ACCELERATED PUBLICATION

Spatial characterisation of ryanodine-induced calcium release in mouse pancreatic acinar cells

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In pancreatic acinar cells, agonists evoke intracellular Ca^{2+} transients which are initiated in the apical region of these polarized cells. There are contradictory experimental data concerning Ca^{2+} release from ryanodine receptors (RyRs) in the apical region. In the present study, we have used low doses of ryanodine to open RyRs leading to the release of Ca^{2+} from intracellular stores. Ryanodine causes Ca^{2+} release that is initiated in the apical region of the cell but is dependent upon functional

inositol 1,4,5-trisphosphate receptors (IP_3Rs). These results suggest that co-ordinated release from co-localized RyRs and IP_3Rs underlies the increased sensitivity of the apical region to initiation of intracellular Ca^{2+} transients.

Key words: calcium wave, exocrine, pancreatic acinar cell, receptor localization, ryanodine receptor.

INTRODUCTION

Pancreatic acinar cells are structurally and functionally polarized [1–4]. Secretory granules, containing digestive enzymes, are found in the apical part of the cell [5]. In contrast, the endoplasmic reticulum, the major Ca^{2+} -releasing store, concentrates in the basal area with only thin strands of endoplasmic reticulum projecting into the secretory granule region [5,6]. The secretagogues acetylcholine and cholecystokinin trigger and regulate secretion in pancreatic acinar cells via the generation of complex Ca^{2+} transients. Both local spikes (localized to the secretory granule region) and global Ca^{2+} transients (originating in the secretory region and then propagating as waves through the cytoplasm of the cell) can be induced by physiological doses of secretagogues [4,7]. Importantly, in all cases, these polarized Ca^{2+} signals all begin at the extreme apex of the cell, in an area termed the ‘trigger zone’ [4].

Strong evidence exists for the release of Ca^{2+} by the action of inositol 1,4,5-trisphosphate (IP_3) on IP_3 receptors (IP_3Rs) during secretagogue-induced signals [3]. IP_3Rs have been localized to the apical pole of the cells by immunostaining [8], and introduction of IP_3 causes Ca^{2+} release predominantly in the apical region [4,7]. The other major group of Ca^{2+} -release channel, ryanodine receptors (RyRs), have also been detected in pancreatic acinar cells by reverse-transcriptase PCR and Western blotting [9,10], and ryanodine has been shown to affect Ca^{2+} fluxes in endoplasmic reticulum preparations from acinar cells [11]. Immunocytochemical data indicate that RyRs seem to be evenly located throughout the basal area of the cell, but are excluded from parts of the apical region and, in particular, are completely absent from the region where agonist-evoked signals are initiated [9,10]. Another study [12], which used a fluorescent version of ryanodine, revealed preferential ryanodine binding in the basal region near to the edge of the granular area (close to the majority of mitochondria [13,14]). It is proposed that RyRs located in this region play a part in propagation of Ca^{2+} waves into the basal region [12,15]. However, infusion of cADP-ribose, a putative

activator of RyRs, triggers Ca^{2+} spiking in the apical part of the cell [16]. Also, there is experimental evidence that IP_3 and RyRs must co-ordinate their activity in the apical region to produce agonist-evoked Ca^{2+} spikes [17] and to initiate Ca^{2+} -induced Ca^{2+} -release (CICR) waves which start in the apical region [18,19]. The apparent contradiction between the Ca^{2+} -release data and the reported localization of RyRs prompted us to experimentally localize the functional RyRs in pancreatic acinar cells.

As implied by its name, the RyR was identified on the basis of its affinity for binding the plant-derived alkaloid ryanodine [20,21] – a substance long known to have complicated pharmacological effects [22]. Subsequently, the RyR was shown to be the Ca^{2+} -sensitive Ca^{2+} -release channel that had been characterized in muscle cells [23–28]. Experiments on purified, reconstituted RyRs and on microsomal preparations have shown that ryanodine binds to the RyR at two or more sites, which can be broadly identified as high (nM) and low (μM) affinity (for review see [29]). These sites seem to correlate with low- and high-affinity effects of ryanodine on flux through the RyR [30]. In cellular preparations, at concentrations $\leq 10 \mu\text{M}$, the RyR is opened in a subconductance state which results in activation of Ca^{2+} flux through the channel [31–33]. However, at concentrations above approx. $10 \mu\text{M}$, ryanodine has an inhibitory effect on RyR activity and completely blocks the channel [29,34–36]. In many cell types, elevated doses of ryanodine have been used to inhibit RyRs and reduce Ca^{2+} release from stores [37–39].

In pancreatic acinar cells, $50\text{--}100 \mu\text{M}$ ryanodine inhibited agonist-induced local spiking, slowed propagation of agonist-induced global waves and completely abolished global CICR waves [12,17,19]. Despite the inhibitory effects of these doses of ryanodine, there is still controversy concerning the cellular location of the site of action of ryanodine and therefore the location of the functional RyRs. In the present study, we show that low doses of ryanodine ($10 \mu\text{M}$) are able to release Ca^{2+} in intact isolated mouse pancreatic acinar cells, while confirming that elevated doses do not. Therefore this lower concentration of

Abbreviations used: AM, acetoxymethyl ester; CICR, Ca^{2+} -induced Ca^{2+} -release; IP_3 , inositol 1,4,5-trisphosphate; IP_3R , IP_3 receptor; RyR, ryanodine receptor.

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ryanodine appears to bind only the low affinity site of RyR, resulting in activation of the channel. Analysis of the spatio-temporal characteristics of the ryanodine-induced release showed that the transient was manifested as a wave which was always initiated at the extreme apex of the cell and then propagated throughout the cell. Intriguingly, pharmacological inhibition of IP₃Rs, which are predominantly localized to the apical region, also completely inhibited ryanodine-induced Ca²⁺ release. This suggests that activation of measurable Ca²⁺ release through RyRs is dependent upon co-ordinated release from IP₃Rs. Our results show that functional RyRs exist in pancreatic acinar cells. There appears to be a close relationship between RyRs and IP₃Rs in the apical region of these cells which allows the channels to co-ordinate their function and thereby control the initiation of intracellular Ca²⁺ transients.

EXPERIMENTAL

Cell preparation and chemicals

Freshly isolated mouse pancreatic acinar cells (individual cells or small clusters) were prepared as described previously [7] and used within 4 h. Fluo-4 acetoxymethyl ester (AM) and Fura Red AM and were from Molecular Probes, collagenase was obtained from Worthington and all other chemicals, including ryanodine (catalogue number R4267), were purchased from Sigma. The cell cytoplasm was loaded with fluo-4 AM by incubation at 22–24 °C in darkness for 25 min in solutions containing 2.5–5 µM fluo-4 AM. All experiments were performed at 22–24 °C in the presence of continuously perfused extracellular solutions. The extracellular solution contained (in mM): 140 NaCl, 4.7 KCl, 1.13 MgCl₂, 10 Hepes, 10 glucose, 1 CaCl₂; pH was 7.2 (adjusted by NaOH) and ryanodine at concentrations and times indicated.

Confocal Ca²⁺ imaging

Fluorescence images were obtained using either a Zeiss LSM 510 confocal microscope with 63 × water immersion objective (NA = 1.2). Cells adhered to poly(L-lysine)-coated slides were imaged on the stage of an inverted microscope (Axiovert 100M, Zeiss). Fluo-4 was excited by a 488 nm laser line and emission collected through a bandpass filter of 505–550 nm. Fura Red was excited similarly but emission collected above 560 nm. Fura Red fluorescence values were normalized by initial resting levels (F_0) and shown as F_0/F .

RESULTS AND DISCUSSION

Low doses of ryanodine can cause Ca²⁺ release

In order to characterize the effects of ryanodine, we used confocal microscopy to image isolated pancreatic acinar cells loaded with Ca²⁺-sensitive fluorescent indicator, either fluo-4 or Fura Red. We applied various concentrations of the drug by rapid superfusion. We found that the release of Ca²⁺ induced by ryanodine showed a dose-dependency which matches the predicted actions of ryanodine on the RyR. While ryanodine did not trigger Ca²⁺ release in all cells, we found that 10 µM ryanodine was the most efficient dose that triggered responses in 35.3 % of cells ($n = 218$; Figure 1). The release triggered by ryanodine caused a large and transient increase in cytosolic Ca²⁺ (for examples, see Figures 2 and 3). Lower doses of ryanodine (2 µM) triggered release on very few occasions (3.5 %, $n = 55$; Figure 1). More striking, though, is that fact that doses above 10 µM were also less likely to trigger release. Ryanodine at 50 µM triggered a change in the cytosolic Ca²⁺ level in relatively few cells (12.9 %, $n = 62$; Figure 1). Changes in Ca²⁺ were never observed following

Figure 1 Dose-dependency of ryanodine-induced Ca²⁺ release

The percentage of cells responding to different ryanodine concentrations is shown. Note the biphasic nature of the dose-dependency, with maximum release efficiency at 10 µM ryanodine.

application of ryanodine above 50 µM ($n = 35$; Figure 1). This results in a biphasic dose dependency where release triggered by ryanodine becomes less likely at higher concentrations (Figure 1).

This dose-dependency of release correlates closely with the action of ryanodine on isolated purified RyRs. It is thought that when ryanodine binds directly to a high-affinity site on the RyR, it forces the channel into an open configuration. At higher concentrations, however, ryanodine can bind to a lower-affinity site which closes the channel [29,30]. The similarity of the effects of ryanodine in pancreatic acinar cells suggests that the lower doses of ryanodine trigger Ca²⁺ release by binding to RyRs in the pancreatic acinar cell and keeping them open. Although the dose-dependency of ryanodine action in acinar cells parallels that on isolated RyRs, it should be noted that the concentrations required in acinar cells are relatively high. The affinity of the activating site is thought to be in the low-nanomolar range, whereas 10 µM causes release in acinar cells. In fact, in many other cellular systems, similar elevated concentrations are

Figure 2 Profile of cytosolic Ca²⁺ transients induced by low doses of ryanodine

The trace shows fluorescence from a cell loaded with Fura Red (region shown in accompanying fluorescence image). Application of 10 µM ryanodine to the cell causes a rapid, large and transient increase in the cytoplasmic Ca²⁺ level. All three cells in the cluster showed similar responses.

Figure 3 Ryanodine preferentially releases Ca^{2+} in the apical region

The trace shows fluo-4 fluorescence from the regions overlaying the transmitted image of the cells. Application of $10 \mu\text{M}$ ryanodine triggers a large Ca^{2+} transient. In this case, both cells responded to ryanodine application, although fluorescence recordings are shown only from apical (red trace) and basal (blue trace) regions of the upper cell. The area surrounded by a broken-line box is expanded in the inset. The expanded trace clearly shows that the fluorescence begins to rise in the apical region before the basal level increases. Sequential pseudocolour fluorescence images from time-points marked by arrows in inset are shown in the lower panel. The images show the first increase in Ca^{2+} occurs at the extreme apex of the upper cell. The increase in Ca^{2+} then spreads as a wave from the apical towards the basal membrane.

Figure 4 Inhibition of IP_3Rs by caffeine completely abolishes ryanodine-induced Ca^{2+} release

The trace shows normalized fluorescence from two separate pancreatic acinar cells loaded with Fura Red. Application of 20 mM caffeine causes a slight change (step decrease in normalized value) in the fluorescence of the dye which is reversed upon removal of the drug. This is due to well-documented effects of caffeine on the dye independent of Ca^{2+} . Application of $10 \mu\text{M}$ ryanodine in the presence of caffeine, which inhibits IP_3Rs , does not cause any change in the cellular Ca^{2+} level. In contrast, later application of the same dose of ryanodine, following removal of caffeine, triggers large Ca^{2+} transients in both cells.

required to cause release and it is generally considered that the transition between the activating and inhibitory effects of ryanodine occurs at approx. $10 \mu\text{M}$ [30] in intact cells. It seems that, in pancreatic acinar cells using this particular ryanodine, the activating/inhibiting transition occurs close to $50 \mu\text{M}$, since this concentration still releases Ca^{2+} in some cells. Furthermore, concentrations above $50 \mu\text{M}$ have recently been used to inhibit RyR-dependent signals in this cell type [12,17,19]. In other

studies and in different cell types, similar pharmacological effects of ryanodine have been shown, but over slightly varying concentration ranges [16,38]. The concentration required is likely to depend upon several factors, such as the permeability of the plasma membrane and the purity and content of the ryanodine mixture. Indeed, we have found clear differences in potency of ryanodine from different suppliers. To avoid confusion, one source of ryanodine was used in the present study (Sigma; catalogue number R4267). It is likely that these variables underlie the apparent disparity in concentration-dependency. Most importantly, though, the fact that ryanodine is able to cause release indicates that RyRs in the pancreatic acinar cell are functional Ca^{2+} -release channels and, when activated, can trigger release from an important Ca^{2+} store.

The Ca^{2+} level during the ryanodine-evoked transient peaked within 5 s and then decreased towards resting levels over the next 35–75 s. The shape of the transients were similar whichever concentration of ryanodine was used to release Ca^{2+} . These characteristics mirror those of transients triggered by elevated doses of agonist [40]. Such agonist-evoked signals have been shown to mobilize approx. 60–70% of the measurable Ca^{2+} stored inside the cell [41]. This suggests that the RyRs which are activated by ryanodine binding are able to trigger release of a very substantial proportion of the stored Ca^{2+} .

Ryanodine triggers Ca^{2+} waves which are initiated in the apical region

The evidence concerning the localization of RyRs in pancreatic acinar cells is somewhat contradictory. Immunocytochemistry places the channels mainly in the basal region and shows them to be excluded from the apical 'trigger zone' [9,10]. In contrast with this, cADP-ribose causes Ca^{2+} release in the apical region of the cell [16]. Since we have shown that low doses of ryanodine can trigger Ca^{2+} release from RyRs, we analysed the spatio-temporal

characteristics of this release in an attempt to localize the site of action of ryanodine. Cells loaded with fluo-4 were imaged rapidly (400–800 ms per frame) and treated with 10 μ M ryanodine. A representative trace is shown in Figure 3. As is also shown in Figure 2, ryanodine caused a rapidly activated, large cytosolic Ca^{2+} elevation. Regional analysis of the changes in fluorescence shows that the cytosolic Ca^{2+} concentration rises first in the apical region (Figure 3; inset, red trace) and subsequently rises in the basal region (Figure 3; inset, blue trace). The images in the lower part of the Figure show clearly that the first rise in fluorescence occurs at the extreme apex of the upper cell close to the luminal membrane. The Ca^{2+} rise then spreads throughout the cell as a wave travelling in an apical to basal direction ($n = 10$).

Manifestation of ryanodine-induced Ca^{2+} release is dependent on functional IP_3Rs

The initiation of ryanodine-induced Ca^{2+} release occurs in the region which is known to contain the majority of functional IP_3Rs [4,7,8,42]. As is the case with RyRs, release of Ca^{2+} through IP_3Rs is modulated by Ca^{2+} itself [43] and it is been proposed that Ca^{2+} can co-ordinate the activity of IP_3Rs and RyRs in the apical region of pancreatic acinar cells [17,19]. We therefore tested whether IP_3Rs play some role in generation of the release transients triggered by ryanodine. To do this we assessed the ability of ryanodine to induce Ca^{2+} release when IP_3Rs were pharmacologically blocked by application of elevated doses of caffeine. Elevated (10–20 mM) doses of caffeine are known to inhibit Ca^{2+} flux through isolated IP_3Rs [32]. In pancreatic acinar cells, caffeine is the only reliable membrane permeable antagonist of IP_3Rs and has been shown to rapidly and reversibly block IP_3 - and agonist-evoked Ca^{2+} signals [19,44]. Caffeine is also well known as an activator of RyRs, since it sensitizes the channel to CICR. However, in pancreatic acinar cells, this sensitization does not trigger Ca^{2+} release through RyRs unless cytoplasmic Ca^{2+} buffering is artificially reduced [44]. Therefore, we applied 20 mM caffeine to inhibit IP_3Rs and found that 10 μ M ryanodine was never able to trigger Ca^{2+} release under these conditions ($n = 113$ cells; Figure 4).

One possible interpretation of this inhibition is that ryanodine may directly activate IP_3Rs to cause the release we have described. However, it is reported that ryanodine has no effects on the activity of isolated IP_3R channels, so it is unlikely that the Ca^{2+} release could be due to a non-specific action of ryanodine [32]. Therefore, our results point to the suggestion that activation of Ca^{2+} flux through RyRs by ryanodine is not itself directly responsible for creating the Ca^{2+} elevations. Instead, it would appear that activation of RyRs by ryanodine leads to activation of IP_3Rs and that the co-ordinated release from the two channels generates the large transient which is observed. This correlates well with the dependence upon IP_3Rs of Ca^{2+} release induced by the intracellular messengers, cADP-ribose and nicotinic acid-adenine dinucleotide phosphate (NAADP) [18]. Such co-ordination of release is similar to that proposed to underlie the initiation of agonist-evoked signals [17,18]. In this previous model, the co-ordination of the channels is mediated by Ca^{2+} and CICR-based activation. This type of Ca^{2+} -mediated co-ordination is intuitive, since both channels can be activated by CICR. However, it is difficult to provide direct evidence that Ca^{2+} does underlie the communication between the channels, because there is no measurable rise in Ca^{2+} induced by ryanodine when the IP_3Rs are inhibited. However, the fact that we cannot measure a Ca^{2+} rise under these conditions does not preclude the possibility that there is a small undetectable elevation in Ca^{2+} due to activation of RyRs. Indeed, the low doses of

ryanodine open the RyRs in a subconductance state which probably induces a relatively slow leak of Ca^{2+} through the channel. This kind of leak may not be detectable, owing to high levels of Ca^{2+} buffering in the cytoplasm and strong Ca^{2+} removal mechanisms. However, such Ca^{2+} release from the RyR may still be able to activate IP_3Rs if the channels are very closely co-localized in a Ca^{2+} microdomain. If they were not very close to each other, then we would be able to detect the Ca^{2+} that co-ordinates their function.

IP_3Rs are almost exclusively found in the apical pole of pancreatic acinar cells [4,8,42]. Therefore, the dependency on IP_3Rs for triggering of ryanodine-induced Ca^{2+} transients probably underlies the fact that the waves are always initiated in the apical pole. This makes it difficult to infer very much about the density of RyRs in different regions of the cell. However, if the co-ordination of the channels is mediated by Ca^{2+} (as described above), then functional RyRs must exist near to the IP_3Rs at the extreme apex of the cell, where the wave is initiated. It is more difficult to draw conclusions about amplification and propagation of the transient. All the ryanodine-induced responses propagated to the basal membrane without any reduction in the rate of Ca^{2+} rise (see Figure 3, inset). However, these data cannot tell us whether the ryanodine-induced wave is propagated by CICR, by the delayed action of ryanodine on basal RyRs or spreading of the wave purely as a result of Ca^{2+} diffusion. There is substantial evidence that RyRs exist in the basal region of pancreatic acinar cells, so it seems likely that there should be some ryanodine-induced Ca^{2+} release through RyRs in the basal region. These basal receptors should be able to contribute by CICR to propagation of the Ca^{2+} wave, since recent results suggest that the channel sensitivity is actually sensitized to Ca^{2+} when in the ryanodine-induced subconductance state [33]. There may also be some contribution of Ca^{2+} entry (following store depletion) to the latter stages of the transient. However, the effect of Ca^{2+} entry on agonist-evoked transients of a similar size is small.

In the present study, we have demonstrated Ca^{2+} release in pancreatic acinar cells triggered by direct activation of functional RyRs by low doses of ryanodine. The present data further suggests that the initiation of Ca^{2+} transients in the apical region of pancreatic acinar cells is dependent upon a functional interaction of co-localized Ca^{2+} -release channels. The spatio-temporal profile of ryanodine-induced release indicates that functional RyRs must exist in the apical part of the cell. However, it seems that it is the co-localization of both IP_3Rs and RyRs exclusively in the apical region which makes this part of the cell the most sensitive area for initiation of intracellular Ca^{2+} transients.

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