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Spatial and temporal distribution of agonist-evoked cytoplasmic Ca^{2+} signals in exocrine acinar cells analysed by digital image microscopy

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High resolution digital video imaging has been employed to monitor the spatial and temporal development of agonist-induced cytosolic Ca^{2+} signals in fura 2-loaded exocrine acinar cells. Enzymatically isolated mouse pancreatic and lacrimal acinar cells or small acinar cell clusters were used. These retain their morphological polarity so that the secretory granules in individual cells are located at one pole, the secretory pole. In acinar cell clusters the granules are located centrally, oriented to surround what would be *in situ* referred to as the lumen. In pancreatic and lacrimal acinar cells inositol-1,4,5-triphosphate-generating agonists [acetylcholine (ACh) and cholecystokinin octapeptide (CCK) for the pancreas and ACh in the lacrimal gland] give rise to a rapidly spreading Ca^{2+} signal that is initiated at the secretory pole of the cells. The initial increase in $[Ca^{2+}]_i$ in the luminal pole is independent of extracellular Ca^{2+} indicating that the earliest detectable intracellular Ca^{2+} release is specifically located at the secretory pole. In lacrimal acinar cells ATP acts as an extracellular agonist, independent of phosphoinositide metabolism to activate a receptor-operated calcium influx pathway which, as for ACh, gives rise firstly to an increase in intracellular Ca^{2+} concentration in the secretory pole. We propose that this polar rise in intracellular Ca^{2+} concentration is due to Ca^{2+}-induced Ca^{2+} release. By contrast, when Ca^{2+} release and Ca^{2+} influx are induced in the absence of receptor activation by thapsigargin and ionomycin, the Ca^{2+} signal develops diffusely and slowly with no localization to the secretory pole. The receptor-mediated cytoplasmic Ca^{2+} signals in exocrine acinar cells arise at the secretory pole reflecting the functional polarization within these cells which operates to achieve the unidirectional secretion of electrolytes and proteins specifically at the luminal membrane.

Key words: acetylcholine/adenosine triphosphate/cholecystokinin/lacrimal acinar cells/pancreatic acinar cells/polarized cytoplasmic Ca^{2+} signal

Introduction

Exocrine acinar cells are structurally and functionally polarized. At the structural level most of the rough endoplasmic reticulum (RER) and the nucleus are localized at the base of the cell, the Golgi cisternae are in a supranuclear position whereas the zymogen granules are at the cell apex (luminal or secretory pole) (Amsterdam and Jamieson, 1974). The plasma membrane is divided by the tight junctions into a small luminal (apical) and a large basolateral domain. These two domains have different particle densities when studied by freeze-fracture electron-microscopy and disruption of the tight junctions causes the gradual disappearance of the morphological differences (Meldolesi *et al.*, 1978). Functionally, the acinar cells secrete fluid and enzymes into the lumen and exocytosis occurs specifically at the luminal membrane (Palade, 1975). The membrane receptors for stimulating agonists, for example the hormone cholecystokinin (CCK) in the case of pancreatic acinar cells, are localized all over the basolateral membranes, but are not found on the luminal membrane (Rosenzweig *et al.*, 1983). There are also differences in the distribution of membrane transport proteins with the Na^+-K^+ pump and Ca^{2+}-activated cation channels in the basolateral and Ca^{2+}-activated anion channels in the luminal plasma membrane (Petersen and Gallacher, 1988; Petersen, 1992).

An agonist-evoked rise in the free cytoplasmic Ca^{2+} concentration ($[Ca^{2+}]_i$) is one of the most clearly observable early events in acinar cells leading to a secretory response (Petersen and Gallacher, 1988; Petersen, 1992). In view of the structural and functional polarization of the exocrine acinar cells, it is important to have reliable information about the spatial distribution of the intracellular Ca^{2+} signal.

Unfortunately, the information available is contradictory. Kasai and Augustine (1990) reported for the rat pancreatic acini that the initial increase in $[Ca^{2+}]_i$ is localized towards the luminal pole of the cells, whereas in another study in the same tissue the increase in $[Ca^{2+}]_i$ appeared to begin at a region beneath the basolateral membrane (Habara and Kanno, 1991). In parotid acinar cells, based on electrophysiological evidence, Foskett *et al.* (1989) suggested the presence of a receptor-sensitive intracellular Ca^{2+} pool localized near the basolateral membrane, whereas using digital imaging Dissing *et al.* (1990), reported a synchronous increase in $[Ca^{2+}]_i$ at both luminal and basolateral membranes. The aim of the present study was to clarify this issue. By use of digital video imaging of fura-2 fluorescence at a high speed frame capture mode, which provides ratio images at time intervals of 160 ms, we now show in both pancreatic and lacrimal acinar cells that the initial intracellular release of Ca^{2+} occurs at the secretory pole and that this initial event is followed by increases of $[Ca^{2+}]_i$ along the lateral and basal membranes of the cells. Our study also shows that this spatio-temporal distribution can be evoked by different types of receptor-activated Ca^{2+} mobilization and that this phenomenon does not appear to be related to the acinar architecture as it is also present in single, isolated cells. The initial cytoplasmic Ca^{2+} signal generation in the secretory pole appears to be a specific effect

triggered by receptor activation, since the non-specific increases in $[Ca^{2+}]_i$ induced by the Ca^{2+}-ATPase inhibitor thapsigargin or the ionophore ionomycin were diffusely distributed.

Results

The characteristic morphological features of the pancreatic acinar cell, as examined under a differential interference contrast (DIC) optical system, are shown in Figure 1. The isolation procedure described in the Materials and methods section yields a large variety of acinar structures, consisting of single, isolated cells (Figure 1A), clusters of two (Figure 1B) to four cells (Figure 1C) and large acini, incompletely dispersed (not shown). For both pancreatic (Figure 1) and lacrimal acinar cells (not shown), the characteristic polarization is easily observable. The zymogen granules are packed towards the apical side of the cell, which from here on will be referred to as the 'secretory pole'. This area is surrounded by the basolateral cytoplasm, which by convention will be called the 'basolateral pole'.

Figure 2 illustrates the spatial development of the cytoplasmic Ca^{2+} signal evoked by extracellular perifusion with 0.5 μM ACh. Since the time taken from the initiation of the cytoplasmic Ca^{2+} signal to its full development is very short (<5 s), the 340 and 380 nm frames were captured alternatively at high speed, without frame averaging, to provide a true ratioed image every 160 ms. Under resting conditions, $[Ca^{2+}]_i$ in the four-cell cluster (shown in Figure 2A) was uniformly low throughout the cytoplasm (Figure 2C, a). In all the cells tested (n = 20), the first increase in $[Ca^{2+}]_i$ was recorded in the secretory pole. We also noted frequently that very localized and transient increases in $[Ca^{2+}]_i$ appeared in the basolateral periphery of the cell (Figure 2C, b and c). Following the initial rise in the secretory pole, the Ca^{2+} signal did not propagate uniformly across the cytoplasm towards the basal pole. Instead, the initial increase was followed by a rise in $[Ca^{2+}]_i$ along the lateral membranes (Figure 2C, c and d) and then under the membrane corresponding to the basal pole. This often generated a ring-like pattern of high $[Ca^{2+}]_i$, with central cytoplasmic areas displaying lower $[Ca^{2+}]$ values (Figure 2C, e–g). Nevertheless, after 4–5 sec when the ACh-evoked Ca^{2+} response was fully developed, the cells presented a relatively uniform high $[Ca^{2+}]_i$ (Figure 2C, h). A graphic presentation of the time-dependent increases in $[Ca^{2+}]_i$ in the secretory versus basal pole of the acinus is shown in Figure 2B. The plot clearly shows that the rise in $[Ca^{2+}]_i$ is initiated in the central region of the secretory pole and that the increase at the basal pole (the regions being defined as illustrated in the inset of Figure 2B) starts only about 0.5 s later. Once the process is initiated, the rate of $[Ca^{2+}]_i$ rise in both regions appears to be similar. If the same type of analysis is applied to the individual cells shown in Figure 2A rather than to the entire acinus, the delay in $[Ca^{2+}]_i$ increase between the two poles is even more pronounced. This observation can be explained by the fact that in each individual cell the spreading of the Ca^{2+} signal is slightly different (compare the top right with the bottom left cell of the acinus). In the absence of extracellular Ca^{2+}, ACh also evoked an initial rise in $[Ca^{2+}]_i$ in the secretory pole and within the time-frame of our investigations, 10–20 s after agonist stimulation, this Ca^{2+} signal was seen subsequently to spread in the same manner as in the presence of external Ca^{2+} (n = 5).

The response to another agonist, CCK (100–200 pM), was similar to that described for ACh (n = 8) and Figure 2D shows a typical example of the development of the cytoplasmic Ca^{2+} signal after stimulation with 200 pM CCK. The initial $[Ca^{2+}]_i$ rise in the secretory pole (Figure 2D, c), the subsequent spreading along the lateral and basal membranes (Figure 2D, d) and the final development of the Ca^{2+} signal throughout the whole of the cytoplasm (Figure 2D, e–h) are observed.

The same pattern of spatial organization of the agonist-induced Ca^{2+} response has been observed in lacrimal cells. Figure 3 illustrates the results obtained for a doublet of lacrimal acinar cells (Figure 3A) and similar patterns have

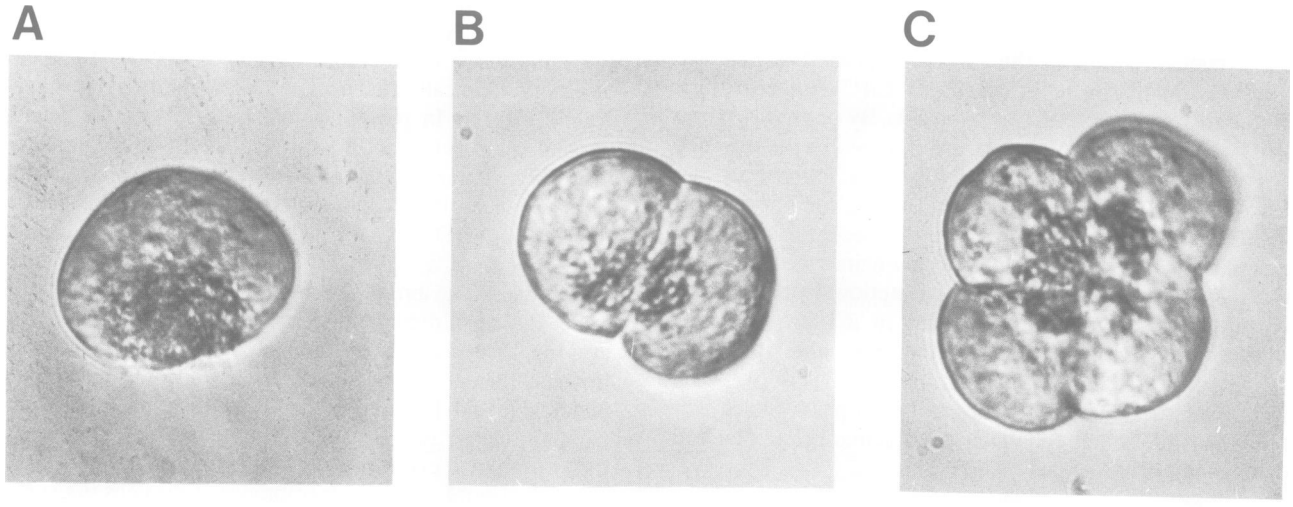

Fig. 1. Differential interference contrast (DIC) images of pancreatic acinar cells showing the morphological polarity of the isolated cells and cell clusters. The zymogen granules are clustered at one pole of the cell, the secretory pole. Calibration bar, 25 μm.

been recorded for single cells or small acini (not shown). The initial focal point of the cytoplasmic Ca^{2+} increase following exposure to 0.5 μM ACh (n = 10) is again the secretory pole (Figure 3B, b). 0.6 s later (Figure 3B, c), the $[Ca^{2+}]_i$ had increased along the lateral membranes and under the basal membrane in one of the cells. With a slight

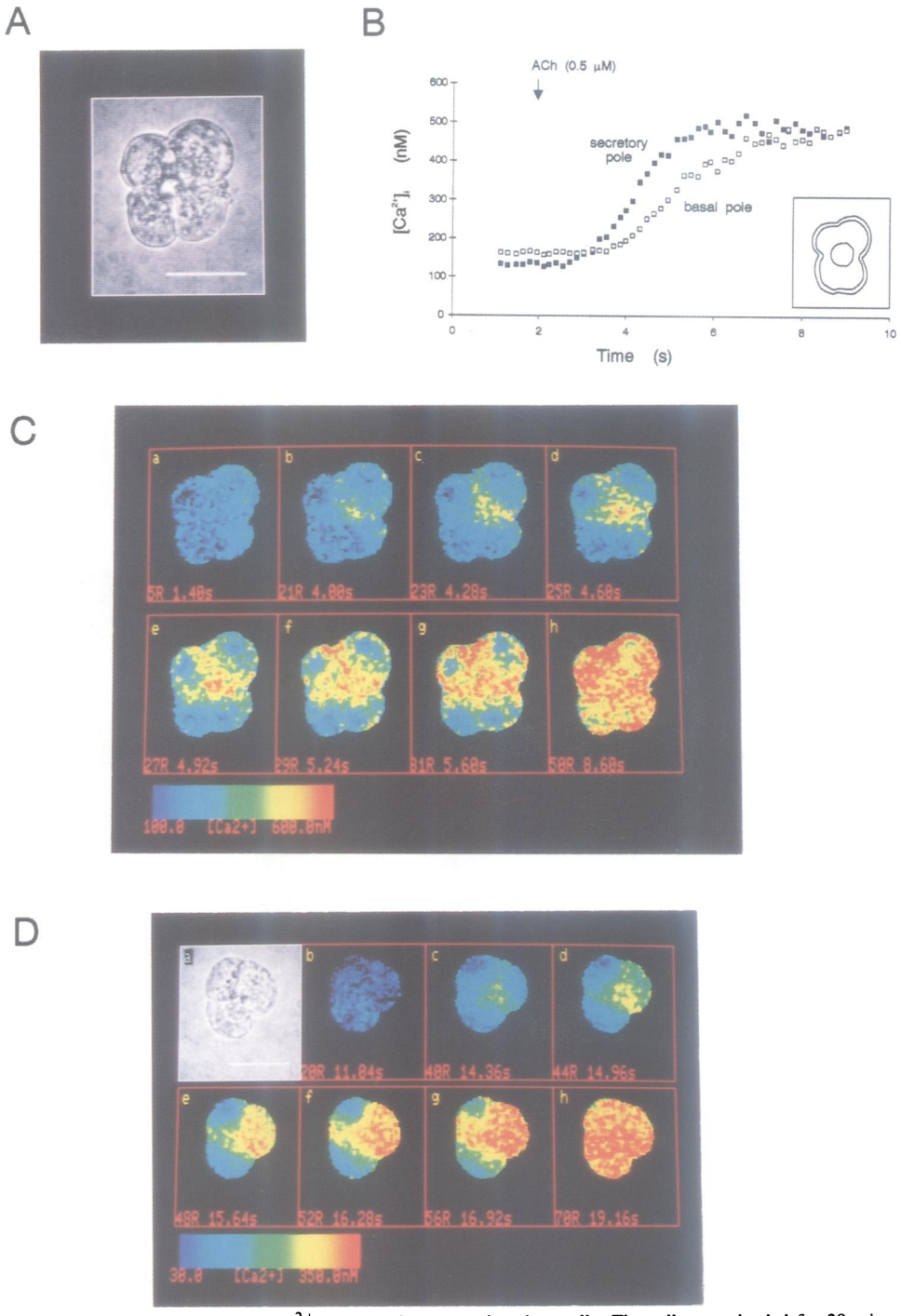

Fig. 2. Digital image analysis of the agonist-evoked Ca^{2+} response in pancreatic acinar cells. The cells were loaded for 30 min with fura-2−AM and digital imaging of the Ca^{2+}-dependent fluorescence changes was performed as described in Materials and methods. **A**. Digitized bright field micrograph of a small acinar fragment composed of four cells. The resolution of the image is adequate for the localization of the secretory and basolateral poles of each individual cell. Calibration bar 25 μm. **B**. Time-course of changes of the $[Ca^{2+}]_i$ in the small acinus following addition of 0.5 μM ACh (arrow). Two areas were defined on morphological criteria, using the bright-field image shown in A. The areas schematically shown in the inset represent the secretory pole (central region and closed squares on the plot) and the basal pole (peripheral region and open squares on the plot). Each point shows the average $[Ca^{2+}]_i$ value at different time points within each of these two areas. **C**. Digital images of the spatio-temporal evolution of $[Ca^{2+}]_i$ changes during exposure to 0.5 μM ACh. Each frame represents a ratioed (340 nm/380 nm) image, obtained after the subtraction of the respective background image as described in Materials and methods and was pseudo-coloured in accordance with the scale presented. The numbers at the bottom of each image represent the ratio frame number and the time at which it was recorded. Frame a shows $[Ca^{2+}]_i$ distribution in the resting state. Frame b shows the initial increase in $[Ca^{2+}]_i$ in the secretory pole and frames c−g follow the evolution of the $[Ca^{2+}]_i$ signal. When the Ca^{2+} response is fully developed (frame h), $[Ca^{2+}]_i$ is homogenously increased across the acinus. **D**. Digital images of a pancreatic acinar triplet. Frame a, digitized bright-field; frame b, $[Ca^{2+}]_i$ distribution in resting state; frame c, the initial increase of $[Ca^{2+}]_i$ in the luminal pole after CCK (200 pM) stimulation; frames d−h the spreading of the Ca^{2+} signal. Calibration bar, 25 μm.

temporal difference the Ca^{2+} signal evolved in the same manner for both cells, generating in this case a very similar pattern to that described for the pancreatic acinar cells (compare Figure 2C, e–g with Figure 3B, d). When the ACh-evoked Ca^{2+} response is fully developed, $[Ca^{2+}]_i$ appears to be homogenously distributed across the cell cytoplasm (Figure 3B, e).

In lacrimal acinar cells, Sasaki and Gallacher (1990, 1992) have recently found that an increase in $[Ca^{2+}]_i$ can be evoked by extracellular ATP, which activates a receptor-operated cation channel. Figure 3C shows that 1 mM ATP generates a Ca^{2+} signal with spatio-temporal characteristics very similar to the ACh-evoked response in the same doublet (n = 7). The initial increase of $[Ca^{2+}]_i$ in the secretory pole (Figure 3C, b) is followed by a lateral spread (Figure 3C, c) and later by a rise of Ca^{2+} at the basal membranes of both cells (Figure 3C, d). Again, the fully developed agonist response is seen as a homogenous increase of $[Ca^{2+}]_i$ throughout the cytoplasm (Figure 3C, e). A graphic illustration of the increases in $[Ca^{2+}]_i$ as a function of time after agonist stimulation is shown in Figure 3D. In the absence of extracellular Ca^{2+}, stimulation with ATP did not evoke any change of $[Ca^{2+}]_i$ (n = 6) whereas the response to ACh was retained (n = 5).

In order to investigate the specificity of the agonist-evoked initial $[Ca^{2+}]_i$ increase in the secretory pole, we tested the spatial distribution of receptor-independent $[Ca^{2+}]_i$ increases. Thapsigargin (TG), a tumour-promoting sesquiter-

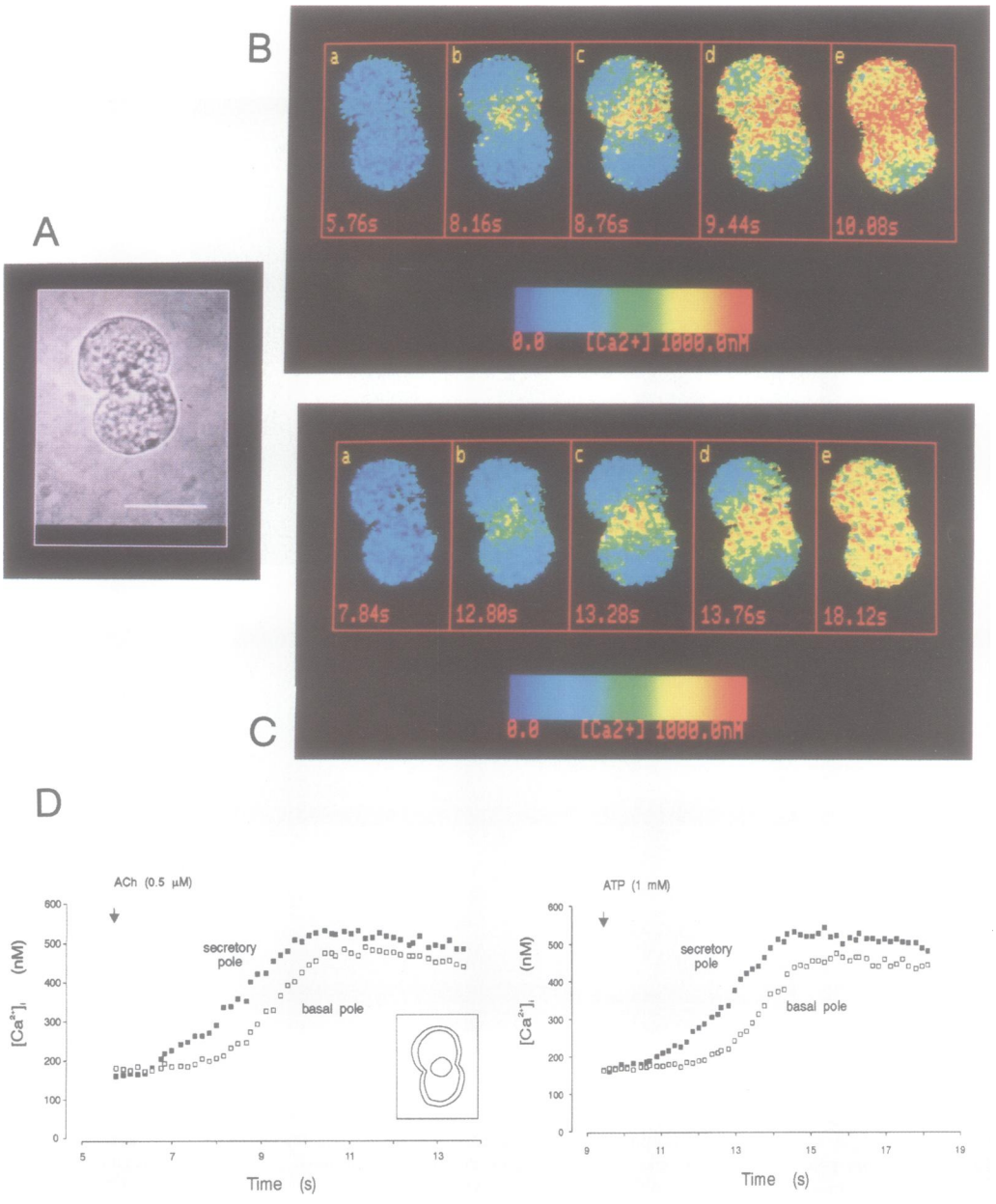

Fig. 3. Effect of Ca^{2+}-mobilizing agonists on the Ca^{2+} signal in lacrimal acinar cells. **A**. Digitized bright-field image of a doublet of acinar cells. Calibration bar, 25 μm. **B**. Evolution of the $[Ca^{2+}]_i$ signal following addition of 0.5 μM ACh: frame a, the homogenous distribution of $[Ca^{2+}]_i$ before agonist admission; frames b–d, generation and spreading of the Ca^{2+} signal; e, fully developed Ca^{2+} response. **C**. Ca^{2+} response evoked by 1 mM ATP in the same doublet of lacrimal cells. Frames a–e represent the same stages as for B. **D**. Time-course of the increase of $[Ca^{2+}]_i$. The analysis was performed as described in the legend to Figure 2. The inset shows schematically the two areas defined for analysis: the central, secretory pole (closed squares) and the peripheral, basal pole (open squares). ACh and ATP were added at the time point indicated by the arrows.

pene lactone, has been shown to raise $[Ca^{2+}]_i$ in various cell types, an action mediated by a specific inhibition of the endoplasmic reticulum Ca^{2+} pumps (Thastrup *et al.*, 1990). Figure 4A compares the spatial distribution of the Ca^{2+} signal evoked by ACh and TG in the same single, isolated pancreatic acinar cell (Figure 4A, a). In contrast to the initiation of the Ca^{2+} response induced by ACh which was clearly limited to the secretory pole (Figure 4B, c), 1 μM TG evoked (n = 8) a diffuse increase in $[Ca^{2+}]_i$ (Figure 4A, e). The rate of increase of $[Ca^{2+}]_i$ induced by TG was much slower than the agonist-evoked increases (<5 s in the case of ACh for the development of the full Ca^{2+} response and >2 min in the case of TG). Another agent used to increase $[Ca^{2+}]_i$ in a non-specific manner was ionomycin (n = 7), an antibiotic which facilitates the transport of Ca^{2+} across the plasma membrane. Figure 4B shows the effect of 0.2 μM ionomycin on a single isolated pancreatic acinar cell (Figure 4B, a) in the presence of 1.1 mM extracellular Ca^{2+}. The increase of $[Ca^{2+}]_i$ evoked by ionomycin (shown in Figure 4B, c) was initially distributed in the peripheral cytoplasm before flooding the whole cell.

Discussion

Our observations using digital imaging fluorescence analysis show that in both lacrimal and pancreatic acinar cells stimulation by several secretagogues evokes a cytoplasmic Ca^{2+} signal with a characteristic spatial organization: an initial increase of $[Ca^{2+}]_i$ in the secretory pole, followed by subsequent increases along the lateral and basal membranes.

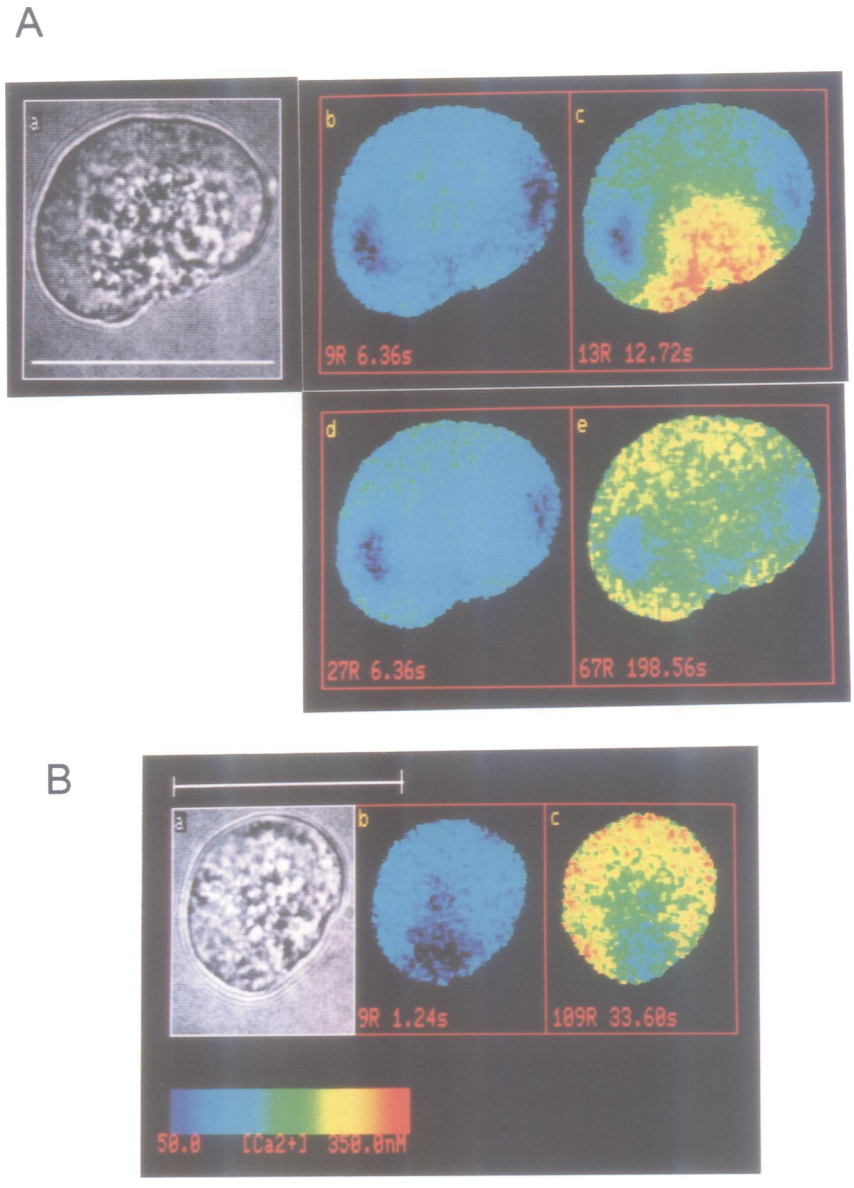

Fig. 4. Spatio-temporal distribution of $[Ca^{2+}]_i$ following increases in $[Ca^{2+}]_i$ evoked by agents which do not involve activation of specific receptors. **A**. Effect of 1 μM thapsigargin on a single, isolated pancreatic acinar cell, shown under bright-field conditions in a. Addition of 1 μM ACh (at time t = 11.5 s) induces a sharply defined increase of $[Ca^{2+}]_i$ in the secretory pole (frame c). Removal of ACh allows $[Ca^{2+}]_i$ to return towards resting levels (frame d), comparable with the prestimulus values (frame b). Addition of thapsigargin (at time t = 164 s) induced a slow increase of $[Ca^{2+}]_i$, diffusely distributed across the cytoplasm (frame e). **B** Effect of 0.2 μM ionomycin, added at time t = 10 s, on the $[Ca^{2+}]_i$. Frame a, bright-field image of a single pancreatic acinar cell; frame b, distribution of $[Ca^{2+}]_i$ before application of ionomycin; and frame c, basolateral increase of $[Ca^{2+}]_i$ after addition of ionomycin. The pseudo-colour scale applies to both figures.

This spatial pattern of Ca^{2+} signal generation appears to be triggered specificially by receptor-dependent mechanisms, since agents which induce a non-specific increase of $[Ca^{2+}]_i$ such as thapsigargin or ionomycin only evoke a diffuse increase in the cytoplasmic Ca^{2+} concentration.

ACh and CCK receptors on pancreatic acinar cells are functionally coupled by different G proteins to phospholipase C (Schnefel *et al.*, 1988) and activation of both these receptors therefore results in the formation of the Ca^{2+} releasing messenger inositol-1,4,5-trisphosphate ($InsP_3$) (Streb *et al.*, 1985; Berridge, 1987). Although the precise pattern of cytoplasmic Ca^{2+} signals evoked by submaximal ACh and CCK concentrations differ (Petersen *et al.*, 1991a; Yule *et al.*, 1991) both agonists primarily evoke release of Ca^{2+} from intracellular pools and these responses can also be observed in the absence of external Ca^{2+} (Yule and Gallacher, 1988; Wakui *et al.*, 1989; Yule *et al.*, 1991; Wakui *et al.*, 1991). Both the CCK and ACh-evoked Ca^{2+} signals are inhibited by the $InsP_3$ antagonist heparin (Wakui *et al.*, 1990, 1991), and direct intracellular infusion of $InsP_3$ or its non-metabolizable analogue inositol-1,4,5-trisphosphorothioate ($InsPS_3$) can, depending on the concentration used, generate various cytoplasmic Ca^{2+} signal patterns similar to those evoked by different ACh and CCK concentrations (Petersen *et al.*, 1991b). In this study we have deliberately chosen concentrations of ACh and CCK that give rapidly developing and sustained responses and have shown that both these agonists primarily evoke Ca^{2+} release into the cytosol in the secretory pole of the cells, further emphasizing the basic similarity in the actions of these two agonists. In the lacrimal acinar cells, the effect of ACh is clearly also mediated by $InsP_3$ (Evans and Marty, 1986; Changya *et al.*, 1989). However, the present results show that the initial mobilization of Ca^{2+} from the Ca^{2+} pools located in the secretory pole is not a phenomenon restricted to the effect of agonists which generate $InsP_3$. Recent studies (Sasaki and Gallacher, 1990, 1992) show that in lacrimal acinar cells, extracellular ATP directly activates a receptor-operated cation channel, permeable to both Na^+ and Ca^{2+}. This is the most likely mechanism responsible for the ATP-evoked elevation of $[Ca^{2+}]_i$ demonstrated by direct measurements of $[Ca^{2+}]_i$ in parotid acinar cells (McMillan *et al.*, 1988) and murine thymocytes (El-Moatassim *et al.*, 1989), since measurements of phosphoinositide metabolism in parotid acinar cells indicate that the elevations of $[Ca^{2+}]_i$ that result from ATP stimulation appear in the absence of $InsP_3$ production (McMillan *et al.*, 1988).

We have previously shown that intracellular Ca^{2+} infusion in pancreatic acinar cells evokes local cytosolic Ca^{2+} spikes in the immediate vicinity of the Cl^--conducting plasma membrane which is most likely the luminal cell membrane (Osipchuk *et al.*, 1990; Petersen, 1992). These Ca^{2+}-induced spikes do not depend on $InsP_3$ receptor activation since they can also be induced in the presence of intracellular heparin (Wakui *et al.*, 1990). In lacrimal acinar cells there is evidence that the ACh-evoked Ca^{2+} signal is due to $InsP_3$-mediated Ca^{2+}-induced Ca^{2+} release (Marty and Tan, 1989). Both ACh (acting via $InsP_3$ formation) and ATP (opening Ca^{2+}-permeable non-selective cation channels) (Sasaki and Gallacher, 1990, 1992) would therefore most likely evoke the observed rise in $[Ca^{2+}]_i$ in the secretory pole via Ca^{2+}-induced Ca^{2+} release. The stores in the secretory pole might have a higher Ca^{2+} sensitivity than in other parts of the cell. Furthermore, CCK receptors are localized not only along the basal membranes, but they are also found deep in the lateral space between two adjacent cells, close to the junctional area between the lateral and apical membrane (Rosenzweig *et al.*, 1983). Since it is probable that other receptors would have a similar distribution and that in the isolated acinar preparation large areas of lateral membranes are exposed to extracellular fluid, it is conceivable that both in the case of ACh and ATP the increase of $[Ca^{2+}]_i$ in the secretory pole could be evoked by activation of regional receptors. However, it is clear from the experiments with thapsigargin and ionomycin, that a slow increase of cytoplasmic Ca^{2+} is not sufficient to evoke a burst of Ca^{2+} release from the secretory pole. It is known that the time-course of Ca^{2+} entry or $[Ca^{2+}]_i$ rise is crucial in evoking Ca^{2+}-induced Ca^{2+} release (Fabiato, 1985; Marty and Tan, 1989).

There is considerable heterogeneity of intracellular Ca^{2+} stores in non-muscle cells (Meldolesi *et al.*, 1990) and the existence of at least two different types of rapidly exchanging Ca^{2+} pools sensitive to either $InsP_3$ or caffeine (and ryanodine) has been inferred from studies on a number of different cell types including exocrine acinar cells (Malgaroli *et al.*, 1990; Osipchuk *et al.*, 1990; Dehlinger-Kremer *et al.*, 1991; Foskett and Wong, 1991). The secretory granules are clearly the most conspicuous organelles in the secretory pole of exocrine acinar cells and contain high amounts of Ca^{2+} (Meldolesi *et al.*, 1990), but at least in PC12 cells Ca^{2+} can only be released from this pool by the combination of ionomycin with a substance that collapses intracellular pH gradients such as monensin or NH_4Cl (Fasolato *et al.*, 1991). The secretory granules are therefore an unlikely source for the Ca^{2+} released into the cytosol in the secretory pole.

In bovine adrenal chromaffin cells it has been shown that $InsP_3$-mobilizing agonists evoke a polarized cytoplasmic Ca^{2+} signal (O'Sullivan *et al.*, 1989) and that secretion can only occur in the cell pole experiencing the rise in $[Ca^{2+}]_i$, whereas nicotinic receptor activation, inducing a rise in $[Ca^{2+}]_i$ along the whole of the plasma membrane, evokes secretion from the entire surface of the cells (Cheek *et al.*, 1989). In the exocrine acinar cells where secretion normally only occurs in the luminal pole (Palade, 1975) we have now shown that the primary receptor-activated cytoplasmic Ca^{2+} signal is generated at this location and then preferentially spreads along the rest of the surface cell membrane. The Ca^{2+} signal is therefore initially directed towards acutely regulated sites in the cell allowing a rapid activation of exocytosis and opening of ion channels in both the luminal and basolateral membranes.

Materials and methods

Preparation of cells

A mixture of individual cells and small clusters of acinar cells were obtained from isolated mouse pancreas and lacrimal glands by enzymatic dispersion in a water bath at 37°C as previously described (Wakui *et al.*, 1989; Sasaki and Gallacher, 1990). The intact glands were injected with a collagenase solution (200 units/ml, Worthington) and in most experiments with isolated pancreas, 2 mg/ml trypsin inhibitor was used. The glands were incubated for 25–30 min, with regular vigorous manual agitation every 5–10 min. At the end of the incubation stage the glandular structure was loosened and final manual agitation yielded the isolated cell preparation, which was subsequently washed twice with a solution (NaHEPES) containing NaCl 140 mM, KCl 4.7 mM, $CaCl_2$ 1.1 mM, $MgCl_2$ 1.13 mM, glucose 10 mM and HEPES 10 mM (pH 7.2, adjusted with NaOH).

Intracellular Ca^{2+} imaging

After isolation, cells were resuspended in NaHEPES medium and loaded with fluorescent dye at a concentration of 1 μM fura-2-AM (acetoxymethyl ester) for 30-40 min at room temperature. For facilitating a rapid and homogenous loading (Poenie *et al.*, 1986) the stock fura-2 solution contained 25% Pluronic F127 detergent (w/w in dimethyl sulfoxide, final concentration 0.025%). After loading, the cells were washed and used within the next 3 h.

For investigation, the cells were placed on a glass coverslip attached to an open perspex perifusion chamber and were continuously perifused with NaHEPES solution. The perifusion setup consisted of a set of four polythene tubes (internal diameter: 0.5 mm, Portex, UK) glued together and mounted on a micromanipulator (Yule and Gallacher, 1988).

The hardware used for image capture consisted of a Nikon Diaphot inverted microscope (using either the 40× or 100× quartz oil immersion objective), MagiCal computer hardware (Joyce Loebl, UK) with a dedicated image analysis system (TARDIS) and an intensified charge couple device (CCD) camera (Photonic Science Inc.). Excitation wavelengths were selected by two interference filters centred at 340 and 380 nm (half-bandwidth 10 nm) placed in the lightpath of a 150 W Xenon lamp, and a quartz neutral density filter was used to reduce the excitation light intensity when necessary. The interference filters were mounted on a rotating filter wheel. The rotation of the wheel was driven by the computer and the software allowed changes of the mode of rotation, from continuous video frame rate capture (25 frames/s) to discontinuous mode, in which the interval between successive captures could be varied from 0.5 s to several minutes. The autofluorescence measured in cells not loaded with fura-2 was not significant at the intensifier and camera gain employed. Background subtraction was carried out independently at each of the two wavelengths.

The images were ratioed (340:380) pixel by pixel and the values, proportional to intracellular free Ca^{2+}, were colour-coded by use of a look-up table. Fura-2 fluorescence was calibrated by using cells loaded with the dye and exposed either to 10 mM EGTA or 10 mM Ca^{2+} in the presence of 1.5 μM ionomycin, in a manner similar to that described by Grynkiewicz *et al.* (1985) and using a dissociation constant for Ca^{2+}-fura-2 complex of 224 nM. Bright field images were used to correlate $[Ca^{2+}]_i$ increases with the morphological structures in each experiment. Time dependent changes in mean $[Ca^{2+}]_i$ could be measured over the whole cell or over any region of interest by defining, with a light pen, a pixel data set.

All experiments were carried out at ambient temperature (25-27°C).

Materials

All chemicals were purchased from BDH and Sigma (UK), with the exception of ionomycin and thapsigargin which were obtained from Calbiochem and fura-2/AM and Pluronic F-127, obtained from Molecular Probes.

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