Spontaneous \([\text{Ca}^{2+}]_i\), Fluctuations in Rat Chromaffin Cells Do Not Require Inositol 1,4,5-Trisphosphate Elevations but Are Generated by a Caffeine- and Ryanodine-sensitive Intracellular \(\text{Ca}^{2+}\) Store*

(Received for publication, October 4, 1989)
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A considerable fraction (65%) of single rat chromaffin cells loaded with the fluorescent \([\text{Ca}^{2+}]_i\), indicator fura-2 exhibited spontaneous rhythmic fluctuations with an average period of \(\approx 100\) s. Parallel patch clamp experiments as well as fura-2 experiments carried out in \([\text{Ca}^{2+}]_i\)-free and other modified media in the presence of \([\text{Ca}^{2+}]_i\) and \(\text{Na}^+\) channel blockers indicated an origin from intracellular stores. Appropriate concentrations of agonists (bradykinin and histamine) for receptors \((\text{B}2\) and \(\text{H}1\)) that trigger generation of inositol 1,4,5-trisphosphate induced increased fluctuation frequency, recruitment of silent cells, and large \([\text{Ca}^{2+}]_i\) changes at high doses. These effects were blocked by cell pretreatment with neomycin, a drug that inhibits inositol 1,4,5-trisphosphate generation. In contrast, spontaneous fluctuations and the effects of another drug, caffeine, which also induced increased frequency and recruitment, were unaffected by neomycin. Ryanodine caused first a prolongation and then (\(\approx 10\) min) a block of both spontaneous fluctuations and caffeine effects, where the single transients after bradykinin and histamine were maintained. Caffeine and ryanodine are known to affect selectively the process of calcium-induced \(\text{Ca}^{2+}\) release; this is the first demonstration of \([\text{Ca}^{2+}]_i\), fluctuation activity arising from \(\text{Ca}^{2+}\)-induced \(\text{Ca}^{2+}\) release in nonmuscle cells with no strict requirement for inositol 1,4,5-trisphosphate involvement.

In a variety of nonmuscle cell types, rhythmic fluctuations of cytosolic \(\text{Ca}^{2+}\), \([\text{Ca}^{2+}]_i\), were described following moderate stimulation with agents inducing a \([\text{Ca}^{2+}]_i\), increase, usually via the generation of inositol 1,4,5-trisphosphate (Ins-P3) and CICR, \([\text{Ca}^{2+}]_i\)-induced \(\text{Ca}^{2+}\) release (CICR), initially identified in striated muscle (9) and later described in neurons (10) and smooth muscle fibers (11). \([\text{Ca}^{2+}]_i\), imaging results confirming the existence of CICR in neurons (12) and even in bovine chromaffin cells (13) have indeed been recently reported.

In the present work, the origin of \([\text{Ca}^{2+}]_i\), fluctuations has been investigated in primary cultures of rat chromaffin cells, where a large fraction (over 65%) fluctuates spontaneously (from intracellular stores) under resting conditions. Fluctuation frequency and occurrence in the cell population were increased by stimulation of either Ins-P3 generation or CICR (with receptor agonists and caffeine, respectively). However, only a CICR blocker, ryanodine (and not a blocker of Ins-P3 generation, neomycin), was able to block spontaneous fluctuations. In contrast, in the ryanodine-pretreated cells the Ins-P3-induced \([\text{Ca}^{2+}]_i\), transients were maintained.

**MATERIALS AND METHODS**

Chromaffin cells (from 175-200-g female Sprague-Dawley rats) were dissociated from dissected adrenal medullae by a combination of mechanical and enzymatic (collagenase IA and D-Nase type III, Sigma) treatments (14). Yield was 2-4 \(\times\) 10^6 cells/rat. Suspended cells were plated over thin coverslip slides coated with polyornithine \((100\) mg/ml) and cultured for 1-4 days in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum.

**[\text{Ca}^{2+}]_i** Measurement—At the beginning of the experiments the cell-bearing slides were transferred to an incubation medium (KRH) containing (mmol/liter): NaCl, 125; KCl, 5; MgSO4, 1.2; CaCl2, 2; Hepes, 4-(2-hydroxyethyl)-l-piperazineethanesulfonic acid; EGTA, ethylenediamine\(\text{N}^\text{N}^\prime\)-tetraacetic acid; PIP3, phosphatidylinositol 4,5-bisphosphate; BK, bradykinin.

**RESULTS**

Fig. 1 illustrates examples of the spontaneous fluctuations observed in 663 active cells (out of 1007) investigated in the present work. The average fluctuation period was \(\approx 100\) s, and the average increase (above the interspike \([\text{Ca}^{2+}]_i\), = 58 \(\pm\) 38 nm) was 469 \(\pm\) 334 nm. \(\text{Ca}^{2+}\) influx across the plasma mem-
brane was investigated first as a process possibly responsible for the fluctuations. Either excess of the Ca\(^{2+}\) chelator, EGTA, or various types of Ca\(^{2+}\) channel blockers were added to the medium bathing spontaneously fluctuating cells. As can be seen in Fig. 1, D and E, excess EGTA did not immediately suppress the activity. In many cases (Fig. 1E) a progressive decrease of the fluctuation size was observed, with increased frequency and disappearance of the activity within 1–3 min after addition of excess EGTA (E,panels D and E). In EGTA-pretreated cells that had stopped fluctuating BK (5 nM) was still able to trigger an individual peak (panel E). Panel F illustrates slow voltage fluctuations recorded in a chromaffin cell by the patch clamping technique (whole cell configuration). The line below the trace indicates that only a single pulse (marked to the right) was delivered, which evoked an action potential (notice the difference in the rising phases of fluctuations and the action potential).

The spontaneous electrical activity of resting chromaffin cells was investigated by patch clamp (whole cell configuration). In the experimental conditions employed (current clamp, estimated pipette [Ca\(^{2+}\)] \(\approx 1\) \(\mu\)M, dissolved in dimethyl sulfoxide, panel D) and continued for 1–3 min after addition of excess EGTA (E, panels D and E). In EGTA-pretreated cells that had stopped fluctuating BK (5 nM) was still able to trigger an individual peak (panel E). Panel F illustrates slow voltage fluctuations recorded in a chromaffin cell by the patch clamping technique (whole cell configuration). The line below the trace indicates that only a single pulse (marked to the right) was delivered, which evoked an action potential (notice the difference in the rising phases of fluctuations and the action potential).

In contrast to extracellular recordings (14, 18), spontaneous action potentials were not observed except for occasional spikes during electrode apposition and membrane perforation. The nature of the channels responsible for the observed slow fluctuations has not been investigated yet.

The results reported above exclude the direct involvement of Ca\(^{2+}\) action potentials in the generation of the [Ca\(^{2+}\)]i fluctuations and point to intracellular rapidly exchanging Ca\(^{2+}\) store(s) as the source of these events. The possible role of Ins-P3 was therefore investigated by using agonists addressed to receptors coupled to phosphatidylinositol 4,5-bisphosphate (PIP\(_2\)) hydrolysis. As can be seen in Fig. 2A, addition of low concentrations (0.05–5 nM) of bradykinin (BK, working through the B\(_1\) receptor, Ref. 19) induced the frequency of the [Ca\(^{2+}\)]i fluctuations to increase. These BK concentrations, when applied to silent chromaffin cells, caused fluctuations to appear (Fig. 2B) after a lag period. In contrast, high concentrations (\(\approx 100\) nM) applied to fluctuating chromaffin cells induced [Ca\(^{2+}\)]i, to increase promptly to very high levels (in the micromolar range) and then decline to plateaus which were maintained for several minutes without distinct fluctuations (Fig. 2C). [Ca\(^{2+}\)]i transient peaks were induced by BK even in cells which had stopped fluctuating after EGTA chelation of extracellular Ca\(^{2+}\) (Fig. 1E).

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Results similar to those with BK were obtained following activation of the H1 receptor (by histamine, 0.1–10 μM, not shown).

BK and histamine were also administered to chromaffin cells pretreated with neomycin. When this antibiotic penetrates within the cells it is known to inhibit Ins-P3 generation (20, 21) and to block the intracellular Ins-P3-induced Ca2+ release (22). Neomycin-treated cells (n = 25) were found to fluctuate similar to controls. Shortly after exposure to antibiotic they also responded normally to both BK and histamine. Within a few minutes, however, they became entirely unresponsive (Fig. 2D). Spontaneous fluctuations (but no BK-induced transients) were observed also in cells in which neomycin had been loaded intracellularly (together with fura-2 free acid) by the ATP permeabilization procedure (23) (not shown). Similar to controls, the spontaneous [Ca2+]i fluctuations of neomycin-treated cells slowly declined after excess EGTA addition and reappeared after a lag when Ca2+ was reintroduced into the medium (not shown).

CICR was next investigated. In striated muscle fibers and other types of cells caffeine is known to facilitate the release evoked by Ca2+ (12, 24, 25), possibly by lowering the activation threshold of the intracellular channel involved. If fluctuations were indeed due to CICR, then the application of caffeine was expected (with other factors unchanged) to increase frequency. This was indeed observed. In particular, in most fluctuating cells treated with 1–5 mM caffeine (n = 42) the period was found to decrease markedly (down to 10 s or less, Fig. 3A), while in many (8/10) of the cells initially silent the drug (5 mM) caused fluctuations to appear (Fig. 3B). When higher concentrations (10 mM) were used, two types of responses were recorded: increased fluctuation frequency (not shown) or single large peaks followed by persistent plateaus (Fig. 3C). If excess EGTA was added to the medium after caffeine, [Ca2+]i fluctuations declined and then stopped, as in control cells (Fig. 3A). When caffeine was applied to cells that had stopped fluctuating after excess EGTA (such as those of Fig. 1, D and E), it induced either a very small [Ca2+]i increase or (most often) no change whatsoever (not shown). The effect of caffeine was investigated also in cells pretreated with neomycin where it was still able to markedly increase the fluctuation frequency (Fig. 3D). This might differ from skeletal muscle fibers; in fact neomycin has been reported to block 45Ca release induced by caffeine from preparations of sarcoplasmic reticulum vesicles (26). All the effects of caffeine were rapidly reversed by washing.

A final series of experiments was carried out using ryanodine, a drug known to block the muscle sarcoplasmic reticulum Ca2+ channel in the open state (11, 27, 28). Results obtained in 16 out of 20 cells are illustrated in Fig. 3, E and F. In the first few minutes the [Ca2+]i, fluctuations appeared unchanged (Fig. 3F). Soon thereafter, however, fluctuations became longer, apparently composed by multiple [Ca2+]i microspikes. Moreover, the interfluctuation [Ca2+]i increased slightly. Finally, a state was reached in which fluctuations ceased and [Ca2+]i was much higher than the initial interfluctuation value (Fig. 3, E and F). As with caffeine, such ryanodine-induced [Ca2+]i plateaus decreased (although slowly) after EGTA addition (not shown). Ryanodine-blocked cells were completely insensitive to caffeine (2–10 mM), applied in either Ca2+-containing (Fig. 3F) or Ca2+-free medium. In contrast, they were still able to respond to high concentrations of BK (Fig. 3E) or histamine (not shown), which induced single [Ca2+]i transients, smaller however than those described in control cells (see Fig. 2). Different from caffeine, blockade by ryanodine was not reversed by washing.

**DISCUSSION**

Spontaneous [Ca2+]i oscillations are not a unique property of rat chromaffin cells. In most (but not all, see Ref. 4) other systems, however, this activity is limited to a small fraction of the cells and becomes widespread only after moderate

Fig. 3. [Ca2+]i fluctuations in rat chromaffin cells; effects of caffeine and ryanodine. Low concentrations (2 mM) of caffeine (C) cause a rapid increase of frequency of [Ca2+]i, fluctuations in spontaneously fluctuating cells (panel A) or their appearance in silent cells (panel B). The application of excess EGTA (E, 4 mM) causes fluctuations to cease only after a delay (over 2 min in panel A), like in control cells (Fig. 1, D and E). Higher concentrations of caffeine (10 mM, panel C) can induce a rapid [Ca2+]i transient followed by a plateau. Panel D shows the increased fluctuation frequency triggered by caffeine (2 mM) in a cell pretreated with neomycin (1 mM, 10-min pretreatment). Panels E–F illustrate the effects of ryanodine (R, 10 μM) progressive increase of the fluctuation duration followed by a high noisy plateau. In the cell of panel E, 20 min of the record was cut out (W). Notice that application of BK (1 mM) to ryanodine-pretreated cells triggers a distinct [Ca2+]i transient (panel E), whereas caffeine (C, 10 mM) is ineffective (panel F). Experimental conditions as in Fig. 1, A–E.
stimulation, e.g. by low concentrations of agonists addressed to PIP2; hydrolysis coupled receptors or by Ins-P3 microinjection (1–3, 5–7). In contrast, in rat chromaffin cells over 65% of the cells were found to fluctuate spontaneously.

Spontaneous action potentials were reported to occur frequently in cultured rat chromaffin cells investigated by extracellular recording (14, 18). The possibility of a causal relationship between these events and the [Ca2+]i fluctuations had therefore to be considered. Whole cell patch clamping of our cells, however, failed to reveal spontaneous action potentials and revealed in contrast the occurrence of slow depolarizations in a fraction of cells (10/15) similar to the fraction showing [Ca2+]i fluctuations. The observed depolarizations appear too slow to be dependent on voltage-gated ion channels. They might be due to activation of membrane channels (Cl− or unspecific cationic channels) sensitive to [Ca2+]i, i.e. not the cause but a consequence of the spontaneous [Ca2+]i fluctuations. The different periodicity of the slow depolarizations versus [Ca2+]i fluctuations could be due to the relatively high pipette [Ca2+]i (around 1 μM). These observations, together with the results with tetrodotoxin, Na+−free medium, EGTA, and Ca2+ channel blockers, tend to exclude the direct involvement of action potentials and extracellular Ca2+ in the generation of the fluctuations.

Our data seem also to exclude the possibility that each [Ca2+]i fluctuation is induced by an Ins-P3 fluctuation. In fact, in neomycin-pretreated cells, we observed dissociation between spontaneous [Ca2+]i fluctuations that were apparently unchanged and receptor-triggered [Ca2+]i events, that were blocked, most probably as a consequence of the inhibition of Ins-P3 generation and/or function (20–22). The requirement for Ins-P3 fluctuations that was hypothesized (1–3) and extensively discussed (5–7) appears incompatible also with the recent demonstration that [Ca2+]i fluctuations are induced by the microinjection of the nonhydrolyzable trisphosphatidylcholine analog of Ins-P3 (29). The opposite dissociations, i.e. alteration and then blockade of [Ca2+]i fluctuations, with the persistence of receptor-triggered [Ca2+]i transients, was observed after treatment with ryanodine, a drug addressed to the intracellular Ca2+ channel responsible for the CICR process in the muscle (11, 27, 28). The stimulatory effect of caffeine also appears mediated by CICR since it was blocked, most probably as a consequence of the inhibition of the intracellular Ca2+ channel responsible for the CICR process in the muscle (11, 27, 28). The stimulatory effect of caffeine also appears mediated by CICR since it was blocked, most probably as a consequence of the inhibition of the intracellular Ca2+ channel responsible for the CICR process in the muscle (11, 27, 28).

Whether the Ca2+ and Ins-P3-sensitive stores reside in two separate organelles is still unclear. Results in neurons (12, 30) and bovine chromaffin cells (13), apparently consistent with this possibility, have been recently reported. On the other hand, the [Ca2+]i transients elicited by BK and histamine in rat chromaffin cells pretreated with ryanodine were always smaller than in controls. A possible explanation for this result is that part of the Ca2+ storage organelles coexpresses the Ins-P3- and Ca2+-sensitive release systems, as suggested in smooth muscle fibers (11). However, a more likely explanation is that in control cells the receptor-triggered responses are sustained not only by the Ins-P3-sensitive store, stimulated directly, but also by CICR, activated indirectly via the Ins-P3-inhube [Ca2+]i increase. Whatever the situation, it is clear that the functioning of the two Ca2+ stores must necessarily be strictly interconnected. CICR as a source of Ca2+ fluctuations could also explain the property of Ins-P3s, either generated at the receptor level by microinjection, to initiate fluctuations or to modify their frequency, as observed in practically all cell systems in which [Ca2+]i fluctuations have been reported.

Acknowledgments—We thank E. W. Westhead (University of Massachusetts) for participating in the initial part of this work, A. Peres and T. Pozzan (Universities of Milan and Ferrara, Italy) for helpful suggestions and support, and L. Di Giorgio and S. Monti for secretarial assistance.

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Spontaneous [Ca2+]i fluctuations in rat chromaffin cells do not require inositol 1,4,5-trisphosphate elevations but are generated by a caffeine- and ryanodine-sensitive intracellular Ca2+ store.

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