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Both RyRs and TPCs are required for NAADP-induced intracellular Ca^{2+} release

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ABSTRACT

Intracellular Ca^{2+} release is mostly mediated by inositol trisphosphate, but intracellular cyclic-ADP-ribose (cADPR) and nicotinic acid adenine dinucleotide phosphate (NAADP) are important messengers in many systems. Whereas cADPR generally activates type 2 ryanodine receptors (RyR2s), the NAADP-activated Ca^{2+} release mechanism is less clear. Using knockouts and antibodies against RyRs and Two-Pore Channels (TPCs), we have compared their relative importance for NAADP-induced Ca^{2+} release from two-photon permeabilized pancreatic acinar cells. In these cells, cholecystokinin-elicited Ca^{2+} release is mediated by NAADP. TPC2-KO reduced NAADP-induced Ca^{2+} release by 64%, but the combination of TPC2-KO and an antibody against TPC1, significantly reduced Ca^{2+} release by 86% (64% vs. 86%, $p < 0.0002$). In RyR3-KO, NAADP-evoked Ca^{2+} release reduced by ~50% but, when combined with antibodies against RyR1, responses were 90% inhibited. Antibodies against RyR2 had practically no effect on NAADP-evoked Ca^{2+} release, but reduced release in response to cADPR by 55%. Antibodies to RyR1 inhibited NAADP-induced Ca^{2+} liberation by 81%, but only reduced cADPR responses by 30%. We conclude that full NAADP-mediated Ca^{2+} release requires both TPCs and RyRs. The sequence of relative importance for NAADP-elicited Ca^{2+} release from the all stores is RyR1 > TPC2 > RyR3 > TPC1 » RyR2. However, when assessing NAADP-induced Ca^{2+} release solely from the acidic stores (granules/endosomes/lysosomes), antibodies against TPC2 and TPC1 virtually abolished the Ca^{2+} liberation as did antibodies against RyR1 and RyR3. Our results indicate that the primary, but very small, NAADP-elicited Ca^{2+} release via TPCs from endosomes/lysosomes triggers the detectable Ca^{2+}-induced Ca^{2+} release via RyR1 and RyR3 occurring from the granules and the ER.

1. Introduction

Exocrine gland cells have provided the most important models for elucidating the mechanisms underlying hormone- or neurotransmitter-evoked intracellular Ca^{2+} release. Early work on salivary glands provided evidence for Ca^{2+} pump-mediated Ca^{2+} uptake into intracellular stores [1] as well as neurotransmitter-elicited liberation of Ca^{2+} from such stores [2]. Ca^{2+} signalling studies on pancreatic acinar cells led to many important findings including the discovery of inositol trisphosphate (IP_3) as a messenger releasing Ca^{2+} from intracellular stores [3], localized Ca^{2+} signal generation in the apical granular pole of the cells [4–6] and intracellular Ca^{2+} tunnels [7]. It has been shown that IP_3 induces responses from the endoplasmic reticulum ER [8], suggesting that IP_3 receptors (IP_3Rs) are located in the ER including the apical area of acinar cells [8–10]. However, it was also found that IP_3 can release Ca^{2+} from a different organelle containing a vacuolar H^+-ATPase [11] followed up by the discovery of IP_3-evoked Ca^{2+} release from bovine adrenal medullary secretory vesicles [12]. Work on isolated pancreatic zymogen granules (ZGs) demonstrated directly both IP_3 and cADPR-induced Ca^{2+} release from this organelle [13] and was later confirmed by a study of tracheal goblet cells [14]. Apart from the ZGs, other acid organelles (endosomes and lysosomes) can also release Ca^{2+} [15–17]. Under normal physiological conditions

Abbreviations: RyR, ryanodine receptor; TPC, two-pore channel; NAADP, nicotinic acid adenine dinucleotide phosphate; cADPR, cyclic-ADP-ribose; IP_3, inositol trisphosphate; KO, knockout; ZG, zymogen granules; ER, endoplasmic reticulum; CCK, cholecystokinin; ACh, acetylcholine.

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the apical Ca^{2+} spikes regulating exocytotic secretion of digestive enzymes are mainly due to Ca^{2+} release from the ER strands in the secretory granular area [18], which are functionally connected to the lumen of the bulk of the ER, allowing diffusion of Ca^{2+} inside the ER throughout the cell [7,19]. In contrast, release of Ca^{2+} from the acid stores may be of particular significance for the initiation of acute pancreatitis [20,21]. The acid stores, like the ER, have been shown to respond to all the three intracellular Ca^{2+} releasing messengers IP_3, cADPR and NAADP [18,22]. Previous work has emphasized the importance of the RyRs for the action of CCK, which is primarily mediated by NAADP, in contrast to the action of acetylcholine (ACh), which is primarily mediated by IP_3 [17,18,20,23,24].

There are conflicting hypotheses about the mechanism underlying NAADP-induced Ca^{2+} signalling [25]. Two-pore channels (TPC) [26,27] have been suggested as the main route for NAADP-induced Ca^{2+} release from the lysosomes. Calcium-induced calcium release (CICR) via IP_3 or ryanodine receptors has been proposed as an explanation for the subsequent amplification of the response by Ca^{2+} release from the ER [26,27]. However, our group has emphasized the primary importance of ryanodine receptors for NAADP-induced Ca^{2+} release in both the secretory granule stores and the ER [22]. Any primary Ca^{2+} release from endosome/lysosome stores in pancreatic acinar cells would be extremely difficult to detect due to the small size of the endo/lysosomal Ca^{2+} store [15,28]. Currently, many aspects of NAADP-elicited Ca^{2+} signalling are very unclear [29,30] and it is therefore necessary to examine experimentally the different potential mechanisms [25,31]. Here we report a comprehensive comparison of the role of different types of RyRs and TPCs in NAADP-induced Ca^{2+} release in pancreatic acinar cells.

2. Materials and methods

2.1. Materials

Most of the reagents were purchased from Sigma (UK). Ned-19 was obtained from Tocris (UK). Thapsigargin and ATP were from Merck Millipore (UK). Rabbit anti-TPC1 and anti-TPC2 polyclonal antibodies were purchased from Lifespan BioSciences (UK). Rabbit anti-RyRs polyclonal antibodies were obtained from Merck Millipore (UK).

2.2. Pancreatic acinar cells isolation

Pancreatic acinar cells (single, or clusters of two or three cells) were isolated from the pancreas of adult C57BL/6 mice (control wt and mutant male or female mice with C57Bl/6JJmsSlc origin) as described previously [4]. Briefly, animals were killed according to UK Schedule 1 regulations and after dissection the pancreas was transferred into a collagenase-containing solution (200 U/ml, Worthington, UK), and incubated at 37 °C water bath for 14–15 min. After digestion, the tissue was mechanically disrupted in Na-Hepes based extracellular buffer, containing (mM): NaCl, 140; KCl, 4.7; Hepes KOH, 10; $MgCl_2$, 1; glucose 10; $CaCl_2$ 1; pH 7.2. Cells were then loaded with an appropriate fluorescent dye (Fluo-5N AM or Fluo-4 AM) following the manufacturer's instruction. All experiments were carried out at room temperature using freshly isolated cells, attached to the poly-L-lysine coverslips of the perfusion chamber.

2.3. Cell permeabilization protocol

Two-photon permeabilization was performed as described previously [22] using Leica multiphoton system SP5 with Mai-Tai two-photon laser tuned to 738 nm. Fluorescence intensity of Fluo-5N was recorded using excitation at 476 nm and emission >500 nm. During permeabilization and experimental protocols with permeabilized cells an intracellular solution was used based on K-Hepes, containing (mM): KCl, 127; NaCl, 20; Hepes KOH, 10; ATP, 2; $MgCl_2$, 1; EGTA, 0.1; $CaCl_2$ 0.05; pH 7.2.

2.4. Data analysis

To enable comparison, confocal images were recorded using identical laser power, photomultiplier sensitivity, and were processed using identical values for contrast and brightness. Statistical significance for the comparison between two groups of data Student's *t*-test was used (statistical significance was taken as $p < 0.05$).

3. Results

3.1. Inhibitory effect of NAADP antagonist Ned-19 on Ca^{2+} signal generation in pancreatic acinar cells

Previously it has been shown that Ca^{2+} responses elicited by physiological concentrations of CCK can be specifically blocked by inactivation of the NAADP receptor, while responses to ACh were unaffected [18,23]. The relationship between CCK action and NAADP signalling was shown by measurements of the intracellular level of NAADP that were increased in a dose-dependent manner upon stimulation with physiological concentrations of CCK. In contrast, ACh did not change the intracellular NAADP concentrations [17]. We employed the cell-permeable NAADP analogue and selective antagonist, Ned-19 [32] in order to explore differences between the mechanisms of ACh- and CCK-evoked Ca^{2+} signal generation. In a previous study, Ned-19, at a high concentration (100 μM), was shown to block NAADP-mediated Ca^{2+} release and binding to the NAADP receptor [33]. In our experiments on intact acinar cells, exposure of the cells to 100 μM Ned-19 was able to block completely cytosolic Ca^{2+} responses induced by 5 pM CCK (Fig. 1A and C), but was unable to inhibit Ca^{2+} oscillations produced by 20 nM ACh (Fig. 1B and D).

In two-photon permeabilized pancreatic acinar cells, pre-incubation with a high concentration of Ned-19 (100 μM) inhibited NAADP-elicited Ca^{2+} responses by 67.8% (Fig. 2A, B, and E), whereas responses to cADPR (10 μM) (Fig. 2C–E) and IP_3 (Fig. 2E) were not significantly affected, indicating that the action of Ned-19 is specific for NAADP-induced responses.

3.2. Both TPCs are involved in NAADP-elicited Ca^{2+} responses with predominant role of TPC2 in endosomes/lysosomes stores

Involvement of TPCs in NAADP-elicited Ca^{2+} signalling has been shown by many groups [26,34]. To further investigate this issue, we have performed experiments using pancreatic acinar cells isolated from TPC2 KO mice [26]. NAADP (100 nM) induced Ca^{2+} release from stores was reduced by 64% in cells from TPC2 KO as compared to the release in cells from wt mice (Fig. 3A, B, and F). A similar (72%) level of inhibition was achieved by treatment of permeabilized cells from wt mice with a TPC2 antibody (Fig. 3C and F). In contrast, an antibody against TPC1 was only able to reduce the response to a minor degree (by 24.5%) (Fig. 3F). Responses to cADPR and IP_3 were not affected by the TPC2 antibody (Fig. 3E). Pre-incubation of permeabilized cells from TPC2 KO with the TPC1 specific antibody resulted in significant ($p < 0.0002$) inhibition of the NAADP-elicited Ca^{2+} release by 85.7% (Fig. 3D and F). Application of a mixture of both antibodies against TPC1 and TPC2 reduced NAADP-induced Ca^{2+} response by 80.6% (Fig. 3F).

These data indicate that both TPCs are involved in NAADP-induced Ca^{2+} signalling in pancreatic acinar cells, but also suggest that TPC2 is far more important than TPC1.