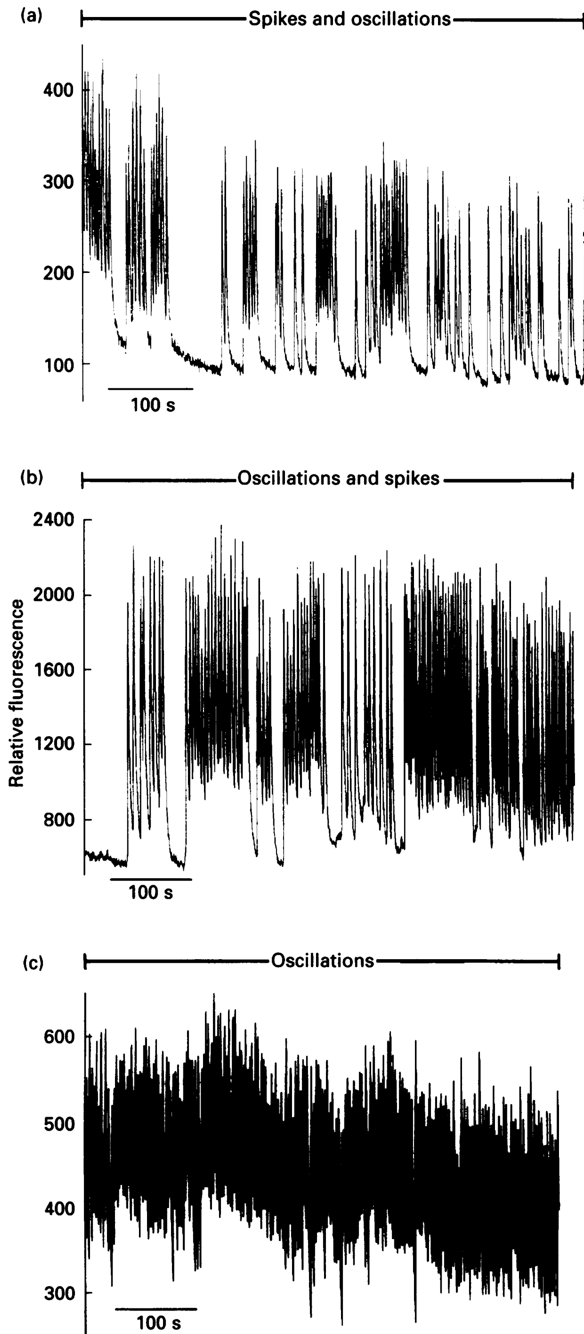

Figure 1 Spontaneous [Ca^{2+}]$_i$ profiles of representative single unstimulated GH_4C_1 cells

(**a**) and (**b**): Single Fluo-3-loaded GH_4C_1 cells exhibited spontaneous fluctuations in [Ca^{2+}]$_i$ which were described by three modes of [Ca^{2+}]$_i$ dynamics: individual spikes, oscillations and periods of quiet (see Figure 2). The two cells shown here were mostly in the spike and oscillation modes. (**c**) An example of a cell that exhibited [Ca^{2+}]$_i$ oscillations during the entire period of observation. These three [Ca^{2+}]$_i$ profiles are representative of over 50 cells studied.

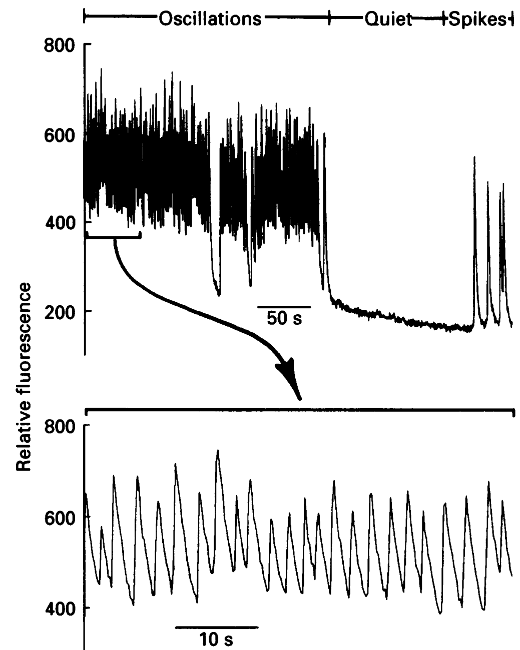

Figure 2 [Ca^{2+}]$_i$ oscillations in unstimulated GH_4C_1 cells were regular in nature

Top panel: spontaneous [Ca^{2+}]$_i$ profile of an unstimulated single Fluo-3-loaded GH_4C_1 cell that exhibited all three modes of [Ca^{2+}]$_i$ dynamics. Bottom panel: the first 50 s of the oscillatory phase is expanded to demonstrate that [Ca^{2+}]$_i$ oscillations were approx. 2 s in duration and asymmetric. Measurements were taken every 200 ms.

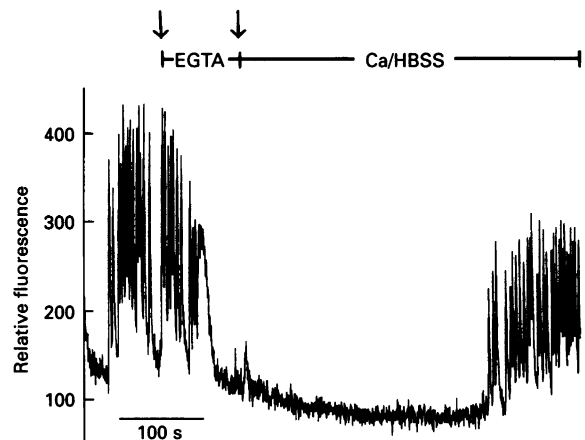

Figure 3 [Ca^{2+}]$_i$ fluctuations in unstimulated GH_4C_1 cells required extracellular Ca^{2+}

Spontaneous [Ca^{2+}]$_i$ fluctuations in a single Fluo-3-loaded GH_4C_1 cell were abolished shortly after the extracellular Ca^{2+} was chelated by replacing the Ca/HBSS bathing the cell with EGTA/HBSS (first arrow; $n > 50$ cells). Several minutes after extracellular Ca^{2+} was restored by replacing the EGTA/HBSS buffer with Ca/HBSS (second arrow), spontaneous [Ca^{2+}]$_i$ fluctuations returned ($n > 10$ cells). In similar experiments in which cells were exposed to EGTA/HBSS for periods of time up to 15 min, oscillations were not observed before extracellular Ca^{2+} was replaced.

present. Removal of KCl from the Ca/HBSS buffer caused [Ca^{2+}]$_i$ to return to the basal level (results not shown). Prior incubation of cells with EGTA/HBSS, in order to inhibit spontaneous fluctuations in [Ca^{2+}]$_i$, did not interfere with the rapid generation of an elevated [Ca^{2+}]$_i$ plateau in response to simultaneous treatment with KCl and extracellular Ca^{2+} (Figure 4b). KCl (35 mM) had no effect on [Ca^{2+}]$_i$ when the experiment was conducted in EGTA/HBSS rather than Ca/HBSS buffer (results not shown). These findings indicate that extracellular Ca^{2+} is indeed rapidly available for influx through VOCC immediately after replacement of EGTA/HBSS with Ca/HBSS.

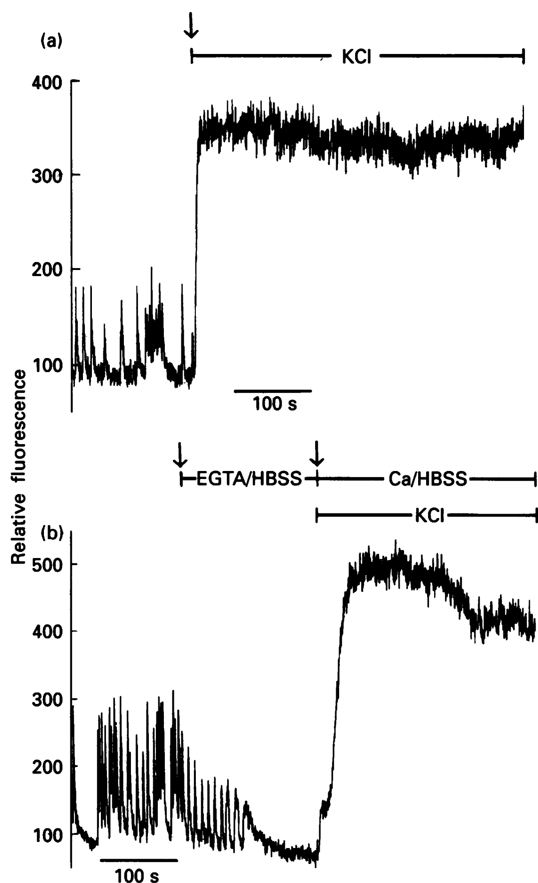

Figure 4 Depolarization of the plasma membrane by KCl resulted in a rapid increase in $[Ca^{2+}]_i$ to a sustained plateau which was not affected by previous exposure to EGTA/HBSS

(**a**) Depolarization of a single Fluo-3-loaded GH_4C_1 cell by treatment with 35 mM KCl in Ca/HBSS buffer resulted in a rapid and sustained increase in $[Ca^{2+}]_i$ ($n > 15$ cells). (**b**) Although spontaneous $[Ca^{2+}]_i$ fluctuations were abolished after extracellular Ca^{2+} was removed by replacing the Ca/HBSS buffer with EGTA/HBSS (first arrow), restoration of extracellular Ca^{2+} by replacing EGTA/HBSS with Ca/HBSS plus 35 mM KCl (second arrow) caused a rapid increase in $[Ca^{2+}]_i$. Compare the kinetics of this $[Ca^{2+}]_i$ increase with the slow return of oscillations under the conditions described in the legend to Figure 3.

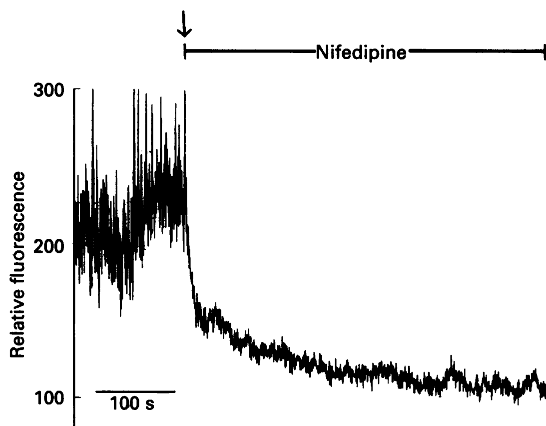

Figure 5 Spontaneous fluctuations in $[Ca^{2+}]_i$ required Ca^{2+} influx through L-type VOCC

Spontaneous $[Ca^{2+}]_i$ fluctuations in a single unstimulated Fluo-3-loaded GH_4C_1 cell were abolished by addition of 2.5 μM nifedipine (arrow, $n > 20$ cells).

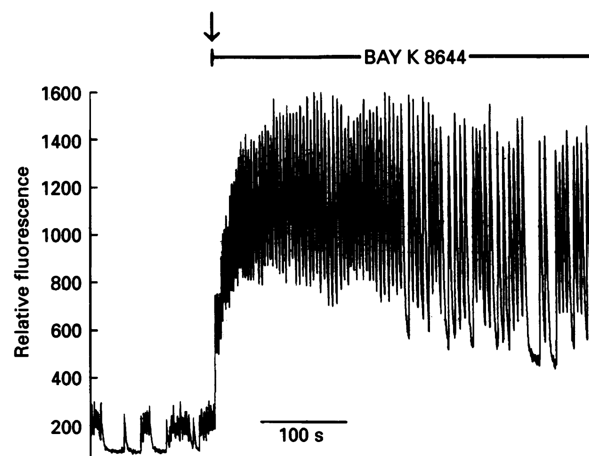

Figure 6 The L-type Ca^{2+}-channel agonist BAY K 8644 stimulated $[Ca^{2+}]_i$ oscillations in single GH_4C_1 cells

Addition of 1 μM BAY K 8644 (arrow) induced large regular $[Ca^{2+}]_i$ oscillations in a single Fluo-3-loaded GH_4C_1 cell ($n > 20$ cells). This experiment was performed in Ca/HBSS buffer.

Incubation of cells with the L-type Ca^{2+}-channel antagonist nifedipine resulted in the rapid cessation of spontaneous $[Ca^{2+}]_i$ fluctuations (Figure 5). Since 1 mM extracellular Ca^{2+} was present throughout the experiment, these data indicate that Ca^{2+} influx through L-type VOCC was essential for the maintenance of spontaneous $[Ca^{2+}]_i$ fluctuations. Because the fluctuations inhibited by EGTA/HBSS returned several minutes, rather than seconds, after re-introduction of extracellular Ca^{2+}, we considered the possibility that an intracellular Ca^{2+} pool had to be replenished before spontaneous $[Ca^{2+}]_i$ fluctuations could recommence. Such a Ca^{2+} pool would be predicted to empty or become inactivated in the absence of extracellular Ca^{2+}.

BAY K 8644-induced $[Ca^{2+}]_i$ oscillations required both extracellular and intracellular Ca^{2+}

The L-type VOCC agonist BAY K 8644 stimulated large regular oscillations in $[Ca^{2+}]_i$ (Figure 6). This effect was uniformly observed in cells exhibiting spontaneous $[Ca^{2+}]_i$ spikes, oscillations or quiet periods before addition of BAY K 8644. Extracellular Ca^{2+} was required for the oscillatory response in $[Ca^{2+}]_i$ to BAY K 8644, since $[Ca^{2+}]_i$ did not change when the agonist was added to cells in EGTA/HBSS buffer (results not shown). Pretreatment of cells with EGTA/HBSS also prevented BAY K 8644-induced $[Ca^{2+}]_i$ oscillations upon simultaneous addition of agonist and re-introduction of extracellular Ca^{2+} (Figure 7). Under these conditions, addition of BAY K 8644 produced either a plateau (Figure 7a; 8/30 observations), a single large spike (Figure 7b; 14/30 observations), or no apparent change (Figure 7c; 8/30 observations) in $[Ca^{2+}]_i$. Despite inhibition of sustained oscillations in response to Ca/HBSS plus BAY K 8644, the Ins(1,4,5)P_3 signal-transduction pathway and the Ins(1,4,5)P_3-sensitive $[Ca^{2+}]_i$ pool were fully intact and able to respond to TRH in such cells (Figure 7c). In cells observed for longer periods of time, BAY K 8644-induced oscillations resumed 5–15 min after the re-introduction of extracellular Ca^{2+} (results not shown). These results indicate that the oscillations induced by BAY K 8644 required not only the influx of extracellular Ca^{2+}, but also the integrity of an intracellular Ca^{2+} pool that was affected by EGTA/HBSS pretreatment.

In populations of GH_4C_1 cells it has previously been demon-

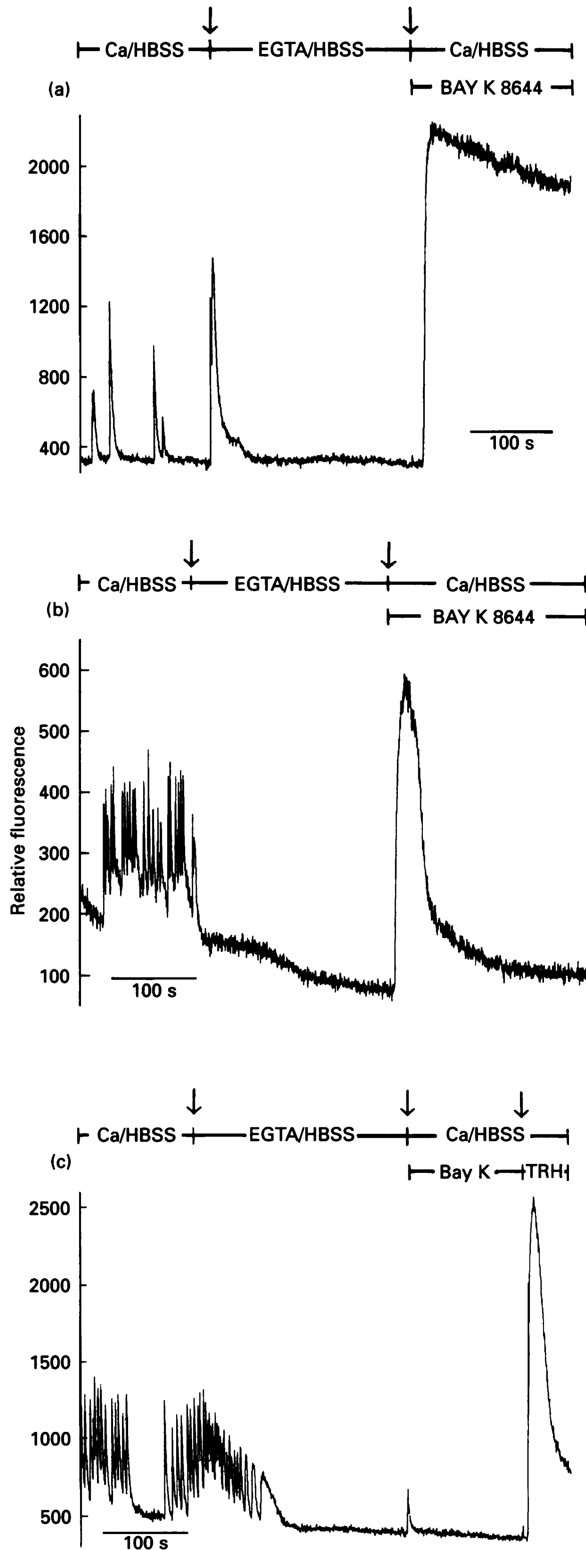

Figure 7 BAY K 8644 did not induce [Ca^{2+}]$_i$ oscillations in GH_4C_1 cells that had been incubated in the absence of extracellular Ca^{2+}

(**a**) and (**b**): As previously shown (Figure 3), spontaneous [Ca^{2+}]$_i$ fluctuations in single unstimulated Fluo-3-loaded GH_4C_1 cells were abolished when extracellular Ca^{2+} was removed by replacing the Ca/HBSS buffer with EGTA/HBSS (first arrow). Restoration of extracellular Ca^{2+} by replacing the EGTA/HBSS with Ca/HBSS plus 1 μM BAY K 8644 (second arrow) resulted in a [Ca^{2+}]$_i$ profile that displayed one of three patterns ($n = 30$ cells): an elevated [Ca^{2+}]$_i$ plateau (**a**), a broad, transient, [Ca^{2+}]$_i$ spike (**b**), or no response (**c**). In (**c**) Ca/HBSS containing BAY K 8644 was replaced with Ca/HBSS containing 100 nM TRH at the third arrow.

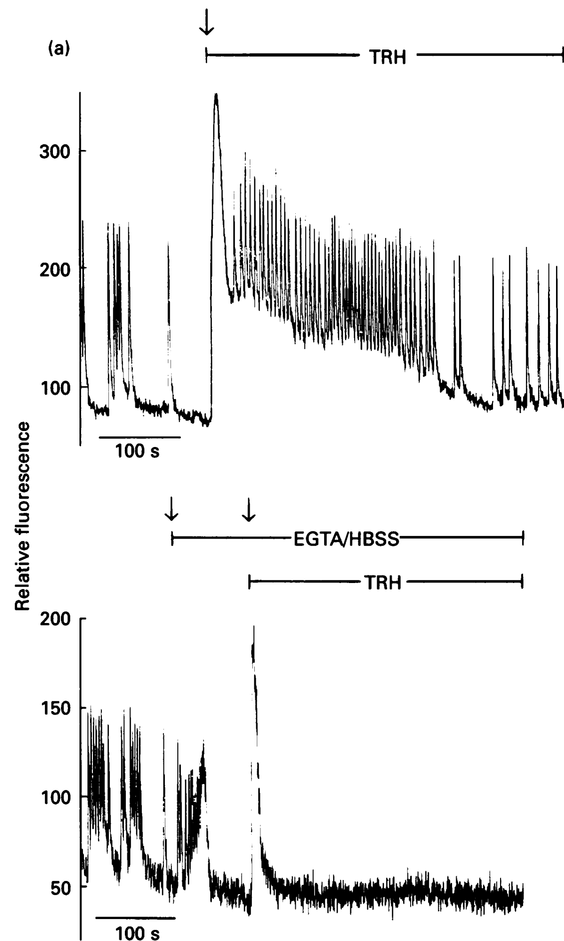

Figure 8 The TRH-induced [Ca^{2+}]$_i$ spike persisted in the absence of extracellular Ca^{2+}

(**a**) Addition of 50 nM TRH (arrow) elicited a transient [Ca^{2+}]$_i$ spike, followed by a series of [Ca^{2+}]$_i$ oscillations in a single Fluo-3-loaded GH_4C_1 cell ($n > 50$ cells). (**b**) As previously shown (Figure 3), spontaneous [Ca^{2+}]$_i$ fluctuations in a single unstimulated Fluo-3-loaded GH_4C_1 cell were abolished when extracellular Ca^{2+} was removed by replacing the Ca/HBSS buffer with EGTA/HBSS (first arrow). Addition of 50 nM TRH in EGTA/HBSS (second arrow) induced a [Ca^{2+}]$_i$ spike but no subsequent [Ca^{2+}]$_i$ oscillations ($n > 10$ cells).

strated that TRH stimulates a biphasic [Ca^{2+}]$_i$ response, characterized by a large spike followed by a plateau [10]. The spike phase has been reported to result from an Ins(1,4,5)P_3-induced release of intracellular Ca^{2+}, and the plateau phase to depend on an influx of extracellular Ca^{2+} [10,25–27]. In single cells, TRH also induced a biphasic [Ca^{2+}]$_i$ response that consisted of a spike followed by oscillations in most cells (Figure 8a). As previously reported [14], some cells exhibited only one of the two [Ca^{2+}]$_i$ phases (results not shown). The spike, but not the oscillatory component, of the TRH response persisted in the absence of extracellular Ca^{2+} (Figure 8b).

TRH and BAY K 8644 discharged different intracellular Ca^{2+} pools

TRH induced a spike in [Ca^{2+}]$_i$ under conditions in which BAY K 8644 was incapable of eliciting sustained [Ca^{2+}]$_i$ oscillations (Figure 7c). This phenomenon was observed regardless of which of the three BAY K 8644-induced [Ca^{2+}]$_i$ profiles was exhibited (results not shown). Pretreatment of GH_4C_1 cells with 1 μM thapsigargin for 10 min abolished the TRH-stimulated

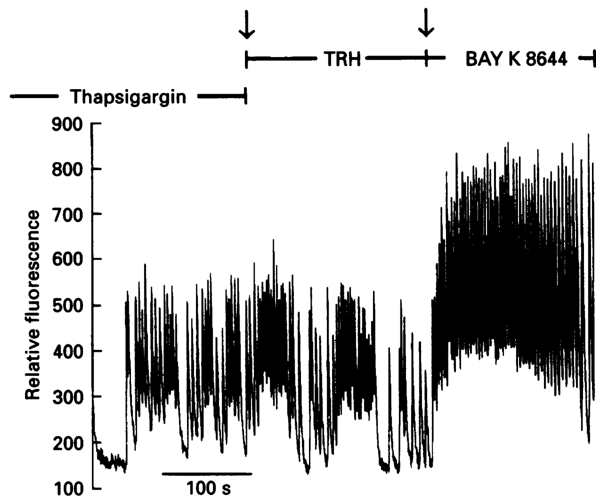

Figure 9 Thapsigargin pretreatment abolished the TRH-induced $[Ca^{2+}]_i$ spike, but not the BAY K 8644-induced $[Ca^{2+}]_i$ oscillations

After a 10 min pretreatment with 1 μM thapsigargin, which did not abolish spontaneous oscillations, addition of 100 nM TRH (first arrow) did not elicit a transient spike in $[Ca^{2+}]_i$. Subsequent addition of 1 μM BAY K 8644 (second arrow) resulted in enhanced $[Ca^{2+}]_i$ oscillations in a single Fluo-3-loaded GH_4C_1 cell ($n = 6$ cells).

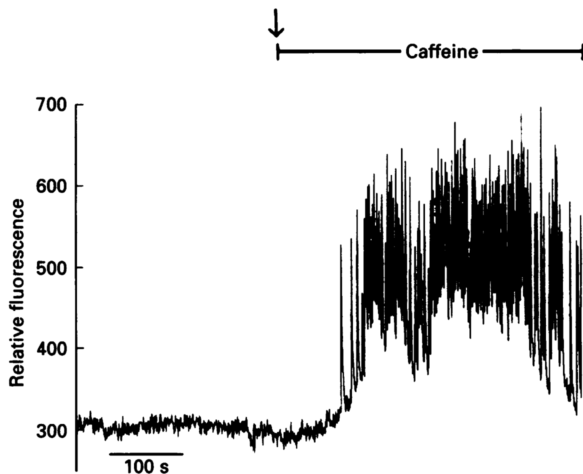

Figure 10 Caffeine elicited $[Ca^{2+}]_i$ fluctuations in quiet single GH_4C_1 cells

Addition of 2 mM caffeine in Ca/HBSS (arrow) caused $[Ca^{2+}]_i$ fluctuations in a single quiescent Fluo-3-loaded GH_4C_1 cell ($n > 30$ cells).

Ins(1,4,5)P_3-induced $[Ca^{2+}]_i$ spike (Figure 9; compared with Figure 8a). Nevertheless, subsequent exposure of the same cells to 1 μM BAY K 8644 induced regular $[Ca^{2+}]_i$ oscillations. These results support a model involving two functionally distinct intracellular Ca^{2+} pools: one pool that releases Ca^{2+} in response to Ins(1,4,5)P_3, and a second pool that discharges Ca^{2+} after BAY K 8644 treatment. Because the TRH-induced response is Ins(1,4,5)P_3-mediated, it follows that the intracellular mechanism of BAY K-induced oscillations is unlikely to involve Ins(1,4,5)P_3 or an Ins(1,4,5)P_3-sensitive Ca^{2+} pool.

Role of CICR in the generation of spontaneous $[Ca^{2+}]_i$ fluctuations

In several cell types, caffeine (1–10 mM) has been shown to stimulate CICR by lowering the $[Ca^{2+}]_i$ threshold for CICR [28–34]. Treatment of GH_4C_1 cells with 2 mM caffeine caused an increase in $[Ca^{2+}]_i$ oscillations in 29/68 cells studied; more importantly, caffeine initiated oscillations in 26/37 previously quiet or low activity cells, but only in 3/31 previously oscillating cells (Figure 10). This result would be expected if caffeine were acting to lower the threshold for CICR. Since caffeine would be expected to elicit the generation of oscillations in quiet cells by increasing the Ca^{2+}-sensitivity of the CICR channel to a level at which the ambient $[Ca^{2+}]_i$ was sufficient to sustain CICR, these findings are consistent with a model in which CICR is required for the generation of spontaneous $[Ca^{2+}]_i$ fluctuations. In contrast, for cells exhibiting spontaneous $[Ca^{2+}]_i$ oscillations, a marked caffeine response would not be expected, since the CICR channels would be active in such cells in the absence of caffeine.

DISCUSSION

GH_3 cells, a strain closely related to the GH_4C_1 cells used in the present studies [8], have been reported to exhibit spontaneous action potentials resulting in transient $[Ca^{2+}]_i$ increases due to Ca^{2+} influx through VOCC [9,15]. In this paper we extended that finding by demonstrating that spontaneous fluctuations in $[Ca^{2+}]_i$ require not only the influx of extracellular Ca^{2+} but also the release of Ca^{2+} from an intracellular store. Further, we found that the relevant intracellular Ca^{2+} store exhibited CICR-like activity and was functionally distinct from the Ins(1,4,5)P_3-sensitive Ca^{2+} store that is released by incubation with TRH.

Although population studies are technically simpler than single-cell $[Ca^{2+}]_i$ measurements, the modulation of spontaneous $[Ca^{2+}]_i$ fluctuations can be investigated only by studying individual cells. Individual GH_4C_1 cells exhibited highly dynamic fluctuations in $[Ca^{2+}]_i$. We observed significant variations both among individual cells (Figure 1) and within the same cell (Figure 2). Unlike some other cell types [35], individual GH_4C_1 cells did not appear to have a unique, reproducible, signature pattern. That is, an individual cell did not exhibit a single type of $[Ca^{2+}]_i$ profile that distinguished it uniquely from other cells in the population.

Spontaneous $[Ca^{2+}]_i$ fluctuations ceased within approx. 2 min ($t_{\frac{1}{2}} = 54$ s) after chelating extracellular Ca^{2+} with EGTA (Figure 3), and the inhibition of $[Ca^{2+}]_i$ fluctuations persisted throughout the duration of Ca^{2+} chelation. These kinetics are similar to those for the so-called 'superficial' Ca^{2+} pool previously described in GH_4C_1 cells [36]. A curious finding was that the last oscillation was often longer in duration than previous spikes (note Figures 3, 4b, 7b, 7c and 8b). Furthermore, even when extracellular Ca^{2+} was replaced almost immediately after the $[Ca^{2+}]_i$ oscillations subsided, spontaneous oscillations did not return for several minutes (Figure 3). Two possible explanations for this latter observation were as follows: First, residual EGTA could have chelated the added Ca^{2+}, thereby making extracellular Ca^{2+} unavailable to the cell. Second, an intracellular Ca^{2+} store could have participated in the generation of $[Ca^{2+}]_i$ fluctuations; such a store would have to be acutely dependent on extracellular Ca^{2+} to remain in a functional state. On re-introduction of extracellular Ca^{2+}, such a store would need to re-fill before re-commencement of $[Ca^{2+}]_i$ oscillations. The first explanation was eliminated by demonstrating that KCl-induced depolarization elicited a rapid increase in $[Ca^{2+}]_i$ after oscillations had previously been inhibited by EGTA/HBSS; these findings indicated that extracellular Ca^{2+} was available for influx into the cell. The hypothesis that an intracellular Ca^{2+} pool was critically involved in the generation of spontaneous $[Ca^{2+}]_i$ fluctuations was then investigated.

It has been reported that the L-type VOCC antagonist

nifedipine abolishes spontaneous [Ca^{2+}]$_i$ fluctuations in Fura-2-loaded GH_3B_6 cells [9]. This finding was confirmed here in Fluo-3-loaded GH_4C_1 cells (Figure 5). We observed two differences between [Ca^{2+}]$_i$ profiles generated when the fluctuations were abolished by nifedipine and those produced by chelation of extracellular Ca^{2+} with EGTA: first, the oscillations ceased more quickly in response to nifedipine than to EGTA; second, the last oscillation produced by nifedipine did not display the characteristic broad peak seen in the EGTA-treated cells. These findings suggest that nifedipine and EGTA inhibited spontaneous [Ca^{2+}]$_i$ fluctuations by different mechanisms. The first observation could be explained by postulating that EGTA chelation of extracellular Ca^{2+} occurred more slowly than antagonism of L-type VOCC by nifedipine. The observation that EGTA and nifedipine produced final oscillations of different durations may be due to the presence of more than one Ca^{2+}-efflux mechanism. For example, plasma-membrane efflux pumps and/or ion exchangers could be inhibited, in the absence of extracellular Ca^{2+} or upon emptying of an intracellular Ca^{2+} pool, leaving uptake into intracellular Ca^{2+} stores as the predominant mechanism for decreasing elevated [Ca^{2+}]$_i$. If Ca^{2+} uptake into intracellular compartments is not as efficient as plasma-membrane Ca^{2+}-efflux mechanisms, the time required for [Ca^{2+}]$_i$ to return to baseline would increase. The experiments utilizing nifedipine were conducted in 1 mM Ca^{2+} medium. Under these conditions, it is likely that plasma-membrane efflux pathways would function, the elevated [Ca^{2+}]$_i$ from the last oscillation would be decreased by a combination of efflux from the cell and uptake into intracellular compartments, and a typical oscillation of 1–2 s duration would result. Alternatively, nifedipine has been reported to inhibit Ca^{2+} release from an intracellular store in neutrophils [37]. If a similar mechanism is present in GH_4C_1 cells, the rapidity of the nifedipine response and the lack of a final broad peak could be due to the simultaneous block of both plasma-membrane L-type VOCC and an intracellular Ca^{2+}-release channel. At present these hypotheses are speculative. Additional studies are required to explain fully the mechanistic differences between the EGTA- and nifedipine-induced [Ca^{2+}]$_i$ profiles.

The role of the L-type VOCC was investigated by using the agonist BAY K 8644. Exposure to BAY K 8644 produced a sustained series of large [Ca^{2+}]$_i$ oscillations (Figure 6). To determine whether these oscillations required an intracellular Ca^{2+} store, experiments were performed in cells after inhibition of the spontaneous [Ca^{2+}]$_i$ fluctuations by EGTA. In such cells, [Ca^{2+}]$_i$ fluctuations were not elicited by BAY K 8644 exposure and re-introduction of extracellular Ca^{2+} (Figure 7). Instead, BAY K 8644 treatment resulted in an elevated [Ca^{2+}]$_i$ plateau, or a broad [Ca^{2+}]$_i$ spike, or no apparent change in [Ca^{2+}]$_i$. One possible explanation for the three different responses is that all cells do not regulate [Ca^{2+}]$_i$ identically. Those cells most able to accommodate a rapid influx of extracellular Ca^{2+} would exhibit no net increase in [Ca^{2+}]$_i$ (Figure 7c), whereas those least able to adapt to the influx would exhibit an elevated [Ca^{2+}]$_i$ plateau (Figure 7a). The absence of sustained oscillations supports the hypothesis that a series of [Ca^{2+}]$_i$ fluctuations is initiated by an influx of Ca^{2+} through VOCC only in the presence of a critical, intact, intracellular Ca^{2+} store.

One well-defined intracellular Ca^{2+} compartment in GH_4C_1 cells is the Ins(1,4,5)P_3-sensitive pool regulated by TRH. In population studies, the [Ca^{2+}]$_i$ response to TRH consists of an initial spike phase, primarily mediated by the release of Ca^{2+} from an Ins(1,4,5)P_3-sensitive pool [25–27], followed by a plateau phase that requires entry of extracellular Ca^{2+} [10]. A biphasic [Ca^{2+}]$_i$ response has also been reported in individual GH_3B_6 cells [14] and was confirmed here (Figure 8a). As observed in populations of GH_4C_1 cells [10], the initial spike phase was insensitive to removal of extracellular Ca^{2+} by EGTA/HBSS (Figure 8b). It was particularly informative that TRH elicited a [Ca^{2+}]$_i$ spike in cells that were unable to respond to BAY K 8644 after exposure to EGTA/HBSS (Figure 7c). This finding indicates that BAY K 8644-induced oscillations do not involve the production of Ins(1,4,5)P_3 or require the TRH-stimulated Ins(1,4,5)P_3-sensitive pool.

Thapsigargin inhibits intracellular Ca^{2+}-ATPases, some of which are responsible for the refilling of depleted intracellular Ca^{2+} stores [38]. Pretreatment of GH_4C_1 cells for 10 min with 1 μM thapsigargin did not inhibit spontaneous or BAY K 8644-induced [Ca^{2+}]$_i$ fluctuations, but thapsigargin did inhibit the TRH-stimulated [Ca^{2+}]$_i$ spike (Figure 9). In population studies utilizing a clone of GH_3 cells, thapsigargin was reported to inhibit the TRH-induced [Ca^{2+}]$_i$ spike, but to have no effect on TRH-stimulated production of Ins(1,4,5)P_3 [28]. Therefore, the absence of a TRH-stimulated [Ca^{2+}]$_i$ spike after thapsigargin treatment is the result of an empty Ins(1,4,5)P_3-responsive calcium pool. Thapsigargin-insensitive [Ca^{2+}]$_i$ pools have been reported in smooth muscle [39] and salivary acinar [40] cells as well as in brain microsomes [41]. Taken together, these results indicate that the TRH-stimulated Ins(1,4,5)P_3-sensitive pool is not responsible for the [Ca^{2+}]$_i$ oscillations observed in response to BAY K 8644.

A candidate for the BAY K 8644-regulated Ca^{2+} pool is a CICR-modulated compartment. Although a CICR pool has not previously been described in GH_4C_1 cells, a caffeine-responsive pool is present in a GH_3 clone [28]. In our cells, caffeine preferentially stimulated [Ca^{2+}]$_i$ fluctuations in cells that exhibited little or no spontaneous [Ca^{2+}]$_i$ activity (Figure 10), supporting a model in which a CICR mechanism is involved in the generation of spontaneous [Ca^{2+}]$_i$ fluctuations. Caffeine stimulates CICR by lowering the Ca^{2+} threshold for Ca^{2+} release [42]. If CICR were part of the generating system for [Ca^{2+}]$_i$ fluctuations, it is likely that spontaneously active cells would have a [Ca^{2+}]$_i$ level sufficient to induce Ca^{2+} release. Stimulation of CICR by caffeine would therefore be unnecessary and no increase in [Ca^{2+}]$_i$ fluctuations would be observed. In inactive cells, however, caffeine could lower the threshold for CICR; ambient [Ca^{2+}]$_i$ levels would become sufficient to support CICR. An increase in [Ca^{2+}]$_i$ fluctuations in response to caffeine would be expected in such cells. This prediction of a differential effect of caffeine on [Ca^{2+}]$_i$ fluctuations in spontaneously active and quiet cells was observed in the present studies.

It has not been possible to test this proposal directly by inactivating the CICR channel. Ruthenium Red is often used as an inhibitor of the ryanodine/CICR channel [31,42,43]. Although Ruthenium Red did decrease Fluo-3 fluorescence and apparent [Ca^{2+}]$_i$ fluctuations, subsequent control experiments revealed that Ruthenium Red has a strong absorbance that spans the emission spectra of all presently available Ca^{2+}-indicator dyes (results not shown). Support for a CICR mechanism was found in the present studies, however (Figures 6, 7 and 10). Contraction-coupling in cardiac muscle cells is modulated via a sarcoplasmic-reticulum CICR channel that is stimulated by extracellular Ca^{2+} influx through VOCC [6,33,42,43]. An analogous system may be present in GH_4C_1 cells, whereby spontaneous action potentials or pharmacological agents such as BAY K 8644 cause Ca^{2+} influx from the extracellular environment, and the resulting elevation in [Ca^{2+}]$_i$ stimulates a series of subsequent [Ca^{2+}]$_i$ fluctuations via discharges from a Ca^{2+}-sensitive Ca^{2+} pool(s).

Considering all the available data, we propose a working model illustrated in Figure 11. GH_4C_1 cells contain at least two functionally distinct intracellular Ca^{2+} stores. One of these stores (1) is Ins(1,4,5)P_3-sensitive and releases Ca^{2+} in response to TRH.

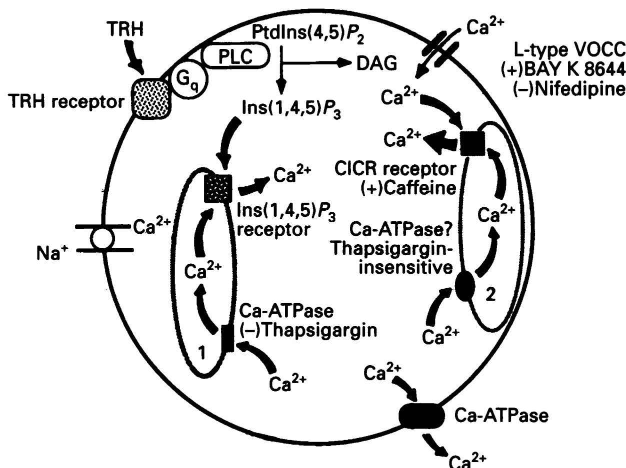

Figure 11 Working model for the modulation of $[Ca^{2+}]_i$ in GH_4C_1 cells

GH_4C_1 cells contain at least two functionally distinct intracellular Ca^{2+} stores. The first store (1) is Ins(1,4,5)P_3-sensitive and releases Ca^{2+} in response to TRH. This store is sensitive to emptying by thapsigargin, but maintains its ability to respond to TRH in the absence of extracellular Ca^{2+}. The second pool (2) is not sensitive to emptying by thapsigargin, but is reversibly inactivated by exposure to EGTA/HBSS. BAY K 8644 and spontaneous action potentials are able to initiate a series of $[Ca^{2+}]_i$ fluctuations by producing Ca^{2+} influx through L-type VOCCs. These transient influxes of extracellular Ca^{2+} then stimulate a series of oscillations via a CICR-like mechanism. GH_4C_1 cells are able to decrease $[Ca^{2+}]_i$ by at least three different mechanisms: Ca^{2+} uptake into intracellular stores, Ca^{2+} efflux via a Na^+/Ca^{2+} exchanger, and Ca^{2+} efflux via a plasma-membrane Ca^{2+}-ATPase. Abbreviations: DAG, 1,2-diacylglycerol; PLC, phospholipase C; G_q, G-protein coupling TRH receptor to PLC.

This pool is depleted by thapsigargin, but maintains its ability to function, at least temporarily, in the absence of extracellular Ca^{2+}. The second pool (2) is functionally separate from pool 1, reversibly inactivated upon exposure of the cells to EGTA/HBSS and insensitive to thapsigargin. Incubation of GH_4C_1 cells with TRH results in an increase in Ins(1,4,5)P_3. The binding of Ins(1,4,5)P_3 to its receptor on the Ins(1,4,5)P_3-sensitive Ca^{2+} pool (1) stimulates the release of Ca^{2+} into the cytosol, resulting in the spike phase of the TRH response. This Ca^{2+} spike may, in turn, stimulate additional oscillations via Ca^{2+} release from the CICR pool (2). BAY K 8644 and spontaneous action potentials are each able to initiate a series of $[Ca^{2+}]_i$ fluctuations by producing Ca^{2+} influx through L-type VOCC. These transient influxes of extracellular Ca^{2+} then stimulate a series of oscillations via a CICR-like mechanism. Nifedipine inhibits $[Ca^{2+}]_i$ fluctuations by antagonizing L-type VOCC and thereby inhibiting the necessary trigger for the fluctuations. Caffeine stimulates $[Ca^{2+}]_i$ fluctuations by lowering the Ca^{2+} threshold of the CICR channel to a level triggered by the ambient $[Ca^{2+}]_i$. This model does not address directly the mechanism used by cells to decrease $[Ca^{2+}]_i$ during the falling phase of each oscillation. GH_4C_1 cells possess a plasma-membrane Ca^{2+}-ATPase as well as a Na^+/Ca^{2+} exchanger [10,22]. Therefore, the falling phase of the Ca^{2+} spike is probably due to the combined activity of uptake into intracellular Ca^{2+} pools, Na^+/Ca^{2+} exchange and efflux via a plasma-membrane Ca^{2+}-ATPase (see [22]). Although other models could be postulated to explain the observations reported herein, the pharmacological and physiological data presently available support this working model.

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