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Measurement of cytosolic free Ca^{2+} in individual pancreatic acini

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The kinetics of changes in cytosolic free Ca^{2+} ($[Ca^{2+}]_i$) were determined in individual rat pancreatic acini by microfluorimetry. Three major findings are reported. First, at maximal stimulatory concentrations for amylase release, both caerulein and bombesin induced an initial rise in $[Ca^{2+}]_i$ followed by prolonged secondary oscillations of smaller amplitude. The latter effect was not observed with supramaximal doses of caerulein. Second, these cyclic changes were dependent, at least in part, on extracellular Ca^{2+}. Finally, comparison of the threshold doses for $[Ca^{2+}]_i$ mobilization and enzyme discharge demonstrated that pathways independent of an elevation of $[Ca^{2+}]_i$ control the secretory activity of pancreatic acini at low, picomolar agonist concentrations.

Ca^{2+}; Fura-2; Amylase secretion; Caerulein; Bombesin; Microfluorimetry

1. INTRODUCTION

Changes in cytosolic free Ca^{2+} concentrations ($[Ca^{2+}]_i$) have long been considered as key events in the control of several biological responses following cell activation (see for review [1,2]). Experimental evidence for a primary role of $[Ca^{2+}]_i$ in regulating the exocytosis of enzymes from pancreatic acinar cells was originally supported mainly by indirect experiments [3,4]. A direct measurement of $[Ca^{2+}]_i$ has now been rendered possible by the generation of intracellularly trappable fluorescent indicators like quin-2 [5] and fura-2 [6]. Thus, the definitive demonstration that pancreatic secretagogues such as caerulein, bombesin and carbachol mobilize $[Ca^{2+}]_i$ from intracellular stores has been obtained by several groups [7–12].

Detailed studies of the quantitative relationships between changes in $[Ca^{2+}]_i$ and enzyme secretion, however, have challenged the assumption that a rise in $[Ca^{2+}]_i$ is a necessary and/or sufficient event for the stimulation of secretion [11,13,14]. Since those measurements averaged the fluorescence signal of millions of cells, the possibility that the absence of a rise in $[Ca^{2+}]_i$ during stimulation of enzyme release reflected the inadequacy of the technique to detect a small and/or localised elevation of $[Ca^{2+}]_i$ could not be ruled out. In addition, pancreatic acini have been shown to form a heterogeneous population in terms of their ability to secrete amylase under both resting and stimulated conditions [15]. Thus, it could be argued that the observation of enzyme release at resting $[Ca^{2+}]_i$ is merely the consequence of the activation of a small fraction of acini whose $[Ca^{2+}]_i$ response would escape detection.

The elucidation of these issues seems to be crucial in defining the molecular features of the exocytotic pathway in pancreatic acini. To address these questions, therefore, we have measured the kinetics of $[Ca^{2+}]_i$ changes in individual acini loaded with fura-2, using fluorescence microscopy with dual excitation wavelength [16,17]. Three major findings are reported: (i) concentrations of caerulein and bombesin which are maximal for amylase release induce prolonged oscillations of $[Ca^{2+}]_i$, although of reduced amplitude in comparison with the initial discharge from intracellular stores; (ii) these sustained oscillations are depen-

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dent, at least in part, on the presence of extracellular Ca^{2+}; (iii) in spite of their stimulatory effect on enzyme secretion, picomolar concentrations of both secretagogues do not evoke a detectable elevation of $[Ca^{2+}]_i$.

2. EXPERIMENTAL

Pancreatic acini were isolated from ad lib fed male Wistar rats, weighing about 200 g [14,18]. Following isolation, acini were suspended in RPMI 1640 culture medium and loaded in the presence of 1 μM fura-2/acetoxymethyl ester, as detailed elsewhere [13,14]. Under these conditions it was estimated that the intracellular concentration of fura-2 approached 15 μM, with the assumption of a volume of 1 μl/10^6 acinar cells [13]. The loaded acini were resuspended in a Krebs-Ringer-Hepes buffer (KRBH), pH 7.4 [11] and then plated onto glass coverslips mounted in a Petri dish (equipped with a stainless steel ring placed on top of the coverslide) which served as the superfusion chamber. Acini were allowed to adhere for 15–20 min at room temperature and then studied under the microscope until 2–3 h after isolation.

Dual wavelength excitation microfluorimetry was performed with a SPEX modular fluorimeter (Glen Creston Corp., London, England) coupled to an inverted microscope (DIAPHOT-TMD, Nikon, Tokyo, Japan) used in the epifluorescence mode (objective: Nikon F40) [16]. The equipment available in our laboratory has been described in greater detail in previous publications [19,20]. The microscope, connected to a photometre and a photomultiplier tube, is contained in a thermostatted box at 37°C. This allows for the equilibration of the superfusion medium (KRBH) by a passage through a coil before reaching the acini. The flow of the superfusion was kept constant at 1 ml/min. Stimuli, dissolved at the final concentration in KRBH supplemented with 0.1% bovine serum albumin, were injected in the close vicinity of the acinus through a wide-mouthed (100 μm) micropipette connected to a side-arm pump. The pipette was positioned perpendicularly to the direction of the stream of superfusion, so that under these conditions only one acinus was challenged with the infused secretagogue. Furthermore, this experimental setup allows for the rapid change of the acinar environment from one to another test condition. To ensure that this technique permits to 'clamp' adequately the cells under observation, preliminary experiments were performed with the insulin-secreting B-cells (Pralong, W.F. and Wollheim, C.B., unpublished). In these cells, which possess voltage-operated Ca^{2+}-channels [21,22], depolarizing concentrations of K^+ induce a rapid increase in $[Ca^{2+}]_i$. Such an effect was fully prevented by 'clamping' a single B-cell with control medium infused via the closely positioned micropipette. Stopping the infusion of control, low-K^+ buffer through the micropipette, resulted in the immediate rise of $[Ca^{2+}]_i$ in the cell now exposed to the high K^+ concentrations of the superfusion medium.

The fluorescence emitted by an acinus was monitored through a diaphragm defining the zone of observation and a cut-off filter (500 nm) and eventually measured by photon counting. Fura-2 signals were calibrated by determining the ratio between the fluorescence emission intensities (at 505 nm) of the probe excited at two different wavelengths: 340 nm (F_{340}) and 380 nm (F_{380}) [6,17,23]. From the ratio $R = F_{340}/F_{380}$, values of $[Ca^{2+}]_i$ were calculated according to the equation used in [19]:

$$[Ca^{2+}]_i = K_d \times \beta \times (R - R_{min})/(R_{max} - R)$$

where K_d is the dissociation constant of the Ca^{2+}/fura-2 complex, estimated to be 225 nM in the cytosolic environment [6]; R is the ratio of the fluorescence intensities with the two excitation wavelengths, determined in separate experiments also at low and saturating Ca^{2+} concentrations (R_{min} and R_{max}, respectively); β is $F_{380}(min)/F_{380}(max)$ (see also [20]).

Secretion studies and measurements of amylase release were performed with fura-2-loaded acini, following exactly the experimental protocols previously detailed [11,13,18].

Results are presented as means ± SE. The materials and their sources were the same as those reported in [11,13,19,20].

3. RESULTS AND DISCUSSION

The distribution of fura-2 within pancreatic acini was analysed under fluorescence microscopy. With the loading conditions employed in this study, the cells were diffusely labelled and did not show any spotty accumulation of the dye within discrete subcellular compartments. This pattern, which was not modified by any experimental treatment, represents an essential condition for accurate determinations of $[Ca^{2+}]_i$ [17]. Recordings were performed on well polarized acini, comprising usually 8–10 cells with no signs of membrane blebbing.

It has been documented that some cell types are characterized by the presence of spontaneous $[Ca^{2+}]_i$ oscillations [19,24–26]. Under basal conditions, i.e. control KRBH medium, however, none of the acini studied (n = 54) displayed significant changes of resting $[Ca^{2+}]_i$ levels (figs 1–3). A typical fluorescence recording from an individual acinus stimulated with caerulein 100 pM is shown in fig.1. The upper panel illustrates the emission traces obtained at F_{340} and F_{380}, with the characteristic antiparallel changes indicative of true $[Ca^{2+}]_i$ variations [6,16,19,23]. A slight decrease in the fluorescence intensity was observed at the two excitation wavelengths, a phenomenon probably due to photobleaching of fura-2, since basal fluorescence of acini did not decrease markedly with increasing the time elapsed from loading. The lower panel of fig.1 illustrates the ratio of F_{340}/F_{380} for the same recording. It is apparent that the artifactual change of fura-2 fluorescence due to photobleaching is cancelled

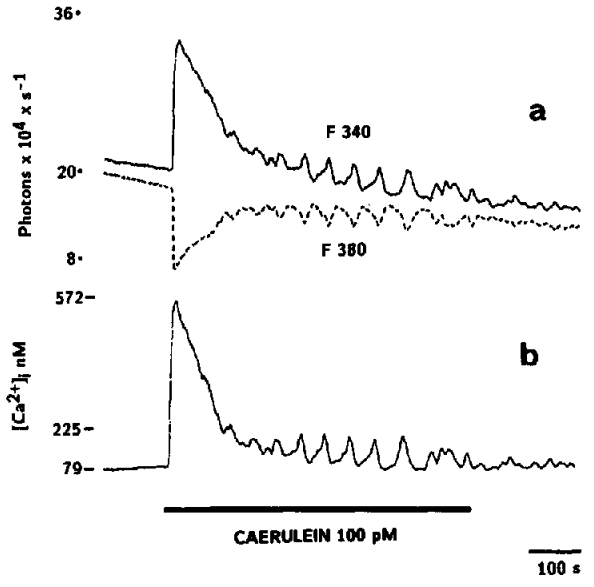

Fig.1. Effect of caerulein on $[Ca^{2+}]_i$ in individual pancreatic acini. (a) Fluorescence of an acinus recorded simultaneously (with an integration time of 1 s) at excitation wavelengths of 340 nm (F_{340}) and 380 nm (F_{380}). (b) Ratio of fura-2 fluorescence (F_{340}/F_{380}) from the same experiment, calibrated for $[Ca^{2+}]_i$. The bar indicates for both panels the time during which caerulein was present at the final concentration of 100 pM. The trace is representative for at least three experiments.

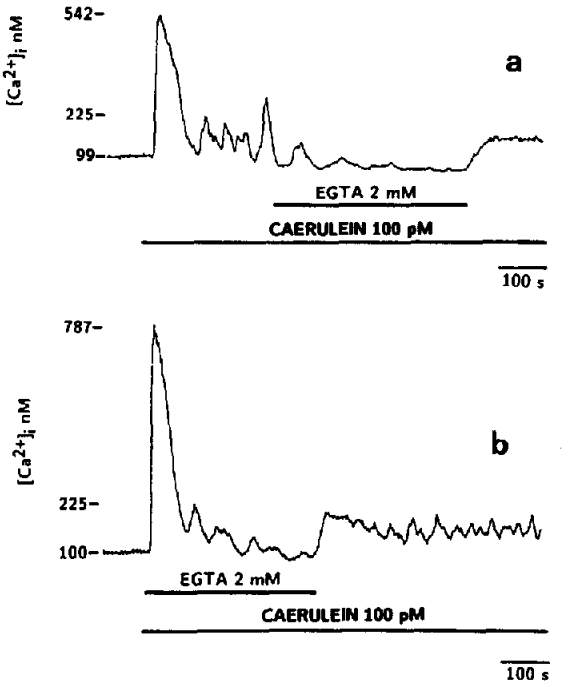

Fig.2. Effect of removal of extracellular Ca^{2+} on the oscillations of $[Ca^{2+}]_i$ induced by caerulein. The superfusion medium contained 1 mM Ca^{2+}, supplemented with 2 mM EGTA where indicated. Bars indicate the duration of the stimulus. These traces are representative for at least three experiments.

out by the ratio method, so that basal values of $[Ca^{2+}]_i$ were not reduced at the end of the trace.

Stimulation of one acinus with caerulein 100 pM elicited an initial, rapid elevation of $[Ca^{2+}]_i$ (fig.1b). This transient was followed by a progressive decay towards prestimulatory levels. About 90% of acini challenged with caerulein 100 pM, a concentration which is maximal for amylase secretion but submaximal in terms of $[Ca^{2+}]_i$ rises [8,10,11], were characterized by the presence of sustained oscillations of smaller amplitude (i.e. averaging 60–70 nM above resting levels). These secondary transients usually disappeared following removal of the agonist (fig.1b). This pattern represents a novel finding which was not observed in previous studies carried out on acinar cell suspensions [7–14]. The failure to detect such prolonged oscillations lies probably on the fact that not all acini are oscillating synchronously and, therefore, the average change in $[Ca^{2+}]_i$ of the total population may well be hidden within the noise of the recorded trace. This behaviour of pancreatic acini is in keeping with the presence of agonist-induced $[Ca^{2+}]_i$ oscillations documented in other cell types [26–28].

The next series of experiments was designed to determine the source of Ca^{2+} mobilized in response to caerulein (fig.2). To this end individual acini were stimulated with caerulein 100 pM, either in the presence or absence of 2 mM EGTA. The trace shown in fig.2a indicates that chelation of extracellular Ca^{2+} by an excess of EGTA, following the initial $[Ca^{2+}]_i$ transients, caused a progressive fading of the secondary oscillations. Reintroduction of the normal Ca^{2+}-containing medium, in the continuous presence of caerulein, resulted in a gradual increase of $[Ca^{2+}]_i$, thus suggesting an augmented permeability of the plasma membrane to Ca^{2+}. The experiment illustrated in fig.2b documents that the initial elevation of $[Ca^{2+}]_i$ induced by caerulein was unaffected by the removal of extracellular Ca^{2+}, in agreement with previous

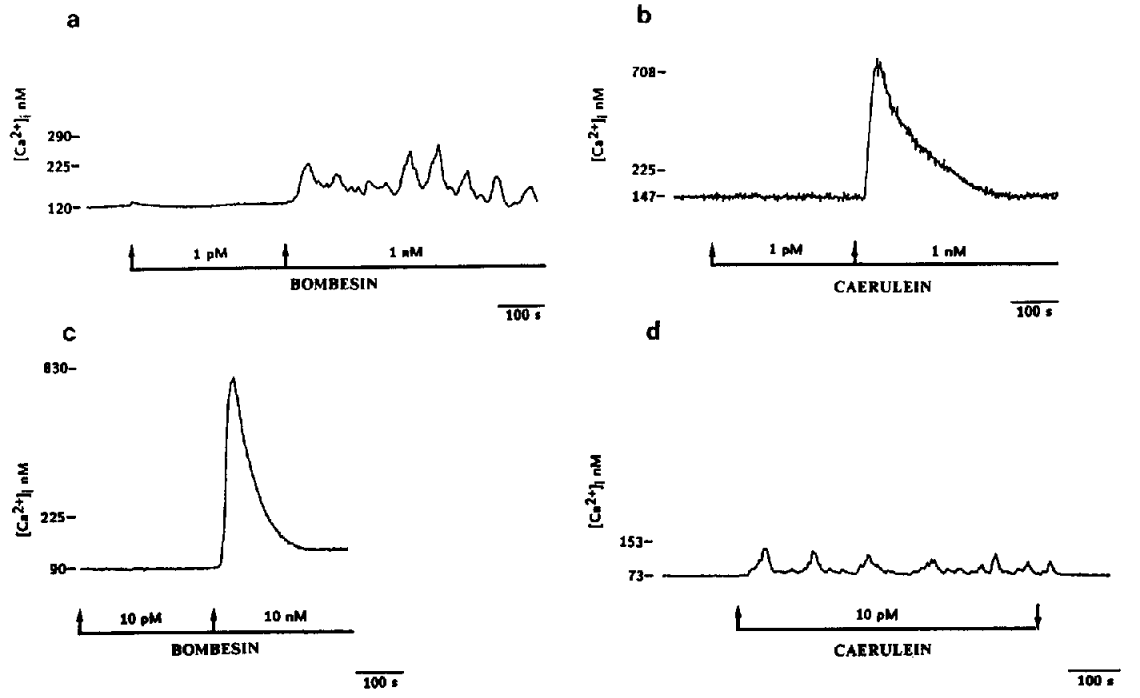

Fig.3. Dose response of the effect of bombesin (a,c) and caerulein (b,d) on $[Ca^{2+}]_i$ in individual pancreatic acini. Bars indicate the duration of the stimulus. These traces are representative of at least two similar observations.

observations on populations of acini [9]. While under these conditions the cyclic changes were not eliminated, a rapid fading of the secondary oscillations occurred. Upon reintroduction of Ca^{2+} in the superfusing medium, in the continuous presence of caerulein, there was a progressive increase in the levels of $[Ca^{2+}]_i$, followed by the appearance of repeated oscillations. In contrast, the sequential exposure of acini to Ca^{2+}- and EGTA-containing media in the absence of caerulein, did not cause any change in fluorescence (not shown). Taken together, these findings indicate that, as previously suggested in other systems [26,28,29], the rhythmic changes in agonist-stimulated pancreatic acini are due, at least in part, to Ca^{2+} cycling by the intracellular stores. The partial dependence on extracellular Ca^{2+} may reflect the need of refilling the agonist-sensitive Ca^{2+} pool to sustain the cycling phenomenon. Although the precise mechanism(s) underlying this pattern of response remain(s) unknown, these observations are compatible with a role for the sustained accumulation of inositol 1,4,5-trisphosphate reported in this tissue [13,14,29]. Moreover, it appears that, following readdition of extracellular Ca^{2+}, the steady-state level of $[Ca^{2+}]_i$ was higher than that maintained in EGTA-containing medium. This finding suggests that refilling of intracellular stores occurs via increased Ca^{2+} influx into the cytosol and subsequent uptake into the agonist-sensitive pool [30].

In spite of the fact that current models of stimulus-secretion coupling in pancreatic acini emphasize the role of Ca^{2+} for certain secretagogues [3,4], it has been recently questioned that an elevation of $[Ca^{2+}]_i$ represents a necessary and/or sufficient event to initiate and maintain enzyme secretion [11,13,14,31]. We sought, therefore, to establish a dose response of the effect of bombesin and caerulein on $[Ca^{2+}]$, using the more powerful and sensitive technique of microfluorimetry. This set of experiments is summarized in fig.3. At concentrations ranging between 1 and 10 pM, bombesin did not evoke any appreciable change in $[Ca^{2+}]_i$ (fig.3a and c), thus supporting the evidence generated in acinar cell populations [14]. It is im-

portant to stress that amylase release is already stimulated 2- to 4-fold above control levels with these peptide concentrations [14]. At higher doses two patterns of response were noticed. The first (bombesin 1 nM) was characterized by prolonged oscillations in $[Ca^{2+}]_i$, similar to those reported for caerulein (fig.3a). The second type (bombesin 10 nM) displayed a rapid elevation of $[Ca^{2+}]_i$ followed by a progressive decay which approached prestimulatory levels (fig.3c), thus mimicking the patterns observed in cell suspensions [9,14]. The fact that both 1 nM and 10 nM of bombesin represent maximal stimulatory concentrations for amylase release [4,9,14] further suggests that there is no direct correlation between changes in $[Ca^{2+}]_i$ and activation of the secretory machinery. The dose response studies with caerulein indicated that concentrations below 10 pM were not stimulatory for $[Ca^{2+}]_i$ (fig.3b). In contrast to previous studies from this laboratory [11,13], minor rises in $[Ca^{2+}]_i$ occurred in about 65% of acini tested with 10 pM caerulein (fig.3d). The amplitude of these oscillations was, however, comparable only to the secondary transients induced by 100 pM of this peptide, albeit less frequent (cf. figs 1b and 3d). When acini were challenged with supramaximal doses of caerulein (1 nM) a typical biphasic pattern was observed (fig.3b). The latter finding, similar to the response evoked by bombesin 10 nM, may represent either a more rapid homologous desensitization or may be the consequence of a further enhanced activation of Ca^{2+}-extruding pumps.

From the above data it follows that, if an elevation of $[Ca^{2+}]_i$ is a prerequisite to trigger exocytosis, then caerulein 10 pM should be the threshold dose capable of stimulating amylase release. The experiments illustrated in fig.4 unambiguously demonstrate that a 4-fold stimulation was elicited already by 1 pM caerulein, which does not increase $[Ca^{2+}]_i$. While it is plausible that the greater release of amylase evoked by caerulein 10 pM could be attributed to the concomitant small oscillations of $[Ca^{2+}]_i$, these experiments provide a strong argument for the hypothesis that signals other than a variation in $[Ca^{2+}]_i$ control the secretion of enzymes from pancreatic acini. Finally, it should be added that a rise of $[Ca^{2+}]_i$ in the range of that observed under stimulation with 10 pM caerulein is not, by itself, sufficient to initiate enzyme secretion [7,10]. We speculate that

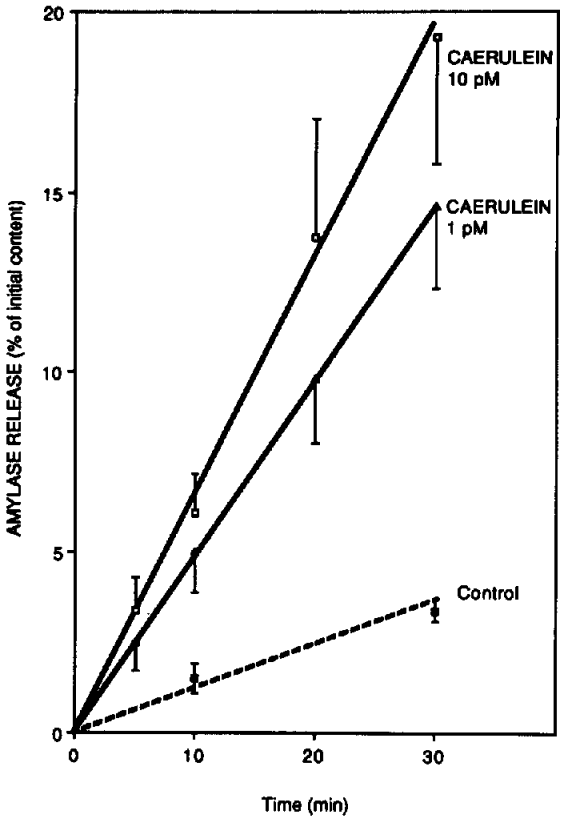

Fig.4. Time course of the effect of caerulein on amylase release in fura-2-loaded pancreatic acini. Results are shown as means ± SE of three independent experiments.

the interaction of an agonist with its specific receptors could generate an additional signal which markedly sensitizes the exocytotic machinery to the ambient $[Ca^{2+}]_i$, as recently suggested [32,33]. In this perspective, we have recently shown in pancreatic acini that caerulein can activate protein kinase C [34] at basal $[Ca^{2+}]_i$, thus attributing a primary role for this enzyme in the control of amylase release [13].

In summary, by using fluorescence microscopy with dual excitation wavelength we have provided the first demonstration that $[Ca^{2+}]_i$ oscillates in individual pancreatic acini in response to concentrations of bombesin and caerulein which evoke maximal amylase discharge. These secondary, sustained oscillations are independent, at least in part, of extracellular Ca^{2+}. The presence of cyclic variations in $[Ca^{2+}]_i$ indicates that the control of Ca^{2+} homeostasis is probably more complex than hither-

to postulated and suggests a frequency- rather than an amplitude-dependence of Ca^{2+}-regulated pathways. Finally, by applying this novel and sensitive technique, we have further documented that secretion can be also stimulated in the absence of a detectable elevation of $[Ca^{2+}]_i$.

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