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$[Ca^{2+}]_i$ oscillations from internal stores sustain exocytic secretion from the chromaffin cells of the rat

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A large (65%) fraction of in vitro cultured rat chromaffin cells exhibit spontaneous $[Ca^{2+}]_i$ oscillations, and the rest can be recruited to oscillate by appropriate stimulations. Based on fura-2 single cell $[Ca^{2+}]_i$ measurements, evidence is provided that these oscillations originate, via the activation of Ca^{2+}-induced Ca^{2+}-release, from intracellular Ca^{2+} stores in rapid equilibrium with extracellular Ca^{2+}. By combining $[Ca^{2+}]_i$ measurements with a specific plaque secretion assay we demonstrate that oscillating cells exhibit a spontaneous exocytic secretory activity whereas the cells with stable $[Ca^{2+}]_i$ do not. $[Ca^{2+}]_i$ oscillations appear therefore to account for the high unstimulated catecholamine release, an activity typical of the chromaffin cells of the rat.

Ca^{2+}; Chromaffin cell; Exocytosis; Ca^{2+}-induced Ca^{2+}-release

1. INTRODUCTION

Oscillations of the cytosolic free Ca^{2+} concentration, $[Ca^{2+}]_i$, have been observed (by single cell measurement, using fura-2 or other $[Ca^{2+}]_i$ dyes) in a variety of cell types, but their mechanisms and physiological importance are still debated (reviews [1,2]). In most studies, oscillations have been reported to be rare or absent at rest, and to become common after moderate stimulation of receptors coupled to the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2). Our previous results [3] demonstrated however that, in a high proportion (~65%) of primarily cultured rat chromaffin cells, $[Ca^{2+}]_i$ oscillates spontaneously at rest (frequency ~ 1/min) and that appropriate receptor stimulation causes an increase of frequency and the recruitment of initially silent cells to oscillate. Rat chromaffin cells appear therefore favourable for the study of oscillation mechanisms. Based on physiological and pharmacological experiments, an intracellular Ca^{2+} store sensitive to caffeine and ryanodine has been proposed to directly sustain the oscillations [3]. A store of this type, analogous to the sarcoplasmic reticulum of muscle fibers, is now believed to exist also in non-muscle cells, both excitable [4–6] and non-excitable [7], and to be activated by adequate changes of $[Ca^{2+}]_i$ (Ca^{2+}-induced Ca^{2+}-release, CICR [8]). If indeed oscillations were sustained by CICR, they would be expected to be stimulated not only by the activation of PIP_2 hydrolysis-coupled receptors, but by any treatments that induce, and blunted by any treatments that buffer adequate $[Ca^{2+}]_i$ increases. Here we demonstrate that this is indeed the case in rat chromaffin cells and, in addition, we report evidence indicating that $[Ca^{2+}]_i$ oscillations sustain spontaneous exocytic secretion, an activity typical of this cell type [9].

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Abbreviations: BK, bradykinin; CICR, Ca^{2+}-induced- Ca^{2+}-release; PIP_2, phosphatidylinositol 4,5-bisphosphate

2. MATERIALS AND METHODS

Single cell $[Ca^{2+}]_i$ measurements were carried out in a large group (over 100) of cells prepared as described in [3], loaded with fura-2 and investigated at 32°C by single cell microfluorimetry [10]. The incubation medium employed contained (in mmol/l): NaCl 125, KCl 5, $MgSO_4$ 1.2, $CaCl_2$ 2, glucose 6, and HEPES-NaOH buffer 25, pH 7.4. Fluorescence ratios (340/380 nm excitation wavelengths) calculated in most experiments at the rate of 1/s, were converted into $[Ca^{2+}]_i$ values as recommended by Grynkiewicz et al. [11]. Reverse hemolytic plaque assays [12] were employed to investigate secretion from single cells while analyzed for $[Ca^{2+}]_i$. By this technique we revealed the appearance around individual cells of chromogranin A, a secretory protein contained within secretory granules together with catecholamines. To this end, sparse rat chromaffin cells, loaded with fura-2 while in suspension, were mixed with a 20% preparation of protein A-coated red blood cells and infused into a Cunningham chamber [12] pretreated with poly-L-lysine. After 30 min (the time needed for the cells to settle) the asay was started by adding a rabbit immune serum against chromogranin A (gift of Dr P. Rosa of our Department [13], used at the final dilution of 1 : 25). The cells were then incubated at 32°C on the fluorescence microscope stage for 4 h during which $[Ca^{2+}]_i$ was assayed in individual cells for 1.5–5 min periods separated by 40 ± 5 min dark intervals. At the end of the incubations, guinea pig complement was added as described in [12]. Fura-2/AM and BAPTA/AM were purchased from Calbiochem (La Jolla, CA, USA). The other materials (of the highest purity) were from commercial sources (see [3]).

3. RESULTS AND DISCUSSION

Fig. 1A shows an example of the caffeine- and ryanodine-sensitive $[Ca^{2+}]_i$ oscillations of cultured rat chromaffin cells. As shown in [3] the frequency of these oscillations increases after application to the cells of low concentrations of agents (bradykinin, BK; histamine) addressed to receptors coupled to PIP_2 hydrolysis. Fig. 1B demonstrates that this same result can be achieved by a completely different type of treatment, i.e. with 5 mM KCl, applied on top of the 5 mM K^+ contained in the standard incubation medium. This effect was prevented by pretreatment of the cells with the Ca^{2+} channel blocker, nitrendipine (1 μM) indicating that the increase of $[K^+]_o$ works by increasing the activation probability of voltage-gated Ca^{2+} channels. Notice in this panel the small, but appreciable and consistent, $[Ca^{2+}]_i$ step that follows immediately KCl addition, and which might correspond to the CICR trigger event at strategic site(s) of the cytoplasm. Higher $[K^+]_o$ additions (>10 mM) were also investigated. In these cases, however, only biphasic responses consisting of a large initial peak followed by a persistent plateau, with suppression of the oscillatory behaviour, were observed (not shown). Interestingly, these responses resembled those obtained when large concentrations of agonists addressed to PIP_2-hydrolysis coupled receptors were administered [3]. Fig. 1C illustrates the effects on $[Ca^{2+}]_i$ oscillations of a high, yet non-toxic concentration of a completely different agent, BAPTA/AM. The latter is a hydrophobic acetoxymethyl derivative rapidly translocated across the plasma membrane to the cytosol, where unspecific esterases convert it into the high affinity Ca^{2+} chelator, BAPTA. Accumulation of the latter substance increases the cytosolic Ca^{2+} buffering capacity. After a few minutes delay (presumably the time needed for the ester translocation and hydrolysis) the frequency of the oscillations declined and their size decreased progressively, with complete arrest within 3-4 min. These results appear consistent with the idea that the frequency of the oscillations is controlled by cytosolic $[Ca^{2+}]_i$, as expected for a CICR-based process. We conclude that any moderate increase, no matter what its origin (intracellular stores sensitive to inositol 1,4,5-trisphosphate or the extracellular medium), shortens the time between successive oscillations

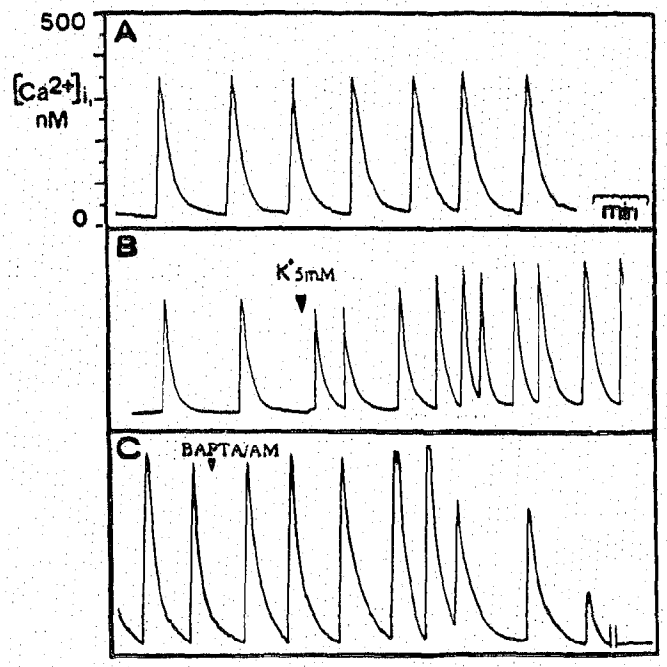

Fig. 1. $[Ca^{2+}]_i$ oscillations in rat chromaffin cells: effects of $[K^+]_o$ doubling and of increased cytosolic Ca^{2+} buffering by cell loading with BAPTA. (A) Illustrates spontaneous $[Ca^{2+}]_i$ oscillations in a resting control cell, the bar to the right marks 1 min. (B) Shows the increased frequency (2.5-fold) following addition to the medium of 5 mM KCl. Note in addition the small $[Ca^{2+}]_i$ step and the slowly developing increase (+35%) in the amplitude of the oscillations. (C) Illustrates the delayed decrease in oscillation size and frequency followed by arrest in a cell incubated with 50 μM BAPTA/AM. The break in the trace to the right side represents a 10 min interruption in the recording. Data in (B) and (C) are representative of 9 and 6 cells, respectively.

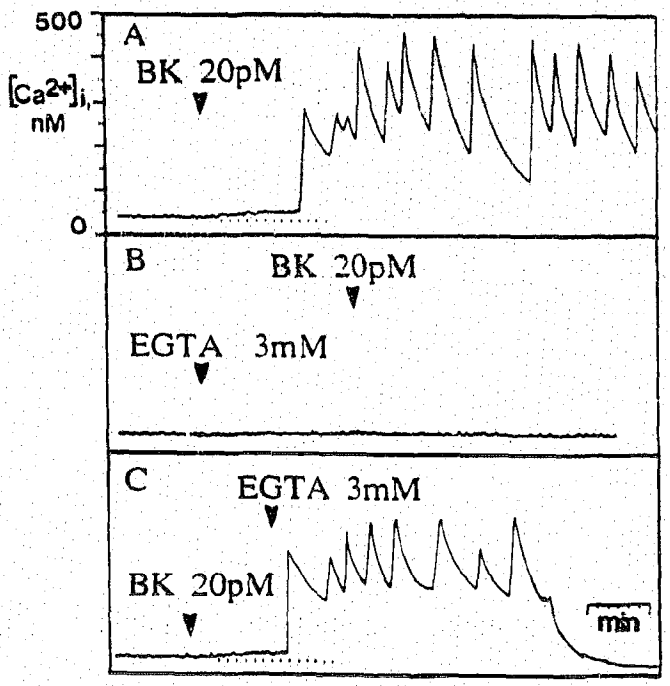

Fig. 2. Induction of $[Ca^{2+}]_i$ oscillations in silent cells: dependence on $[Ca^{2+}]_o$. (A) Shows the induction of an oscillatory behaviour by the addition of a very low concentration of bradykinin. Note the small ramp increase of $[Ca^{2+}]_i$ during the lag phase preceding the first oscillation. (B) Shows the lack of effect of bradykinin when administered in a medium containing excess EGTA (calculated $[Ca^{2+}]_o = 10^{-8}$ M). In contrast, when excess EGTA was applied after BK, i.e. during the lag phase, a burst of $[Ca^{2+}]_i$ oscillations was triggered, followed by arrest only after several min (C). Data in (A) and (B) representative of more than 20 cells, in (C) of 7 cells.

presumably by inducing faster filling of the relevant store and by stimulating its discharge [1,3,14]. Conversely, the accumulation of BAPTA increases the cytosolic Ca^{2+} buffering capacity, dumps $[Ca^{2+}]_i$ changes and thus the probability of the CICR process to be activated.

A subsequent group of experiments was carried out to characterize the $[Ca^{2+}]_i$ oscillation recruitment in the fraction of initially silent chromaffin cells. Fig. 2 shows results with a very small concentration (20 pM) of BK, but similar data were obtained with 5 mM KCl. Consistent with results in [3], oscillations were induced by the peptide; however, after delays of 1–4 min, small $[Ca^{2+}]_i$ increases (usually ramps, panels A and C) were observed. When, however, BK was applied 0.5–5 min after excess EGTA (to chelate $[Ca^{2+}]_o$), no appreciable $[Ca^{2+}]_i$ changes and no oscillations were observed (panel B). This result does not indicate direct dependence of oscillations on $[Ca^{2+}]_o$ because, when the order of additions was changed (i.e. when excess EGTA was applied after BK, during the delay phase preceding the start of the activity) oscillations were induced and lasted several minutes thereafter (panel C). We conclude therefore that involvement of $[Ca^{2+}]_o$ is indirect and probably consists of the supply of Ca^{2+} for complete filling of intracellular rapidly exchanging stores, which appears to be already stimulated at BK concentrations too low to induce Ca^{2+} release from intracellular stores.

Our final problem concerned secretion. In various cell systems [15–18] doubts have been raised about the possibility that $[Ca^{2+}]_i$ oscillations and receptor-triggered Ca^{2+}-release, both originating from intracellular stores, could sustain that activity. Up to now, however, no direct experiments were reported in which $[Ca^{2+}]_i$ and secretion were measured simultaneously in individual cells for extended periods of time. Fig. 3 illustrates our plaque assay and $[Ca^{2+}]_i$ results in rat chromaffin cells, investigated without any stimulation. All the 10 oscillating cells investigated, in-

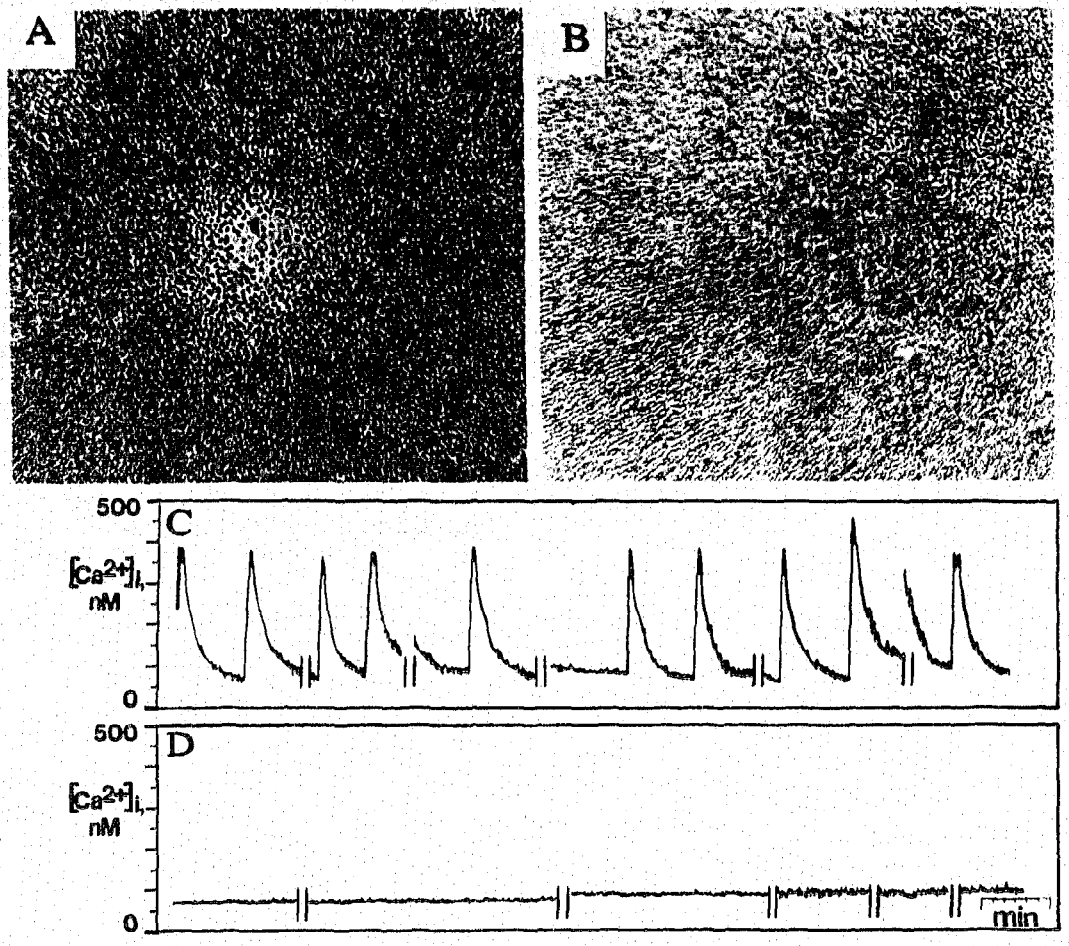

Fig. 3. Concomitant measurement of exocytic secretion and $[Ca^{2+}]_i$ in two individual rat chromaffin cells. (A) Shows the positive reverse hemolytic plaque assay for the secretory protein, chromogranin A, in a cell exhibiting the spontaneous $[Ca^{2+}]_i$ oscillations illustrated in (C). The negative plaque assay of panel B refers to the non-$[Ca^{2+}]_i$ oscillating cell of (D). The position of the analyzed cells is marked by arrowheads in (A) and (B). The breaks of the traces in (C) and (D) indicate interruptions in $[Ca^{2+}]_i$ measurements lasting 40 ± 5 min. Results of (A) and (C) are representative of 10 cells, those in B and D of 6 cells.

cluding that shown in panels A and C, induced hemolysis of the surrounding erythrocytes sensitized for the secretory protein, chromogranin A, whereas the six non-oscillating cells did not (panels B and D). Protein secretion from chromaffin cells is known to occur by exocytosis of granules containing also high amounts of catecholamines [19]. Our present results demonstrate therefore that spontaneous $[Ca^{2+}]_i$ oscillations from internal stores [3] can sustain the unstimulated catecholamine release typical of chromaffin cells of the rat [9]. Interestingly, in the cells of other species (e.g. bovine) where unstimulated catecholamine release is low, $[Ca^{2+}]_i$ oscillations are rare or absent unless appropriate stimuli are applied [3,6]. A role of $[Ca^{2+}]_i$ oscillations in secretion could thus be not a peculiarity of rat chromaffin cells but a more general property, that deserves to be systematically investigated in the various types of secretory cells.

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