Chapter 12

Two-Photon Permeabilization and Calcium Measurements in Cellular Organelles

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Abstract

Inositol trisphosphate and cyclic ADP-ribose, main intracellular Ca^{2+} messengers, induce release from the intracellular Ca^{2+} stores via inositol trisphosphate and ryanodine receptors, respectively. Recently, studies using novel messenger nicotinic acid adenine dinucleotide phosphate (NAADP) releasing Ca^{2+} from calcium stores in organelles other than endoplasmic reticulum (ER) have been conducted. However, technical difficulties of Ca^{2+} measurements in relatively small Ca^{2+} stores prompted us to develop a new, more sensitive, and less damaging two-photon permeabilization technique. Applied to pancreatic acinar cells, this technique allowed us to show that all three messengers – IP_3, cADPR, and NAADP – release Ca^{2+} from two intracellular stores: the endoplasmic reticulum and an acidic store in the granular region. This chapter describes a detailed procedure of using this technique with pancreatic acinar cells.

Key words: Ca^{2+} stores, Ins(1,4,5)P_3, Ryanodine, Pancreas and pancreatic cells, Two-photon permeabilization, Secretory granules.

1. Introduction

Hormone induced Ca^{2+} release from intracellular stores into the cytosol (1) and intracellular Ca^{2+}-releasing messenger inositol (1,4,5-trisphosphate (IP_3) are the most important discoveries in the Ca^{2+} signaling field (2, 3). IP_3-induced Ca^{2+} release is currently accepted as the principal mechanism for generation of Ca^{2+} signals in non-excitable cells (4, 5). While it is generally accepted that the endoplasmic reticulum (ER) is the main organelle for Ca^{2+} release (6, 4, 7–9), other organelles can also serve as a possible Ca^{2+} store. Several candidates have been suggested

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including the mitochondria, which play an important role in shaping cytosolic Ca^{2+} signals (10–13). Other organelles such as the nuclear envelope (14–19), the Golgi apparatus (20–22), the secretory granules (23–26), and the endosomes (27) have been reported to store and release Ca^{2+}.

In pancreatic acinar cells, the bulk of the ER Ca^{2+} store is located in the basal part of the cell (28) with thin ER projections into the secretory granule area (29) where Ca^{2+} release is usually initiated (30). Application of intracellular Ca^{2+} messenger inositol 1,4,5-trisphosphate (IP_3) or cyclic ADP ribose (cADPR) produces Ca^{2+} release specifically localized in the secretory granular area (30, 31), including novel Ca^{2+} releasing messenger nicotinic acid adenine dinucleotide phosphate (NAADP) (32–35). Several hypotheses explaining possible mechanisms of NAADP-induced Ca^{2+} release have been suggested recently (36–39).

To study effects of intracellular Ca^{2+} messengers, researchers have to use permeabilized cells which are of particular importance for studies of internal stores (2, 3). Chemical permeabilization often damages intracellular membranes and makes it difficult to detect other stores apart from the largest ER. We have tried several permeabilizing agents (digitonin, saponin, streptolysin) until we came up with the idea to use two-photon laser approach (40, 41) for permanent cellular permeabilization (42). This method is much less damaging than chemical permeabilizations. Permeabilization has been achieved by localized perforation of the membrane using two-photon (tuned to 740–750 nm) high intensity laser pulses. Two-photon light when directed to a small area of cell membrane can perforate plasma membrane allowing successful intracellular delivery of foreign DNA (41). We have modified this technique to achieve permanent permeabilization of pancreatic acinar cells (**Fig. 12.1a–d**). The result of permeabilization is the hole of approximately 2 μm in size, with most of plasma membrane and intracellular organelles intact.

We have studied Ca^{2+} release elicited by the intracellular Ca^{2+} releasing messengers NAADP, cADPR, and IP_3 and found that all three messengers can release Ca^{2+} from two separate internal stores: thapsigargin-sensitive (ER type) store and thapsigargin-insensitive acidic Ca^{2+} store (**Fig. 12.2**) (42, 43). With the help of two-photon permeabilization technique we have shown that in both stores NAADP and cADPR likely activate RyRs, whereas IP_3 activates IP_3Rs. The use of antibodies and/or large proteins also allowed us to modulate specific intracellular function(s) (**Fig. 12.3**). This technique serves as a reliable and convenient method to permeabilize cells with the minimal damage to cellular membranes and organelles.

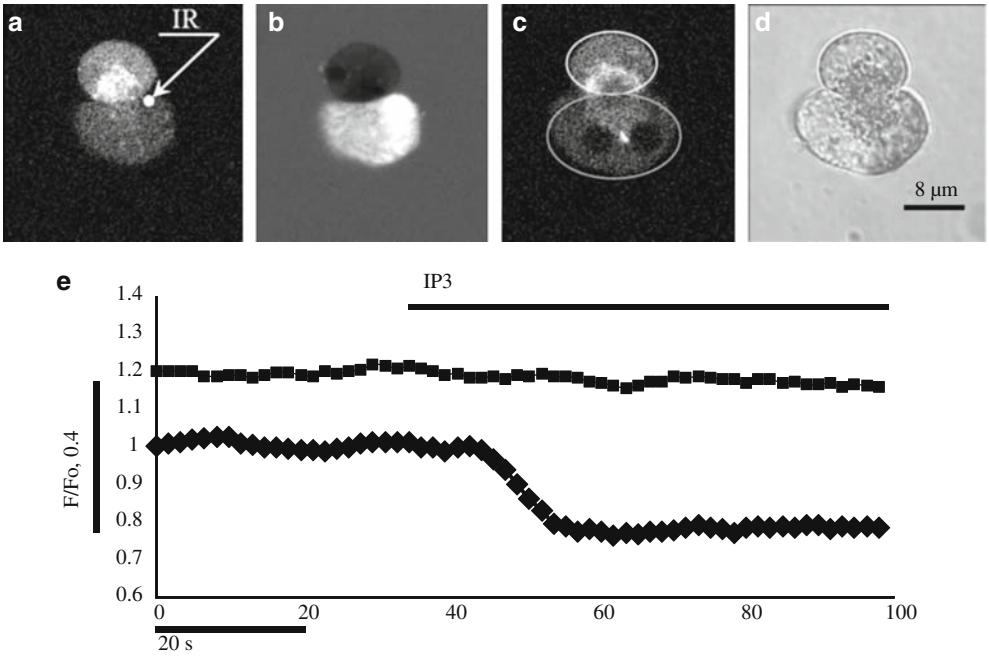

Fig. 12.1. Two-photon permeabilization of pancreatic acinar cells and the effects of Ca^{2+} releasing messengers (modified from Gerasimenko et al. (42)). **a**. A doublet of pancreatic acinar cells loaded with Fluo-5 N AM before permeabilization. Arrow shows the position of two-photon light application. **b**. Same cell doublet after permeabilization and perfusion with Texas Red dextran (3×10^3 M_r). Only the lower cell has been permeabilized and is therefore bright due to diffusion of Texas Red dextran into the cytoplasm. **c**. Same cell doublet after washing out of Texas Red dextran. Note reduced fluorescence of Fluo-5 N in the lower permeabilized cell. **d**. Transmitted light picture of the doublet (after permeabilization) shown in **a–c**. **e**. IP_3 (10 μM) was applied to the doublet shown in **a-d**. Note that IP_3 elicited a reduction in $[Ca^{2+}]$ in the intracellular stores in the lower (permeabilized) cell, whereas there was no response in the upper (intact) cell.

2. Materials

2.1. Mouse Pancreatic Acinar Cells Preparation

1. Buffer I for cell isolation, which contains 140 mM NaCl, 4.7 mM KCl, 10 mM HEPES, 1 mM $MgCl_2$, 10 mM Glucose, 1 mM $CaCl_2$, pH 7.2 (adjusted with NaOH). Store at 4°C; pre-warm to 37°C before use.
2. Solution of collagenase (Worthington, UK; 200 u/mL; 1 mL/pancreas) in buffer I for pancreas digestion. Store at –20°C; pre-warm to 37°C before use.
3. Water-bath at 37°.
4. Centrifuge with swing rotor (Denley, UK) or any centrifuge capable of $100 \times g$ with swing rotor for 15 mL tubes.
5. Animal facility for mice handling (schedule 1).

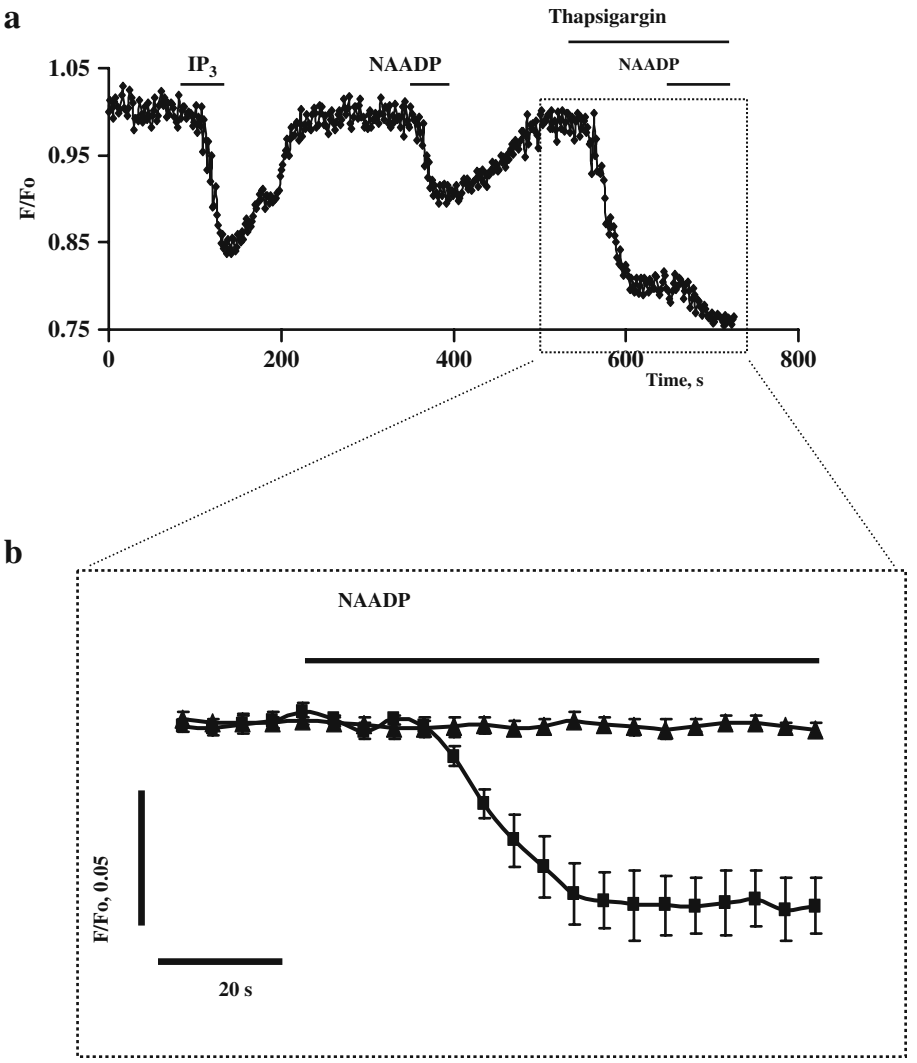

Fig. 12.2. NAADP, IP_3, or cADPR elicit Ca^{2+} release from both thapsigargin-sensitive and thapsigargin-insensitive intracellular stores (modified from Gerasimenko et al. (42)). **a**. Ins(1,4,5)P_3 and NAADP induce large Ca^{2+} responses from whole cell (before thapsigargin), while NAADP can induce only small Ca^{2+} release after high dose of thapsigargin. **b**. Same experiment as shown in 12.2a with the ROI in the granular area (*lower trace*, ■) and basal area (*upper trace*, ▲). NAADP (100 nM) induces Ca^{2+} release from the store in the secretory granule area in the presence of thapsigargin (10 μM) but not in the basal area.

2.2. Two-Photon Permeabilization

1. Buffer II for cell permeabilization, which contains 128 mM KCl, 20 mM NaCl, 10 mM HEPES, 2 mM ATP, 1 mM $MgCl_2$, 0.1 mM EGTA, 0.075 mM $CaCl_2$, pH 7.2 (adjusted with KOH). Store at 4°C. Pre-warm to 37°C before use. (see **Note 2**).

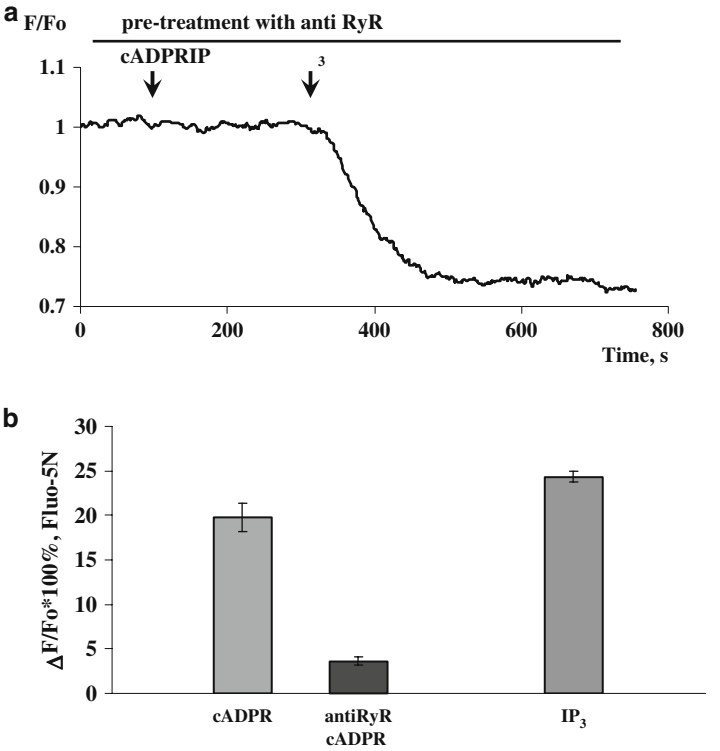

Fig. 12.3. Antibodies against RyRs completely block functional release induced by cADPR, but not by Ins(1,4,5)P_3. **a.** Permeabilized pancreatic acinar cells have been incubated with anti-RyRs antibodies (Millipore, UK) for 20 min and then cADPR has been added with no effect. After that Ins(1,4,5)P_3 induced Ca^{2+} release similar to observed in control experiments. **b**. Comparison of responses induced in the absence of antibodies to cADPR (*first column*) and in the presence of anti-RyRs antibodies to cADPR (*second column*) and to I(1,4,5)P_3 (*third column*).

2. Microscope cover slips (borosilicate glass, thickness no.1) (VWR International, UK), coated with poly-L-lysin (Sigma, UK).
3. Two-photon femtosecond laser Millennia (power 8 W) (Spectra-Physics).
4. Leica SP2 confocal two-photon microscope (similar results were also obtained using Olympus two-photon confocal microscope).
5. Perfusion chamber (self-made or from any supplier).

2.3. Fluorescent Calcium Dyes

1. Fluorescent dyes Mag-Fura-2 AM, Fluo-5 N AM, Texas Red dextran (3×10^3 M_r) (all Invitrogen, UK). Prepared as 1 mM stock solutions in DMSO, stored at –20°C.
2. 1 mM solution of the "near–membrane" Ca^{2+} indicator FFP-18 (K^+) salt (TEFLabs, USA) in buffer II, stored at –20°C.

3. Methods

3.1. Cell Preparation

1. Isolate mouse pancreatic acinar cells by collagenase digestion as described previously (29) (using schedule 1 procedure for handling CD1 mice, according to Animals (Scientific Procedures) Act). Briefly, inject extracted pancreas with 1 mL of collagenase solution, place it in 1.5 mL eppendorf tube, and incubate 15 min in a water-bath at 37°C.
2. Dissociate cells by pipetting through a large diameter tip (cut tip end if necessary).
3. Collect cells in a separate tube filled with buffer I.
4. Centrifuge cells for 1 min at 100×*g* using swing rotor at room temperature.
5. Gently resuspend the cell pellet in 2 mL of buffer I, add about 10 mL of the same buffer, and wash cells by pipetting several times.
6. Centrifuge cells for 1 min at 100×*g* using swing rotor at room temperature.
7. Resuspend cells in 2 mL of buffer I.
8. Prepare stock of indicator dye for cell loading by desiccation of Mag Fura-2AM or Fluo-5 N AM in DMSO in concentration of 2 mM according to manufacturer's instructions (Invitrogen).
9. After isolation, load cells with low affinity Ca^{2+}-sensitive dyes: Mag Fura-2 AM (5 μM) or Fluo-5 N AM (5 μM) by adding an aliquot (5 μL to 2 mL of cells) of concentrated stock and incubate for 30–45 min at 37°C in water-bath.
10. Centrifuge for 1 min at 100×*g* using swing rotor at room temperature. Resuspend cells in 2 mL of buffer I.

3.2. Two-Photon Permeabilization

1. Place 200 μL of cells on poly-L-lysine-coated cover slips attached to perfusion chamber under the microscope. Perform all experiments at room temperature. Prior to permeabilization, perfuse the cells with buffer II (K^+ rich, low Ca^{2+}, containing EGTA).
2. Stain cell membrane with the "near-membrane" Ca^{2+} indicator FFP-18 (K^+ salt) (1 μM) for 5 min to help the formation of a single, site-specific perforation in the cell membrane using the two-photon laser beam.
3. Apply a high intensity two-photon laser beam in pulse mode at 740–750 nm from Spectra-Physics (8 W Millennia femtosecond laser) to a small area of the cell membrane

(**Fig. 12.1a**) (*see* **Note 1**). This results in heating of this small membrane area and subsequent hole formation.

4. Permeabilization can be confirmed by monitoring the fluorescence of Texas Red dextran added to the extracellular medium. Upon permeabilization Texas Red dextran penetrates into the cytoplasm of targeted cell (**Fig. 12.1b**).
5. After permeabilization, perfuse cells with intracellular solution (buffer II) for 5–10 min to wash out the cytosolic component of the fluorescent dye (*see* **Note 3**): Experiments shown in **Fig. 12.1 w**ere conducted in the same solution as above except that $CaCl_2$ was reduced to 0.05 mM.
6. Observe washing of Texas Red dextran from the extracellular solution, which confirms successful and stable permeabilization (**Fig. 12.1c**).
7. After perforation, cells should be able to respond to intracellular Ca^{2+} releasing messengers IP_3, NAADP, or cADPR, (**Fig. 12.1e–g**).

3.3. Fluorescent $[Ca^{2+}]$ Measurements

1. Acquire fluorescent images using the Leica SP2 MP two-photon confocal microscope with objective 63× NA 1.2.
2. Excitation and emission wavelengths for Mag Fura-2 are 430 nm (2–5% power) and 460–590 nm, respectively. Alternatively, for excitation of Mag Fura-2 use the two-photon wavelength ~745 nm.
3. Excitation and emission wavelengths for Fluo-5 N are 488 nm (Argon Ion laser, 1–2% power) and 510–590 nm, respectively.
4. Collect fluorescent images with a frequency of 0.6–1.0 frame/s.
5. Excitation and emission wavelengths for Texas Red dextran are 543 nm and 580–650 nm, respectively.
6. Calculate free Ca^{2+} concentrations assuming that the K_d of Fluo-5 N for Ca^{2+} is 90 μM (*see* **Note 4**).
7. Perform the calibration procedure by applying ionomycin (10 μM) and nigericin (7 μM) with 2 mM EGTA or 10 mM $CaCl_2$.
8. Perform statistical analysis using Microsoft Excel software. Determine *P* values for statistical significance between sets of data using Student's *t*-test.

3.4. Conclusions

We compared responses to the messengers in cells permeabilized by two-photon light with responses obtained from saponin (as well as digitonin and streptolysin) permeabilized acinar cells. We found that two-photon permeabilization has two principal advantages, namely better preserved morphology (including polarity)

and responsiveness. The amplitudes of the responses to IP_3 were approximately 1.5 times higher in the two-photon permeabilized cells than in cells permeabilized by saponin (even more difference for digitonin and streptolysin). Two-photon permeabilization resulted in a formation of a hole with a diameter of ~2 μm at any site selected on the surface of the cell. This hole did not close after permeabilization, as in previously published work (41), perhaps due to the larger size and/or the exposure of the cell to an intracellular solution before the two-photon pulse. The success rate with two-photon permeabilization was high (>50%) and we propose this technique as a reliable and convenient method for studies of intracellular stores and organelles.

4. Notes

1. The choice of permeabilization region on cell membrane should be preferably away from the bulk of the ER. Permeabilization can damage not only plasma membrane but also intracellular membranes and organelles which potentially can cause the dramatic loss of Ca^{2+}. In our experiments best results were obtained by placing target of laser beam near granular area (**Fig. 12.1a**).
2. Concentration of ATP can be increased to 3 mM to ensure high activity of SERCA pumps.
3. Permeabilizations were conducted at first in buffer II with calcium concentration (0.075 mM $CaCl_2$) and then before experiment calcium was reduced (by perfusion with buffer II containing 0.05 mM $CaCl_2$).
4. The pH-dependence of the K_d of Fluo-5 N for Ca^{2+} was tested. There was no significant difference between the results at pH 7.2 and at pH 6 (27).

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