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Cyclic ADP-ribose regulation of ryanodine receptors involved in agonist evoked cytosolic Ca^{2+} oscillations in pancreatic acinar cells

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We have investigated the role of the ryanodine-sensitive intracellular Ca^{2+} release channel (ryanodine receptor) in the cytosolic Ca^{2+} oscillations evoked in pancreatic acinar cells by acetylcholine (ACh) or cholecystokinin (CCK). Ryanodine abolished or markedly inhibited the agonist evoked Ca^{2+} spiking, but enhanced the frequency of spikes evoked by direct internal inositol trisphosphate ($InsP_3$) application. We have also investigated the possibility that cyclic ADP-ribose (cADP-ribose), the putative second messenger controlling the ryanodine receptor, plays a role in Ca^{2+} oscillations. We found that cADP-ribose could itself induce repetitive Ca^{2+} spikes localized in the secretory pole and that these spikes were blocked by ryanodine, but also by the $InsP_3$ receptor antagonist heparin. Our results indicate that both the ryanodine and the $InsP_3$ receptors are involved in Ca^{2+} spike generation.

Key words: acetylcholine/Ca^{2+} oscillations/cholecystokinin/cyclic ADP-ribose/inositol trisphosphate/ryanodine

Introduction

In many cell types, agonist evoked cytosolic Ca^{2+} oscillations are primarily due to repetitive release of Ca^{2+} from intracellular stores through inositol trisphosphate ($InsP_3$) and/or ryanodine receptors and reuptake via thapsigargin-sensitive Ca^{2+} pumps (Berridge, 1993a,b; Petersen *et al.*, 1993, 1994). In pancreatic acinar cells there is strong evidence for the involvement of $InsP_3$ receptors in agonist evoked Ca^{2+} oscillations (Wakui *et al.*, 1989; Thorn *et al.*, 1993), and also indications that a caffeine- and Ca^{2+}-sensitive Ca^{2+} release channel is present (Wakui *et al.*, 1990; Nathanson *et al.*, 1992; Kasai *et al.*, 1993). The secretory pole of the pancreatic acinar cells contains both these channels and plays a particularly important role in Ca^{2+} signalling (Kasai *et al.*, 1993; Thorn *et al.*, 1993), but the functional inter-relationship between the two types of Ca^{2+} release channels is unknown and there is little information about the nature and control of the $InsP_3$-insensitive Ca^{2+} release channel. Here, we show that ryanodine rapidly abolished or severely inhibited the agonist evoked Ca^{2+} spiking, but enhanced the frequency of the spikes evoked by intracellular $InsP_3$ infusion. We also demonstrate that cyclic adenosine 5′-diphosphoribose (cADP-ribose), the putative second messenger regulating the ryanodine receptor (Clapper *et al.*, 1987; Berridge, 1993b; Galione, 1993), can by itself induce repetitive Ca^{2+} spikes localized in the secretory pole and that these spikes are abolished by ryanodine. The cADP-ribose induced Ca^{2+} spiking is also blocked by the $InsP_3$ receptor antagonist heparin as are the responses to agonists, $InsP_3$ and caffeine. We propose that both types of Ca^{2+} release channel are intimately involved in the Ca^{2+} spiking mechanism and that both $InsP_3$ and cADP-ribose may act as the physiologically relevant controlling messengers.

Results

The effects of ryanodine and ruthenium red on agonist evoked responses

In acutely isolated single mouse pancreatic acinar cells, low concentrations of acetylcholine (ACh) (20–50 nM) evoke repetitive short-lasting cytosolic Ca^{2+} spikes, due to Ca^{2+} release from internal stores, confined to the secretory pole (secretory granule area) and these give rise to repetitive spikes of Ca^{2+}-sensitive ion currents as measured in patch–clamp whole-cell current recording experiments (Osipchuk *et al.*, 1990; Petersen *et al.*, 1991a; Thorn and Petersen, 1992; Thorn *et al.*, 1993) (Figure 1a and b). Very low (just suprathreshold) concentrations of cholecystokinin (CCK) ($\leq$5 pM) evoke mostly repetitive short-lasting spikes of the type also induced by low ACh concentrations (Figure 1c) whereas at slightly higher concentrations (10–20 pM) a mixture of short-lasting spikes and broader transients is seen (Petersen *et al.*, 1991a).

Application of ryanodine (10 μM) in the extracellular solution, rapidly and reversibly inhibited the short-lasting spikes induced by low concentrations of either ACh (n = 3 cells, Figure 1b) or CCK (n = 3, Figure 1c). This blockade was not due to ryanodine binding to the extracellular agonist receptor as inclusion of ryanodine in the patch pipette also blocked the agonist response (n = 2 of 3). Rapidly reversible effects of ryanodine have been shown in anterior pituitary GH_3 cells (Kramer *et al.*, 1994). Ruthenium red, an agent that blocks the ryanodine receptor (Lai *et al.*, 1988; Marty and Tan, 1989), was infused into the cells through the patch pipette and completely blocked the agonist evoked short-lasting spikes (n = 3, Figure 1d). Agonists at higher, but still physiological concentrations, evoke oscillations of global Ca^{2+} waves across the cell of longer duration than the local Ca^{2+} spikes (Figure 1e) (Thorn *et al.*, 1993). Ryanodine applied to the extracellular solution substantially inhibited these agonist responses (Figure 1f, n = 6). Intracellular perfusion with ruthenium red was also effective in inhibiting the agonist evoked global

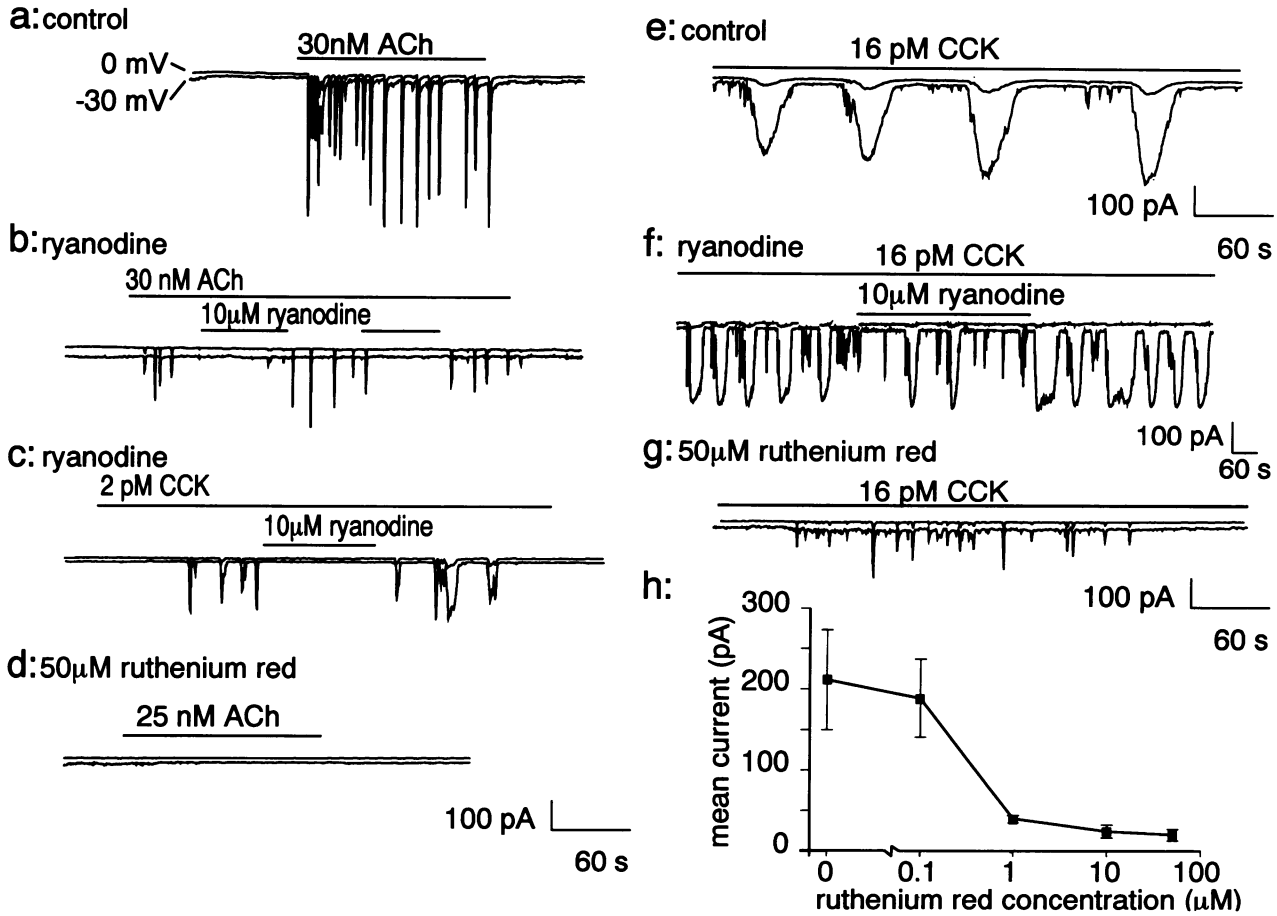

Fig. 1. Effects of ryanodine and ruthenium red on agonist evoked Ca^{2+} spiking monitored as Ca^{2+}-dependent current changes. (**a**) Control response to a low ACh concentration eliciting a train of spikes. Ryanodine reversibly blocked these short-lasting spikes evoked by either ACh (**b**) or a very low CCK concentration (2 pM, only 10 μM EGTA in pipette) (**c**). Intracellular perfusion with ruthenium red in the patch pipette solution blocked the short-lasting spikes induced by ACh (**d**). CCK (16 pM) evokes both short-lasting spikes and broad transient current responses (**e**) that are a reflection of a global Ca^{2+} increase (Thorn *et al.*, 1993). Ryanodine applied against this higher agonist concentration substantially inhibited the responses (**f**). Intracellular perfusion of ruthenium red also inhibited the Ca^{2+} oscillations, an example of which is shown in **g**. At each concentration of ruthenium red used, the mean current response (n = 4 at each point) to 16 pM CCK was plotted in **h**. The graph in h shows the block with a K_d of ~500 nM ruthenium red.

Ca^{2+} oscillations (Figure 1g, n = 4). A graph of the mean of the CCK (16 pM) evoked oscillatory current response plotted against the ruthenium red concentration is shown in Figure 1h. Very high (pharmacological) ACh concentrations (500 nM) evoke sustained current responses that are not inhibited by ryanodine (10 μM, n = 2). Such responses are, in contrast to the short-lasting spikes evoked by low ACh concentrations, not sustained by Ca^{2+} release from internal stores, but acutely dependent on external Ca^{2+} and therefore due to Ca^{2+} entry from the external solution (Yule and Gallacher, 1988).

These results suggest a crucial role for a ryanodine receptor in the oscillatory response of pancreatic acinar cells to low and physiological agonist concentrations. It has previously been shown that both the ACh and the CCK evoked Ca^{2+}-dependent current responses are blocked by the $InsP_3$ receptor antagonist heparin (Wakui *et al.*, 1990; Thorn and Petersen, 1993; Thorn *et al.*, 1993). Our new results, taken together with the earlier data, indicate a complex interplay of the two types of intracellular Ca^{2+} release channels in the generation of cytosolic Ca^{2+} oscillations.

The effect of cyclic ADP-ribose and its sensitivity to ryanodine and heparin

Substantial evidence has recently accumulated indicating that cADP-ribose is an endogenous regulator of the non-skeletal muscle type ryanodine receptor (Berridge, 1993b; Galione, 1993; Meszaros *et al.*, 1993). We therefore tested the effects of internal application of cADP-ribose. Figure 2a demonstrates Ca^{2+}-dependent current responses elicited by the infusion of cADP-ribose. The lowest concentration tested was 100 nM which in 2 cells evoked no effect and in one case (Figure 2a) elicited two single spikes. At 1 μM, cADP-ribose typically evoked repetitive spikes (Figure 2a and b; n = 5) in those cases where the acinar cells were used within 1.5 h after isolation. Older cells only rarely responded. The responses at 10 μM cADP-ribose were similar to those obtained at 1 μM (Figure 2a; n = 5). The highest concentration tested was 100 μM and in these cases sustained responses were obtained (Figure 2a; n = 4).

Extracellular application of ryanodine reversibly inhibited the cADP-ribose induced spikes (n = 3, Figure 2b). When cADP-ribose was infused together with heparin, repetitive spiking did not occur except for an initial transient (n = 4,

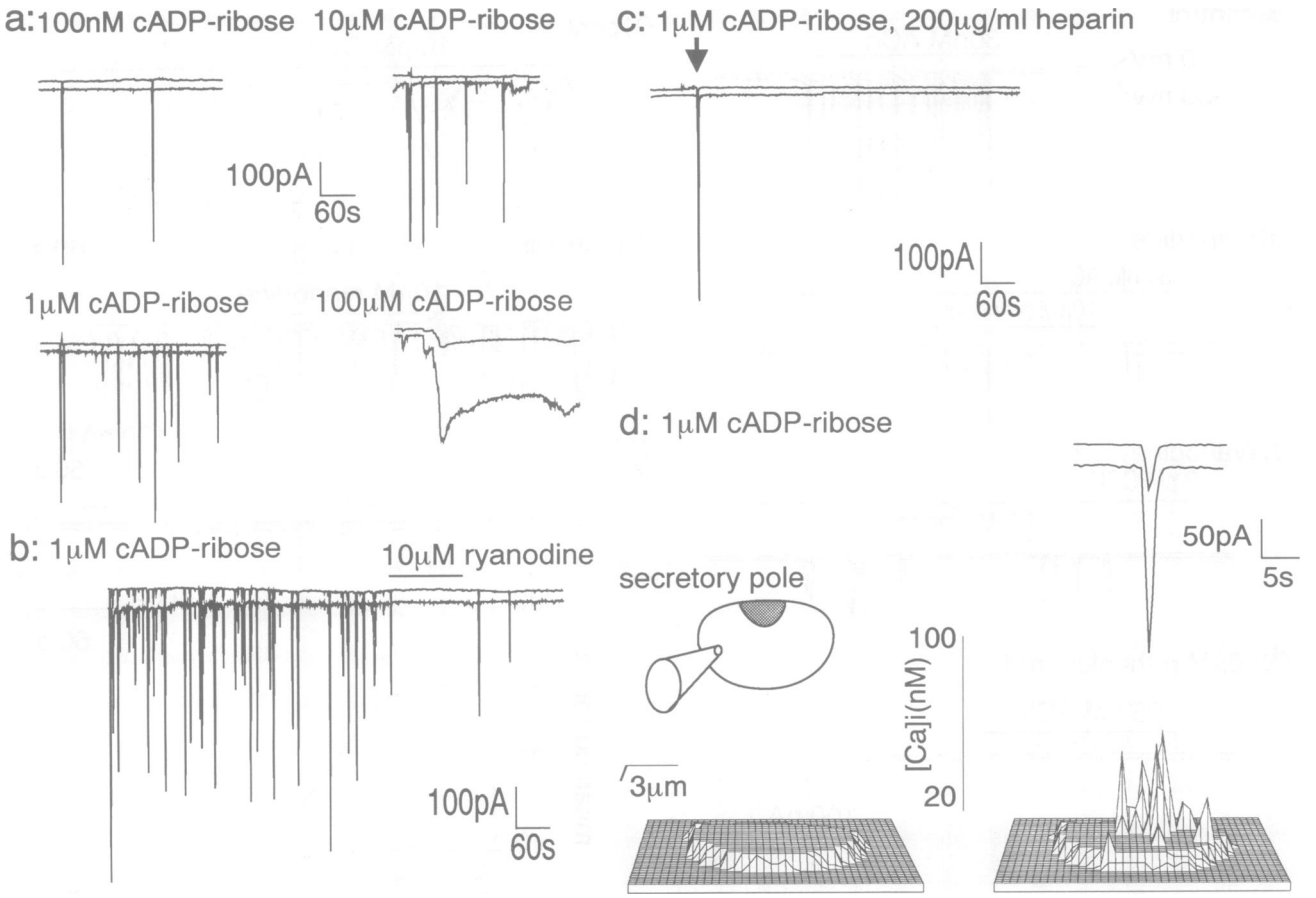

Fig. 2. cADP-ribose evokes Ca^{2+} spiking. (**a**) Illustrative responses to a range of concentrations of cADP-ribose applied through the solution of the patch pipette. At 100 nM the current trace from the one cell that did respond is shown. 1 and 10 μM cADP-ribose elicited trains of short-lasting spikes and 100 μM induced sustained Ca^{2+}-dependent current responses. Application of ryanodine to the extracellular solution reversibly inhibited the short-lasting spikes induced by 1 μM cADP-ribose (**b**). Intracellular perfusion of heparin and cADP-ribose only evoked a single transient (**c**). Combined patch–clamp and Ca^{2+} imaging demonstrated that the cADP-ribose induced current spikes were due to local Ca^{2+} rises restricted to the secretory pole of the acinar cells (**d**). The Ca^{2+}-dependent current spike is shown along with two three-dimensional maps of the $[Ca^{2+}]_i$ level taken during an interspike period (left) and at the peak of the current spike.

Figure 2c) probably as a result of the delayed entry of heparin into the cell due to its relatively high molecular weight (Thorn and Petersen, 1993). In combined patch–clamp and Fura-2 Ca^{2+} imaging experiments, we determined that the Ca^{2+} signal associated with the spikes is localized to the secretory pole of the cells (n = 5, Figure 2d), similar to the agonist induced spikes (Thorn *et al.*, 1993).

The effects of caffeine, inositol trisphosphate and GTP-γ-S and the sensitivity of these responses to ruthenium red, heparin and ryanodine

Caffeine, an exogenous activator of the ryanodine receptor can (in low concentrations and with low levels of intracellular Ca^{2+} buffer) acutely induce Ca^{2+} spiking (Wakui *et al.*, 1990). Figure 3a illustrates an example of caffeine induced spikes (n = 11 of 14). These spikes were blocked by the intracellular infusion of ruthenium red (Figure 3b, n = 7 of 9) and also by the infusion of heparin (Figure 3c, n = 3). The heparin block of the spikes induced by caffeine and cADP-ribose suggests that in addition to the activation of the ryanodine receptor there is a role for the $InsP_3$ receptor in these responses.

We know that infusion of $InsP_3$ elicits spikes that are also blocked by heparin (Wakui *et al.*, 1990), but the possible role of a ryanodine receptor in the $InsP_3$ induced response has not been investigated. The extracellular application of 10 μM ryanodine during responses to intracellular $InsP_3$ infusion did not block the trains of spikes; on the contrary, the frequency of spiking increased (Figure 3d, n = 10 of 12). Even at a higher concentration (100 μM), ryanodine could not block the spiking (n = 3). Ryanodine (10 μM) applied alone, in the absence of $InsP_3$ infusion, failed to elicit spiking (n = 3). Ruthenium red (50 μM), when infused together with $InsP_3$ did not block the spikes, but caused a decrease in spike frequency to 53% of control ($InsP_3$ alone; n = 6). Intracellular perfusion of a combination of $InsP_3$ (10 μM) and cADP-ribose (1–10 μM) resulted in spikes of much greater frequency than in control $InsP_3$ infusion experiments (n = 4), resembling the effects of ryanodine on the $InsP_3$ induced spikes. Combined patch–clamp and Ca^{2+} imaging experiments localized the ryanodine potentiated $InsP_3$ spikes to the secretory pole of the cell (Figure 3f, n = 4).

Intracellular application of GTP-γ-S or GMP-PNP can generate Ca^{2+} spiking, presumably via activation of phospholipase C inducing $InsP_3$ formation (Osipchuk *et al.*, 1990). Figure 3e shows that a low concentration of GTP-γ-

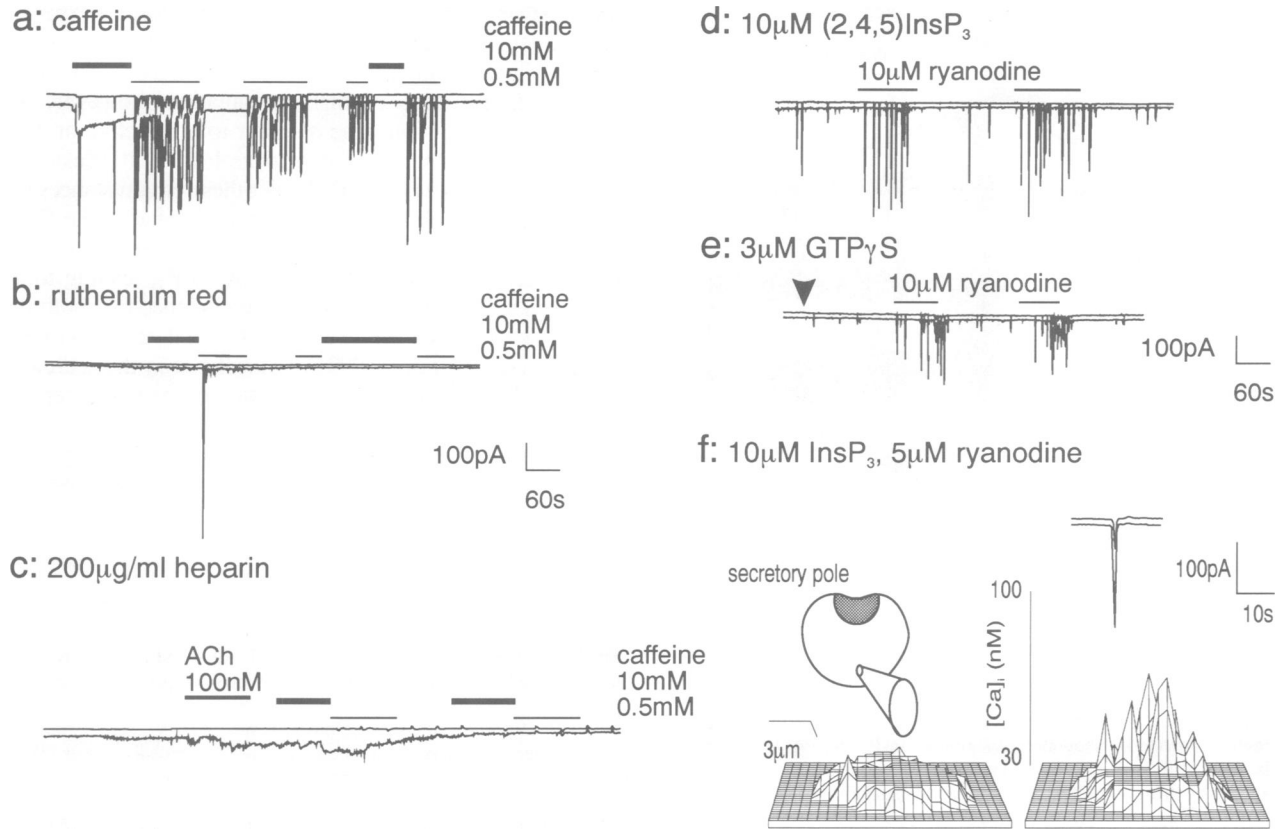

Fig. 3. Effects of ruthenium red, heparin and ryanodine on Ca^{2+} spikes evoked by caffeine, $InsP_3$ and GTP-γ-S. (**a**) Typical response to caffeine applied to the extracellular medium at concentrations of 10 and 0.5 mM. It has previously been observed that after the application of a high caffeine concentration short-lasting spikes can be repeatedly elicited by the subsequent application of low caffeine concentrations (Wakui *et al.*, 1990). We used this protocol (**a**) in the presence of ruthenium red in the pipette solution (**b**) which substantially blocked the oscillations and in the presence of heparin which also blocked the caffeine induced oscillations (**c**). (**d**) Typical enhancement of $InsP_3$ (2,4,5-$InsP_3$) induced spiking during the extracellular application of ryanodine. A very similar effect was observed when ryanodine was applied during the spiking induced by low concentrations of non-hydrolysable GTP analogues (**e**). The spatial localization of a spike induced in the presence of both $InsP_3$ and ryanodine was determined using combined Ca^{2+} imaging and patch–clamp current recording (**f**). The three-dimensional maps (f) show the Ca^{2+} distribution just before a spike and at the peak of the current spike. The Ca^{2+} rise is restricted to the secretory pole of the cell.

S evoked short-lasting spikes of a low frequency and that ryanodine markedly accelerated the spiking (n = 6, GTP-γ-S or GMP-PNP).

Discussion

Our data demonstrate that both $InsP_3$ and cADP-ribose can evoke cytosolic Ca^{2+} oscillations. Unlike the results recently obtained in sea urchin eggs, where there is clear evidence of redundancy since inhibition of either pathway had no effect on Ca^{2+} signalling (Galione *et al.*, 1993a), our data show that both $InsP_3$- and cADP-ribose-sensitive Ca^{2+} release channels are required for agonist evoked Ca^{2+} spiking (Figure 4). Since the cADP-ribose evoked short-lasting Ca^{2+} spikes are localized in the secretory pole of the acinar cells (Figure 2), like those evoked by agonists and $InsP_3$ (Thorn *et al.*, 1993), both the ryanodine and $InsP_3$ receptors are likely to be present in this part of the cell. This is in agreement with the data of Kasai *et al.* (1993) showing that the secretory pole is the site of a heparin-insensitive, Ca^{2+} induced Ca^{2+} release. Co-operation of $InsP_3$ and ryanodine receptors has previously been suggested (Wakui *et al.*, 1990; D'Andrea *et al.*, 1993) and within the secretory pole, the two receptors may be colocalized (Giannini *et al.*, 1992) or located in separate pools (Malgaroli *et al.*, 1990).

The finding that ryanodine blocks agonist and cADP-ribose evoked Ca^{2+} spiking, but enhances $InsP_3$ induced Ca^{2+} spiking shows that although intracellular $InsP_3$ application can mimic the effect of agonists (Petersen *et al.*, 1991b) it is very unlikely that the agonists act by generating the relatively high $InsP_3$ levels (>3 μM) required to induce spiking in intracellular infusion experiments. There is evidence showing that high concentrations of ACh and CCK do evoke $InsP_3$ production (Streb *et al.*, 1985), but it is far from clear that, for example, CCK at physiological levels actually causes $InsP_3$ formation (Matozaki *et al.*, 1989). Nevertheless, it is possible that physiological CCK levels evoke a small and unmeasurable formation of $InsP_3$ in microdomains.

Our data indicate that a messenger in addition to $InsP_3$, that can activate the ryanodine receptor, is needed in order to explain the generation of agonist evoked Ca^{2+} spikes. cADP-ribose could be this messenger, but it is not clear how cADP-ribose generation could be controlled in the pancreatic acinar cells. Recent evidence shows that cGMP can stimulate cADP-ribose production in sea urchin eggs (Galione *et al.*, 1993b). A similar role for cGMP in pancreatic acinar cells is unlikely, despite the fact that agonist stimulation evokes an increase in cGMP concentration (Christophe *et al.*, 1976). Exogenous dibutyryl cGMP has no effect on ^{45}Ca transport

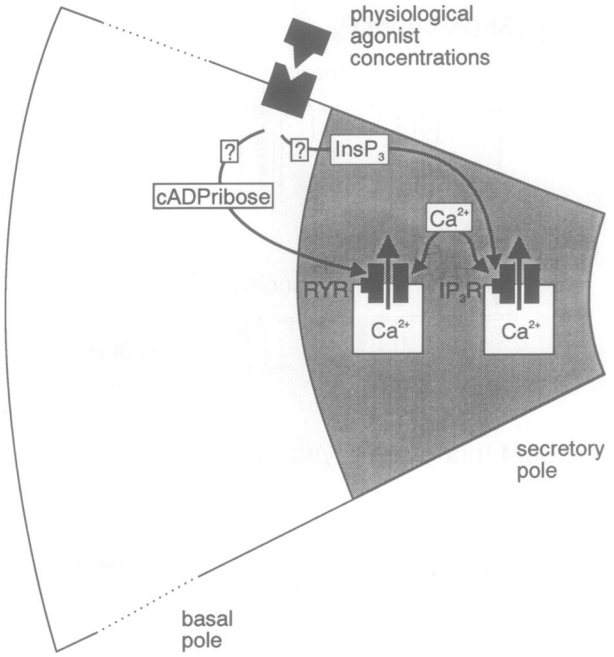

Fig. 4. Working model for agonist evoked Ca^{2+} spiking in secretory pole of pancreatic acinar cell. The question marks indicate that there is at present no evidence showing regulation of $InsP_3$ and cADP-ribose levels in these cells using low (physiological) concentrations of the main agonists ACh and CCK. For further explanation see text.

(Christophe *et al.*, 1976) and also fails to induce any membrane potential or resistance changes in pancreatic acinar cells, apart from very selectively blocking CCK responses acting as a competitive hormone receptor antagonist (Philpott and Petersen, 1979).

The effect of ryanodine on its receptor is to block the channel or promote an open 'lock' of the channel into a sub-conductance state (Rousseau *et al.*, 1987). In skeletal muscle this open locked state is still capable of regulation by Ca^{2+} (Oyamada *et al.*, 1993). These reported effects of ryanodine would either block an oscillatory mechanism that was dependent on the ryanodine receptor (Friel and Tsien, 1992) or at least substantially modify the kinetics of the oscillations. In the case of $InsP_3$ induced oscillations, ryanodine application only increased the spike frequency, suggesting that the ryanodine receptor plays a secondary role in this activity. This conclusion is further supported by the inability of ruthenium red infusion to block $InsP_3$ evoked spiking. In contrast, the actions of cADP-ribose and caffeine are primarily to sensitize the ryanodine receptor, since Ca^{2+} spiking can be blocked by ryanodine and ruthenium red. Ca^{2+} release from the ryanodine-sensitive pool may in turn sensitize and recruit $InsP_3$ receptors into the spiking mechanism. The action of ryanodine at the ryanodine receptor may be different under different circumstances; for instance, ryanodine binding is enhanced in the presence of cADP-ribose (Meszaros *et al.*, 1993). However, if we assume that under all conditions the concentration of ryanodine we used is acting to promote an open channel low conductance block of the ryanodine receptor, we can explain our data as follows. In the case of stimulation by cADP-ribose and caffeine, the $InsP_3$ receptor may be sensitized to basal levels of $InsP_3$ by a small local elevation of the Ca^{2+} concentration due to the opening of the ryanodine receptor (Bezprozvanny *et al.*, 1991) and would therefore be expected to be open. The action of ryanodine would reduce Ca^{2+} current flow through the ryanodine receptor and block spiking. In the case of $InsP_3$ stimulation, however, we would expect the ryanodine receptor to be inactive or only active at a very low level due to its low Ca^{2+} sensitivity (Bezprozvanny *et al.*, 1991). Under these circumstances the open channel block by ryanodine would enhance the Ca^{2+} leak through the ryanodine receptor and further sensitize the $InsP_3$ receptor, already stimulated by $InsP_3$, leading to an enhanced spike frequency. We have evidence that both the $InsP_3$ receptor (Thorn *et al.*, 1993) and the ryanodine receptor (Kasai *et al.*, 1993) are localized in the secretory pole and therefore the close interaction of the two receptors proposed here is quite feasible.

The action of agonists most closely resemble the actions of cADP-ribose and caffeine suggesting that agonists act primarily through a sensitization of the ryanodine receptor possibly through the production of cADP-ribose.

Materials and methods

Single pancreatic acinar cells were acutely isolated and investigated using the whole cell patch-clamp configuration, at room temperature, as described previously (Osipchuk *et al.*, 1990; Petersen *et al.*, 1991a; Thorn and Petersen, 1992). From a holding potential of −30 mV, steps were made to 0 mV, the reversal potential of the two Ca^{2+}-dependent currents (Thorn and Petersen, 1992). The extracellular Na^+-rich solution contained (mM): 140 NaCl, 4.7 KCl, 1.13 $MgCl_2$, 11 glucose, 1 Ca^{2+}, 10 HEPES-NaOH pH 7.2. The standard intracellular solution (pipette solution) contained (mM): 140 KCl, 1.13 $MgCl_2$, 0.1 EGTA, 2 ATP, 10 HEPES-KOH pH 7.2. Ryanodine (Calbiochem), caffeine, ACh and CCK-8 (Sigma) were applied outside the cells by means of a local rapid perfusion system. Ruthenium red, heparin (mol. wt <3000), 2,4,5-$InsP_3$, GTP-γ-S, GMP-PNP (Sigma) and cADP-ribose (Amersham) were added, as required, to the intracellular (patch-clamp pipette) solution. Ca^{2+} imaging experiments used an Applied Imaging Magical System (Thorn *et al.*, 1993). The cells were loaded with Fura-2-AM (Molecular Probes; 1 μM, 10 min, room temperature) or with Fura-2 free acid (100-200 μM) through the patch pipette.

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