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Research Article

A novel pattern of fast calcium oscillations points to calcium and electrical activity cross-talk in rat chromaffin cells

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Abstract. Slow oscillations of cytosolic calcium ion concentration – $[Ca^{2+}]_c$ – typically originate from release by intracellular stores, but in some cell types can be triggered and sustained by Ca^{2+} influx as well. In this study we simultaneously monitored changes in $[Ca^{2+}]_c$ and in the electrical activity of the cell membrane by combining indo-1 and patch-clamp measurements in single rat chromaffin cells. By this approach we observed a novel type of spontaneous $[Ca^{2+}]_c$ oscillations, much faster than those previously described in these cells. These oscillations are triggered and sustained by complex electrical activity (slow action potentials and spike bursts), require Ca^{2+} influx and do not involve release from intracellular stores. The possible physiological implications of this new pathway of intracellular signalling are discussed.

Key words. Calcium oscillations; ionic channels; action-potential clamp; indo-1 microspectrofluorometry; electrophysiology.

Introduction

Cells respond to a variety of hormones and neurotransmitters by generating appropriate physiological responses. Data from a wide variety of cell types has indicated that the cytosolic concentration of free calcium, $[Ca^{2+}]_c$, plays a key role in this transduction process [1]. An increase in $[Ca^{2+}]_c$ frequently occurs when transmitters bind to cellular receptors, either by means of inflow through the plasmalemma or by release from internally stored pools [2]; a rise in $[Ca^{2+}]_c$ from its normal low level causes activation of numerous enzymes and proteins. However, Ca^{2+} signalling is not an 'all or none' process; rather, the response of a cell to an agonist is determined by the duration, pattern and localisation of the $[Ca^{2+}]_c$ rise. Ca^{2+} signalling is also intimately entangled with the electrical activity of the plasma membrane, thereby performing complex electrochemical information processing. In fact, variations of $[Ca^{2+}]_c$ can modulate ion channel activity, while the electrical activity of the plasma membrane in turn regulates Ca^{2+} channels and may trigger significant $[Ca^{2+}]_c$ changes.

Rat chromaffin cells are a well-known and valuable experimental model, as they share many features of mature sympathetic neurons, including voltage-dependent Na^+ and Ca^{2+} channels [3–8], a variety of 'neuron-specific' proteins and the ability to secrete catecholamines in response to elevation of the cytosolic calcium concentration [9]. Receptor activation in chromaffin cells may be translated into a single $[Ca^{2+}]_c$ transient that diffuses all over the cell or into a train of $[Ca^{2+}]_c$ spikes. Interestingly, the range of agonist doses that induce or modulate these slow oscillations is distinctly lower than the concentration needed to elicit a single maximal response. Therefore, os-

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cillatory activity may represent an extremely sensitive 'digital' frequency-encoded signal. In our previous work, we proposed that these $[Ca^{2+}]_c$ oscillations originate from an intracellular store that can be identified as inositol 1,4,5-triphosphate (IP_3) sensitive [10], but we also reported Ca^{2+} entry from the extracellular space, largely occurring through di-hydropyridine (DHP)-sensitive voltage-sensitive calcium channels (VOCCs) [11, 12]. These findings were further supported by the evidence that bradykinin-induced, and seldom spontaneous $[Ca^{2+}]_c$ changes, can be accompanied by electrical activity of the plasma membrane, which, however appeared not to be related to action potentials [13]. In contrast, in some neurosecretory cells, such as pituitary cells and amphibian spinal neurons, Ca^{2+} influx is an established pathway for controlling $[Ca^{2+}]_c$ changes in space and in time [14, 15]. In this study we specifically addressed the issue whether opening and closing of ion channels participate in the generation of $[Ca^{2+}]_c$ oscillatory activity. Changes in $[Ca^{2+}]_c$ and in the electrical activity of the cell membrane were simultaneously investigated in single rat chromaffin cells by combining indo-1 and perforated-patch measurements. By this approach we describe a novel type of spontaneous $[Ca^{2+}]_c$ oscillations, much faster than those previously described in rat chromaffin cells and independent of Ca^{2+} release from intracellular stores. The possible physiological implications of this new pathway of intracellular signalling are discussed.

Material and methods

Cell cultures

Adrenal medulla from rat adrenal glands was dissected free of the cortex, rinsed several times in Krebs-Ringer solution buffered with Hepes (KRH; composition in mmol/l: 125 NaCl, 5 KCl, 1.2 KH_2PO_4, 1.2 $MgSO_4$, 2 $CaCl_2$, 6 glucose and 25 Hepes-NaOH, pH 7.4) and finally cut in small pieces with a pair of fine needles. To dissociate the cells, the adrenal tissue was treated with collagenase type A and DNAase I at 37 °C for 90–120 min, whilst tissue fragments were gently drawn in and out of a Pasteur pipette. After centrifugation, chromaffin cells were finally suspended in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% foetal calf serum and 5% horse serum, plated over thin glass coverslips coated with poly-ornithine and cultured for 1–4 days under a humidified atmosphere containing 5% CO_2.

Indo-1 microspectrofluorometry

The microspectrofluorometric set up was built around a Zeiss inverted Axiovert 135 TV light microscope (Carl Zeiss, Oberkochen, Germany) positioned within a Faraday cage. The monochromatic excitation light (338 nm) was provided by a 150 W Xe arc lamp coupled with a monochromator and conveyed via a liquid light guide to the epifluorescence port of the microscope (optical components from Oriel Instruments, Stratford, CT, USA). The microscope was modified in two ways: (i) a 12-V motorised iris was inserted at a conjugate optical plane of the microscope light path, to define the field where fluorescence intensity is to be measured; (ii) a home-made splitting cube was positioned at the photographic port of the eyepieces; it is equipped with a dichroic mirror and two band-pass emission filters to separate 405- and 495-nm components of the emission light and to convey them to two photomultiplier tubes (PMTs; Thorn EMI, Middlesex. UK). Currents from the PMTs were converted into voltage, filtered at 100 Hz, amplified 10 times and then fed to a computer. A new software (SIGNALS, see below) was specifically designed to record, in parallel, both fluorescence and patch-clamp measurements. Ratioing (data reported as arbitrary units, a.u.) and/or $[Ca^{2+}]$ calculations were carried out on pairs of corresponding 405 and 495 values according to [16]. At the beginning of each experiment, cell-bearing coverslips were rinsed with KRH and loaded by incubation at 37 °C for 45 min with indo-1 pentaacetoxymethylester dissolved in KRH, supplemented with 1% bovine serum albumin. The concentration of indo-1 for incubation was adjusted for each batch of the dye (typically from 2 to 5 µM) to the minimum value that gave reliable signals from both photomultipliers in our setup (405- and 495-nm wavelengths). After this time cells were rinsed with KRH and transferred to the stage of the microscope where they were continuously perfused (at 37 °C) with standard or modified KRH solutions dispensed by a multibarrel system controlled by solenoid operated valves.

Patch-clamp recordings

Recording pipettes were pulled from borosilicate capillaries on a vertical puller, heat polished and filled with 4 µl of a solution containing (in mmol/l): 20 KCl, 120 K aspartate, 2 $MgCl_2$, 0.4 $CaCl_2$, 1 EGTA-KOH and 10 Hepes-KOH. Immediately before connecting the pipette to the end stage of the amplifier, 1 µl of the same solution containing 0.25 M nystatin was added. Electrophysiological measurements were performed in perforated patch, whole-cell recording configuration using a two-phase lock-in amplifier (Henigman SWAM IIC; Celica, Ljubljana, Slovenia). Current/potential signals were filtered at 1 KHz and recorded at 2-KHz sampling rate. In current clamp experiments, signals were digitised (Lab-PC+ board, National Instruments, Austin, TX, USA) and recorded by SIGNALS, a software specifically designed for this application. By this programme it was possible to simultaneously record multiple channels (typically, current and voltage from the patch-clamp amplifier and the voltages from the two PMTs), to perform on-line mathematical processing of signals (typically ratioing 405/495-nm PMT signals to estimate $[Ca^{2+}]$), and

to generate current signals that can be employed to reproduce the electrical conditions previously recorded in the same or another cell. In voltage-clamp experiments, signals were either recorded as described above, or digitised (TL-1 interface and Lab Master DMA; Axon Instruments Foster City, CA, USA) and recorded by the sw Clampex 5.5.1 (Axon Instruments). All data were analysed off-line by the following software: Clampan 5.5.1, Clampfit 6.0.2, Axoscope 1.1 (Axon Instruments); Origin 3.5 (Microcal Software, Northampton, MA, USA).

Materials

DMEM, foetal calf serum, horse serum, antibiotics and glutamine were from Bio-Whittaker (Walkersville, MD, USA), Indo-1AM and tetrodotoxin from Calbiochem (La Jolla, CA, USA), all other chemicals from Sigma (St Louis, MO, USA).

Results

Spontaneous $[Ca^{2+}]_c$ oscillations were observed in the majority of the rat chromaffin cells (87%, n = 75) we studied by indo-1 microspectrofluorometry. Unexpectedly, only half of them (55%) conformed to the previously described typology [10, 17], i.e. displaying $[Ca^{2+}]_c$ oscillations with a frequency of ~0.015 Hz, an amplitude of ~300 a.u. (corresponding to ~700 nM) and a duration of the single transients ranging from 30 to 90 s. In the remaining 45% of the cells showing spontaneous $[Ca^{2+}]_c$ activity, oscillations were far more frequent (~0.3 Hz), rapid (2–5 s) and reduced in amplitude (<100 a.u., ~200 nM). The differences between slow and fast oscillations are illustrated in figure 1. Notice that the time scale of fast oscillations (1B) is more expanded (10 times with respect to (1A) to better describe the $[Ca^{2+}]_c$ transients. In a few cells, the two types of oscillations appeared to be superimposed. Since we did not find any trace in the literature of fast $[Ca^{2+}]_c$ oscillations in chromaffin cells, a series of experiments were performed to investigate their nature. Intracellular Ca^{2+} redistribution generally sustains slow $[Ca^{2+}]_c$ oscillations. In order to test whether membrane currents contributed to generating the fast rhythmic activity observed here, we decided to simultaneously monitor changes in $[Ca^{2+}]_c$ and membrane electrical properties by combining indo-1 and patch-clamp measurements. Because of the extreme sensitivity of $[Ca^{2+}]_c$ oscillations to the smallest perturbations, electrophysiological recordings were performed under whole-cell, nystatin perforated-patch conditions, to minimize interference with intracellular regulation of $[Ca^{2+}]_c$.

In ~25% of the cells studied by current clamp, we observed spontaneous firing of action potentials (fig. 2, middle panel) with kinetics and duration similar to those

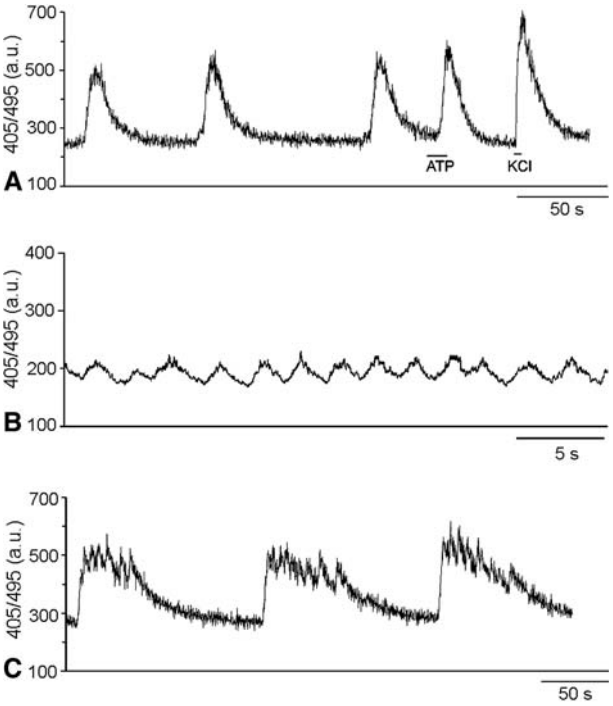

Figure 1. Two types of $[Ca^{2+}]_c$ oscillations in chromaffin cells. Ordinates report the ratio of indo-1 fluorescence emission at two wavelengths (405 and 495 nm); absolute $[Ca^{2+}]_c$ values can be roughly estimated by considering that 100 a.u. corresponds to about 200–250 nM Ca^{2+}. (A) Slow $[Ca^{2+}]_c$ transients occurred spontaneously (about 1/min) in a chromaffin cell and could be triggered by stimulation of receptors coupled to IP_3-induced $[Ca^{2+}]$ release from the endoplasmic reticulum (ER) (ATP 300 µM) or by depolarization (30 mM K^+). (B) Fast $[Ca^{2+}]_c$ oscillations in a different cell. Notice the expanded time scale. The oscillations displayed a regular pattern, a period of about 3 s and a reduced amplitude. (C) Slow and fast $[Ca^{2+}]_c$ oscillations coexisted in a different chromaffin cell. Small-sized and fast oscillations appear to superimpose onto large and slow $[Ca^{2+}]_c$ transients that eventually subsided with their typical time course.

previously described in the literature [4, 18, 19]. Concomitant measurement of $[Ca^{2+}]_c$ in these cells did not reveal variations of the basal levels (fig. 2, upper panel). Thus, Ca^{2+} influxes promoted by individual action potentials were not able to significantly affect the cytosolic concentration of the cation in chromaffin cells.

A different type of electrical activity was observed in all cells exhibiting fast $[Ca^{2+}]_c$ oscillations (40%): prolonged depolarisations with superimposed bursts of fast action potentials. This activity was in phase with $[Ca^{2+}]_c$ changes, and showed a well-defined and reproducible pattern. Figure 3 illustrates the common features of this bursting activity. An initial slow depolarising phase (foot; dV/dT = 32 ± 11.3 mV/s, n = 100, 10 cells) brought the potential to a threshold value (~50 mV) that triggered a rapid spike (upstroke maximum dV/dT = 1.5 ± 0.06 V/s, peak ≈ + 5 ± 6.2 mV, n = 100, 10 cells). The repolarising phase of the spike, however, did not bring the potential

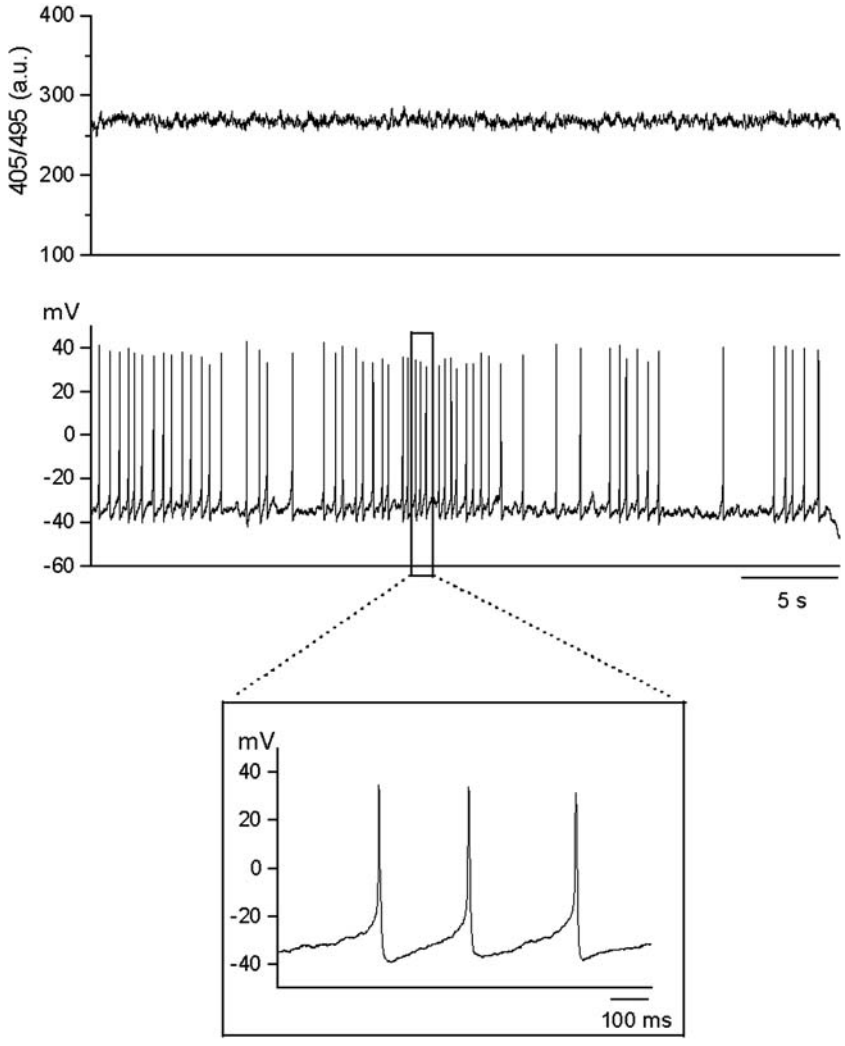

Figure 2. Simultaneous $[Ca^{2+}]_c$ measurement (indo-1 fluorescence emission ratio at 405/495 nm; top trace) and electrophysiological recording (current clamp; middle trace) in a chromaffin cell displaying no $[Ca^{2+}]_c$ activity. Numerous action potentials were recorded, but no $[Ca^{2+}]_c$ changes appeared to be evoked by such activity. A portion of the electrophysiological recording is expanded in the bottom panel to display the precise spike time course.

back to resting values, and a burst of spikes was triggered at progressively more depolarised potentials (from ~–20 to 0 mV), giving rise to a kind of unstable plateau before activity was eventually turned off.

The next question was whether opening and closing of ion channels sustain $[Ca^{2+}]_c$ oscillations or, alternatively, are the consequence of $[Ca^{2+}]_c$ changes, triggered by redistribution from intracellular pools. The fact that electrical activity usually preceded the $[Ca^{2+}]_c$ transients is in favour of the former hypothesis; however, several interpretations are still possible. To address the issue in a more direct and unambiguous way, cells showing fast $[Ca^{2+}]_c$ oscillations and bursting activity were switched from current-clamp to voltage-clamp conditions. Inhibition of the voltage-dependent channel activity caused immediate block of $[Ca^{2+}]_c$ oscillations in all cells examined; the block was promptly relieved by returning to current-clamp recording (fig. 4A).

To further check the dependence of fast $[Ca^{2+}]_c$ oscillations on electrical activity, the membrane potential of spontaneously oscillating chromaffin cells was recorded and used as command voltage waveforms to control membrane potential of silent cells. One example of these experiments is illustrated in figure 4B: bursting electrical activity and $[Ca^{2+}]_c$ variations, in these previously silent cells, were indistinguishable from those observed in spontaneously active cells.

No specific experiments were designed to ascertain the nature of the channels involved in bursting activity. However, all manoeuvres interfering with Ca^{2+} influx through voltage-operated channels, such as removal of extracellular Ca^{2+} (fig. 5A), administration of 500 µM Cd^{2+}

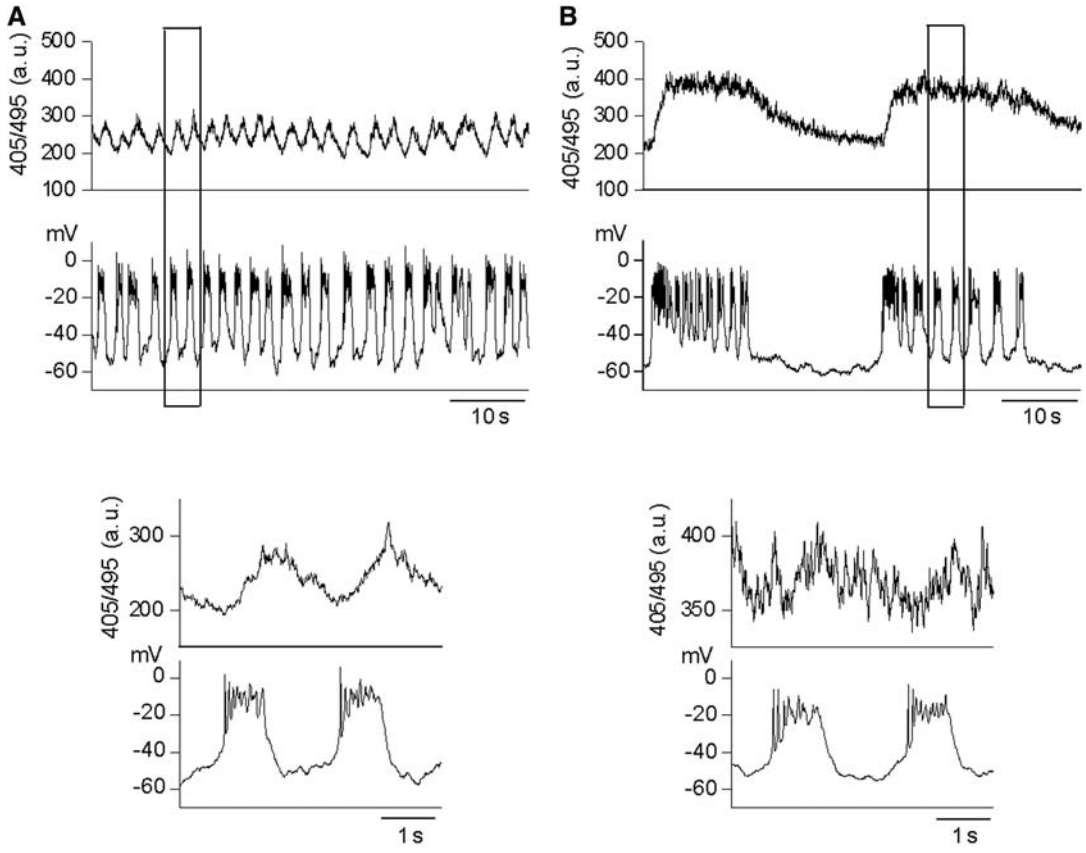

Figure 3. Simultaneous $[Ca^{2+}]_c$ measurement (indo-1 fluorescence emission ratio at 405/495 nm; upper trace) and electrophysiological recording (current clamp; lower trace) in chromaffin cells displaying fast $[Ca^{2+}]_c$ oscillations. Complex and prolonged action potentials were recorded in these cells. The cell illustrated in (A) displayed fast $[Ca^{2+}]_c$ oscillations only. The bursts of action potentials occurred rhythmically and were clearly synchronised with the oscillations. The enlarged traces in the bottom panel show that each complex action potential underlay the rising phase of a $[Ca^{2+}]_c$ oscillation that slowly waned after the electrical activity had subsided. The cell illustrated in (B) displayed fast $[Ca^{2+}]_c$ oscillations superimposed on slow $[Ca^{2+}]_c$ transients. Bursts of complex spikes appeared to underlie fast oscillations that in turn triggered and sustained large slow $[Ca^{2+}]_c$ transients. The fast oscillations stopped, and the transient slowly relaxed when the burst of electrical activity subsided.

(fig. 5B) or treatment with 10 µM nifedipine (not shown) inhibited both electrical and $[Ca^{2+}]_c$ changes.

Although Ca^{2+} influx appeared to be the trigger event of fast oscillations, intracellular Ca^{2+} stores might play a role during this activity. Previous work with ruptured-patch recording showed that electrical activity accompanied calcium transients that were induced by low concentration of bradykinin (Bk, 100 pM) – and occasionally occurred spontaneously – and were sustained by release from intracellular stores [13]. We tested our cells with 100 pM bradykinin, but no effect was observed on electrical activity or fast calcium oscillations (not shown). The classical pharmacological strategy to exclude participation of calcium stores is to deplete their lumenal Ca^{2+} by interfering with the refilling activity of the sarco-endoplasmic reticulum calcium ATPases (SERCAs); to this end thapsigargin (Tg) or cyclopiazonic acid (CPA) can be used. A preliminary series of tests were performed to verify the specificity of the two treatments on SERCA and their possible side effects on voltage-dependent channels. The drug concentration effectively blocking SERCAs was assessed as the concentration required to abolish the response to either 300 µM ATP or 100 nM bradykinin, but not to 30 mM KCl. A depolarising ramp (from a holding potential of –60 mV to +60 mV, during 50 ms) was applied to the cells either under control conditions or in the presence of the drugs. At the effective concentrations, Tg (0.1–1 µM), but not CPA (1–10 µM), caused a marked decrease in voltage-dependent currents (43–63% reduction of inward currents and 22–36% reduction of outward currents, n = 13; data not shown). In light of these results we decided to use only CPA. Pretreatment with this drug (20 µM for 10–15 min) did not abolish fast $[Ca^{2+}]_c$ oscillations. A final series of experiments were aimed at evaluating whether individual $[Ca^{2+}]_c$ transients were sustained by the sole Ca^{2+} influx or were partially reinforced by Ca^{2+} discharge from intracellular stores via Ca^{2+}-induced Ca^{2+} release (CICR). To this end, CPA treat-

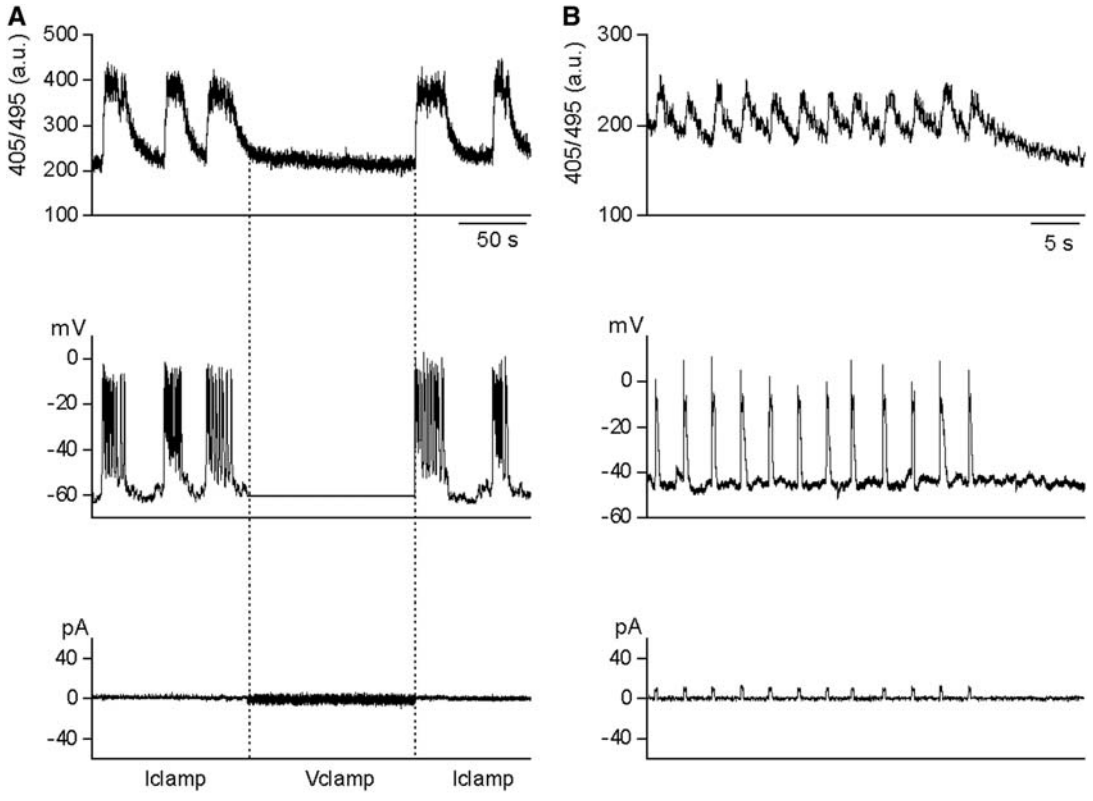

Figure 4. The relationship between electrical activity and fast $[Ca^{2+}]_c$ oscillations in two chromaffin cells. Top traces: $[Ca^{2+}]_c$ measurement (indo-1 fluorescence emission ratio at 405/495 nm); middle traces: membrane potential recordings; bottom traces: membrane current recordings. (A) The panel illustrates a spontaneously active cell that displayed $[Ca^{2+}]_c$ oscillations sustained by bursts of complex electrical activity. When the recording mode was switched from current clamp to voltage clamp, and the electrical activity abolished, $[Ca^{2+}]_c$ activity also subsided. As soon as the cell was returned to current-clamp recording, both activities resumed. (B) Voltage-clamp recording from a spontaneously silent cell is illustrated. The membrane potential time course previously recorded from a cell that displayed complex action potentials and fast Ca^{2+} oscillations was used as the command voltage. The elicited bursts of electrical activity (middle trace) gave rise to $[Ca^{2+}]_c$ oscillations that displayed the frequency and time course typical of spontaneous fast $[Ca^{2+}]_c$ oscillations.

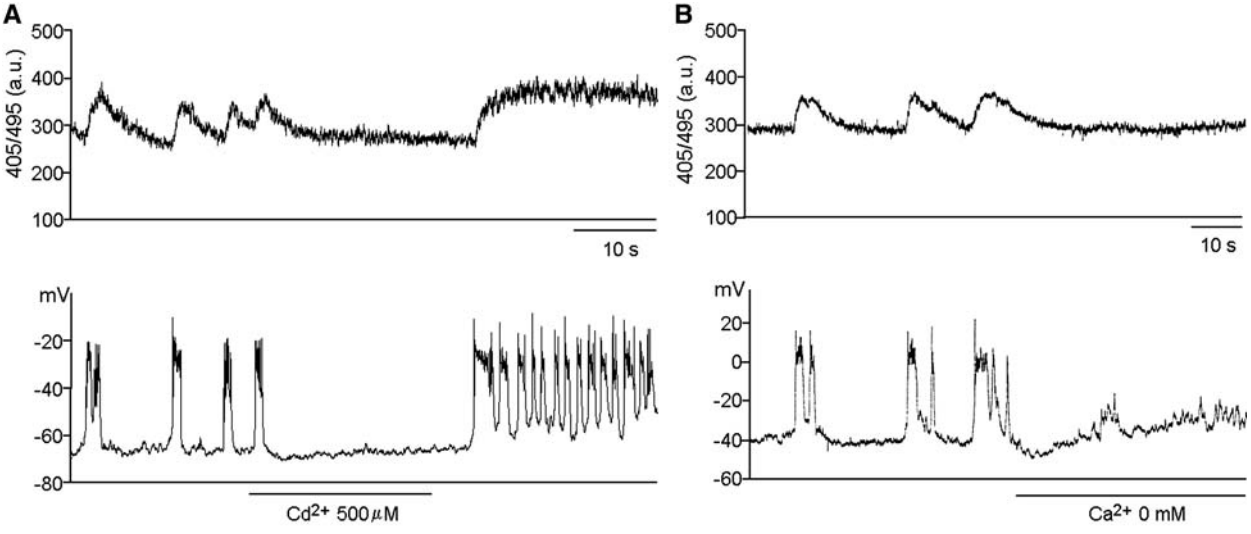

Figure 5. Calcium-channel activity is needed for both complex electrical activity and fast $[Ca^{2+}]_c$ oscillations. Upper traces, $[Ca^{2+}]_c$ measurement (indo-1 fluorescence emission ratio at 405/495 nm); lower traces: membrane potential recording. (A) A chromaffin cell displaying complex action potentials and fast $[Ca^{2+}]_c$ oscillations is illustrated. Washing out Ca^{2+} abolished both the spontaneous, complex action potentials and the concomitant $[Ca^{2+}]_c$ oscillations. The perforated-patch recording rapidly deteriorated in zero Ca^{2+}. (B) Similar recordings in another chromaffin cell. Both the spontaneous, complex action potentials and the concomitant $[Ca^{2+}]_c$ oscillations were abolished by application of Cd^{2+} (500 µM). When Cd^{2+} was washed away, a violent burst of electrical activity ensued that produced a sustained rise in $[Ca^{2+}]_c$.

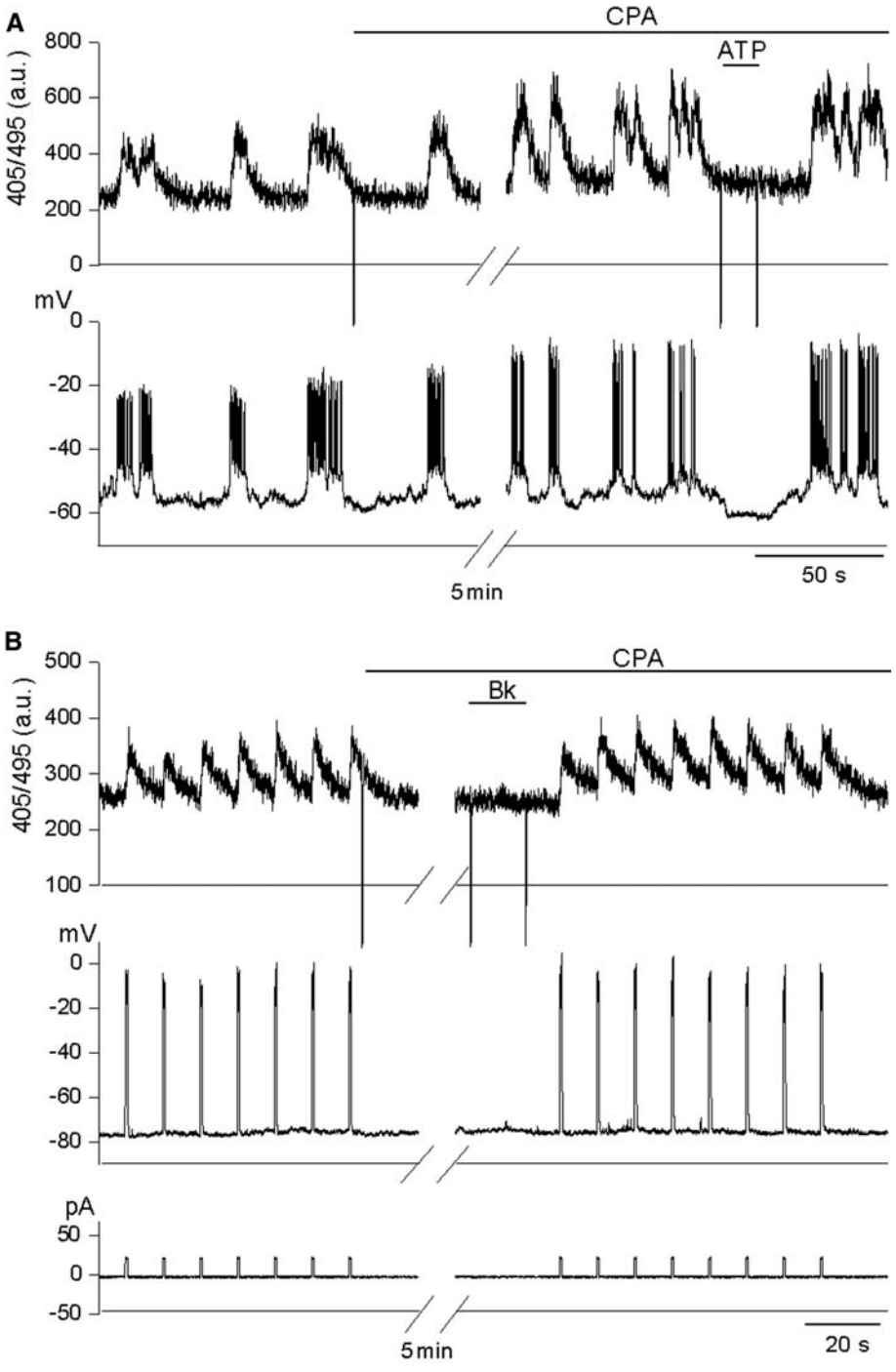

Figure 6. Intracellular Ca^{2+} stores are not needed to sustain fast $[Ca^{2+}]_c$ oscillations. In both panels the top trace illustrates $[Ca^{2+}]_c$ measurements (indo-1 fluorescence emission ratio at 405/495 nm); the second trace illustrates membrane potential; the third trace in the bottom panel illustrates the injected membrane current. (A) Application of CPA to block SERCAs and deplete intracellular stores (see text) did not interfere with either bursts of complex spikes or $[Ca^{2+}]_c$ oscillations in a cell displaying spontaneous activity. Conversely, response to ATP (300 μM) was abolished. (B) Small current pulses elicited in a silent chromaffin cell complex spikes and synchronous $[Ca^{2+}]_c$ oscillations. Application of CPA had no effect, although response to bradykinin (Bk, 100 nM) was lost.

ment was applied to both spontaneously active cells and silent cells stimulated by depolarising pulses. Figure 6, A and B, illustrates the two protocols and clearly shows that $[Ca^{2+}]_c$ transients were not significantly reduced after Ca^{2+} stores were depleted, as documented by the inhibition of responses to either ATP or Bk.

Discussion

Two types of $[Ca^{2+}]_c$ oscillations coexist in rat chromaffin cells

Spontaneous $[Ca^{2+}]_c$ oscillations have long been described in chromaffin cells [17, 20]. They have been proposed to originate from an intracellular store, eventually identified as IP_3 sensitive [10–13]. In the present work we recorded these slow $[Ca^{2+}]_c$ oscillations, but we also unexpectedly identified a different type of oscillatory activity that, to our knowledge, has never been described before. This novel $[Ca^{2+}]_c$ oscillation activity displayed rapid kinetics and was therefore referred to as fast $[Ca^{2+}]_c$ oscillations. This Ca^{2+} transient activity was found in a large percentage of cells; as all procedures were performed according to the same protocols we used in the past, we imagine they may have previously escaped our attention merely for technical reasons: in fact, their frequency was close to the sampling rate we used in our previous work on slow oscillations, and their amplitude was significantly smaller. Indo-1, a dual-emission ratiometric dye, permits simultaneous and continuous measurement of both wavelength values and is therefore more suitable than fura-2 to monitor fast events. Furthermore, in previous studies attention was generally focussed on slow transients, so that moving average and further smoothing of the Ca^{2+} signal were employed to improve signal/noise ratio. A previous attempt at correlating electrical and photometric measurements did not highlight these fast oscillations [13]; in that case, ruptured patch was employed and membrane potential values were in general above –45 mV, so that complex electrical responses (with a clear contribution by repetitive Na^+ spike firing) were not observed. All this suggests that this kind of calcium and electrical activity combination is very sensitive to perturbations of the bioelectrical and biochemical conditions of the cell.

First of all, we addressed the question whether slow and fast oscillations simply reflected a different regulation of the same process or resulted from activation of distinct mechanisms. The whole body of our data argues against the former possibility. The two types of $[Ca^{2+}]_c$ oscillations can coexist and display different sensitivities to external calcium, channel blockers and SERCA inhibitors. In contrast to the fast Ca^{2+}-transient activity studied here, slow oscillations are mostly sustained by release from intracellular stores; they are abolished, or drastically reduced in amplitude, by treatment with SERCA inhibitors; and they may be influenced and complemented, but are not sustained, by calcium inflow through the plasmalemma [10–13]. It should be pointed out that rapid sequences of bursts can generate $[Ca^{2+}]_c$ increases characterized by a sustained plateau, lasting up to several tens of seconds, that might be considered as slow oscillations. However, this kind of activity not only displays a smooth (rather than peaky) shape, but is also dependent on membrane activity (see e.g. fig. 4A). The distinct nature of these two forms of Ca^{2+} signalling raises the question what conditions determine one type of activity rather than another. Although we cannot provide a conclusive answer, in general slow $[Ca^{2+}]_c$ oscillations were observed in the presence of moderate basal $[Ca^{2+}]_c$, while fast ones occurred at higher average values of $[Ca^{2+}]_c$. Still, it is not clear whether basal $[Ca^{2+}]_c$ dictates the type of oscillation, or whether the kind of oscillatory activity itself determines the average $[Ca^{2+}]_c$ level.

Fast $[Ca^{2+}]_c$ oscillations are generated by plasma membrane changes in permeability

Fast $[Ca^{2+}]_c$ oscillations have been suggested to be triggered by plasma membrane Ca^{2+} fluxes [21]; the simple observation, reported here, that electrical activity is in phase with $[Ca^{2+}]_c$ changes supports this hypothesis, but alternative possibilities must be considered. Indeed, in various cell types, including sympathetic neurons $[Ca^{2+}]_c$, oscillations have been reported to be triggered by periodic release from inner stores, although they are largely sustained by a synchronous influx from the extracellular milieu [22–24]. This sequence of events has been thoroughly described in pituitary gonadotrope cells [25]: Ca^{2+} released from IP_3-sensitive stores activates SK_{Ca} channels; the ensuing hyperpolarisation of the plasma membrane removes inactivation of both Na^+ and Ca^{2+} voltage-dependent channels that give rise to action potential firing. All these events are quite rapid; thus, mere temporal correlation between the onset of the electrical activity and the rise in $[Ca^{2+}]_c$ is not conclusive evidence.

To demonstrate the presence of a membrane oscillator, fast $[Ca^{2+}]_c$ oscillations must be abolished by blocking voltage-dependent channels, whereas the induction of the electrical activity in silent cells must be able per se to generate fast $[Ca^{2+}]_c$ transients. Our experiments confirm both these points.

The question remains, what specific electrical activity sustains fast $[Ca^{2+}]_c$ oscillations. In contrast to pituitary cells [14] and amphibian spinal neurons [15], fast $[Ca^{2+}]_c$ oscillations in chromaffin cells are not sustained by individual action potentials but by a bursting activity that bestows sustained depolarisation with prolonged Ca^{2+} entry. A very similar activity has been observed in another neuroendocrine system, β-pancreatic cells; in that case the electrical activity was not the trigger event, but rather the consequence of changes in ATP/ADP cytosolic concen-

trations. We have no clues, at present, to the nature and mode of onset of the bursting activity, so we cannot exclude that the actual trigger might be metabolic oscillations in this system as well. As regards neurons, most of the literature on fast Ca^{2+} transients is focussed on evoked events (by activation of sensory or synaptic receptors), so that comparison with this spontaneous activity may not be appropriate. On the other hand, oscillations of both membrane potential and Ca^{2+} levels have been reported in neurons from several areas of the central nervous system (CNS), and sustain rhythmic activity in many structures of the thalamo-cortical system and basal ganglia. In the various systems, oscillations have been attributed to recurrent activation of T-type calcium channels or complex interplays between voltage-dependent channels and synaptic receptors (see e.g. [1, 26]).

It is rather strange that this electrical activity has escaped attention for so many years of electrophysiological investigation of chromaffin cells. The most reasonable explanation we can propose, based on our observations, is that bursting activity is very sensitive to modifications of cytosol composition, so that it is lost under standard, ruptured-patch, whole-cell recording, a condition where the intracellular milieu is severely altered by dialysis. Electrical transients were previously observed, in this cell type, by ruptured-patch recording [13, 17]; however, they had slower time courses, were not accompanied by repetitive Na^+ spike firing, were evoked by low bradykinin concentration and were associated with (and possibly triggered) massive transients sustained by release from intracellular stores. The overall electrical and calcium activity of chromaffin cells thus appears to be very sensitive to the recording conditions. This is the reason why we preferred to record in perforated-patch conditions, even though this approach is more demanding, and recordings cannot be very prolonged.

Intracellular Ca^{2+} stores are not involved in fast $[Ca^{2+}]_c$ oscillations

The evidence that fast $[Ca^{2+}]_c$ oscillations are generated by changes in plasma membrane permeability does not rule out the possibility that intracellular Ca^{2+} stores participate in individual Ca^{2+} transients. This mechanism of amplification via a CICR mechanism has been described in various cell types [2], and it was reported to markedly reinforce Ca^{2+} entry elicited by action potentials [15, 27–29]. In chromaffin cells, our data indicate that inhibition of Ca^{2+}-store refilling, by blockade of SERCAs, causes depletion of the intracellular stores but does not affect fast Ca^{2+} oscillations. We did not employ thapsigargin, because it interfered with membrane currents. This is a direct effect on the channels: it was also observed in ruptured-patch recordings with high concentrations of EGTA or BAPTA in the patch pipette, so it was not due to $[Ca^{2+}]_c$ elevation following SERCA block.

Taken together, our data indicate that at least two independent mechanisms can sustain $[Ca^{2+}]_c$ independent oscillatory activity in rat chromaffin cells, generating a multiplicity of patterns of Ca^{2+} signalling. This is not surprising in view of the multiple, differentially regulated secretory pathways operating in adrenergic cells, and more generally in synapses, that require a refined and complex dynamic regulation of Ca^{2+} fluxes, local levels and homeostasis. Proper understanding of this complexity is crucial in interpreting the physiological regulation of adrenergic function and the pathogenesis of adrenergic disturbances.

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