Chapter 7

Permeabilization of Cell Membrane for Delivery of Nano-objects to Cellular Sub-domains

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Abstract

Delivery of nano-objects to specific cellular sub-domains is a challenging but intriguing task. There are two major barriers on the way of a nano-object to its intracellular target: (1) the cell membrane and (2) the intracellular barriers. The former is a common issue for all nanomedicine and a matter of very intense research. The latter is the primary problem for targeted delivery of nano-objects to specific cellular sub-domains and can be studied more easily using permeabilized cells. Membrane permeabilization for nano-medical research requires (1) perforation of the outer membrane, (2) development of a solution that will keep cellular sub-domains in the functional state, and (3) modification of the perimembrane cytoskeleton. We developed a very successful model of saponin membrane permeabilization of cardiomyocytes. This allowed us to deliver particles up to 20 nm in size to perinuclear and perimitochondrial space. Here we describe the method.

Key words Saponin permeabilization, Perimembrane cytoskeleton, Gold nanoparticle, Delivery of nano-objects, Intracellular sub-domains, Electron microscopy

1 Introduction

Successful delivery of genes and drugs to intracellular sub-domains depends on solving two major problems: transport of nano-objects through both cellular and intracellular membranes (1, 2). While the former is a common problem for nanomedicine (and a matter of intensive research with multiple very promising results), the intracellular pathways for delivery of nano-objects to certain cellular organelles remain to be elucidated (1). This research could be significantly facilitated with careful (without damage of cellular sub-domains) permeabilization of cell membrane.

Excluding the development of a specific protocol, membrane permeabilization for nanomedical research could be divided into three separate tasks:

1. Cell membrane permeabilization without permeabilization or damage of intracellular membranes.

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2. Development of "intracellular" solution that will keep cellular organelles functional and maintain the intracellular distances unchanged (for instance, will prevent mitochondrial swelling).
3. Modification of the cytoskeleton along the cell membrane to allow particles up to 20 nm to enter the cell.

In our experiments, we used rat ventricular myocytes obtained by enzymatic dissociation (3). Spatio-temporal characteristics and the frequency of elementary Ca^{2+} release events (Ca^{2+} sparks) are very sensitive to any mistake in permeabilization. For example, high $[Ca^{2+}]$ in the intracellular solution results in an almost immediate increase in amplitude and frequency of Ca^{2+} sparks (4), while permeabilization of the endoplasmic reticulum (SR in muscle cells) membrane will abolish the sparks. In our experiments, we monitored the Ca^{2+} sparks, the amount of Ca^{2+} in the SR and contraction of the myocytes with confocal microscopy (4, 5). Intracellular distances in intact and permeabilized cardiomyocytes were measured with calibrated gold nanoparticles using electron microscopy (6, 7).

There are many methods of cell membrane permeabilization, from mechanical skinning to making membrane holes only for special ions with corresponding ionophores. Gentle saponin permeabilization allows the removal of the outer cell membrane without damaging intracellular membranes.

Figure 1 shows saponin permeabilization in cardiac myocytes. The upper panel (**a**) presents the same myocyte before and after permeabilization. Note that the myocyte after permeabilization has the same shape and size. The middle panel (**b**) confirms the permeabilization. The myocyte was preloaded with membrane-permeable form of Ca^{2+}-sensing fluorescent dye. Therefore, before permeabilization (**b** *a*), Ca^{2+} sparks (local event) and waves (global Ca^{2+} release events) could be seen very well. Within 1 min after permeabilization (**b** *b*), the fluorescent dye left the cell and the sparks disappeared. The addition to the bathing solution of the same but membrane-impermeable Ca^{2+}-sensing fluorescent dye (**b** *c, d*) again allows seeing the Ca^{2+} release events, which have the same velocity, frequency, and spatio-temporal properties (**c**). This demonstrates that intracellular membranes were not damaged with our method of saponin permeabilization.

This method of membrane permeabilization is suitable for nano-objects as well. Figure 2 shows saponin permeabilization of the cardiac cell transfected with green fluorescent protein (GFP), which is a 4.2 nm long cylinder with a cylindrical diameter of 2.4 nm (8). The cell was exposed to 0.01% saponin for 30 s. Multiple GFP-filled membrane blebs indicate gentle permeabilization. Therefore, GFP takes much longer to leave the cell after permeabilization (7), and GFP stays within cellular sub-domains (including nuclei; N) even after 5 min. This reminds us that membrane is not the only barrier for free diffusion of nano-objects inside the cell.

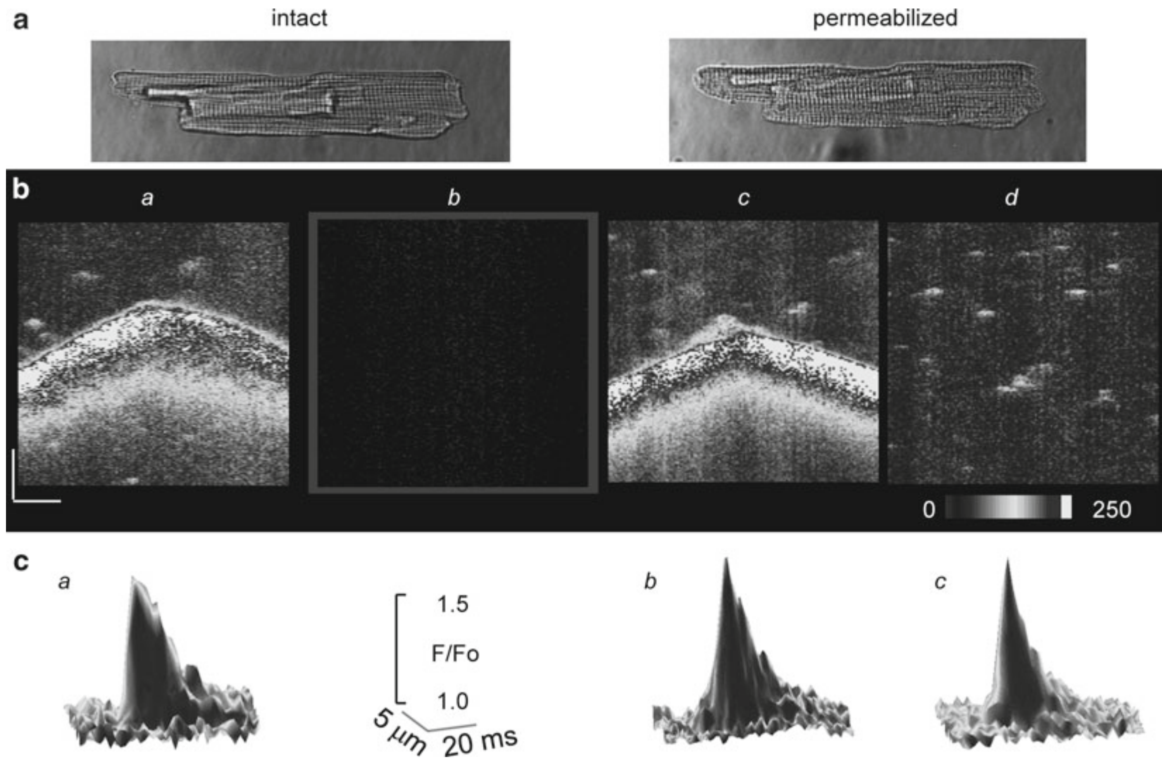


Fig. 1 Saponin permeabilization of cardiomyocytes has no effect on Ca^{2+} release from sarcoplasmic reticulum. (**a**) Images of a cardiac myocyte obtained in transmitted light before and after permeabilization with saponin. (**b**) Line scan images of fluorescence in a portion of the same cell preloaded with fluo-3 AM measured before permeabilization (a), after permeabilization in an internal solution with no dye (b), and after addition to the internal solution 30 μM fluo-3 potassium salt in the presence of 0.1 (c) or 0.5 mM EGTA (d) (pCa 7). *Calibration bars*: horizontal 10 μm, vertical 0.4 s, the pseudo scale bar represents changes in units of absolute fluorescence. (**c**) Surface plots of averaged Ca^{2+} sparks measured before permeabilization (a) and after permeabilization (reproduced from ref. 5 with permission from Wiley-Blackwell)

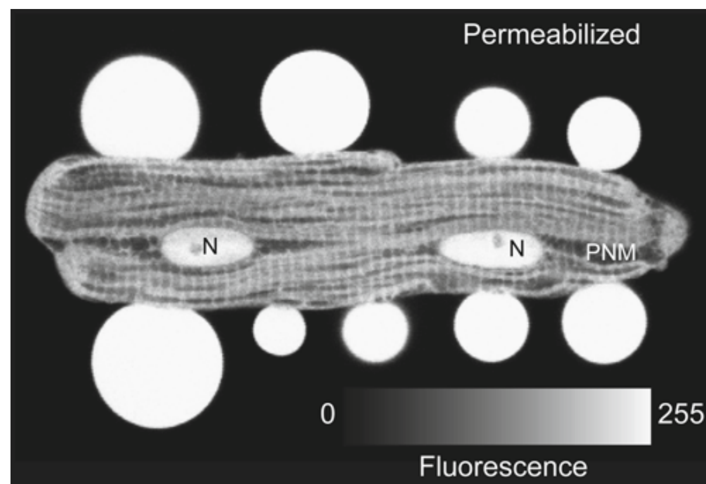


Fig. 2 Light permeabilization. Before the permeabilization, the cardiomyocytes were transfected with GFP. *N* nucleus, *PNM* perinuclear mitochondria (reproduced from ref. 7 with permission from Cell press)

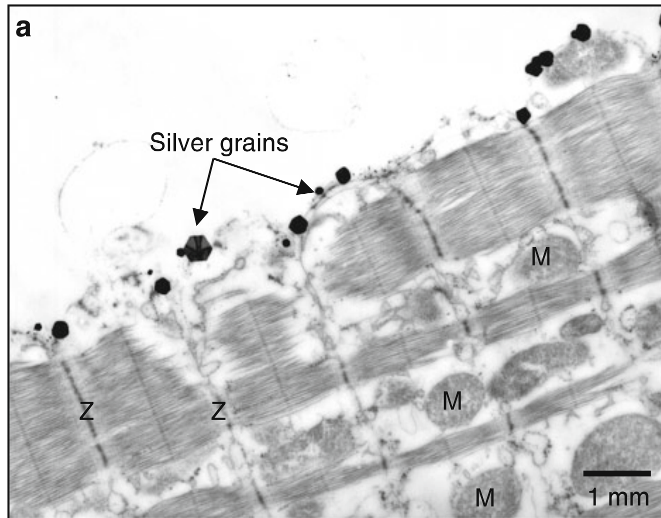
Our experiments showed that the cytoskeleton of permeabilized myocytes does not allow nano-objects ≥11 nm in diameter to enter the cell. We should note that this problem pertains only to permeabilized cells because of a lack of transmembrane transporting mechanisms. In the case of saponin-permeabilized cells, the mesh of actin filaments located along the cell membrane creates an additional barrier for nano-objects. However, short pretreatment of intact cells with cytochalasin D significantly reduces the mesh integrity and allows particles up to 20 nm to diffuse inside of the myocytes (6). Electron micrographs in Fig. 3 show the effect of 40 μM cytochalasin D on the distribution of nanoparticles in permeabilized ventricular myocytes. Note that the silver grains are only markers for the location of calibrated gold nanoparticles (the deeper in the slice the particle was located, the smaller the silver grain produced with the silver enhancement procedure).

Here we describe the method of saponin permeabilization of the cell membrane. The method is shown to be useful for the delivery of nano-objects to perinuclear and perimitochondrial space.

2 Materials

1. Tyrode solution: 140 mM NaCl, 5.4 mM KCl, 0.5 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM Hepes, 0.25 mM NaH_2PO_4, 5.6 mM glucose, pH 7.3 (6).
2. The permeabilization solution: 100 mM K^+ aspartate (see Note 1), 20 mM KCl, 3 mM MgATP, 0.81 mM $MgCl_2$ ($[Mg^{2+}]_{free}$ = ~1 mM), 0.5 mM EGTA, 0.114 mM $CaCl_2$ ($[Ca^{2+}]_{free}$ = ~100 nM), 20 mM Hepes, 3 mM glutamic acid, and 3 mM malic acid, pH 7.2 (see Note 2) (6).
3. 1% saponin in permeabilization solution.
4. The solution for permeabilized cells: 100 mM K^+ aspartate (see Note 1), 20 mM KCl, 3 mM Mg ATP, 0.81 mM $MgCl_2$ ($[Mg^{2+}]_{free}$ = ~1 mM), 0.1 mM EGTA, 0.03 mM $CaCl_2$

Fig. 3 Remodeling of cytoskeleton with cytochalasin D allows particles up to 20 nm to diffuse inside of cells. Representative micrographs show the distribution of the nanoparticles before (**a**) and after (**b**) partial ablation of the cytoskeleton (20 min pretreatment with 40 μM cytochalasin D). (**c**) Graphs representing the density of nanoparticles in intact ventricular cells before (*gray*) and after (*dark gray*, only for particles ≥11 nm) 20 min of pretreatment with 40 μM cytochalasin D. *Asterisks* indicate data that are statistically different from the corresponding control. *M* mitochondrion, *Z* Z line. *Arrows on* (**b**) indicate T-tubules and *black ovals* show nanoparticles located deeper inside the ultrathin section, which therefore have smaller diameters after silver enhancement (reproduced from ref. 6 with permission from Cell press)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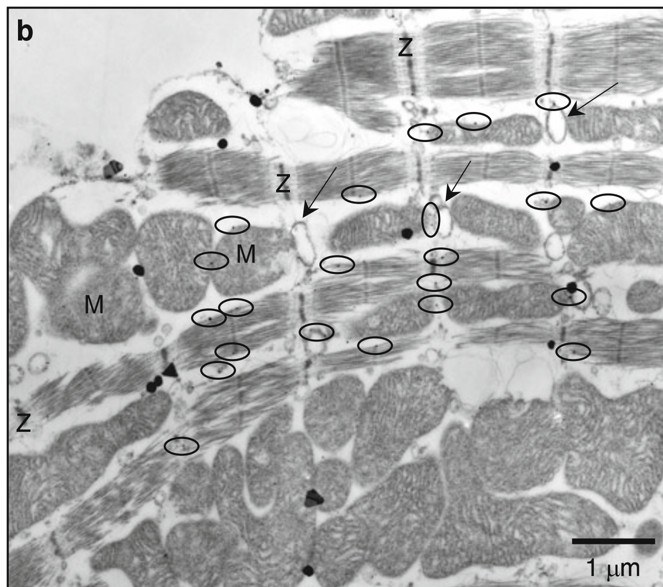
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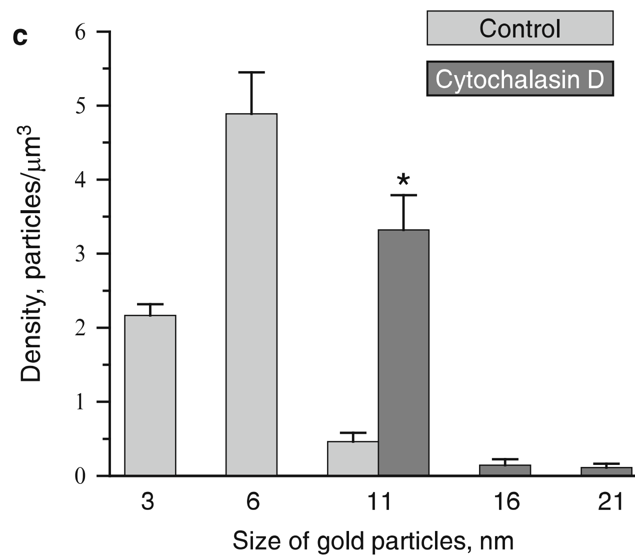
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($[Ca^{2+}]_{free}$ = ~60 nM), 20 mM Hepes, 3 mM glutamic acid, 3 mM malic acid, 10 mM phosphocreatine, 5 U/ml creatine phosphokinase, and 1% polyvinylpyrrolidone (PVP10; MW 10,000), pH 7.2 (see Note 2) (7).

5. Cells have to be attached to the bottom. To do that, we coated cover slips with laminin as recommended by the manufacturer (Molecular Probes; Invitrogen) and allowed cells 30 min to attach.

3 Methods

Carry out all procedures at room temperature.

In our experiments, we used Tyrode solution and primary culture of rat ventricular myocytes (single freshly isolated cells). However, any monolayer cell culture (any confluency) and corresponding media could be used.

1. Pretreat cells 20 min with 40 μM cytochalasin D in 1 ml of Tyrode solution.
2. Replace the Tyrode solution with 1 ml of permeabilization solution for 1 min (see Notes 3 and 4).
3. Replace the permeabilization solution with 1 ml of the same (permeabilization) solution containing 0.01% saponin for 30–60 s.
4. Replace the permeabilization solution with 1 ml of the solution for permeabilized cells containing nanoparticles.

To visualize cells, we used C-Apochromat 63×/1.2 W corr objective. Under the mentioned conditions, intracellular organelles (such as mitochondria and sarcoplasmic reticulum) of cardiac myocytes remain functional at room temperature for at least 2 h (see Note 5).

4 Notes

1. For both permeabilization solution and the solution for permeabilized cells, we used DL-aspartic acid potassium salt.
2. During the preparation of the solution for permeabilized cells, pay attention to the pH. The pH is very important for Ca^{2+} buffering power of EGTA.
3. All steps of the saponin permeabilization have to be performed under visual control. We used an inverted microscope equipped with at least ×40 objective.
4. The moment of permeabilization is seen (within a minute) as a sharp reduction in cell shining (cells become gray; Fig. 1a).

After that, the permeabilization solution should be immediately replaced by the solution for permeabilized cells.

5. We found that entry of 3-nm particles into VDAC pore (located in the outer mitochondrial membrane) is significantly restricted in permeabilized cardiomyocytes in comparison to isolated mitochondria (7).

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