Receptor-mediated intracellular signalling: oscillations and waves of cytosolic calcium

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Introduction
Oscillations and waves of $[\text{Ca}^{2+}]_c$ have come to light after the recent development both of calcium-sensitive dyes, such as fura-2 [1], and of computerized imaging microscopy, an approach that allows changes in intracellular calcium concentration to be followed in real time inside single living cells.

Although the function of $[\text{Ca}^{2+}]_c$ oscillations is still largely unknown, it has been suggested that oscillatory activity may sustain and/or modulate neurotransmitter release [2, 3].

Oscillations of $[\text{Ca}^{2+}]_c$ originate from intracellular rapidly exchanging stores and propagate as $\text{Ca}^{2+}$ waves throughout the cell with no loss of the signal. A number of different models have been proposed to account both for the periodic $[\text{Ca}^{2+}]_c$ release from intracellular stores and for the spatial propagation of the signal [4–6]. Apparently, such models, however, can account only for some of the results that have appeared in the literature. In fact, many of the discrepancies reported so far are difficult to reconcile, suggesting that alternative mechanisms may operate in different cell types. In addition, it is even possible that individual cells, at different physiological states, differ in the number and/or distribution of molecular components (receptors, channels and enzymes) responsible for $\text{Ca}^{2+}$ signalling.

In our work we have investigated the mechanisms involved in the generation and control of the rhythmic $[\text{Ca}^{2+}]_c$ variations in rat chromaffin cells [7] and in $[\text{Ca}^{2+}]_c$ waves in nerve-growth-factor-differentiated PC12 cells. In the first cell system, the frequency of oscillation is extremely sensitive to moderate $[\text{Ca}^{2+}]_c$ variations, thus providing a digital signal encoding the plasma membrane receptor activation.
Materials and methods

Cell cultures
Rat chromaffin cells were prepared according to Brandt et al. [8]. In outline, adrenal medulla from rat adrenal glands were dissected free of the cortex, thoroughly rinsed in Krebs-Ringer solution buffered with Heps and then cut in to small pieces. The adrenal tissue was incubated in 0.5 units/ml collagenase A and 10 μg/ml DNAase at 37°C for up to 2 h, during which tissue fragments were dissociated mechanically into single cells by repeated passages through the opening of Pasteur pipettes. After centrifugation at 500 g for 4 min, the chromaffin cells were finally suspended in Dulbecco’s-modified Eagle’s medium supplemented with 10% (v/v) fetal calf serum, plated on to thin glass coverslips coated with poly(ornithine) and cultured for 1–4 days under a humidified atmosphere containing 5% CO₂.

Fura-2 videomicroscopy
The digital fluorescence-imaging microscopy system is built around a Zeiss inverted ‘IM 35’ light microscope, as described previously [9]. In outline, two independent light pathways, controlled by two photographic shutters, provide the excitation light at 340 nm and 380 nm. Fluorescence images were collected by a low-light level charge coupled device camera and fed into a digital image processor where video frames were digitized and integrated in real time. The digital data were transferred in two 300 Mb hard disks with a maximal rate of acquisition of 4 images/s (2 ratios/s) for images of 128 x 128 pixels and 16 bit depth. The images were then processed to convert fluorescence data in [Ca²⁺] images (340/380 nm excitation wavelength ratio method [1]). Mean values of the pixel intensity in the area of interest were calculated from the entire sequence of frames, thus providing a plot of [Ca²⁺] variations in spatially distinct areas during the experiment. At the beginning of each experiment the cell-bearing coverslips were rinsed with Krebs-Ringer solution buffered with Heps and loaded with fura-2 penta-acetoxymethyl ester (2–5 μM, at 37°C for 45 min) dissolved in the Krebs-Ringer solution supplemented with 1% BSA. At the end of the loading the cells were rinsed in the Krebs-Ringer solution and transferred to a thermostatted (37°C) open micro-incubator on the stage of the microscope.

Results and discussion
Chromaffin cells have many features in common with sympathetic neurons, including cellular line-age, voltage-dependent channels, signalling and secretory pathways [10–14]. Both types of cells also display similar repetitive rises in the intracellular free Ca²⁺ concentration [15, 16]. We have found that, in rat chromaffin cells, oscillations occur spontaneously in a large fraction of the population and can be induced in most of the remaining cells either by moderate activation of receptors linked to inositol 1,4,5-trisphosphate (InsP₃) generation (Figure 1, trace A) or by slight depolarization (Figure 1, trace B). Once a cell is oscillating, no matter whether spontaneously or agonist-induced, moderate elevations of [Ca²⁺], cause the frequency of oscillations to increase (Figure 1, traces A and C). If [Ca²⁺], is elevated further, the oscillations progressively decline in amplitude and are eventually inhibited (Figure 1, trace D); this block can be reversed by treatments that lower [Ca²⁺],. Thus, it appears that oscillations occur only when [Ca²⁺], lies within a critical range of values.

In rat chromaffin cells, [Ca²⁺], oscillations originate from intracellular Ca²⁺ pools; two independent, rapidly exchanging Ca²⁺ stores have been identified in chromaffin cells, one sensitive to InsP₃ and the other to caffeine/ryanodine [7, 15, 17]. The Ca²⁺ pool involved in the oscillatory activity appears to be the same as that released by InsP₃, since (1) administration of sarcoendoplasmic reticulum Ca²⁺-ATPase inhibitor, thapsigargin (which eventually leads to the depletion of the store), causes the immediate block both of spontaneous and of agonist-induced [Ca²⁺], oscillations (Figure 1, trace A), while (2) ryanodine (which locks the Ca²⁺ channel open in the InsP₃-insensitive store) did not prevent the oscillatory activity and, in contrast, sometimes induced oscillations [7].

Experiments in which the extracellular Ca²⁺ is suddenly chelated by EGTA, or voltage-operated Ca²⁺ channels are blocked by nitrendipine, lead to the rapid inhibition of the oscillations. Therefore, despite the central role of an intracellular pool, a constant refilling from the extracellular environment is required. The analysis of subcellular patterns of [Ca²⁺], variations revealed pulsatile hot spots; in ‘silent’ cells they remained discrete whereas in oscillating cells they usually expanded into full-blown oscillations [7]. Recently, support has accumulated for a model in which oscillations are sustained by a mechanism of calcium-induced calcium modulation of the cognate receptor [6, 18]. Indeed, the biphasic, Ca²⁺-dependent regulation of oscillations mirrors the bell-shaped modulation that has been identified recently for the InsP₃ receptor [19]. Little is known at present about the mechanisms that generate
Cross-talk between Receptors and Intracellular Second Messenger Systems

Figure I

Oscillations in [Ca$^{2+}$], observed in rat chromaffin cells

Trace A illustrates the effects of repetitive additions of a low (50 pM) concentration of bradykinin (BK) to a single silent cell; notice the dose-dependent increase in oscillation frequency. Administration of thapsigargin (Tg, 1 μM) causes the immediate block of [Ca$^{2+}$] oscillations. Trace B shows the induction of oscillatory activity elicited by the extracellular application of 5 mM KCl. Trace C shows the stimulatory effect of the same concentration of KCl on the frequency of oscillations that are induced by BK. Trace D shows the blockade of [Ca$^{2+}$] oscillations that follows 5 mM KCl administration to an already active cell; the spike amplitude is progressively reduced, leading eventually to a steady increase in [Ca$^{2+}$]. The bar represents 5 min.

rhythmic Ca$^{2+}$ discharge from a discrete pool of stores and sustain its spreading throughout the cell. As for the latter aspect of the cell signalling, we have evidence that, in nerve-growth-factor-differentiated PC12 cells, maximal stimulation in Ca$^{2+}$-free medium of receptors linked to InsP$_3$ generation gives rise to propagation of the signal in the form of intracellular Ca$^{2+}$ waves. Depending on the agonist, waves were found to spread from the soma to the neurite terminals or in the opposite direction. Such a propagation was non-decremental and independent of extracellular [Ca$^{2+}$], and was present also in PC12 clones that were unresponsive to caffeine and ryanodine. These results suggest that intracellular Ca$^{2+}$ stores, probably InsP$_3$-sensitive, are responsible also for wave propagation. It appears, therefore, that Ca$^{2+}$ waves and oscillations are closely related phenomena, both sustained by the positive modulation of the InsP$_3$ receptor. Such a modulation can thus provide a spatio-temporal encoding of extracellular signals.

The mechanism of neurosecretion from chromaffin cells has been studied fairly extensively [20]. In addition to the activation by a single large calcium influx across the membrane, as is induced by depolarization or by maximal receptor activation, growing evidence indicates that secretion can be sustained also by rhythmic oscillations of intracellular Ca$^{2+}$ levels [3]. Interestingly, in bovine chromaffin cells, spontaneous oscillations are absent and basal neurotransmitter release is low, while the majority of resting rat chromaffin cells show rhythmic changes of [Ca$^{2+}$], and measurable secretory activity [2]. Whether increasing the frequency of oscillations enhances secretion remains to be investigated. In conclusion, the different temporal and spatial organization of [Ca$^{2+}$] transients within the cell cytosol represents one of the most relevant recent findings in the field of signalling that might provide a cue for the comprehension of the control of cellular functions such as secretion or motility.

Cyclic AMP serves as an important second messenger transmitting extracellular signals (including those from a variety of hormones and neurotransmitters) to modulate such diverse phenomena as metabolism, gene transcription and memory. The regulation of adenylyl cyclases, the enzymes that convert ATP to cyclic AMP, is the key step in controlling the intracellular concentration of cyclic AMP. So far, cDNAs encoding six mammalian adenylyl cyclases have been cloned [1-9]. The cloned cDNAs make it possible to study the regulation of each type of enzyme individually. Based on their amino acid sequence similarity, types V and VI adenylyl cyclases are more related to each other than they are to the other four enzymes, as are types I and IV. The tissue distributions of adenylyl cyclases have been examined by estimating the quantity of messenger RNA. Types I, II and III adenylyl cyclases are more related to each other than they are to the other four enzymes, as are types II and IV. The tissue distributions of adenylyl cyclases have been examined by estimating the quantity of messenger RNA. Types I, II and III adenylyl cyclases display a more restricted pattern of expression than do the other three enzymes. Types I and II adenylyl cyclases are more abundant in brain, whereas the type III enzyme is found mostly in olfactory neuroepithelium. The expression of each protein remains to be analysed in detail.

Two signalling pathways are known to regulate the activity of mammalian adenylyl cyclases. Receptors for many hormones and neurotransmitters activate heterotrimeric G-proteins (α-, β- and γ-subunits) that either stimulate (Gi) or inhibit (Gs) adenylyl cyclase activity [10]. Elevation of intracellular Ca2+ concentration can also activate calmodulin-sensitive forms of the enzymes [11]. Each of the six types of adenylyl cyclases has been expressed in Sf9 cells using the baculovirus expression system [12]. Isolated plasma membranes containing each enzyme subtype were reconstituted with the purified regulatory components to study the regulation of their enzyme activity. Each of six types of adenylyl cyclase can be activated by the GTPγS-bound α-subunit of Gs (Gs). Only types I and III adenylyl cyclases are sensitive to stimulation by Ca2+-calmodulin. Interestingly, there is strong synergism between activated Gs and Ca2+-calmodulin on the activity of type I adenylyl cyclase, indicating the potential for cross-talk between two signalling pathways (Figure 3b).

Such synergistic activation has been reported for plasma membranes from rat brain and from Aplysia neurons [13, 14].

Unexpected type-specific regulation of adenylyl cyclase activity by G-protein βγ-subunits

The adenylyl cyclase activities in the S49 cell and in platelet membranes, which were used originally to characterize the effects of G-proteins, are activated by Gs, but are largely insensitive to exogenously added G-protein βγ-subunits [10]. Therefore, as each subtype of adenylyl cyclase was examined, we