Mechanism of $[Ca^{2+}]_i$ Oscillations in Rat Chromaffin Cells

COMPLEX Ca^{2+}-DEPENDENT REGULATION OF A RYANODINE-INSENSITIVE OSCILLATOR*

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In the population of primary cultured rat chromaffin cells, over half exhibited spontaneous $[Ca^{2+}]_i$ oscillations, whereas most others were induced to oscillate by low concentrations of bradykinin or KCl. $[Ca^{2+}]_i$ spots were observed to pulsate in a defined cytoplasmic area (the oscillator). In silent cells those spots remained discrete, whereas in oscillating cells the $[Ca^{2+}]_i$ increase expanded to occupy the entire cytoplasm. Alternation of these discrete and expanded events was observed in a few irregularly oscillating cells. Thapsigargin induced prompt blockade of both pulsations and oscillations and prevented recruitment of silent cells to oscillate. This indicates sarcoendoplasmic reticulum Ca^{2+}-ATPase-type Ca^{2+} pump(s) to be crucial for the functioning of the oscillator. Effects of other treatments were variable, depending on the concomitant $[Ca^{2+}]_i$ changes. Oscillations were blocked when EGTA or nitrendipine decreased Ca^{2+} influx and thus $[Ca^{2+}]_i$; they were also blocked when $[Ca^{2+}]_i$ was markedly increased by excess KCl, bradykinin, or ryanodine. When in contrast the $[Ca^{2+}]_i$ increases induced by the latter agents remained moderate, oscillations were stimulated. The rhythmic activity of rat chromaffin cells appears, therefore, to operate under a complex regulation that requires $[Ca^{2+}]_i$ within an appropriate operative range and does not involve directly the ryanodine receptor but might rely on the activation of IP3 receptors.

In a variety of cell types the basal concentration of the free cytosolic Ca^{2+}, $[Ca^{2+}]_i$, does not remain necessarily stable but can exhibit rhythmic oscillations, with periods ranging from tens of seconds to a few minutes. In most cases, oscillations were shown to be sustained by the repetitive release of a Ca^{2+} pool accumulated within rapidly exchanging Ca^{2+} store(s) (for recent reviews, see Jacob (1990), Meyer and Stryer (1991), and Berridge (1992)). The nature and regulation of the store(s) are still open to question. In brief, the models that have been proposed involve either a single or two separate, but functionally coordinate, Ca^{2+} stores (see Berridge (1991a and 1992), Meyer and Stryer (1991), and Cuthbertson and Chay (1991)). The single store models (at least in their original formulations) predict an increase in the concentration of the inositol 1,4,5-trisphosphate $(IP_3)^1$ following activation of polyphosphoinositide hydrolysis to be both necessary and sufficient to trigger and sustain oscillations. In these models the IP_3-sensitive store is therefore identified as the store responsible for the activity (Parker and Ivorra, 1990; Meyer and Stryer, 1991; Cuthbertson and Chay, 1991). In contrast, in the two store models the oscillator is sensitive not to IP_3 but to appropriate increases of $[Ca^{2+}]_i$, through a process known as Ca^{2+}-induced Ca^{2+} release (CICR) (Berridge and Galione, 1988). In the CICR models, discharge of the store is triggered by $[Ca^{2+}]_i$ elevation, no matter whether induced by IP_3 or by Ca^{2+} influx. CICR is a process typical of the ryanodine (Ry) receptor, a second type of intracellular Ca^{2+} channel, initially identified in striated muscle fibers (Iino et al., 1988) and now known to be expressed also by a variety of other cells (McPherson et al., 1991; Walton et al., 1991; Giannini et al., 1992).

Rat chromaffin cells exhibit spontaneous activity in a large fraction of the population and respond to appropriate stimulations with changes in frequency and recruitment of silent cells (Malgaroli et al., 1990; Malgaroli and Meldolesi, 1991). Since these cells are known to possess separate Ca^{2+} stores, sensitive to either IP_3 or Ry (Malgaroli et al., 1990), they represent a suitable system for the investigation of the mechanisms that underly the oscillatory process. In previous studies of our laboratory, carried out by single cell microfluorimetry, evidence obtained by the application of a variety of treatments suggested identification of the Ry-sensitive store as the intracellular Ca^{2+} oscillator (Malgaroli et al., 1990). The present results reveal, however, that the control of the oscillatory activity is more complex than previously envisaged and document the key role of a Ry-insensitive oscillator operated under continuous $[Ca^{2+}]_i$-dependent modulation.

EXPERIMENTAL PROCEDURES

Cell Cultures—Rat chromaffin cells were prepared according to Brandt et al. (1976). Briefly, adrenal glands were aseptically removed from 175 to 200-g ether-anesthetized female Sprague-Dawley rats. Each adrenal medulla was dissected free of the cortex, rinsed several times in a medium containing 137 mmol/liter NaCl; 3 mmol/liter KCl, 0.7 mmol/liter Na_2HPO_4; 25 mmol/liter HEPES, pH 7.2, 10 mmol/ liter glucose, and 350 units/ml (each) penicillin G and streptomycin and then cut in small pieces with a pair of fine needles. To dissociate the cells, the adrenal tissue fragments were treated at 37 °C for 90–120 min with a mixture of collagenase A and DNase I (0.5 units/ml and 10 µg/ml, respectively, dissolved in the above medium

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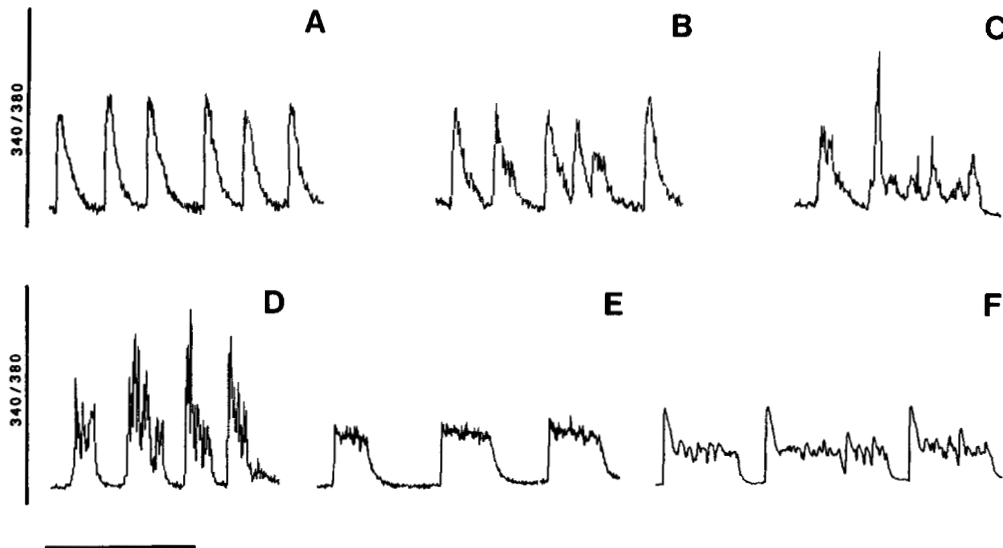*[Ca²⁺]ᵢ Oscillations in Rat Chromaffin Cells*

FIG. 1. **Spontaneous [Ca²⁺]ᵢ oscillations in rat chromaffin cells.** In this and the following figures each trace illustrates the time course of the fluorescence signal ratios (340 nm/380 nm), calculated from the digital images of individual Fura-2-loaded cells. The various oscillatory patterns exhibited by chromaffin cells (Ca²⁺ fingerprints) are shown in *A–F*. *Bar*, 5 min.

supplemented with 1% bovine serum albumin), with gentle up and down drawing in a Pasteur pipette every 15–20 min. The cells were then centrifuged at 500 × *g* for 4 min and rinsed twice with the HEPES-buffered medium. Yield was ~10⁴ cells/medulla. Chromaffin cells were finally suspended in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, plated over polyornithine (100 μg/ml)-coated glass coverslips, and cultured for 1–4 days under a humidified atmosphere containing 5% CO₂.

Fura-2 Loading—The cell-bearing coverslips were rinsed three times with a Krebs-Ringer solution buffered with HEPES (150 mmol/liter NaCl, 5 mmol/liter KCl, 1 mmol/liter MgSO₄, 2 mmol/liter CaCl₂, 10 mmol/liter glucose, 30 mmol/liter HEPES, pH 7.4). Cells were loaded by incubation at 37 °C for 45 min with Fura-2 pentaacetoxymethylester (2–5 μM) (Grynkiewicz *et al.*, 1985), dissolved in the Krebs-Ringer solution supplemented with 1% bovine serum albumin. Cells were then rinsed twice in the Krebs-Ringer solution and transferred to the heated (37 °C) stage of the microscope. Incubations were carried out in 2 ml of Krebs-Ringer solution. Since rapid addition and intermixing were imperative to precisely correlate the actions of various agents, a new delivery procedure was employed. Appropriate volumes of 100–1000-fold concentrated solutions were loaded into a 2-ml syringe connected to the incubation chamber via a small tube. Aspiration into the syringe of 1 ml of incubation medium, followed by reintroduction of this mixture into the chamber, yielded accurate and rapid (<1 s) delivery and mixing of the agents.

Fura-2 Videomicroscopy—The digital fluorescence-imaging microscopy system was built around a Zeiss inverted IM 35 light microscope as described previously (Grohovaz *et al.*, 1991). Briefly, excitation light at 340 or 380 nm was provided by two 150-watt xenon lamp; fluorescence images were collected by a low-light level CCD camera (Hamamatsu Photonics, Herrsching, Germany) and fed into a digital image processor (Argus 100; Hamamatsu Photonics) where video frames were digitized and integrated (five consecutive video images of 128 × 128 pixels × 16 bits) in real time. Digital data were transferred at high rate into a Motorola 68020-based host computer, and stored in two 300-megabyte hard disks. After background and calibration, images were similarly acquired at the two wavelengths, and ratios were calculated pixel by pixel on pairs of corresponding 340 and 380 images. Mean values of pixel intensity in the area of interest could be calculated from frame sequences, thus providing quantitative temporal analyses in spatially distinct areas.

Materials—Most of the fine chemical used were purchased from Sigma. Fura-2, Ry, and thapsigargin (Tg) were from Calbiochem. Nitrendipine was a gift from Bayer. The B₂ receptor antagonist, Arg⁰-[Hyp³,Thi⁵,⁸,D-Phe⁷]BK, was the gift of Dr. D. Regoli (University of Sherbrooke, Quebec, Canada).

RESULTS

Spontaneous and Stimulation-induced [Ca²⁺]ᵢ Oscillations—
In agreement with our previous results (Malgaroli *et al.*, 1990),

50% (*i.e.* 73 out of a population of 141) of our cultured chromaffin cells exhibited spontaneous oscillations. In over half of the active cells, oscillations were rhythmic spikes of almost constant amplitude (see Fig. 1*A*); in others a marked variability of all the oscillation parameters was observed (Fig. 1, *B* and *C*). In approximately one-fourth (19/73) of the oscillating cells, individual transients were more complex, noisy, and long lasting, as illustrated by Fig. 1, *D–F*. When left untreated, individual cells exhibited only one of the spontaneous oscillation patterns shown, which was maintained during up to 2 h of observation (Ca²⁺ fingerprint; Prentki *et al.*, 1988).

In silent cells, oscillations could be induced by low concentration of either BK (0.05–0.2 μM; Malgaroli *et al.*, 1990) or KCl (2–5 mM, Fig. 2*C*), working via activation of polyphosphoinositide hydrolysis or depolarization, respectively. Moreover, each of these treatments did increase the frequency of ongoing oscillations, spontaneous or induced by the other. In contrast, higher concentrations lead to single [Ca²⁺]ᵢ transients followed by sustained plateaus (Malgaroli *et al.*, 1990). Compared with the resting level observed before recruitment, the interspike [Ca²⁺]ᵢ of the cells induced to oscillate was always higher, slightly with BK and more markedly with KCl. When these agents were washed out, the arrest of the oscillations was accompanied by the return of [Ca²⁺]ᵢ to the preoscillatory level (see Fig. 3*B*).

Effects of EGTA and of Ca²⁺ Channel and Ca²⁺ Pump Blockers—Fig. 2 illustrates the blocking effect on spontaneous oscillations of both the Ca²⁺ chelator EGTA (calculated [Ca²⁺]ₒ < 10⁻⁸ M; *trace A*; 20/20 cells) and the voltage-gated Ca²⁺ channel blocker, nitrendipine (*B*, 15/17 cells). Block was not immediate but could be preceded by a single smaller spike. Moreover, the [Ca²⁺]ᵢ level reached after blockade was distinctly lower than the preceding interspike level. Reintroduction of Ca²⁺ into the EGTA-containing medium resulted in the reappearance of the oscillations (*trace A*). With nitrendipine oscillations were resumed after washout (not shown) or, in the presence of the drug, when subnanomolar concentrations of BK were applied (*traces B* and *C*).

Another agent investigated was the sesquiterpene tumor promoter thapsigargin (*Tg*) (Fig. 3), an inhibitor of the intracellular sarcoendoplasmic reticulum-type Ca²⁺ ATPases
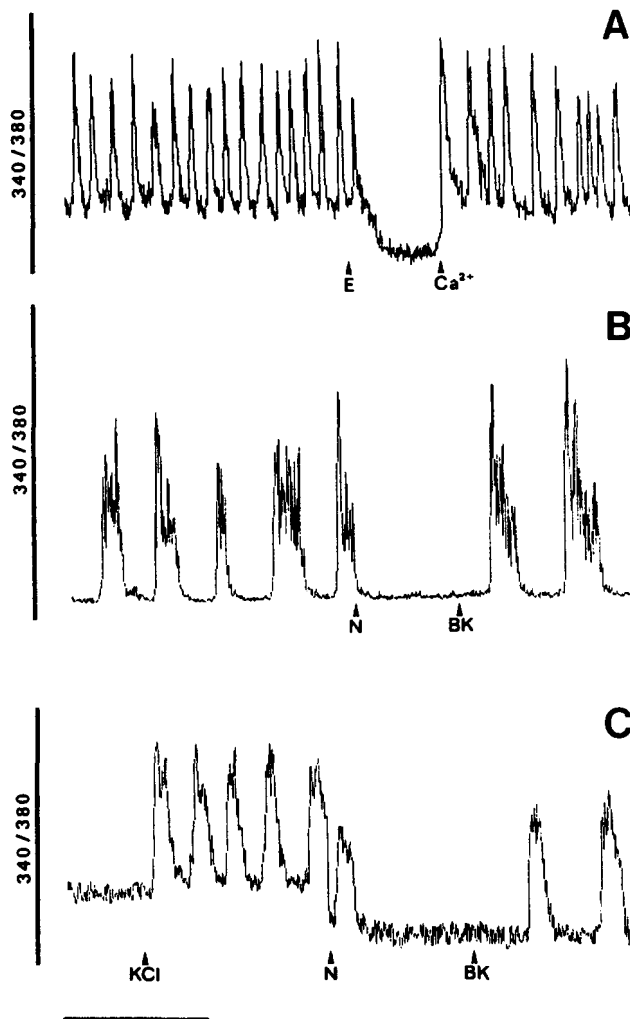
FIG. 2. **Blocking effect of EGTA and nitrendipine on [Ca^{2+}]$_i$ oscillations.** *Trace A* shows that after addition of excess EGTA (*E*, 1 mM final concentration) a single smaller spike precedes the blockade of the oscillation activity. Ca^{2+} reintroduction (2 mM final concentration) into the medium causes oscillations to resume. *Trace B* shows the inhibition by nitrendipine (*N*, 1 μM); in nitrendipine-blocked cells BK (50 pM) is still able to induce oscillations. Similar results are shown in *C*, where the KCl-induced oscillations are blocked by the Ca^{2+} channel inhibitor, an effect accompanied by a pronounced [Ca^{2+}]$_i$ decrease. In the latter cell BK (200 pM) is still capable of restoring oscillations without appreciable effect on the interspike [Ca^{2+}]$_i$. Oscillations resumed after EGTA or nitrendipine blockade maintain their original fingerprint. *Bar*, 5 min.

(SERCAs; Thastrup *et al.*, 1990; Lytton *et al.*, 1991). When administered to oscillating cells the drug caused the abrupt and rapid (within seconds) block of the activity (9/9 cells, *trace A*), without appearance of the smaller spikes described with EGTA. The effect of Tg was reversed neither by washing nor by administration of stimulants (BK or KCl) before or after the wash (not shown). In silent cells, pretreatment with the drug prevented cell recruitment by the above stimulants, administered at the concentrations previously effective in eliciting the oscillation activity (see *trace B*).

Effects of Caffeine and Ryanodine—Caffeine, a drug often employed as an indirect activator of CICR (Rousseau *et al.*, 1987), has been reported to induce additional effects, such as: increase of cAMP levels due to inhibition of 5',3'-phosphodiesterase (Butcher and Sutherland, 1962); negative modulation of the IP$_3$ receptor (Parker and Ivorra, 1991; Berridge, 1991b); and partial inhibition of IP3 generation (Zacchetti *et*

al., 1991). The results obtained with 1–3 mM caffeine administered to oscillating cells were markedly variable (Fig. 4). Out of 40 cells, increase in the frequency of oscillation (*trace A*) was observed in 4; block of the activity (*trace B*) in 13; conversion of the oscillations from the spiky to the prolonged-and-noisy time course (*trace C*), apparently due to slowing down of the [Ca^{2+}]$_i$ decline, in the remaining 23. This last effect was probably due to the cAMP increase, since it was reproduced by the application of either forskolin (*Fk*: 10 μM, Fig. 4C) or the membrane-permeant 8-bromo-cAMP (0.5 mM, not shown). Administration to the cells of higher concentrations of caffeine (10–20 mM) induced either a single [Ca^{2+}]$_i$ spike followed by a slowly declining plateau (see Fig. 5C) or no response whatsoever.

In striated muscle fibers, the intracellular channel that sustains CICR binds with high affinity and specificity the plant alkaloid Ry. Such a binding blocks the channel open in a low conductance state and thus induces Ca^{2+} depletion of the sarcoplasmic reticulum (Rousseau *et al.*, 1987; Smith *et al.*, 1985). In 15/25 chromaffin cells Ry either increased the frequency of oscillations (Fig. 5A) or induced recruitment of silent cells (Fig. 5B), with trains persisting for 10 min or longer. In a few of these cells, oscillations subsided after 5–10 min but could be reestablished by BK administration and then abolished by the B$_2$ receptor inhibitor (Fig. 5B). The oscillations induced or modulated by Ry were blocked by Tg (Fig. 5A). In an additional four cells the alkaloid failed to induce appreciable [Ca^{2+}]$_i$ effects. However, the expected persistent opening of the Ry receptor had apparently occurred, since the stimulatory effects of caffeine were prevented, whereas BK was still capable of inducing oscillations (Fig. 5C). Finally, in the last six cells Ry blocked the oscillations when [Ca^{2+}]$_i$ was significantly higher than the resting [Ca^{2+}]$_i$ level (Fig. 6, *A* and *B*). In two of these cells the increased [Ca^{2+}]$_i$ level was not persistent but reversed after a few min, even in the continuous presence of the drug, and this was accompanied by the resumption of the oscillations (Fig. 6B).

Blockade of the oscillations accompanied by an increased stable [Ca^{2+}]$_i$ level was not specific for Ry but was observed also in a few cells exposed to 5 mM KCl (Fig. 6C). Moreover, the sequential application of Ry and KCl yielded potentiated responses in 3/3 cells. In particular, low concentrations of KCl, that induced oscillations when administered alone, triggered the spike-plateau response, typical of higher [K$^+$]$_o$, when administered to Ry-pretreated cells (Fig. 6D). In contrast, no potentiation was observed when BK was administered to Ry-pretreated cells instead of KCl (Fig. 5C).

Subcellular Distribution of the [Ca^{2+}]$_i$ Events—Our video-imaging analyses revealed subcellular [Ca^{2+}]$_i$ events in both silent and oscillating cells. Fig. 7 shows three series, each composed by five 1-s images of a spontaneously oscillating cell accompanied by two silent cells. The images shown were taken at the beginning of three successive oscillations (see the traces to the *right*). Notice that the early steps of the [Ca^{2+}]$_i$ changes were remarkably similar in the three oscillations, with appearance of localized spots in roughly the same cytosolic area and later spreading of the [Ca^{2+}]$_i$ increase to the rest of the cell.

In Fig. 8, *A* and *B*, the 1-s image series correspond to two dissimilar events occurring in sequence in the same cell. The first is an aborted oscillation and the second a full-blown oscillation. In spite of the different outcome of the events, the initial steps were similar, again with involvement of only a single cytoplasmic area. In the aborted oscillation, however, the local [Ca^{2+}]$_i$ increase failed to invade the rest of cytoplasm, and the discrete signal was dissipated within a few
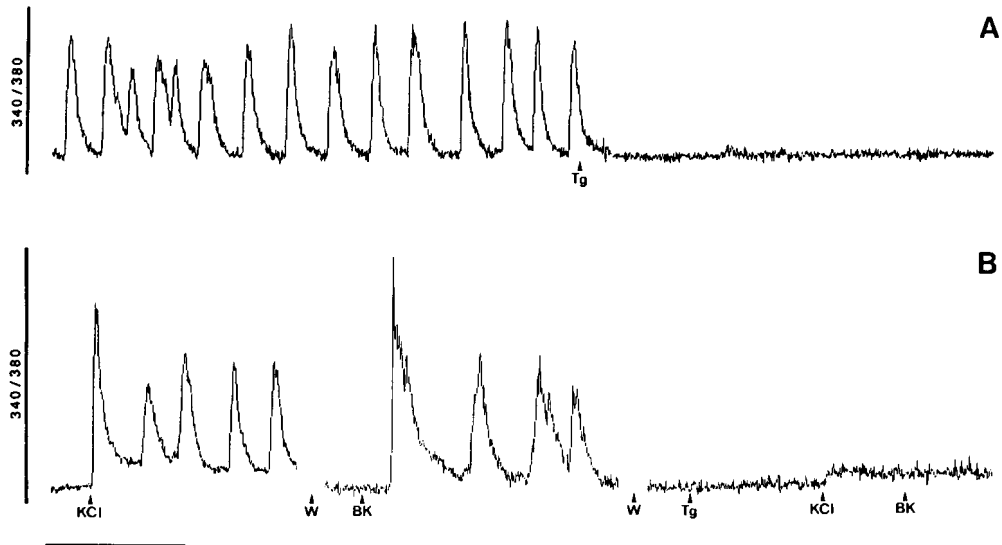
FIG. 3. **Blockade by thapsigargin of [Ca²⁺]ᵢ oscillations.** Administration of the SERCA-type Ca²⁺-ATPase inhibitor, thapsigargin (*Tg*, 1 μM), causes the immediate block of spontaneous [Ca²⁺]ᵢ oscillations (*trace A*). Similarly, Tg pretreatment of a cell previously responsive to KCl (5 mM) and BK (50 pM) prevents the induction of oscillations by the later administration of either stimuli (*trace B*). Washes (*W*) were for 20 min in the Krebs-Ringer solution. *Bar*, 5 min.
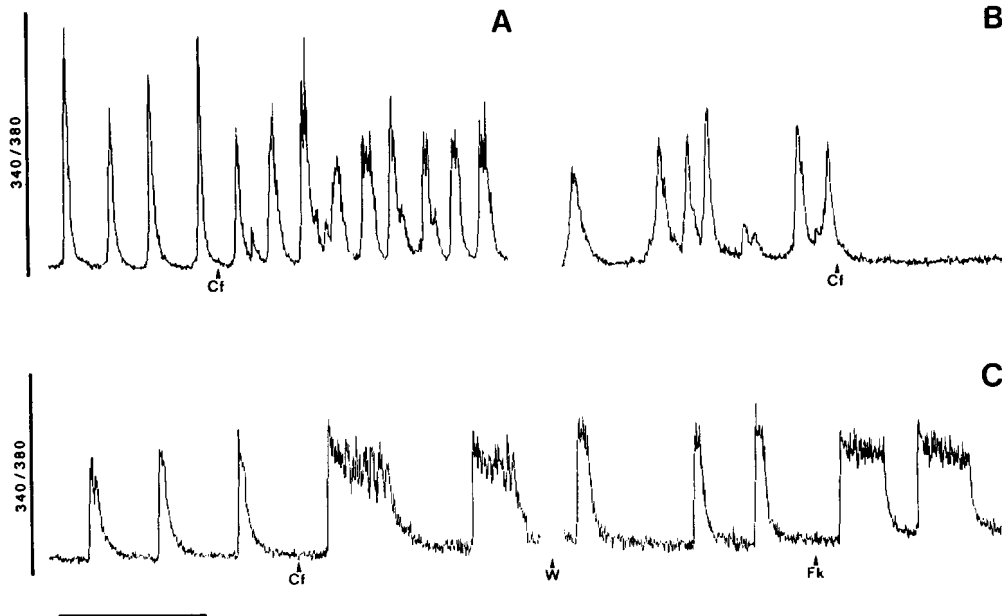

FIG. 4. **Effects of caffeine on [Ca²⁺]ᵢ oscillations.** Administration of caffeine (*Cf*, 1–3 mM) to oscillating cells gives rise to a variety of responses: increase in the oscillation frequency (*trace A*), reversible blockade of oscillations (*trace B*), marked increase in the duration of individual oscillations, with minor effects on their amplitude and frequency (*trace C, left*). This latter effect of caffeine is mimicked by forskolin (*Fk*, 10 μM) (*trace C, right*). Washes (*W*) were for 20 min in the Krebs-Ringer solution. *Bar*, 5 min.

seconds. *C* illustrates a wider [Ca²⁺]ᵢ temporal analysis of the cell shown in *A* and *B*, recorded from two distinct areas labeled * and **. Notice that the full-blown oscillation (*arrowhead*) differs only slightly in the two areas, whereas the aborted oscillation (*arrow*), clearly visible as a small spike in one (*), is almost undetectable in the other (**). In most cases aborted oscillations were not single, but accompanied by other rhythmic events exhibiting the same localized distribution (see the three small spikes preceding the analyzed aborted oscillation in the *trace * of Fig. 8C).

The image series shown in Fig. 9 refers to a single cell, however, nonoscillating. When incubated in the Ca²⁺-containing medium (*A*) the imaged cell showed an irregularly pulsatile discrete spot of [Ca²⁺]ᵢ, that in the averaged image (*right*)

appears distinctly different from the rest of the cell. In the Ca²⁺-free medium (*B*) the spot, although less intense, was still appreciable. Moreover, when the Ca²⁺-free incubated cell was treated with Tg (*C*), the spot was reinforced and no longer pulsatile. Rather, it persisted apparently unchanged for 1–2 min, after which it faded out progressively. [Ca²⁺]ᵢ spots were clearly detected in 15% of the silent rat chromaffin cells and never seen in their tumoral counterpart PC12 (not shown), a cell line unable to develop [Ca²⁺]ᵢ oscillations even when exposed to stimuli (Grohovaz *et al.*, 1991).

DISCUSSION

Dual Ca²⁺ Control of [Ca²⁺]ᵢ Oscillations—[Ca²⁺]ᵢ oscillations originated from intracellular stores, spontaneous as well
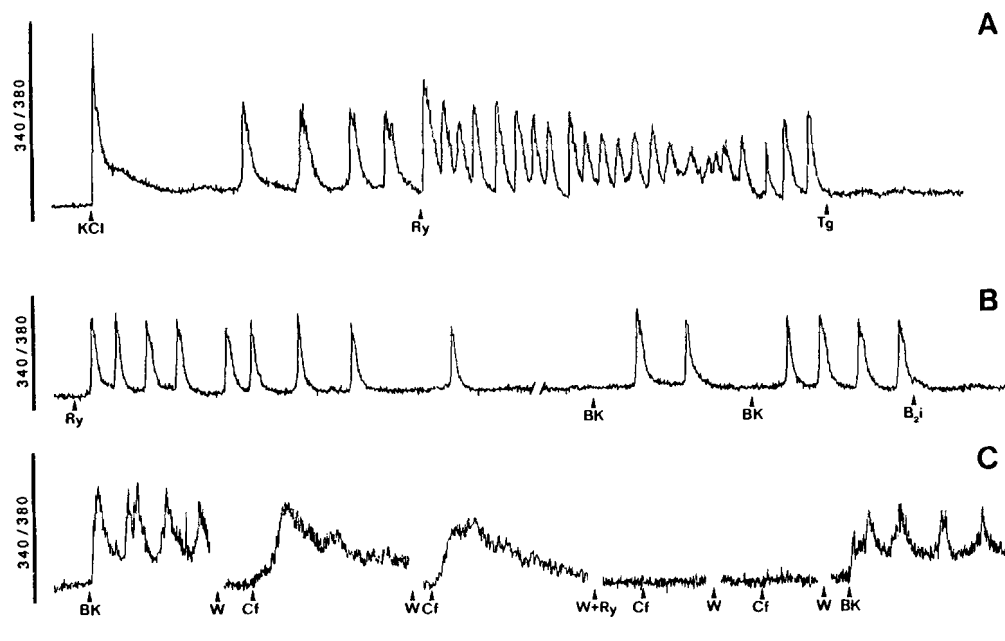
FIG. 5. **Stimulatory effects of ryanodine on [Ca²⁺]ᵢ oscillations.** *Trace A* shows a silent cell where 5 mM KCl induces oscillatory activity. In this cell Ry administration (10 μM) leads to a moderate elevation of the [Ca²⁺]ᵢ interspike level accompanied by an increase of the frequency of the oscillations, until a final block by Tg (1 μM). *Trace B* shows the Ry induction of [Ca²⁺]ᵢ oscillations in a silent cell. Ten minutes later the frequency decreases, and the activity eventually disappears. Oscillations with a similar pattern are reinduced by challenge with BK (50 pM), their frequency is increased by a second challenge and finally blocked by a specific B₂ receptor inhibitor (B₂i, 25 μM). In this trace 15 min of the record were cut out (/ /). In *trace C* a silent cell is first induced to oscillate with BK (50 pM). After washout the cell is challenged twice with a high concentration of caffeine (Cf) (10 mM) and exhibits persistent [Ca²⁺]ᵢ increases. After Ry treatment (10 μM) the same cell is no longer responsive to Cf but still capable of oscillating when stimulated with BK (50 pM). Washes (W) were for 20 min in the Krebs-Ringer solution. *Bar*, 5 min.
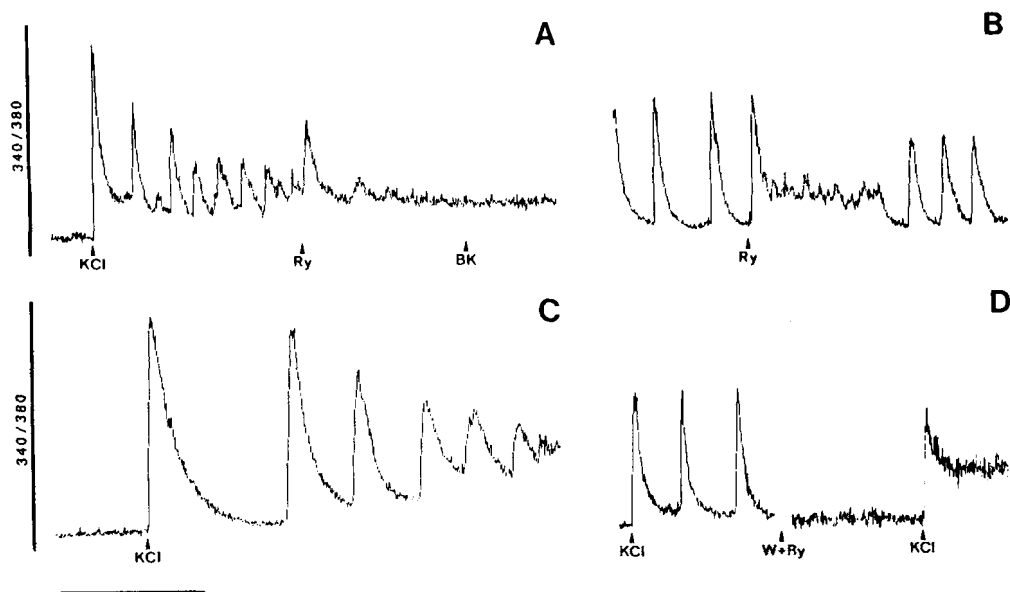
FIG. 6. **Oscillation blockade by high [Ca²⁺]ᵢ increase.** *Trace A* is from a cell where oscillations, initially induced by KCl (5 mM), are then blocked by Ry (10 μM) administration. Blockade is accompanied by a sustained [Ca²⁺]ᵢ plateau. Under these conditions the cell is no longer responsive to BK (100 nM). *Trace B* shows a similar response to Ry administration. In this cell, oscillations are resumed when [Ca²⁺]ᵢ spontaneously resets toward the initial interspike value. *Trace C* reports the response of a cell to KCl (5 mM); blockade of oscillations coincides with a steady increase of [Ca²⁺]ᵢ. *Trace D* shows the different responses of a cell to two 5 mM administrations of KCl, given before and after Ry (10 μM) treatment. The initial KCl induces promptly [Ca²⁺]ᵢ oscillations, which disappear after washing (W). Following incubation with Ry for 20 min, KCl administration fails to induce oscillations but leads to a sustained increase of [Ca²⁺]ᵢ. *Bar*, 5 min.

as triggered by low concentrations of BK, had already been reported in rat chromaffin cells in a previous study of our laboratory (Malgaroli *et al.*, 1990). The results, however, differed in at least two important aspects from those we have now obtained. The first such difference, the slower inactivation of the oscillations observed when Ca²⁺ influx was affected

by either EGTA or the Ca²⁺ channel blocker, nitrendipine (Malgaroli *et al.*, 1990), was probably due to technical reasons, in particular to the mixing problems on the stage of the upright microscope employed at the time. The faster inactivation observed in our present experiments demonstrates that oscillations depend more extensively than previously envis-
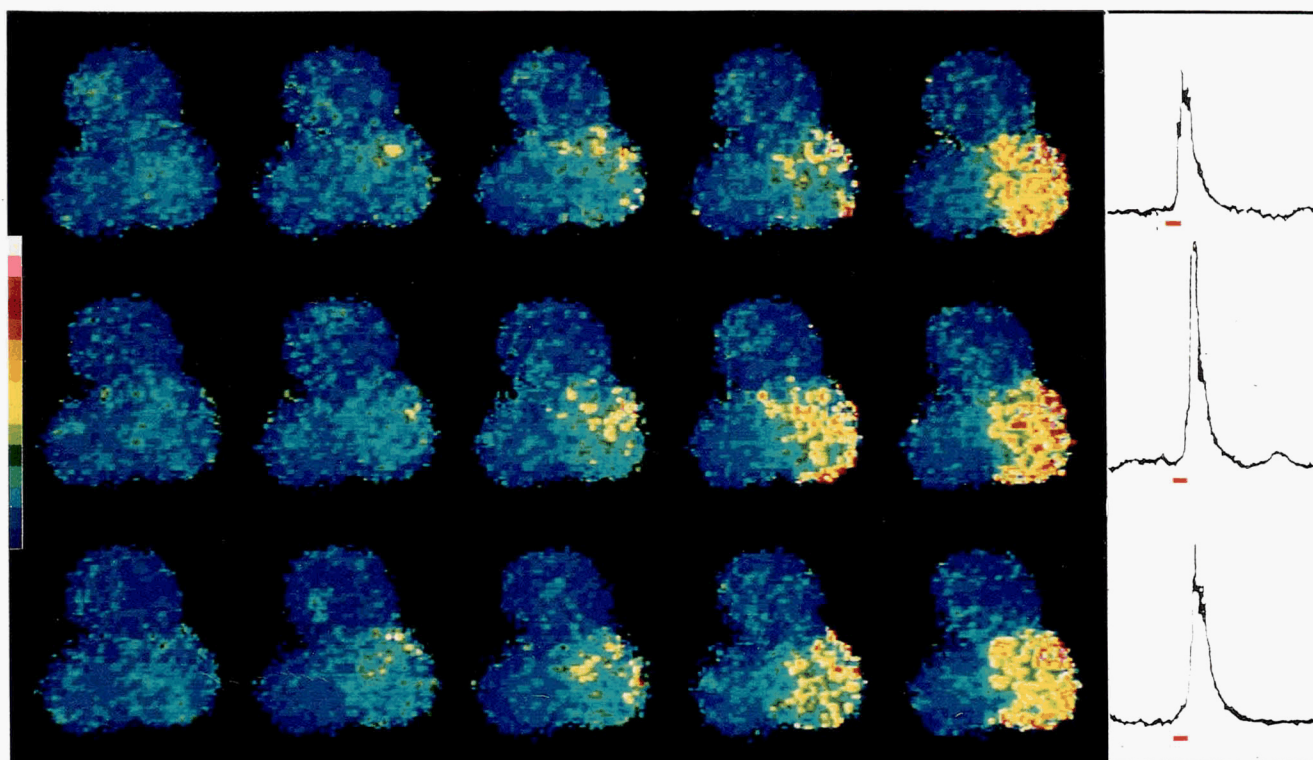
FIG. 7. **Early [Ca²⁺]ᵢ steps in three successive oscillations.** Three series, each composed by five images 1 s apart, illustrate the rising phase of three successive oscillations in a spontaneously active cell accompanied by two silent cells. Early steps of the three oscillations are similar and consist in a [Ca²⁺]ᵢ increase, originated in the same cytoplasmic area, that rapidly spreads to the rest of the cell. The traces to the *right* illustrate the full transients and mark the time corresponding to the image series (*red bars*). Intensities of fluorescence ratio values are represented according to the *calibration bar* to the *left*, where the lowest values are coded *blue*.

aged on the continuous Ca²⁺ supply from the extracellular space, largely occurring through voltage-gated Ca²⁺ channels.

The second discrepancy is more difficult to explain. In 16/20 cells investigated in the previous study Ry was shown to induce marked increases of the basal [Ca²⁺]ᵢ accompanied by block of the oscillations. The latter events were therefore proposed to be sustained by CICR, the process specifically blocked by the plant alkaloid (Malgaroli *et al.*, 1990). Of our cells, however, only six exhibited Ry responses similar to those reported previously, whereas in 15 the basal [Ca²⁺]ᵢ was not markedly increased, and the oscillations were not blocked, but rather stimulated. These results, obtained under conditions in which the Ry receptor was certainly affected, as documented by the disappearance of the responses to caffeine, exclude the Ry receptor to be an integral component of the [Ca²⁺]ᵢ oscillator. Rather, the observed changes of oscillation parameters appear to correlate with the different degrees of [Ca²⁺]ᵢ increase, probably dependent on the decreased Ca²⁺ buffering and/or the stimulation of Ca²⁺ influx that follow the persistent emptying of the Ry-sensitive stores (Cheek *et al.*, 1990; Thayer *et al.*, 1988; Martinez-Serrano and Satrustegui, 1989; Clementi *et al.*, 1992). The variable extent of the latter processes in different cells could induce either moderate or large [Ca²⁺]ᵢ increases, and this ultimately results in either stimulation or blockade of the oscillations. This interpretation appears consistent with a previously reported theoretical model in which the oscillatory activity was predicted to operate exclusively in a defined range of [Ca²⁺]ᵢ (Somogy and Stucki, 1991). The dual [Ca²⁺]ᵢ-dependent control of oscillations appears not specific of Ry but common to other treatments active on [Ca²⁺]ᵢ, including BK and KCl. Interestingly, the responses to low KCl were potentiated in the Ry-pretreated cells, with conversion of the oscillations into the single

spike typical of higher KCl concentrations.

The drug that in rat chromaffin cells induced the most consistent effects was Tg. In all our experiments this SERCA blocker was able, in fact, to rapidly (within seconds) block ongoing oscillations and to prevent their appearance by subsequently administered stimulatory agents. These results, together with the blockade by Tg of the spot pulsations in apparently silent cells, discussed below, suggest not only that SERCAs are responsible for the pumping of Ca²⁺ within the oscillating stores, but also that the filling of those stores up to a critical level, which cannot be reached when the pump is blocked, is an essential step of each oscillation. Alternatively, the immediate blockade by Tg could be due to the increase of [Ca²⁺]ᵢ, up to inhibitory values, in the cytoplasmic rim around the oscillator, sustained by the unmatched leakage of the cation across the limiting membrane.

The properties of oscillation control so far emerged resemble properties recently identified for the IP₃ receptor. In fact, on the one hand, Ca²⁺ release by IP₃ exhibited dependence on the loading conditions of the store (Missiaen *et al.*, 1991, 1992); on the other hand, it was shown to be modulated positively by [Ca²⁺]ᵢ increases up to 250–300 nM and negatively by higher [Ca²⁺]ᵢ (Finch *et al.*, 1991; Bezprozvanny *et al.*, 1991). Thus, although at the moment direct evidence is not yet available, we hypothesize that in rat chromaffin cells the store filled up by SERCAs can be rhythmically discharged through the IP₃ receptor channel operated under a Ca²⁺-dependent control. This hypothesis appears in agreement with results obtained in other cell types (DeLisle and Welsh, 1992; Lechleiter and Clapham, 1992; Iino and Endo, 1992; Miyazaki *et al.*, 1992), as well as with recently proposed models (Somogy and Stucki, 1991; Berridge, 1992). In additional cells, however, the situation might be different. In pancreatic acinar cells the
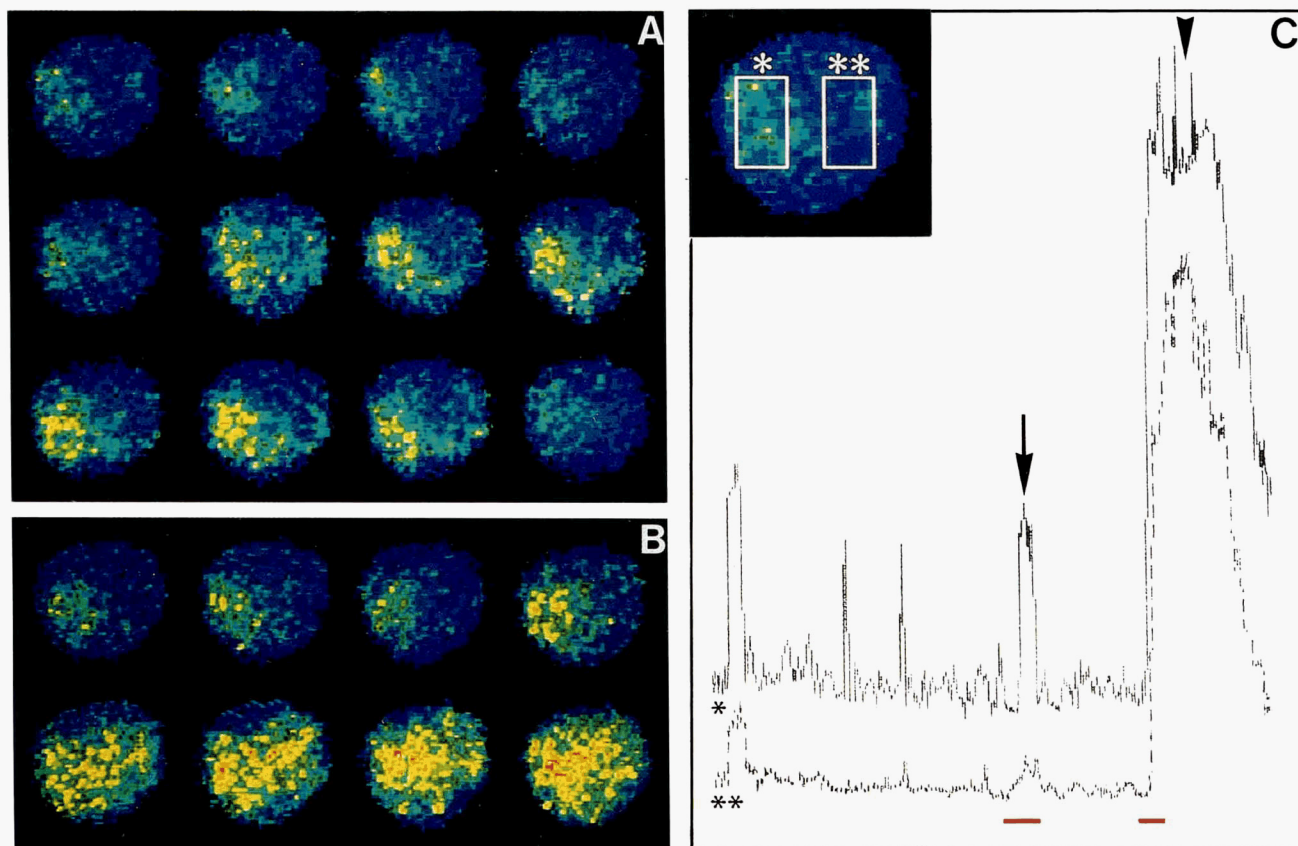
FIG. 8. **Comparison of an aborted oscillation with a full-blown oscillation in a single irregularly active cell.** The figure shows two series of images (1 s apart) from the same spontaneously oscillating cell. Notice that the aborted rapidly dissipated oscillation (*A*) closely resembles the early steps of a subsequent full-blown oscillation (*B*). The traces in *C* illustrate the $[Ca^{2+}]_i$ changes measured in the two windows (* and **) marked over the cell profile shown at the *top*. The full-blown oscillation induces comparable $[Ca^{2+}]_i$ changes (*arrowhead*) in both the cell areas, whereas the illustrated aborted oscillation (*arrow*), as well as the few preceding ones, elicit fully appreciable responses in *panel* * and only marginal signals in *panel* **. *Red bars* (12 and 8 s, respectively) mark in the traces the times when the *A* and *B* series were collected.

FIG. 9. **Discrete $[Ca^{2+}]_i$ pulsations in a silent cell: effect of thapsigargin.** The figure shows three series of images (1 s apart) from the same silent cell. *A* shows the highly localized pulsatile $[Ca^{2+}]_i$ spot in the cell when bathed in the Ca^{2+}-containing Krebs-Ringer solution. *B* illustrates successive images of the same cell after addition of excess EGTA to the extracellular medium, and *C* shows the response to Tg (1 μM) of the same cell maintained in the EGTA-containing medium. Notice that Tg treatment converts the pulsatile into a persistent $[Ca^{2+}]_i$ spot. On the *right* of each panel are the average images of 64 consecutive frames.
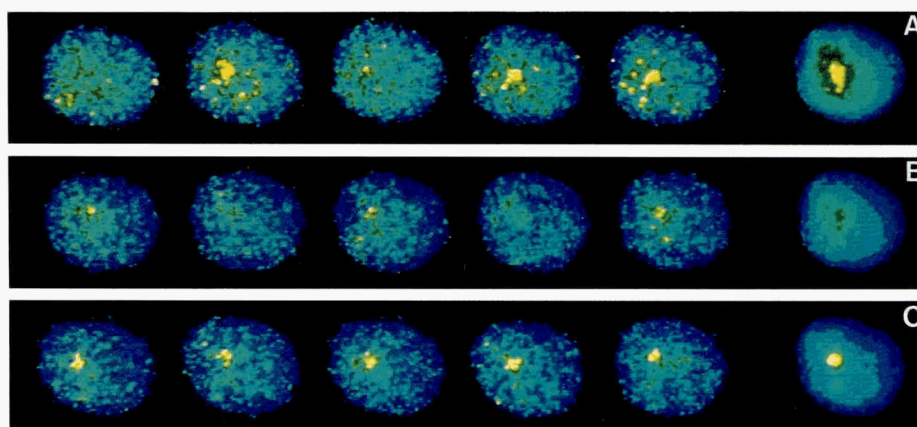
IP$_3$ receptor appears not to be involved (Wakui *et al.*, 1990). Moreover, in sympathetic neurons and parotid acinar cells the channel directly involved in oscillations has been identified as the Ry receptor (Friel and Tsien, 1992; Foskett and Wong, 1991). In addition, in the latter cells the effects of Tg are similar to those we have observed with Ry in rat chromaffin cells, thus excluding the participation of SERCA pumps in the oscillator (Foskett and Wong, 1991; Foskett *et al.*, 1991). We conclude that, although Ca^{2+} regulation might be a property of oscillations in all cells, the molecular components of the oscillator, in particular pumps and channels, can apparently be different.

Oscillation Fingerprint—One of the various effects of caffeine we have observed deserves attention, because it had never been reported, *i.e.* the change of the oscillation fingerprint, from the spike to the prolonged-and-noisy type. Because of its duplication by other agents that increase cAMP (forskolin, cAMP analogues), this effect seems to depend on a modulation by the cyclic nucleotide, presumably via activation of protein kinase A. The IP$_3$ receptor is indeed phosphorylated by the kinase, and this can induce modulations of its activation (Ferris and Snyder, 1992a; Burgess *et al.*, 1990). The fingerprint of oscillations could thus be due to variable levels of the kinase activity, corresponding therefore to a form of

cross-talk between the cAMP and Ca^{2+}-signaling systems.

Pulsatile $[Ca^{2+}]_i$ Spots—The spots that we have observed in rat chromaffin cells resemble those described previously in other systems (Parker and Yao, 1991; Rooney *et al.*, 1990), with the additional interesting feature that the process did not evolve the same in all cells but could remain discrete (pulsatile spots; aborted oscillations) or proceed to full-blown oscillations. Initial steps were similar; however, in the case of the discrete events, the positive modulation, induced by Ca^{2+} release from the oscillator, was apparently not enough to trigger discharge of the Ca^{2+} stores in the surrounding cytoplasm. In that case the released Ca^{2+} was presumably reaccumulated into the stores and extruded across the plasmalemma. When in contrast the initial $[Ca^{2+}]_i$ increase from the oscillator was sufficient to activate adjacent stores, a nondecremental recruitment did invade the entire cytoplasm.

An important question to be asked concerns the nature of the oscillator. Why is it stimulated under conditions where the other Ca^{2+} stores remain quiescent? The colocalization we have observed with the Tg-induced $[Ca^{2+}]_i$ increase area suggests a concentration of SERCA-filled structures within which Ca^{2+} storage appears to be unstable. Among properties that could sustain localized pacemaking are a higher concentration (or responsiveness) of the pump and/or the channel with respect to the rest of the cell. In the case of the IP_3 receptor, both high concentration in a peculiar endoplasmic reticulum subcompartment and molecular heterogeneity, with more sensitive isoforms, have been described, however, in cells different from chromaffin cells (Ferris and Snyder, 1992a, 1992b; Meldolesi *et al.*, 1992). The variable degree of expression of these components among the cells, together with their variable distribution in the Ca^{2+} stores, could also explain why in some cells the oscillator activity remains discrete, whereas in others it can evolve into full-blown oscillations. New experimental approaches, in particular the combination in individual cells of Ca^{2+} imaging with immunocytochemistry, might ultimately provide adequate answers.

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