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Review

Nanosecond electric pulses: A mini-review of the present state of the art

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ABSTRACT

Nanosecond electric pulses (nsEPs) are defined as very short high intensity electric pulses which present great potential for the destabilization of intracellular structures. Their theoretical descriptions first suggested specific effects on organelles that have been confirmed by various observations both *in vitro* and *in vivo*. However, due to their concomitant effects on the plasma membrane, nsEPs can also affect cell functions. In this mini-review, nsEP effects on cells are described following three topics: effects at the plasma membrane level, intracellular effects, and the impact on cell survival. Eventually, a short description of the major results obtained *in vivo* will be presented. This study shows that the use of nsEPs has evolved during the last decade to focus on low voltage for practical applications.

1. Introduction

Since the early 2000s, the emergence of nanosecond electric pulses (nsEPs) paves the attention of the scientific community working on electroporation. The emergence of nsEPs is related to the ability to reliably generate these short pulses. Various nanosecond pulse generators have been implemented which are able to deliver very high intensity (beyond kV/cm) and short (nanosecond) pulses [1–6]. The development of miniaturized applicators to allow very high voltage applications has enhanced the optimization of electrical conditions to trigger biological effects. These applicators are generally suitable for observations under a microscope. It is possible to distinguish in the literature applicators as tungsten electrodes with an inter-electrode gap of 150 μm [7], or flat applicators also having an inter-electrode gap of 100 μm [8–11]. Finally, microfluidic systems have recently been developed.

By extension, the terminology "nsEPs" is used for electric pulses with a duration of less than 1 μs, although the theoretical analyses are carried out experimentally for pulses of a few nanoseconds to a few tens of nanoseconds [12] as well as *in silico* to study the effects of electric fields on the lipid bilayer [13–19]. These theoretical descriptions of effects on phospholipid bilayers quickly led to the prediction that short and intense pulses could affect specifically intracellular membranes due to their small diameter [20,21].

Thus, by a simple analysis of Laplace's equation, it was possible to predict that the membranes of organelles, which have a radius smaller than that of the cell, have a shorter charging time than the plasma membrane, and could be specifically destabilized by high intensities and short pulses [12,22]. As predicted, a pulse of 15 ns could allow the destabilization of the membrane of an object with a diameter of 1 μm when a sufficient strength is applied [23]. The first work on living cells consisted in applying a very small number of pulses (less than 10), but with an intensity of 5.3 kV/cm [24]. These results have shown a destabilization of eosinophil granules without detecting any alteration of the plasma membrane and thus suggested a targeted effect of nsEPs on the internal membranes. However in the next years, many other effects have been observed, both intracellularlyand directly on the plasma membrane, making these nsEPs no longer specific to intracellular components but very versatile. Some threshold values have been determined for cell death, but the variety of voltages and durations studied have not yet resulted in fine definition of their cellular effects depending on their characteristics. This presents the advantage of leaving the research field wide open but the disadvantage of not giving any guidance to the possible cell electro-manipulation that was expected when nsEPs were first introduced [25]. Moreover, the majority of studies do not compare their results with those obtained with "classical" electrical conditions (*i.e.* ms and μs pulses applied at a lower intensity), and therefore it is not possible to assess the "nanopulse" specificity of these effects.

2. Effect at the plasma membrane level

Effects of electric field pulses on membrane permeabilization are classically and indirectly studied through uptake of fluorescent molecules which are able to pass or not this cell barrier. However, the molecules used were having a size which was too large for the membrane to allow crossing in the case of small numbers of nsEPs [21–24].

Abbreviations: NsEPs, nanosecond electric pulses.

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It has thus been accepted that nsEPs did not allow plasma membrane permeabilization as long pulses did (μs, ms duration). However, the detection of permeabilization using smaller molecules [26] or ions was shown next [27]. Thanks to more direct methods of detection, like labeled phospholipids, or patch-clamp, membrane destabilization and nanopore formation have been indirectly observed.

Since 2003 the possibility of nsEPs to affect the plasma membrane has been highlighted [28]. In 2004, the application of a single pulse of 30 ns and 25 kV/cm has been shown to lead to the translocation of phosphatidylserine (PS) from the inner leaflet of the lipid bilayer to the outer leaflet, involving a membrane effect [29]. This translocation was preferably held on the side of the cell facing the anode during the seconds following pulse application [30] demonstrating the dependence of the phenomenon to the orientation of the electric field, as this is the case for classical electropermeabilization [31]. Under the influence of electric fields, the charged polar head of phospholipids changes direction and thus forces the whole phospholipid to change its orientation [32], a mechanism that may involve calcium ions [33]. This effect was directly correlated to the passage of a lipid from one layer to another, directly caused by electric fields. It should be noted that although the PS translocation is a marker of apoptosis, rapid observation of this event, preferentially at the anode in the time scale concerned, did not make it an apoptotic effect but an electric biophysical effect. Therefore the transverse diffusion of PS has led to the assumption that membrane nanopores were constituted (with a duration of the order of a nanosecond, and a diameter of a nanometer), in the absence of entry of large molecules as it was the case for classical electropermeabilization [34]. The mechanism of membrane destabilization is similar in both cases, as it is caused by the change in membrane potential [35,36], but the result is different because of the size of membrane defects. This translocation may arise in the application of pulses of 3 ns and, as shown in previous studies, on different cell types, making the formation of nanopores ubiquitous [22,37]. The threshold values for nanopore formation were established at 6 kV/cm for a 60 ns pulse, and 1 kV/cm for a pulse of 600 ns, *i.e.* a submicrosecond pulse [27,38]. Increasing the number of pulses or their intensity has led to an increase in transmembrane transfer, which could lead to permeabilization of the plasma membrane similar to the one observed with longer classic pulses [26,39–41]. It has to be noted that the formation of these larger defects could be a side effect of nsEPs and not their direct effect. Similarly, a higher number of pulses and increased intensity have led to the passage of calcium across the plasma membrane [42,43].

This nano-membrane destabilization has also been measured directly by detecting ion flow through nanopores by patch-clamp [41,41,44]. The patch-clamp technique has been indeed widely used to study the effects of nsEPs on voltage-gated channels [45,46]. This effect is a side effect, related to the opening of nano-defects that are responsible for ion flux causing a possible cellular regulation by inhibition of calcium and sodium voltage-gated channels. In addition, the regulation of sodium channels may be a calcium-dependent mechanism [45].

Finally, cell swelling [47,48] and the formation of "blebs"[49] have been observed after applying nsEPs, as it is the case for longer pulses [50]. This swelling was observed by an increase of the cell size, directly correlated to an expansion of the membrane, indicating a membrane destabilization. However in the case of "blebs", although their initial formation has been linked to a membrane destabilization, nsEP bleb growth was observed only toward the anode. This growth was subject to the polymerization of actin during the pulse application [49]. These results show major intracellular effects of nsEPs.

3. Intracellular effects

As indicated in the Introduction, nsEPs were first considered for their potential selective effect on the inner organelles of the cell (not only endoplasmic reticulum and mitochondria, but also nuclei, Golgi vacuoles and secretory or endocytic vesicles). The internal effects of nsEPs have been studied using the properties of these envelopes that are designed for compartmentalization, and thus sequestration of molecular species. Few studies have targeted the effect of nanosecond pulses on the cytoskeleton. Actin has been shown to stabilize the plasma membrane of plant cells during nsEP application and to act on its permeabilization [51]. As discussed above, actin has also been shown to be involved in the formation of blebs on animal cells [49].

However, the release of intra-cytoplasmic calcium has been specifically studied. Indeed, in the cell, calcium is present in organelles, mainly in the endoplasmic reticulum and mitochondria. Several studies have shown a release of calcium independently of intra-cytoplasmic membrane calcium channels, which was thus directly linked to the destabilization of organelle envelopes by pulses shorter than 100 ns [21,52]. However, beyond a threshold of 100 kV/cm, an influx of calcium into the cell was observed related to the destabilization of the plasma membrane and not just of the envelopes of intracellular organelles [42,43]. Activation of a voltage dependent calcium channel of excitable cells has also been shown [53]. Finally, the effects on vesicles and vacuoles were reported [23,24,39], demonstrating the ability of very short and relatively intense nanosecond pulses to destabilize intracellular phospholipid bilayers.

Similar specific effects of 4 and 600 ns pulses on mitochondria have been demonstrated due to a decrease in mitochondrial transmembrane potential [54,55]. These results point to the possibility of destabilizing a complex envelope, as a mitochondrionis composed of two lipid bilayers, and it has been shown that the inner membrane is affected with pulses as short as 4 ns in duration [54]. In the case of pulses of several hundred nanoseconds (submicrosecond), apoptotic effects have been reported, not allowing in this case to define whether the permeabilization of the mitochondrial inner membrane was a primary or secondary effect of nsEPs related to the apoptotic process [56,57]. Eventually, a study has brought a new dimension on the effect of nsEPs, adding the rise and fall time of pulses as a determining factor for the targeting of organelles, the shortest time preferentially targeting the internal membranes [55].

The last but not the least organelle of interest is the cell nucleus. However, the structure of the nuclear envelope is more complex than the other ones, not only because it is a double bilayer as that of the mitochondrion, but also because of the continuity of these membranes with the endoplasmic reticulum, the presence of nuclear pores, and the internal genomic and proteic material. Indeed, the nuclear envelope interacts with chromatin and telomeres. Effects of the nuclear envelope were reported indirectly by the ability to increase the level of plasmid expression following electrotransfection [58,59]. Recently it has been shown that under less intense electric field parameters, no effect of nsEPs on gene expression could be detected [60]. Effects on genetic material have also been reported, particularly on DNA strand breaks [61], the consecutive detachment of telomeres attached to the nuclear envelope [62], and also a potential change in chromatin condensation [63]. Changes in the conductivity of the nucleoplasm have also been detected [64]. However actually no experiment shows the destabilization of the nuclear envelope itself by nsEPs and this can be explained first by the size of the nucleus, which is much larger than that of other intracellular organelles and could prevent any effect of the nsEPs on the nuclear envelope; second by the complexity of the envelope; and finally by the technical limitations of detection of this expected destabilization.

4. Impact on cell viability

Any impact on the external or internal membranes of the cell can profoundly affect its performance and therefore can cause its death. This is why a large number of studies have studied the lethal effects of nsEPs and their impact on cellular mechanisms involved in cell proliferation like caspase activation or JNK (c-Jun N-terminal kinases) pathway. This kind of observations must take into consideration that, as for any experiment on a population, the effects are dependent on the phase of the cell cycle in which the cell is [57].