Ca\textsuperscript{2+} dynamics in salivary acinar cells: distinct morphology of the acinar lumen underlies near-synchronous global Ca\textsuperscript{2+} responses

Olga Larina and Peter Thorn*
Department of Pharmacology, University of Cambridge, Tennis Court Road, Cambridge, CB2 1PD, UK

*Author for correspondence (e-mail: pt207@cam.ac.uk)

Accepted 9 June 2005
Journal of Cell Science 118, 4131-4139 Published by The Company of Biologists 2005
doi:10.1242/jcs.02533

Summary
In salivary acinar cells, the pattern of the Ca\textsuperscript{2+} signals that regulates fluid and enzyme secretion has yet to be resolved, as there are conflicting reports in the literature. We have used a two-photon technique to directly visualize the acinar cell lumen in living fragments of exocrine tissue and simultaneously recorded agonist-induced changes in intracellular Ca\textsuperscript{2+}. We show near-synchronous global Ca\textsuperscript{2+} responses in submandibular acinar cells, distinct from the typical apical to basal Ca\textsuperscript{2+} wave usually seen in rodent pancreatic acinar cells. In an effort to explain the basis of these near-synchronous global Ca\textsuperscript{2+} responses we used immunocytochemical experiments to localize luminal proteins and inositol trisphosphate receptors (InsP\textsubscript{3}Rs) in tissue fragments. Zona occludens 1 (ZO-1), a tight junction protein, shows that individual submandibular acinar cells are often nearly completely encircled by a narrow luminal structure. By contrast, in pancreatic fragments, ZO-1 staining shows short luminal branches terminating abruptly at the apical pole of single acinar cells. Co-immunostaining of InsP\textsubscript{3}Rs type 2 and type 3 showed them in the same region as ZO-1 in both exocrine tissues. Functional experiments showed that the near-synchronous global Ca\textsuperscript{2+} responses were still observed in the absence of extracellular Ca\textsuperscript{2+} and also in the presence of ryanodine. We conclude that the elaborate luminal region of submandibular cells leads to a hitherto unrecognized extensive distribution of InsP\textsubscript{3}Rs in a band around the cell and that this underlies the near-synchronous global Ca\textsuperscript{2+} response to agonists. We suggest that this may be a structural adaptation in submandibular cells to support the copious amounts of fluid secreted.

Supplementary material available online at http://jcs.biologists.org/cgi/content/full/118/18/4131/DC1

Key words: Acinar, Salivary gland, Calcium, Lumen

Introduction
The importance of intracellular Ca\textsuperscript{2+} in stimulus-secretion coupling in secretory epithelial cells has been long recognized (Matthews et al., 1973; Hunter et al., 1983; Petersen, 1992). The Ca\textsuperscript{2+} response is primarily the release of Ca\textsuperscript{2+} from intracellular stores and shows complex variations in time and space. Ca\textsuperscript{2+} oscillations in epithelia were first shown in parotid acinar cells (Gray, 1988) and since Kasai and Augustine’s seminal paper on pancreatic acinar cells (Kasai and Augustine, 1990) an apical initiation and basal propagation of Ca\textsuperscript{2+} waves has become dogma in the field (Thorn et al., 1993; Toescu et al., 1992; Elliot et al., 1992; Lee et al., 1997). However, it is not clear if this is the case for salivary acinar cells where the patterns of Ca\textsuperscript{2+} signals in are still not resolved. Some report apical-to-basal Ca\textsuperscript{2+} waves are either not seen [parotid (Dissing et al., 1990)] or are very rapid, giving a near simultaneous Ca\textsuperscript{2+} response across the cell (Giovannucci et al., 2002; Takemura et al., 1999; Liu et al., 1998). By contrast, others report Ca\textsuperscript{2+} waves [submandibular (Lee et al., 1997; Harmer et al., 2005), parotid (Tojyo et al., 1997)] apparently similar to those seen in pancreatic acinar cells. A resolution of these different observations is essential to our general understanding of how Ca\textsuperscript{2+} signals are generated and how they regulate secretion.

There are two major problems with previous imaging studies of Ca\textsuperscript{2+} in polarized epithelia. Firstly, most use preparations of isolated cells, and small clusters of cells (<10 cells), where the characteristic morphology of polarized acinar cells is compromised because of the severing of tight junctions during cell isolation (Park et al., 2004). Secondly, the acinar lumen is not normally visible with light microscopy, so the apical domain is often identified simply as the region containing secretory granules. This distinction is functionally important; the luminal plasma membrane is the only region where exocytosis takes place and, in the generation of Ca\textsuperscript{2+} signals, inositol triphosphate receptors (InsP\textsubscript{3}Rs) are highly enriched, specifically in the endoplasmic reticulum immediately adjacent to the acinar lumen (Lee et al., 1997; Yule et al., 1997; Zhang et al., 1999).

We have overcome both of these problems using live-cell two-photon microscopy. Ca\textsuperscript{2+} responses from single cells, within large fragments of exocrine tissue that retain the morphological features of the intact gland (Park et al., 2004), were recorded with Fura-2. We visualized the ducts and acinar lumen by the simultaneous imaging of an extracellular fluorescent dye (Thorn et al., 2004). Our experiments compare salivary and pancreatic tissue and
show that in submandibular acinar cells agonists evoke a near-synchronous Ca\(^{2+}\) rise across the cell comparable to the apical to basal Ca\(^{2+}\) waves seen in pancreatic acinar cells. We show the acinar lumen in submandibular tissue is significantly more extensive than in pancreas, often leading to an encircling of single acinar cells with a band of InsP\(_3\)Rs. This hitherto unrecognized three-dimensional organization of InsP\(_3\)Rs explains the near-synchronous global Ca\(^{2+}\) signals in submandibular acinar cells, and the extensive luminal area is probably an adaptation in these cells that secrete copious amounts of fluid.

### Materials and Methods

#### Cell preparation

Lobules and fragments (~50-100 cells) of mouse submandibular gland were prepared by collagenase digestion in normal Na\(^+\)-rich extracellular solution using the method of Thorn et al. (Thorn et al., 1993) modified to reduce the time in collagenase and limit mechanical trituration and cells were then plated onto poly-L-lysine-coated glass coverslips.

Na\(^+\)-rich extracellular solution contained (mM): 135 NaCl, 5 KCl, 1 MgCl\(_2\), 10 Hepes, 10 glucose, 2 CaCl\(_2\); pH 7.4. K\(^+\)-rich solution (mM): 5 NaCl, 135 KCl, 1 MgCl\(_2\), 10 Hepes, 10 glucose, 2 CaCl\(_2\); pH 7.4.

#### Immunofluorescence

Cells attached to glass coverslips were washed in PBS, fixed in methanol for 10 minutes at –20°C. After 1 hour in 2% donkey serum plus 2% fish skin gelatin in PBS, cells were incubated in primary antibody for 1 hour and then secondary antibodies for 30 minutes. The antibody dilutions were as follows: InsP\(_3\)R2 (pAb; Chemicon, Temecula, CA, USA); 1:20; InsP\(_3\)R3 (mAb; BD Transduction Laboratories, San Jose, CA, USA) 1:100; ZO-1 (mAb; Zymed Laboratories Inc., USA; 1:75 dilution), which showed no significant conjugated donkey anti-mouse IgG (Jackson ImmunoResearch Laboratories Inc., USA; 1:200 dilution) and FITC-conjugated goat anti-mouse IgM (Jackson ImmunoResearch Laboratories Inc., USA; 1:200 dilution) and fluorescence decreases in response to acetylcholine (ACh), consistent with a Ca\(^{2+}\) rise. However, experiments with submandibular tissue often failed to show a decrease in Fura-2 fluorescence. We postulated that a reduction in cell volume, and subsequent concentration of dye, might be a major factor contributing to the observed fluorescence changes.

#### Single-cell fluorescence measurements

Cell fragments, loaded with Indo-1 AM for 30 minutes were washed, and plated onto coverslips. Single cells were illuminated (Nikon Diaphot inverted microscope, 40\(\times\) oil immersion NA 1.3 objective) at 360 nm and emitted light collected at 410-430 nm and 430-600nm. The ratio of the two intensities was calibrated and expressed as Ca\(^{2+}\) concentration (Phocal, Strathclyde, UK).

Student's t-tests were used to test for significance and the probability (P) is quoted.

#### Results

Large volume changes in submandibular acinar cells

Lobules and fragments of exocrine tissue, loaded with Fura-2AM, were imaged on a two-photon microscope. With two photon excitation at 810 nm, elevated Ca\(^{2+}\) decreases Fura-2 emission measured at 490-540 nm (see Nemoto et al., 2001). Our experiments with pancreatic tissue showed large Fura-2 fluorescence decreases in response to acetylcholine (ACh), consistent with a Ca\(^{2+}\) rise. However, experiments with submandibular tissue often failed to show a decrease in Fura-2 fluorescence. We postulated that a reduction in cell volume, and subsequent concentration of dye, might be a major factor contributing to the observed fluorescence changes.

Fig. 1. Agonist-evoked shrinkage of submandibular acinar cells; effect of high extracellular K\(^+\). A control cluster of submandibular acinar cells (A; approx. eight cells), washed in Na\(^+\)-rich extracellular medium containing the fluorescent dye, SRB and imaged with two-photon microscopy. (B) Fluorescent dye is excluded from the cell cytosol and dramatic decreases in cells volume in response to 10 \(\mu\)M ACh can readily be observed (B). (C) Superfusion with a K\(^+\)-rich extracellular solution (trace i, black) prevented cell swelling but had no effect on the early intracellular Ca\(^{2+}\) response to ACh compared with control (trace ii, grey) as measured in Indo-1 loaded cells. Scale bar: 10 \(\mu\)m.
problem in imaging submandibular acinar cells. To test this idea we used the two-photon microscope to image extracellular fluorescent dye (OG or SRB) and define the outside edge of cells (Fig. 1A). Apparent changes in cell volume were then determined assuming symmetrical cell dimensions. Stimulation of submandibular cells with 10 μM ACh (Fig. 1B) significantly decreased cell volume by 28.69±5.87% (mean±s.e.m., n=17, P<0.01). By contrast, ACh stimulation of pancreatic acinar cells did not produce a cell volume change (–0.24±0.84%, n=12, P=0.85; data not shown). To limit volume changes in submandibular cells we applied a K+-rich extracellular solution; a condition expected to depolarize the cell and limit ion movements across the plasma membrane. Under these conditions the ACh-induced cell volume decrease in submandibular acinar cells was considerably attenuated; only a 6.54±1.71% decrease was observed (n=15).

The effect of K+-rich extracellular solution on the Ca2+ signal was then determined in cells loaded with the ratiometric Ca2+-sensitive dye Indo-1, a method chosen because the ratio is independent of changes in cell volume. We observed no significant difference in the peak Ca2+ response to 10 μM ACh (Fig. 1C, 0.69±0.15 μM n=10, in control; 0.75±0.25 μM, n=6 in K+-rich solution, P=0.4). However, the plateau Ca2+, measured at 300 seconds after the peak, was significantly smaller (0.21±0.03 μM, n=10 control; 0.10±0.01 μM in K+-rich solution, n=9, P<0.01) consistent with cell depolarization reducing the driving force for Ca2+ entry. Since the initial Ca2+ response was unaffected, all subsequent experiments were carried out in K+-rich solutions.

Near-synchronous global Ca2+ responses in submandibular acinar cells
Release of Ca2+ through InsP3Rs is the major trigger for Ca2+ waves in acinar tissue. InsP3Rs are found throughout the cell (Fogarty et al., 2000) but highly enriched in the endoplasmic reticulum adjacent to the luminal plasma membrane (Lee et al., 1997; Yule et al., 1997; Zhang et al., 1999). To identify the luminal region in living cells we added the fluorescent probe SRB to the extracellular media. The effective optical slice (~1 μm depth) of two-photon excitation enabled visualization of SRB fluorescence in the ducts and acinar lumen.

Fig. 2. Simultaneous identification of acinar lumens and agonist-evoked intracellular Ca2+ changes show near-synchronous global Ca2+ signals in submandibular acinar cells. Extracellular SRB fluorescence and intracellular Fura-2 fluorescence were recorded with two-photon microscopy to identify the acinar lumen and intracellular Ca2+ changes, respectively, in pancreatic fragments (10 μM ACh; A,B) and submandibular fragments (10 μM ACh C,D; 300 nM ACh; E,F). All Fura-2 self ratio images have been overlaid with a binary mask (in white) obtained from the SRB image. (A) In the top left panel, SRB outlines pancreatic acinar cells (image shows approx. seven cells on the edge of a tissue fragment) and fills the acinar lumens allowing placement of ROIs on the apical (ROI 1) and basal (ROI 2) pole of the cell. The pseudocolour images show ACh-induced Fura-2 self-ratio changes taken at the time points (i, ii, iii) shown on the graphs in B, which plot the average Fura-2 self-ratio changes over time in the ROIs. (C) In the top left panel, SRB outlines submandibular acinar cells (image shows approx. five cells on the edge of a tissue fragment). Acinar lumens are apparently elongated structures. ROIs were placed in the narrow, apparent apical pole of the cell (ROI 1) and wider, apparent basal (ROI 2) pole of the cell. The pseudocolour images show 10 μM ACh-induced Fura-2 self-ratio changes taken at the time points (i, ii, iii) shown on the graphs in D, which plot the average Fura-2 self-ratio changes over time in the ROIs. (E) In the top left panel, SRB outlines submandibular acinar cells (image shows approx. six cells on the edge of a tissue fragment). The pseudocolour images show 300 nM ACh-induced Fura-2 self-ratio changes taken at the time points (i, ii, iii) shown on the graphs in F, which plot the average Fura-2 self-ratio changes over time in the ROIs. Scale bars: 10 μm.
simultaneously with measurements of Fura-2 fluorescence inside acinar cells (Fig. 2A).

Within pancreatic fragments the acinar lumen, filled with fluorescent dye, appeared as a discrete region, usually close to the narrowest (apical) pole of the cell (Fig. 2A). Application of 10 μM ACh invariably led to a Ca\textsuperscript{2+} wave that was measured as the spread of the Ca\textsuperscript{2+} signal from a region of interest (ROI) placed adjacent to the acinar lumen to a ROI placed in a distant region in the basal pole (Fig. 2B, apparent wave velocity of 9.5±1.67 μm/second, \( n=30 \)). By contrast, in submandibular fragments the acinar lumen often appeared as elongated regions not especially close to the narrow pole of the cell (Fig. 2C). ACh always elicited a Ca\textsuperscript{2+} response but obvious Ca\textsuperscript{2+} waves were not observed. Instead the Ca\textsuperscript{2+} signal in ROIs placed close to the apparent acinar lumen was nearly synchronous with those in ROIs placed on the opposite side of the cell (Fig. 2D, apparent wave velocity of 31.4±2.98 μm/second, \( n=39 \); significantly faster than the apparent wave velocity in pancreas \( P<0.01 \)).

It has previously been shown in parotid acinar cells that the Ca\textsuperscript{2+} wave velocity slows at lower concentrations of agonist (Tojyo et al., 1997). This is probably due to a decrease in excitability when lower concentrations of InsP\textsubscript{3} are generated. It is therefore possible that submandibular acinar cells might generate more InsP\textsubscript{3} than pancreatic cells, through possession of either more cell-surface receptors or a more efficient signal cascade and this in turn might generate faster Ca\textsuperscript{2+} waves. We therefore tested the effects of lowered agonist concentrations in submandibular cells. In our hands 100 nM and 200 nM ACh rarely induced Ca\textsuperscript{2+} waves (2/15 cell clusters and 2/10 cell clusters, respectively). Although some cells did show noisy fluorescence signals, which might be indicative of localized Ca\textsuperscript{2+} responses as recently reported in submandibular cells (Harmer et al., 2005). At 300 nM ACh, Ca\textsuperscript{2+} waves were seen (9/15 cell clusters) and analysis of the responses showed an apparent Ca\textsuperscript{2+} wave velocity of 18.5±2.51 μm/second (\( n=29 \), bars: 10 μm). We conclude that even at a concentration of ACh that is threshold for inducing a Ca\textsuperscript{2+} wave, the apparent wave velocity in submandibular cells is still much faster (nearly double) than that in pancreatic acinar cells, consistent with a fundamental difference in the Ca\textsuperscript{2+} signalling machinery in the salivary cells.

The acinar lumen is highly elaborated in submandibular cells

SRB labelling of the acinar lumen suggested that submandibular tissue might have a much more extensive, luminal structure than that of pancreatic tissue. Indeed early studies on salivary glands describe extensions to the main acinar lumen as intercellular canaliculi running between the cells with a morphology similar to the main acinar lumen (Tamarin and Sreebny, 1965). In subsequent studies intercellular regions were immunostained positive for InsP\textsubscript{3}Rs and aquaporins (Lee et al., 1997; Matsuzaki et al., 1999; Takemura et al., 1999). However, these studies employed thin tissue sections in which three-dimensional structure is lost. To directly compare the luminal structures in pancreatic and submandibular tissue we therefore used tissue fragments that retain the three-dimensional structure of the intact gland. To identify the acinar lumen we immunostained for the tight junction protein zona occludens (ZO-1). In tissue fragments matched for the number of nuclei (stained with Hoechst 33358) we found a clear difference in the staining pattern of ZO-1 between the pancreas and the submandibular gland. In submandibular glands ZO-1 was much more extensively arborized compared with the simple branching structure found in the pancreas (Fig. 3A). Comparing magnified images of secretory endpieces, ZO-1 staining is apparent as a thin band (presumably, in reality, a tube) that in pancreatic tissue ends at a discrete region in one pole of the cells (Fig. 3B) but in submandibular tissue wraps around and almost completely encircles individual acinar cells (Fig. 3C,D). We quantified these differences, measuring the length of the terminal branches stained with ZO-1 (i.e. the last branch onto individual acinar cells). In pancreatic tissue branches were 6.44±0.35 μm in length (\( n=28 \)), significantly shorter than branches in submandibular tissue, which were 11.67±0.64 μm (\( n=62 \)) in length (\( P<0.01 \); see Movies 1 and 2 in supplementary material).

The elaborated acinar lumen in submandibular cells is enriched in InsP\textsubscript{3}R

Our use of tissue fragments and three-dimensional reconstruction methods contrast the complexity of the elaborated lumens in submandibular tissue with the relatively simple structures found in the pancreas. To test for differences in InsP\textsubscript{3}R distribution we immunostained tissue fragments for InsP\textsubscript{3}R-type 3 (InsP\textsubscript{3}R3)
and counter immunostained for ZO-1. Once again, submandibular cells (Fig. 4B) showed elaborate ZO-1 staining compared with that in pancreatic acinar cells (Fig. 4A). In both tissues immunostaining for InsP$_3$R3 localized closely with ZO-1 staining. InsP$_3$R2 is also present in salivary acinar cells (Lee et al., 1997; Takemura et al., 1999) and here we show that immunostaining for InsP$_3$R2 directly corresponds to immunostaining for InsP$_3$R3 (Fig. 4C). This indicates that the whole length of the ZO-1 structures in both tissues is associated with InsP$_3$Rs. To determine if other luminal proteins are associated with ZO-1, we also immunostained for aquaporin-5 (AQP-5) (Matsuzaki et al., 1999), which was also found to follow the elaborated acinar lumens in submandibular cells (data not shown).

The near-synchronous global Ca$^{2+}$ responses of submandibular cells are probably due to encircling of cells with InsP$_3$Rs

Since a band of InsP$_3$Rs nearly encircle submandibular acinar cells then this would provide an explanation for the near-synchronous Ca$^{2+}$ increases across the cell. However, other possible explanations might include a role for Ca$^{2+}$ influx. Ca$^{2+}$ entry might be a larger component of the response in submandibular cells than in pancreatic acinar cells and as a consequence the Ca$^{2+}$ wave might appear faster. However, two-photon experiments under the same conditions as shown in Fig. 2, but in the absence of extracellular Ca$^{2+}$, still showed a near-synchronous Ca$^{2+}$ rise across the cell (Fig. 5A, Ca$^{2+}$ wave velocity 24.30±3.85 μm/second, n=23).

Another possible difference is that ryanodine receptors (RYRs), thought to be present in acinar cells [submandibular (Lee et al., 1997), parotid (Zhang et al., 1999), pancreatic (Thorn et al., 1994)] may be differentially recruited in the two tissues. However, after pre-incubation of the cells in 20 μM ryanodine (RY), a concentration known to block RYRs, we still observed near-synchronous Ca$^{2+}$ increases across the cell induced by the application of 10 μM ACh (Fig. 5B, apparent Ca$^{2+}$ wave velocity 21.20±2.04 μm/second, n=52).

To further investigate a potential role for RYRs we conducted experiments with 200 mM ryanodine (observed apparent wave velocity 29.92±3.21 μm/second, n=18) and with ryanodine from another source (Tocris) at 20 μM (observed apparent wave velocity 30.94±2.81 μm/second, n=26) and 200 μM (observed apparent wave velocity 28.27±3.48 μm/second, n=16). In none of these experiments was there evidence for an action of ryanodine on the response induced by 10 μM ACh.

These experiments support the idea that it is the distribution of InsP$_3$Rs that underlies the faster wave velocities seen in submandibular cells.
Isolated single cells show slower apparent Ca\(^{2+}\) wave velocities

Our data is in contradiction to previous work on submandibular cells, in which slower Ca\(^{2+}\) wave velocities were recorded (Lee et al., 1997). One possible explanation is that previous studies often use single cells and clusters of small numbers of cells. To test for this possibility we measured Ca\(^{2+}\) responses from single submandibular cells. Our results showed Ca\(^{2+}\) responses to 10 \(\mu M\) ACh with an apparent Ca\(^{2+}\) wave velocity of 18.80±4.17 m/second (\(n=15\), Fig. 6). This is significantly slower than that recorded in tissue fragments, suggesting that the procedure used to isolate single cells may result in damage to the signal transduction machinery.

Discussion

Our imaging methods have allowed us to identify the acinar cell lumen in living exocrine tissue fragments and to simultaneously record acinar cell Ca\(^{2+}\) signal characteristics. In pancreatic tissue the lumen abuts the apical region of the cell and single acinar cells showed the typical agonist-evoked apical to basal Ca\(^{2+}\) waves. By contrast, in submandibular tissue the lumen was more extensive and was even found close to the apparent basal pole. In submandibular cells, agonists evoked near-synchronous global Ca\(^{2+}\) signals.
To investigate the basis of the differences in the Ca\textsuperscript{2+} signal between the two tissues we immunostained fixed tissue fragments. Immunostaining for the tight junction protein, ZO-1, defined the acinar lumen in pancreatic tissue as a branching system that terminates abruptly at the narrow apical pole of acinar cells. By contrast, in submandibular tissue ZO-1 immunostaining was much more extensive and single acinar cells were nearly encircled by a band of ZO-1 staining. These patterns of staining in the two tissues are entirely consistent with our live-cell two-photon imaging of the exocrine lumen with extracellular dyes. Immunostaining for InsP\textsubscript{3}R2 and InsP\textsubscript{3}R3 showed that both colocalize with ZO-1, as does AQP-5, indicating that the elaborated luminal structures of the submandibular acinar cells are probably of functional importance in generating the Ca\textsuperscript{2+} signal and in fluid secretion.

We show that the fast apparent Ca\textsuperscript{2+} wave velocity in submandibular acinar cells is retained in the absence of extracellular Ca\textsuperscript{2+} and also in the presence of ryanodine. These observations show that neither Ca\textsuperscript{2+} influx nor RYRs are important in the generation of the rapid global Ca\textsuperscript{2+} response and strongly implicate the more extensive distribution of InsP\textsubscript{3}Rs in submandibular acinar cells as the fundamental factor in generating the fast Ca\textsuperscript{2+} wave. We conclude that the elaborated lumen and the near-encircling of the submandibular acinar cell with InsP\textsubscript{3}Rs initiates the Ca\textsuperscript{2+} response at multiple sites around the cell, giving rise to a much higher apparent Ca\textsuperscript{2+} wave velocity than in the pancreas.

Comparative anatomy of the acinar lumen in submandibular and pancreatic tissue

Two independent lines of evidence, imaging of extracellular dyes and immunostaining of ZO-1, show a much more extensive acinar lumen in submandibular than in pancreatic tissue. This data is absolutely consistent with observation of intercellular canaliculi in salivary acinar cells that have been reported previously (Tamarin and Sreebny, 1965; Matsuzaki et al., 1999). However, to our knowledge, no direct comparative studies across different exocrine tissues has previously been performed, and it is this side-by-side comparison of submandibular with pancreatic tissue that highlights the contrasting anatomy of the two exocrine glands. These differences are further highlighted in our study through our use of 3D reconstruction techniques (see Fig. 4 and Movies 1 and 2 in supplementary material) where the near-encircling of submandibular acinar cells with InsP\textsubscript{3}Rs is immediately apparent in a way not obvious in single thin sections.

Ca\textsuperscript{2+} wave velocity measurements

The dramatic tissue-type differences we see in Ca\textsuperscript{2+} wave velocity (9.5 \mu m/second in pancreatic and 31.4 \mu m/second in submandibular tissue) are consistent with most previous reports (Thorn et al., 1992; Giovannucci et al., 2002; Takemura et al., 1999; Liu et al., 1998; Tojo et al., 1997). In other reports, slower Ca\textsuperscript{2+} waves were seen in submandibular tissue (Lee et al., 1997; Harmer et al., 2005). In Harmer et al.’s (Harmer et al., 2005) study this may be due to the relatively high imposed Ca\textsuperscript{2+} buffering in their patch-clamp experiments (500 \mu m EGTA plus 100 \mu m Fura-2) which would tend to slow wave velocities (see Kidd et al., 1999) and in Lee et al.’s (Lee et al., 1997) study it may be due to the use of small clusters of acinar cells, which we show does slow Ca\textsuperscript{2+} wave speeds (Fig. 6).

What underlies the higher apparent Ca\textsuperscript{2+} wave velocity in salivary acinar cells?

Giovannucci et al. (Giovannucci et al., 2002) have explicitly investigated tissue differences between pancreatic and parotid glands. They showed that the parotid gland acinar cell Ca\textsuperscript{2+} response was more sensitive than the pancreas cells to small increases in InsP\textsubscript{3} concentration: low concentrations of InsP\textsubscript{3} that induced local Ca\textsuperscript{2+} responses in pancreatic acinar cells induced global Ca\textsuperscript{2+} responses in the parotid acinar cells. They suggested that these differences might be due to different levels of InsP\textsubscript{3}R expression and showed that the parotid gland contained fourfold more InsP\textsubscript{3}Rs, as measured by radioligand binding and western blotting experiments than the pancreas (Giovannucci et al., 2002). This data is entirely consistent with our own observations. It is likely that the extended distribution of InsP\textsubscript{3}Rs we report in salivary glands, distributed along the extended acinar lumen, requires the higher levels of receptor expression seen by Giovannucci et al. (Giovannucci et al., 2002).

However, alone, these lines of evidence do not directly link InsP\textsubscript{3}R number and distribution with the differences in the Ca\textsuperscript{2+} response between pancreatic and salivary acinar cells. What we have done is to directly demonstrate that neither Ca\textsuperscript{2+} influx nor possible Ca\textsuperscript{2+} release from RYR-sensitive Ca\textsuperscript{2+} stores can explain the fast Ca\textsuperscript{2+} wave in submandibular cells. Since RYRs are known to be present in salivary acinar cells (Lee et al., 1997; Zhang et al., 1999) it might be expected that they do play a role. However, we have previously shown in pancreatic acinar cells that ryanodine is most effective in blocking the Ca\textsuperscript{2+} responses induced by low agonist concentrations (Thorn et al., 1994). This presumably is a circumstance where relatively modest levels of InsP\textsubscript{3} only partially recruit the InsP\textsubscript{3}R pool and where the total Ca\textsuperscript{2+} response may depend on a parallel recruitment of RYRs. By contrast, at high agonist concentrations, sufficient InsP\textsubscript{3} is generated to recruit the total InsP\textsubscript{3}R pool and the majority of the Ca\textsuperscript{2+} response will therefore be derived from the InsP\textsubscript{3}Rs. Whatever the explanation, ryanodine, even at the extremely high concentration of 200 \mu M, failed to affect the Ca\textsuperscript{2+} response of submandibular cells (Fig. 5) and we can conclude that the differences in the Ca\textsuperscript{2+} signal between the pancreas and submandibular acinar cells are therefore most probably dependent on the different distributions of the InsP\textsubscript{3}Rs.

What we are actually observing when we measure Ca\textsuperscript{2+} wave velocities is not clear. The measured Ca\textsuperscript{2+} wave velocity is much higher than would be expected by simple Ca\textsuperscript{2+} diffusion. These high velocities are therefore likely to be a reflection of either the regenerative spread of Ca\textsuperscript{2+} along InsP\textsubscript{3}Rs by the positive feedback process of Ca\textsuperscript{2+}-induced Ca\textsuperscript{2+} release (CICR active on InsP\textsubscript{3}Rs) or the temporally coordinated release of Ca\textsuperscript{2+} from InsP\textsubscript{3}Rs. It is not clear which mechanism dominates the response in submandibular cells, but in either case it is the distribution of InsP\textsubscript{3}Rs that would be critical to shaping of the Ca\textsuperscript{2+} signal.
Physiological relevance

In terms of the physiological response, the rodent pancreatic acinar cell is thought to have a relatively poor fluid secretory output (Argent et al., 1986) compared with the submandibular acinar cell (Foskett et al., 1989). This is shown in our measurements of cell volume changes, which directly reflect ion movements during the first stages of fluid secretion. Here we show significant changes in volume in submandibular cells but not in pancreatic cells, indicating a greater ion flux in the salivary tissue [see Fig. 1, and also Foskett (Foskett, 1990)].

Fluid secretion is, in the first instance, instigated by the opening of apical Cl− channels but activation of a K+ channel is required to maintain cell hyperpolarization and to provide a route for K+ exit (Petersen, 1992; Hayashi et al., 1995; Takeo et al., 1998). Salivary acinar cells have a robust Ca2+-dependent K+ conductance (Maruyama et al., 1983; Smith and Gallacher, 1992) and the fact that this is missing in rodent pancreatic acinar cells (Kidd and Thorn, 2001) may explain their weaker secretory response. The identity of the Ca2+-dependent K+ channel, recruited during fluid secretion in salivary acinar cells, is not known (Hayashi et al., 1995) but most models of secretion would place the K+ channel on the basal plasma membrane. This positioning is supported by the work of Harmer et al. (Harmer et al., 2005), which does show evidence that the Cl− current can, at low levels of cell stimulation, be activated alone. However, much work on salivary acinar cells shows a nearly simultaneous activation of K+ and Cl− current on cell stimulation (Foskett et al., 1989; Takeo et al., 1998).

This might arise because of an apical location of K+ channels, but our work suggests an alternative hypothesis. Unlike the classical view, derived from the pancreatic acinar cells, where the basal plasma membrane can be some distance from the discrete apical region, we show that in submandibular acinar cells the luminal region encircles the cells and thus the basal plasma membrane is actually close to the apical domain. We would therefore expect that the extended luminal region associated with an extended area of InsP3Rs would rapidly activate luminal Cl− channels but the near-synchronous global Ca2+ responses that arise from this distribution of InsP3Rs would also trigger the rapid activation of Ca2+-dependent K+ channels even if they were exclusively in the basal plasma membrane. Further work will be required to directly compare fluid secretion in pancreatic and submandibular tissues and test these ideas.

Conclusions

In conclusion, our data show an extended acinar lumen in submandibular glands nearly encircles individual acinar cells and is associated with a characteristic near-synchronous global Ca2+ response. We speculate that these are important adaptations to support copious fluid secretion in these cells.

This work was funded by a Wellcome Trust Overseas Fellowship to O.L. and Medical Research Council (UK) project grants (G0000214, G0400669 to PT). We thank Ian Parker for help with the movies.

References


Ca\textsuperscript{2+} signalling in salivary acinar cells


