Correlations between the functional integrity of the endoplasmic reticulum and polarized Ca^{2+} signalling in mouse lacrimal acinar cells: a role for inositol 1,3,4,5-tetrakisphosphate

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Ca^{2+} signalling in exocrine acinar cells has been shown to be both polarized and pulsatile in all cell types tested, except acutely isolated mouse lacrimal acinar cells. Lacrimal cells are also unusual in that they display a very low sensitivity to $\text{Ins}(1,4,5)\text{P}_3$ (InsP_3) that may be enhanced by placing the cells in primary culture for 12–72 h or by intracellular infusion of a low concentration of $\text{Ins}(1,3,4,5)\text{P}_4$ (InsP_4). We have proposed previously that this atypical behaviour stemmed from vesiculation of the endoplasmic reticulum (ER) incurred during isolation of the cells and, furthermore, that time in culture or InsP_4 increased sensitivity to InsP_3 by increasing ER integrity [Smith, Harmer, Letcher and Irvine (2000) *Biochem. J.* 347, 77–82]. We have measured the half time for fluorescence recovery after photobleaching (FRAP) of a fluorescent marker (Mag-fluo 4) loaded into the ER lumen in order to determine directly the functional

integrity of the ER in lacrimal cells. The half-time for FRAP was increased (indicating a reduction in the functional integrity of the ER) following exposure to anti-microtubule agents (taxol and nocodazole) known to perturb ER structure and decreased (indicating an increase in the functional integrity of the ER) by time in culture and exposure to InsP_4 . The action of InsP_4 was particularly pronounced because it occurred under patch-clamp whole-cell conditions that were themselves found to reduce ER functional integrity. These data show that ER remodelling could be a physiological regulator of Ca^{2+} signalling and indicate a role for InsP_4 in control of this process.

Key words: Ca^{2+} tunnelling, fluorescence recovery after photobleaching (FRAP), InsP_4 , membrane remodelling, microtubule.

INTRODUCTION

Exocrine acinar cells are both structurally [1] and functionally polarized [2]. The functional polarization extends to the Ca^{2+} signalling process that controls fluid and electrolyte secretion and is manifest in an apical localization of the initiation site for these Ca^{2+} signals [3]. The observation that infusion of $\text{Ins}(1,4,5)\text{P}_3$ (InsP_3) via a patch-clamp pipette at the basal pole of the cell gives rise to a Ca^{2+} signal that spreads from the apical pole [3] indicates that the polarity of the Ca^{2+} signal is not a simple function of localized InsP_3 production, but is rather the result of variation in the Ca^{2+} -release properties of the primary Ca^{2+} store, the endoplasmic reticulum (ER) [2]. The extent to which the properties of the ER can dictate the characteristics of the Ca^{2+} signal has only recently been appreciated [4]. For example, recent evidence that the Ca^{2+} taken into the ER at the basal pole of the cell may be released at the apical pole (' Ca^{2+} tunnelling') [5] is feasible only if the ER exists as a continuous network. Furthermore, local Ca^{2+} -signalling domains may also depend on ER structure, because they are abolished by agents which disrupt the ER by preventing microtubule turnover [6].

In previous studies we have characterized signal transduction in mouse lacrimal acinar cells [7–11]. Like all other exocrine cells studied to date, these cells have muscarinic acetylcholine receptors which act via increased InsP_3 levels to mobilize Ca^{2+} , preferentially from the apical pole of the cell [3,12]. Unlike other

acinar cells, acutely isolated lacrimal acinar cells have a very low sensitivity to InsP_3 infused via the patch-clamp pipette [10,13] and an almost complete inability to produce the Ca^{2+} spikes or oscillations that characterize local Ca^{2+} signalling in other exocrine acinar cells [14]. Furthermore, lacrimal acinar cells are the only acinar cells in which a clear response to InsP_4 has been shown [7,8,11,15]. Most recently, we have shown that both InsP_3 -sensitivity and the capacity to produce oscillatory Ca^{2+} signals can be increased by maintaining the cells in primary culture [11,16]. We have hypothesized that the lack of responsiveness of acutely isolated cells is the result of vesiculation of the ER that occurs during the process of cell isolation [17] and that the effect of primary culture is to allow time for the recovery of the functional integrity of the ER [11].

We have used fluorescence recovery after photobleaching (FRAP) to test our hypothesis. Most previous FRAP protocols have used green-fluorescent-protein-tagged proteins localized to the ER [18–20]. Our experiments depend on the ability of acetoxymethyl ester (AM)-linked fluorescent probes to load into membrane-bound intracellular compartments, coupled with the low Ca^{2+} -affinity of the Mg^{2+} -sensitive probe Mag-fluo 4 (K_d for Ca^{2+} 22 μM), which fluoresces strongly in the presence of the high Ca^{2+} found within the ER and thus functions as a fluorescent marker of the ER lumen [4]. The functional integrity of the ER may be determined by bleaching the dye in a small area of the ER using a brief high-energy pulse from a carefully focused UV

Abbreviations used: AM, acetoxymethyl ester; $[\text{Ca}^{2+}]_i$, intracellular Ca^{2+} concentration; CICR, Ca^{2+} -induced Ca^{2+} release; ER, endoplasmic reticulum; FRAP, fluorescence recovery after photobleaching; GAP, GTPase-activating protein; InsP_3 , $\text{Ins}(1,4,5)\text{P}_3$; InsP_4 , $\text{Ins}(1,3,4,5)\text{P}_4$; IP4BP, InsP_4 -binding protein.

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laser and then measuring the kinetics of fluorescence recovery with high resolution confocal imaging. The speed with which fluorescence returns to the photobleached region is dependent on how rapidly dye can move from unbleached regions of the ER and this in turn is dependent on the degree of connectivity in the ER [4,18]. FRAP will occur rapidly in a well-integrated ER and slowly, if at all, in a highly vesiculated one. FRAP has been used previously in pancreatic acinar cells to show a high degree of connectivity in the ER of these cells [4], but not to measure relatively rapid changes in the functional integrity of the ER. We have used anti-microtubule agents known to perturb the ER to show, first, that this technique has the resolution to respond to changes in ER structure. We have then measured FRAP following a 24 h period in primary culture or following exposure to $\text{Ins}(1,3,4,5)P_4$ ($\text{Ins}P_4$) to determine whether these treatments altered the degree of connectivity in the ER of lacrimal acinar cells.

EXPERIMENTAL

Adult male CD1 mice were killed by cervical dislocation and lacrimal cells were isolated by collagenase (Worthington Biochemical Corp.) digestion in extracellular media containing 1 mM Ca^{2+} [10].

For primary culture, cells were re-suspended in serum-free Dulbecco's minimal essential medium/F12 medium (1:1, v/v) containing antibiotics and antimycotics (Life Technologies) and cultured for 24 h at 37 °C under a CO_2/O_2 (1:19) atmosphere on glass coverslips coated with a thin (≈ 1 mm) layer of a basement membrane matrix (Matrigel; Becton–Dickinson) [11].

Cells were loaded by incubation with 5 μM Mag-fluo 4-AM for 30–35 min at 37 °C. This protocol optimizes loading of fluorescent marker to the ER lumen [4].

For experimental purposes, freshly isolated cells were allowed to settle on to coverslips identical with those used for primary culture. The coverslips formed the base of a perfusion chamber which was placed on the stage of an inverted microscope (Axiovert 100M; Zeiss).

The patch-clamp whole-cell configuration was achieved with single cells using 2.5–3 M Ω patch-clamp pipettes as described previously [10]. Electrophysiological measurements were made using an EPC-8 patch-clamp amplifier and pulse-fit (Heka) software. The high-resistance 'seal' between patch-pipette and membrane was monitored continuously in all whole-cell experiments, as was the Ca^{2+} -dependent K^+ current. K^+ -channel activity, and by inference the intracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$), did not alter during FRAP experiments (results not shown).

Fluorescence images were captured every 100 ms using a Zeiss LSM 510 laser scanning confocal microscope. Mag-fluo 4-loaded cells were excited using the 488 nm laser line at low power (emission filter 505–550 nm). For FRAP experiments, both the UV (lines 351 and 364, 80 mW) and visible (488 nm, 15 mW) lasers were used simultaneously at near maximal power to selectively photobleach small regions of the cells.

Images were analysed using the Zeiss LSM confocal software. Non-linear regression was performed using GraphPad Prism (GraphPad Software Inc., San Diego, CA, U.S.A.) software.

Results in the text and in Figures are shown as means $\pm$ S.E.M., where n is the number of observations.

The patch-clamp pipette contained (in mM) 140 KCl, 1.13 MgCl_2 , 10 glucose, 0.5 EGTA and 1 ATP, buffered to pH 7.4 with 10 mM Hepes and, where indicated in the text and Figure legends, $\text{Ins}P_4$. $\text{Ins}P_4$ was kindly donated by Professor Robin F.

Irvine (Department of Pharmacology, University of Cambridge, Cambridge, U.K.).

The extracellular bathing solution contained (in mM) 140 NaCl, 4.7 KCl, 1.13 MgCl_2 , 1.2 CaCl_2 and 10 glucose, buffered to pH 7.4 with 10 mM Hepes.

RESULTS

Figure 1(A) shows a single, acutely isolated, mouse lacrimal acinar cell under bright-field illumination. Acutely isolated cells were chosen for the bulk of these experiments to provide a baseline for subsequent examination of the effect of time in culture and because the effects of $\text{Ins}P_4$ on Ca^{2+} mobilization are most pronounced in these cells. This cell has adopted a characteristic 'kidney' shape in which the apical pole lies on the inner curve. The visible presence of secretory vesicles confirms this orientation. The pseudo-colour image of the same cell shown in

Figure 1 FRAP in intact acutely isolated (control) lacrimal acinar cells

(A) Bright-field image of a single, isolated lacrimal acinar cell oriented so that the apical pole is at the left of the image. The blue circle on this and all subsequent images indicates the region within which Mag-fluo 4 was photobleached and measurements of fluorescence recovery were made. (B)–(D) Pseudocolour images of the cell in (A) showing Mag-fluo 4 fluorescence from the ER before photobleaching (B), immediately following photobleaching (C) and approximately 10 s following photobleaching (D). (E) Changes in Mag-fluo 4 fluorescence measured within the blue circle shown on (A)–(D) plotted on an arbitrary scale against time (orange trace) and the best fit of FRAP to a mono-exponential (black trace). Inset is the $t_{1/2}$ value for FRAP for this experiment. The arrows indicate the time point at which the images shown in (B)–(D) were obtained.

Figure 2 Effects of taxol and nocodazole on FRAP in intact acutely isolated lacrimal acinar cells

(A) Representative time courses taken from paired experiments using the same batch of cells showing FRAP in control cells compared with cells that had been exposed to 100 μ M taxol for at least 30 min. (B) Averaged data from paired experiments showing the $t_{1/2}$ for FRAP measured under control conditions ($n = 11$) and following exposure to 100 μ M taxol ($n = 11$). (C) Representative time courses taken from paired experiments using the same batch of cells showing FRAP in control cells compared with cells that had been exposed to 66 μ M nocodazole for at least 30 min. (D) Averaged data from paired experiments showing the $t_{1/2}$ for FRAP measured under control conditions ($n = 14$) and following exposure to 66 μ M nocodazole ($n = 19$).

Figure 1(B) displays the distribution of Mag-fluo 4. The areas of greatest fluorescent intensity are in the basolateral regions of the cell and co-localize with the bulk of the ER. There was little or no fluorescence in the apical and nuclear regions of the cell. The distribution of fluorescence is in contrast with that seen using fura 2 to measure $[Ca^{2+}]_i$ and itself indicates that the Mag-fluo 4 is marking a different cellular compartment. Photobleaching a small area of the cell (indicated on the Figure) produced a dark area in the cell (Figure 1C) that persisted for less than 5 s. After this period, fluorescence was again evenly distributed across the basolateral pole of the cell (Figure 1D). As might be expected, overall fluorescence was reduced following irreversible photobleaching of a significant fraction of total dye. The time course of the recovery of fluorescence into the photobleached region is shown in Figure 1(E). These data fit well to a mono-exponential increase that yields a $t_{1/2}$ for recovery of 0.92 s. On average, the time-dependence of fluorescence recovery in intact, untreated, freshly isolated (control) cells was 0.89 ± 0.029 s ($n = 41$). This rapid fluorescence recovery demonstrates a high degree of connectivity within the basolateral pole of even freshly isolated lacrimal cells.

The form and function of the ER is actively maintained by the cytoskeleton; therefore agents that inhibit normal microtubule turnover would be expected to have an impact on ER structure. The results shown in Figure 2 demonstrate this point and, more importantly, show that perturbations in the functional integrity of the ER can be measured using FRAP of Mag-fluo 4. The results shown in Figure 2(A) and summarized in Figure 2(B) show that the $t_{1/2}$ for fluorescence recovery was significantly increased compared with paired control experiments ($P < 0.01$) by exposure of lacrimal cells to 100 μ M taxol for at least 30 min.

Figure 3 FRAP in intact lacrimal acinar cells maintained in culture for 24 h

(A) Bright-field image of a single isolated lacrimal acinar cell previously maintained in primary culture for 24 h oriented so that the apical pole is towards the bottom left of the image. The blue circle on this and all subsequent images indicates the region within which Mag-fluo 4 was photobleached and measurements of fluorescence recovery were made. (B)–(D) Pseudocolour images of the cell in (A) showing Mag-fluo 4 fluorescence from the ER before photobleaching (B), immediately following photobleaching (C) and approx. 10 s following photobleaching (D). (E) Changes in Mag-fluo 4 fluorescence measured within the blue circle shown on (A)–(D) plotted on an arbitrary scale against time (orange trace) and the best fit of FRAP to a mono-exponential (black trace). Inset is the $t_{1/2}$ value for FRAP for this experiment. The arrows indicate the time point at which the images shown in (B)–(D) were obtained. (F) Averaged data showing the $t_{1/2}$ for FRAP measured under control conditions ($n = 41$) and following 24 h in primary culture ($n = 24$).

In a comparable series of experiments, 66 μ M nocodazole appeared less effective than taxol, but nevertheless we were able to measure a significant ($P < 0.05$) increase in $t_{1/2}$ following exposure to 66 μ M nocodazole for at least 30 min. These data

Figure 4 FRAP in acutely isolated lacrimal acinar cells under patch-clamp whole-cell conditions

(A) Changes in Mag-fluo 4 fluorescence measured in a cell maintained under patch-clamp conditions for 5 min plotted on an arbitrary scale against time (orange trace) and the best fit of FRAP to a mono-exponential (black trace). Inset is the $t_{1/2}$ value for FRAP calculated from this experiment. (B) Changes in Mag-fluo 4 fluorescence measured within the blue circle shown on (C)–(F) in a cell maintained under patch-clamp conditions for 10 min, plotted on an arbitrary scale against time (orange trace) and the best fit of FRAP to a mono-exponential (black trace). Inset is the $t_{1/2}$ value for FRAP for this experiment. The arrows indicate the time point at which the images shown in (D)–(F) were obtained. (C) Bright-field image of a single isolated lacrimal acinar cell maintained under patch-clamp conditions for 10 min oriented so that the apical pole is towards the bottom left of the image. The blue circle on this and all subsequent images indicates the region within which Mag-fluo 4 was photobleached and measurements of fluorescence recovery were made. (D)–(F) Pseudocolour images of the cell in (C) showing Mag-fluo 4 fluorescence from the ER before photobleaching (D), immediately following photobleaching (E) and approx. 10 s following photobleaching (F). The dark area at the basolateral pole of the cell is the nucleus.

also provide additional confirmation that Mag-fluo 4 fluorescence is localized to a membrane-bound compartment.

It is not possible to pair cultured cells and control cells in the same way as acutely treated cells, therefore the average $t_{1/2}$ for FRAP measured in a large number of cells ($n = 24$) following a 24 h period in culture was compared with the overall control data shown in Figure 1. It is clear from the bright-field image (Figure 3A) as well as from the distribution of Mag-fluo 4 (Figures 3B–3D) that the polarization of these cells has been preserved through their brief period in culture. Furthermore, the data in Figures 3(E) and 3(D) show that the $t_{1/2}$ for fluorescence recovery in cells maintained in primary culture for 24 h was significantly ($P < 0.01$) more rapid than that seen in acutely isolated cells (Figure 1). The reduction in $t_{1/2}$ is consistent with a

Figure 5 FRAP in acutely isolated lacrimal acinar cells under patch-clamp whole-cell conditions in the presence of InsP_4

(A) Changes in Mag-fluo 4 fluorescence measured in a cell maintained under patch-clamp conditions with $50 \mu\text{M}$ InsP_4 in the patch pipette for 5 min, plotted on an arbitrary scale against time (orange trace) and the best fit of FRAP to a mono-exponential (black trace). Inset is the $t_{1/2}$ value for FRAP calculated from this experiment. (B) Changes in Mag-fluo 4 fluorescence measured within the blue circle shown on (C)–(F) in a cell maintained under patch-clamp conditions with $50 \mu\text{M}$ InsP_4 in the patch pipette for 10 min, plotted on an arbitrary scale against time (orange trace) and the best fit of FRAP to a mono-exponential (black trace). Inset is the $t_{1/2}$ value for FRAP for this experiment. The arrows indicate the time point at which the images shown in (D)–(F) were obtained. (C) Bright-field image of a single isolated lacrimal acinar cell maintained under patch-clamp conditions with $50 \mu\text{M}$ InsP_4 in the patch pipette for 10 min, oriented so that the apical pole is towards the top of the image. The blue circle on this and all subsequent images indicates the region within which Mag-fluo 4 was photobleached and measurements of fluorescence recovery were made. (D)–(F) Pseudocolour images of the cell in (C) showing Mag-fluo 4 fluorescence from the ER before photobleaching (D), immediately following photobleaching (E) and approx. 10 seconds following photobleaching (F). (G) Averaged data showing the $t_{1/2}$ for FRAP measured under control conditions ($n = 41$), following 5 and 10 min under patch-clamp conditions in the absence ($n = 21$ and $n = 11$ respectively) or presence ($n = 22$ and $n = 17$ respectively) of InsP_4 .

reduction in ER vesiculation as a consequence of the time in culture.

The data in Figure 4 show FRAP measured in freshly isolated cells under patch-clamp conditions as a prerequisite to experi-

ments to determine whether Ins P_4 altered the functional integrity of the ER. Unlike previous observations in the pancreas [4], we found, in lacrimal acinar cells, that the patch-clamp whole-cell configuration caused a substantial and progressive increase in the $t_{1/2}$ for FRAP. The $t_{1/2}$ increased to 2.4 s (cf. 0.92 s, Figure 1) 5 min after the breakthrough to whole-cell conditions (Figure 4A) and to 4.78 s after 10 min under whole-cell conditions (Figure 4B). In some of these experiments there was no visible equilibration of fluorescence across the basolateral pole of the cell following the photobleaching pulse. On average, the $t_{1/2}$ of fluorescence recovery in patch-clamped cells was 2.2 ± 0.37 s ($n = 21$) and 4.1 ± 0.91 s ($n = 11$) after 5 and 10 min of whole-cell dialysis respectively ($P < 0.01$ and $P < 0.01$ respectively compared with control).

The data shown in Figure 5 were obtained under conditions identical with those shown in Figure 4, except that 50 μ M Ins P_4 was added to the intracellular medium at the start of the experiment and thus entered the cell as soon as the breakthrough to the whole-cell configuration was achieved. In the presence of Ins P_4 , the $t_{1/2}$ for fluorescence recovery was much more rapid than under whole-cell conditions in the absence of Ins P_4 . On average, the time-dependence of fluorescence recovery in patch-clamped cells dialysed with Ins P_4 was 1.3 ± 0.13 s ($n = 22$) and 1.4 ± 0.28 s ($n = 17$) after 5 and 10 min of whole-cell dialysis respectively. There was no significant difference between the $t_{1/2}$ for FRAP in the presence of Ins P_4 at 5 and at 10 min under whole-cell conditions and little difference between either of these values and the $t_{1/2}$ for FRAP seen in intact cells (Figure 5G).

DISCUSSION

Our results show, first, that we can measure changes in the functional integrity of the ER using agents known to perturb microtubule activity and, secondly, that both of the conditions known to enhance the sensitivity of isolated lacrimal cells to Ins P_3 , namely time in culture and exposure to Ins P_4 , correlate with an increase in the functional integrity of the ER.

Current models for Ca^{2+} signalling in polarized acinar cells has Ca^{2+} release from intracellular stores occurring primarily and preferentially at the apical (secretory) pole of the cell [2]. The model was derived [3] and is supported by direct visualization of apical Ca^{2+} release in a wide variety of acinar cells. The central role of the apical pole of the cell in Ca^{2+} signalling has called into question the morphological identity of the Ca^{2+} store in acinar cells, because the ER, which acts as Ca^{2+} store in other cell types, is almost exclusively located at the basolateral pole, away from the focus of Ca^{2+} release [1]. One possible explanation is that Ca^{2+} release at the apical pole of the cell occurs instead from the secretory vesicles, which are the predominate membrane-bound organelle at this pole of the cell, and this initiating Ca^{2+} signal is propagated by the ER via Ca^{2+} -induced Ca^{2+} release (CICR) [21]. An alternative explanation, supported by the data presented here, is that rapid movement or 'tunnelling' of Ca^{2+} within a continuous ER network that spans both poles of the cell replenishes Ca^{2+} released at the apical pole from the bulk Ca^{2+} store at the basolateral pole [4,22]. A reduction of the functional integrity by increased vesiculation of the ER would therefore separate the primary Ca^{2+} -release site from most of the intravesicular Ca^{2+} , and it follows that, if the part of the Ca^{2+} store most sensitive to Ins P_3 can no longer release Ca^{2+} , the overall sensitivity of the cell to Ins P_3 will be reduced (see Figure 6).

We suggested previously that the low Ins P_3 -sensitivity of lacrimal cells [10], and the inability of these cells to generate oscillatory Ca^{2+} signals, resulted from vesiculation of the ER most likely incurred during cell preparation [11]. We also

predicted that the increased Ins P_3 -sensitivity and appearance of oscillatory Ca^{2+} signals following time in primary culture would be accompanied by an increase in the functional integrity of the ER [11]. The results presented here show that this was an accurate prediction. According to our hypothesis, acutely isolated lacrimal acinar cells respond poorly to Ins P_3 because the most sensitive accumulation of Ins P_3 receptors, at the apical pole of the cell, are not functionally connected to the Ca^{2+} store. The cells respond to high concentrations of Ins P_3 , which can act either by releasing Ca^{2+} from less sensitive regions of the ER that are part of the bulk Ca^{2+} store, or perhaps by stimulating Ca^{2+} release from a sufficiently large number of isolated stores to trigger a global signal by CICR.

The massive reduction in FRAP observed under patch-clamp conditions was unexpected. The only previous such study, using pancreatic acinar cells, reported little change in the $t_{1/2}$ for fluorescence recovery during whole-cell experiments. The $t_{1/2}$ reported here for FRAP following 10 min under whole-cell conditions has little meaning, because there was practically no fluorescence recovery under these conditions. Under all other conditions, the fluorescence level equilibrated over the whole cell during the recovery period, whereas under whole-cell conditions the fluorescence in the photobleached region 'recovered' very slowly to a value much lower than that in adjacent non-photobleached areas. A likely explanation of this phenomenon is that some cellular component necessary for maintenance of ER integrity is lost through the patch pipette. Our data indicate that the corollary of this hypothesis is that inclusion of Ins P_4 in the patch pipette prevents loss of this cellular component. At this point we can only speculate on the nature of this component which both binds Ins P_4 and regulates ER integrity.

These data affirm that acutely isolated mouse lacrimal acinar cells, in response to secretory stimuli, behave in a different manner to other exocrine acinar cells. They support our hypothesis that Ca^{2+} tunnelling through a well integrated ER that connects the apical and basolateral poles is an obligatory component of the mechanism for polarized and oscillatory Ca^{2+} signals in exocrine acinar cells and that the failure of this process in lacrimal cells accounts for their functional differences (Figure 6). We have shown that both time in culture and Ins P_4 are capable of restoring or protecting the ER and that the action of Ins P_4 is particularly evident in acinar cells maintained under patch-clamp whole-cell conditions. At this point it is possible only to speculate as to the nature of the link between Ins P_4 and the ER, but it is not hard to postulate a role for GAP1^{IP4BP} (GAP is GTPase-activating protein; IP4BP is Ins P_4 -binding protein), the putative Ins P_4 receptor [23–25] in this context. GAP1^{IP4BP} shares sequence homology with another protein, GAP1^M [26], which binds PtdIns P_3 . Given the strong evidence that PtdIns P_3 plays a role in membrane trafficking [27–29], it is intriguing to speculate that Ins P_4 plays an analogous role in controlling remodelling of the ER [30]. Thus Ins P_4 potentiates Ins P_3 -induced Ca^{2+} release because it promotes ER integrity which, in turn, facilitates Ca^{2+} tunnelling that effectively couples the Ca^{2+} store to the primary Ca^{2+} -release site. This hypothesis also gives back to Ins P_4 a role in the regulation of Ca^{2+} influx [9], because depletion of the Ca^{2+} stores, which is a necessary prerequisite for Ca^{2+} influx, will occur most effectively and at lower Ins P_3 concentrations when ER integrity is high. There could even be a more direct role for Ins P_4 in the control of Ca^{2+} influx if membrane remodelling plays a significant role in positioning the ER to allow 'conformational coupling' of the Ca^{2+} store to the plasma-membrane Ca^{2+} influx channel [31].

What is not clear at this point is whether there is a physiological role for vesiculation of the ER in the control of Ca^{2+} signalling.

Figure 6 Model showing the implications of ER fragmentation on Ca^{2+} signalling in lacrimal acinar cells

(A) Ca^{2+} release at the apical pole of a cell in which the ER is vesiculated. This signal does not propagate across the cell, because the Ca^{2+} store at the apical pole is small and depletes rapidly. (B) Ca^{2+} release at the apical pole of a cell in which the ER is continuous between the apical and basolateral poles of the cell. This Ca^{2+} signal propagates across the cell because the Ca^{2+} store at the apical pole is replenished by Ca^{2+} tunnelling through the ER from the basolateral pole of the cell. (C) Propagated Ca^{2+} signal in response to a large stimulus to a cell in which the ER is vesiculated. This could be due to (i) release of sufficient Ca^{2+} from the apical pole of the cell to activate the basolateral Ca^{2+} store by CICR or (ii) generation of sufficient InsP_3 to directly activate the less sensitive basolateral Ca^{2+} store. Although both (B) and (C) could support global Ca^{2+} signals originating at, or close to, the apical pole of the cell, (B) is the only model that could support repetitive transient Ca^{2+} signals that are restricted to the apical pole of the cell.

Vesiculation may simply be an artefact of cell preparation or cell permeabilization [32], useful only because some aspects of Ca^{2+} signalling are more apparent in cells that are 'damaged' in this way. Alternatively, the cell could control the degree of vesiculation as part of the Ca^{2+} signalling mechanism [33]. Our data emphasize the role of the microtubular network in the control of ER remodelling [34] and show that the cell *could* alter the functional integrity of the ER network to suit the circumstances. This is an attractive hypothesis in polarized exocrine acinar cells, where a very small increase in ER vesiculation would uncouple the sites at the apical pole responsible for 'initiating' the signal from the Ca^{2+} store located at the basolateral pole. Such a mechanism could shift cells into a quiescent mode, for example to facilitate recovery from periods of intense activity. This last is consistent with the observation that part of the response to sustained high levels of $[\text{Ca}^{2+}]_i$ in several cell types is increased vesiculation of the ER [18,19,35].

Taken together, our results show that the functional integrity of the ER plays an important role in Ca^{2+} signalling in polarized acinar cells, that membrane remodelling could be an additional mechanism for control of Ca^{2+} signalling, and that the potentiating action of InsP_4 could be exerted via an action on endomembrane remodelling.

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