Bioelectrochemistry 121 (2018) 135–141

Contents lists available at ScienceDirect

Bioelectrochemistry

journal homepage: www.elsevier.com/locate/bioelechem

Electropermeabilization of cells by closely spaced paired nanosecond-range pulses

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ARTICLE INFO

Article history:
Received 15 November 2017
Received in revised form 26 January 2018
Accepted 26 January 2018
Available online xxxx

Keywords:
Nanosecond pulses
Electroporation
Electropermeabilization
Nanopores
Membrane permeability

ABSTRACT

Decreasing the time gap between two identical electric pulses is expected to render bioeffects similar to those of a single pulse of equivalent total duration. In this study, we show that it is not necessarily true, and that the effects vary for different permeabilization markers. We exposed individual CHO or NG108 cells to one 300-ns pulse (3.7–11.6 kV/cm), or a pair of such pulses (0.4–1000 μs interval), or to a single 600-ns pulse of the same amplitude. Electropermeabilization was evaluated (a) by the uptake of YO-PRO-1 (YP) dye; (b) by the amplitude of elicited Ca^{2+} transients, and (c) by the entry of Tl^{+} ions. For YP uptake, applying a 600-ns pulse or a pair of 300-ns pulses doubled the effect of a single 300-ns pulse; this additive effect did not depend on the time interval between pulses or the electric field, indicating that already permeabilized cells are as susceptible to electropermeabilization as naïve cells. In contrast, Ca^{2+} transients and Tl^{+} uptake increased in a supra-additive fashion when two pulses were delivered instead of one. Paired pulses at 3.7 kV/cm with minimal separation (0.4 and 1 μs) elicited 50–100% larger Ca^{2+} transients than either a single 600-ns pulse or paired pulses with longer separation (10–1000 μs). This paradoxically high efficiency of the closest spaced pulses was emphasized when Ca^{2+} transients were elicited in a Ca^{2+}-free solution (when the endoplasmic reticulum (ER) was the sole significant source of Ca^{2+}), but was eliminated by Ca^{2+} depletion from the ER and was not observed for Tl^{+} entry through the electropermeabilized membrane. We conclude that closely spaced paired pulses specifically target ER, by either permeabilizing it to a greater extent than a single double-duration pulse thus causing more Ca^{2+} leak, or by amplifying Ca^{2+}-induced Ca^{2+} release by an unknown mechanism.

1. Introduction

Most electroporation treatments, from gene delivery to tissue ablation, require the delivery of multiple pulses to achieve the desired effect. However, even the first pulse(s) in a train disrupts the integrity of the cell membrane, reducing its electric resistance, and likely initiating adaptive biological responses. Therefore, an "already exposed" cell is different from a naïve cell, and its susceptibility to subsequent pulses will likely be different.

In the most widely accepted mechanism of electroporation, an externally applied electric field charges the cell membrane until it reaches a critical potential [1–3]. At this potential, pores (or other defects) form in the membrane, allowing membrane-impermeable solutes and ions to leak in and out of the cell. This leak current should prevent or slow down further build-up of the membrane potential if the external electric field is still applied, and hinder the membrane potential build-up from subsequent pulses. This way, the electroporation efficiency of pulses in a train should decrease with the sequential number of the pulse [4,5]. However, experimental measurements of the uptake of membrane-impermeable dyes (such as propidium or YP) often showed a simple linear dependence on the pulse number [6–9]. The membrane did not reseal between the pulses, as could be evidenced by dye uptake continuing much longer than the interpulse interval. The linear dependence of electroporation on the pulse number was interpreted as opening of a certain constant number of pores by each pulse in the train, no matter whether the membrane was already permeabilized or not [8,9].

Moreover, with long enough interpulse intervals (e.g., 10–30 s), cells become more sensitive to new pulses, a phenomenon known as electrosensitization [10–13]. A pre-exposure to electroporation pulses, or conditioning, can enhance the effect of the next electroporation treatment as much as 2.5 times (25 °C) or even 6 times (37 °C) [10]. Such hypersensitivity results from biological rather than just physical-chemical effects of electroporation, although its exact mechanism remains elusive. Electrosensitization also explains higher efficiency of electric pulses delivered at low repetition rates, when the total treatment

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https://doi.org/10.1016/j.bioelechem.2018.01.013
1567-5394/

becomes lengthy enough to let sensitization develop. Alternatively, longer inter-pulse intervals could allow more time for membrane resealing, so the treatment as a whole becomes more effective [4,5]; the discrimination of this mechanism from the biological effect of sensitization may be difficult since both processes can develop concurrently.

Shortening the time interval between pulses to single microseconds and below should preclude any changes in cells' susceptibility due to active biological responses. Therefore any observed changes could be attributed to physical-chemical effects, such as the formation of short-lived membrane pores. It has long been contemplated that electroporation produces two distinctly different types of pores, namely the short- and long-lived ones [9,14–17]. The short-lived pores are numerous but reseal within milliseconds and thereby contribute little to the transmembrane transport (this concept, however, was recently challenged [18]). The formation and resealing of short-lived pores can be detected by the increased conductance of a dense cell suspension in 10 ms after an electroporating pulse, with almost complete restoration of conductance in 100 ms [9]. The long-lived pores, on the contrary, are too few to significantly increase the membrane conductance, but contribute more to the membrane transport due to their long lifespan, seconds to hours [8,9,17,19–21]. The fraction (number and/or size) of long-lived pores is at least an order of magnitude smaller than short-lived pores [9], so their impact on the membrane conductance is relatively small.

This model suggests that electroporation should be less efficient if a new pulse is applied when the short-lived pores are still open (e.g., within <10 ms after the previous pulse). However, Vernier et al. [22] reported the uptake of propidium and YP only when the interval between 4-ns pulses was reduced from 100 or 10 ms down to 1 or 0.1 ms, as if the cells became more vulnerable within the short time window after the previous pulse. Our recent study showed that a second 300-ns pulse, applied 50 μs after the first one, increases YP uptake twofold [23]. Reducing the inter-pulse interval from 100 ms to 1 ms (sixty 300-ns pulses), or even from 1 or 10 s down to 1 ms (two 9-μs pulses) did not change the survival of electroporated U937 and Jurkat cells [12]. Delivering 10, 600-ns pulses at various intervals from 200 ms down to 1.4 μs caused essentially the same propidium uptake in CHO cells [24], suggesting that the short lived-pores had no impact. The same study also reported that the effect of a train of ten 600-ns pulses delivered at 50, 250 or 500 kHz (i.e., with gaps of 19.4, 3.4, or 1.4 μs) caused less membrane disruption than a single pulse of the same intensity but 10-fold duration (6 μs). For two of the tested permeabilization markers (propidium and FM1–43 dye), the effect of a train of pulses with the shortest gap of 1.4 μs was as much as 50% smaller than after a single 6 μs pulse. A paradox presented by these data is that a single 6 μs pulse can be viewed as ten 600-ns pulses with zero gaps – hence the reduction of the gap from 1.4 μs further towards zero should at some point start producing stronger effects. The only alternative to this expectation would be a reduction of the effect by any transient turn off of the applied electric field, no matter how brief it is.

In fact, the latter idea bridges with a recently discovered phenomenon of bipolar cancellation [23,25–28]. Indeed, the reversal of the pulse polarity, or the delivery of a 2nd pulse of the opposite polarity shortly after the first one, profoundly reduces the efficiency of diverse electroporation treatments. Bipolar cancellation is limited to pulses of nanosecond duration (perhaps up to several μs) and the inter-pulse intervals of up to 10–50 μs. Bipolar cancellation takes place even if the 2nd phase (or the 2nd pulse of the opposite polarity) is reduced to 35% of the first phase [27] or to just 10–20% (unpublished data). Then, one may expect that a brief reduction of the applied field down to zero, even without inverting its polarity, might also have some "canceling effect". The mechanism responsible for the bipolar cancellation has not been established, and the processes of membrane pore formation by nanosecond electric pulses (nsEP) and their elimination are far from being understood. This project aimed at getting a new insight into these processes by analyzing electropermeabilization of cells by closely spaced paired nsEP of the same polarity. We explored inter-pulse intervals shorter than in previous studies (down to 0.4 μs) and established only additive effects on YP uptake, as well as only additive and supra-additive effects on Tl^+ entry and Ca^{2+} entry and mobilization; these findings further challenge the concept that formation of short-lived pores reduces sensitivity to electroporation. We also revealed a previously unknown specific effect of closely spaced pulses on Ca^{2+} mobilization from ER.

2. Materials and methods

2.1. Cells and media

Chinese hamster ovary cells (CHO-K1) and a murine neuroblastoma-rat glioma hybrid (NG108) were purchased from the American Type Culture Collection (ATCC, Manassas, VA). CHO cells were propagated in Ham's F12 K medium (Mediatech Cellgro, Herdon, VA) supplemented with 10% fetal bovine serum (FBS). NG108 cells were grown in Dulbecco's Modified Eagle's medium (Caisson Labs, North Logan, UT) without sodium pyruvate, supplemented with 4 mM L-glutamine, 4.5 g/l glucose, 10% FBS, 0.2 mM hypoxanthine, 400 nM aminopterin, and 0.016 mM thymidine. Both cell lines were cultured at 37 °C with 5% CO_2 in air. 100 I.U./ml penicillin and 0.1 μg/ml streptomycin were added to culture media to prevent contamination. The media supplements were from Sigma-Aldrich (St. Louis, MO) except for the serum (Atlanta Biologicals, Norcross, GA). Cells were seeded onto "0" thickness glass coverslips 12–24 h before the experiments.

2.2. Calcium imaging, YO-PRO-1 and Tl^+ uptake measurements

The cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_i$) was monitored by time lapse ratiometric fluorescence imaging with Fura-2. The detailed procedures can be found in our previous publications [29,30]. Briefly, cells on glass coverslips were loaded with 5 μM of Fura-2AM (Teflabs, Austin, TX) with 0.02% of Pluronic F-127 (Sigma-Aldrich) for 30 min at room temperature in the dark. The coverslips were transferred into a glass-bottomed chamber mounted on an IX71 microscope (Olympus America, Center Valley, PA) and rinsed for 15 min with a physiological solution containing (in mM): 140 NaCl, 5.4 KCl, 1.5 $MgCl_2$, 2 $CaCl_2$, 10 Glucose, and 10 HEPES (pH 7.3, 290–300 mOsm/kg). Afterwards, the solution was refreshed every 5 min using a VC-6 valve controller system (Warner Instruments, Hamden, CT).

Fura-2 was excited alternatively at 340 and 380 nm using a fast wavelength switcher Lambda DG4 (Sutter Instruments, Novato, CA). Fluorescence images at 510 nm were collected with a UApoN340 40×/1.35 objective (Olympus) and an iXon Ultra 897 back-illuminated CCD Camera (Andor Technology, Belfast, UK), and processed with Metafluor v. 7.5 software (Molecular Devices, Sunnyvale, CA). Time lapse recordings at a rate of 10 images/s started 30 s prior to nsEP application and continued for 5 min.

For experiments in Ca^{2+}-free medium, $CaCl_2$ was omitted, and the solution was passed through a column packed with Calcium Sponge S (Thermo Fisher Scientific, Waltham, MA) to remove residual Ca^{2+}. As measured with Fura-2 added to the physiological solution, the residual Ca^{2+} level after Calcium Sponge S treatment did not exceed 100 nM.

In order to isolate nsEP-induced Ca^{2+} influx through the cell membrane, the ER Ca^{2+} stores were depleted by perfusion with 20 μM cyclopiazonic acid (CPA) in the physiological solution. It caused Ca^{2+} leak from ER, seen as a transient increase in the cytosolic Ca^{2+} level, followed by a gradual recovery to the initial level in 15–20 min. Cells with incomplete restoration of the initial Ca^{2+} level were not used for experiments.

To study YP uptake, 1 μM of the dye was added to the physiological solution. Lambda DG4 and FITC filter set (Chroma Technology, Bellows Falls, VT) were utilized to excite fluorescence of YP and to collect the emission of the dye. Recordings of YP uptake started 60 s prior to nsEP application and continued for 5 min after it, once in 20 s. YP