Effects of electroporation on optically recorded transmembrane potential responses to high-intensity electrical shocks

V. P. Nikolski, A. T. Sambelashvili, V. I. Krinsky, and I. R. Efimov. Effects of electroporation on optically recorded transmembrane potential responses to high-intensity electrical shocks. Am J Physiol Heart Circ Physiol 286: H412–H418, 2004. First published October 2, 2003; 10.1152/ajpheart.00689.2003.—The outcome of defibrillation shocks is determined by the nonlinear transmembrane potential ($\Delta V_m$) response induced by a strong external electrical field in cardiac cells. We investigated the contribution of electroporation to $\Delta V_m$ transients during high-intensity shocks using optical mapping. Rectangular and ramp stimuli (10–20 ms) of different polarities and intensities were applied to the rabbit heart epicardium during the plateau phase of the action potential (AP). $\Delta V_m$ were optically recorded under a custom 6-mm-diameter electrode using a voltage-sensitive dye. A gradual increase of cathodal and well as anodal stimulus strength was associated with 1) saturation and subsequent reduction of $\Delta V_m$; 2) postshock diastolic resting potential (RP) elevation; and 3) postshock AP amplitude (APA) reduction. Weak stimuli induced a monotonic $\Delta V_m$ response and did not affect the RP level. Strong shocks produced a nonmonotonic $\Delta V_m$ response and caused RP elevation and a reduction of postshock APA. The maximum positive and maximum negative $\Delta V_m$ were recorded at 170 ± 20 mV/cm² for cathodal stimuli and at 240 ± 30 mV/cm² for anodal stimuli, respectively (means ± SE, n = 8, P = 0.003). RP elevation reached 10% of APA at a stimulus strength of 320 ± 40 mV/cm² for both polarities. Strong ramp stimuli (20 ms, 600 mV/cm²) induced a nonmonotonic $\Delta V_m$ response, reaching the same largest positive and negative values as for rectangular shocks. The transition from monotonic to nonmonotonic morphology correlates with RP elevation and APA reduction, which is consistent with cell membrane electroporation. Strong shocks resulted in propidium iodide uptake, suggesting sarcolemma electroporation. In conclusion, electroporation is a likely explanation of the saturation and nonmonotonic nature of cellular responses reported for strong electric stimuli.
oxygenated modified Tyrode solution at 35°C as previously described (2). Hearts were stained with the voltage-sensitive dye 4-(2-(6-(dibutylamino)-2-naphthalenyl)ethenyl)-1-(3-sulfopropyl)pyridinium inner salt (di-4-ANEPPS) or N-(4-sulfobutyl)-4-(6-(4-(dibutylamino)phenyl)hexatrienyl) pyridinium inner salt (RH-237) (1 μM). Motion artifacts in optical recordings were suppressed by 15 mM of the excitation-contraction uncoupler 2,3-butanedione monoxime (Sigma).

We designed the electrode shown in Fig. 1A to achieve high-current density stimulation over a relatively large area of the epicardium without electrochemical electrode-saline interface artifacts (bubbles) in the optical recordings. A transparent plastic box filled with Tyrode solution with a 6-mm-round hole was gently pressed against the left ventricular free wall (see Fig. 1B) to seal the opening. The 16 × 16 photodiode array optical recording system (2) was focused on the 4 × 4-mm area of epicardial surface inside the hole. A 20 × 20-mm silver wire loop was positioned inside the box 10 mm from the heart surface around the opening, allowing unobstructed optical recordings from the field of view. Stimulating current was injected inside the box through this wire. The reference electrode was a 9-mm-diameter Ag-AgCl disk placed in the bath away from the heart (see Fig. 1). The current was allowed to exit the box only through the hole directly into the epicardium, producing homogeneous transmembrane polarization in the field of view. Figure 2 shows a representative example of optical recordings from all 256 channels. To minimize possible effects of heterogeneous virtual electrode polarization near the edges of the hole, we analyzed recordings only from the center of the mapped area. The average current density of the stimulus was determined as the ratio between the applied current and total cross-sectional area of the hole. The total impedance between the test and reference electrodes was 200–300 Ω.

The heart was paced at a cycle length of 300 ms through a bipolar electrode placed at the apex. Rectangular and ramp stimuli of both polarities and with a duration of 10 or 20 ms were generated by the bipolar operational power supply/amplifier (Kepco) during the plateau phase of the AP (20–40 ms after the upstroke). To calibrate the transmembrane voltage optical signal, we assumed that the APA was 100 mV and the resting potential was −85 mV (5). Applied shock voltage and current waveforms were recorded simultaneously with optical maps. We used two sampling rates: a sampling rate of 1.5 kHz was used during acquisition of all 256 optical channels, and a sampling rate of 5 kHz was used during recordings of only 4 optical channels from the center of the field of view.

To detect sarcolemma electroporation during the shocks, we perfused the heart with 30 μM of propidium iodide (PI; a membrane-impermeable nucleic acid stain) and recorded the increase of its fluorescence after shock application for 10–20 min as a marker of electroporated areas. We then washed out the PI for 30 min, cryosectioned the heart from the base to apex with 1-mm steps in 20-μm-thick slices, and analyzed the sections with an epifluorescent microscope (Nikon Eclipse600FN). To avoid possible spectra overlapping, we did not use voltage-sensitive dye staining in these experiments at all or used RH-237, which has a different emission spectrum than PI.

Student’s t-test for paired data was used to compare strength of stimuli. Values of P < 0.05 were considered significant. All quantitative data are expressed as means ± SD unless otherwise specified.

RESULTS

Figure 2A presents the AP upstroke and shock-induced response recorded at all 256 optical channels within the 4 × 4-mm area during application of a strong anodal shock (280 mA/cm², 20 ms). The isopotential maps of maximum polarizations achieved during such stimulus (Fig. 2B) illustrate stimulation homogeneity in the recorded area.

Figure 3 shows one representative example (of 8 preparations) of optical recordings of the transmembrane potential obtained from one channel in the middle of the field of view. For moderate strength (light gray traces), shock-induced transients (ΔV_m) were monotonic and gradually increased with the increase of the stimuli. However, with a further increase in shock strength, ΔV_m became nonmonotonic. First, they rapidly increased (phase 1) and then saturated and relatively slowly decreased (phase 2). These observations agree with observations in cell culture and isolated cells (9, 18) for hyperpolar-
izing responses but are different for depolarizing ones. Interestingly, with a gradual increase of stimulus strength, the amplitude of the voltage transient decreases, whereas the slope of the second phase increases. The appearance of a second phase in $\Delta V_m$ was accompanied by another transition on a different time scale (Fig. 3B)–appearance of shock-induced diastolic depolarization, suggesting an onset of electroporation.

In each of eight preparations for every shock strength applied, we measured (see Fig. 4, inset) the following: 1) the largest deviation of optical potential from the preshock level during the stimulus application ($V_{max}$); 2) the final deviation at the end of the shock ($V_e$); 3) the postshock diastolic potential (DP) elevation; and 4) the postshock APA ($V_{amp}$). The plot in Fig. 4 shows how these characteristics depend on the shock strength in another representative preparation. Weak rectangular stimuli induced monotonic $\Delta V_m$ transients and did not affect DP. Strong rectangular shocks produced nonmonotonic $\Delta V_m$ (as a result, $V_e$ deviates from $V_{max}$), caused DP elevation, and reduced postshock APA. Figure 5A shows the average data and SDs for the largest positive and negative values of $\Delta V_m$ during shocks ($V_{max}$) and the postshock DP elevation for eight hearts.

To characterize the onset of nonmonotonic $\Delta V_m$ transients and DP elevation, we determined the following from each individual plot (see Fig. 4): 1) the shock strengths causing a DP elevation of 10% of APA ($I_{DP,10\%}$); and 2) the shock strengths inducing the maximum depolarization or hyperpolarization ($I_{V_{max}}$). Figure 5B shows the average and SDs for $I_{V_{max}}$ and $I_{DP,10\%}$ for cathodal and anodal stimuli. A paired Student $t$-test comparison of anodal versus cathodal stimuli gave $P = 0.003$ for $I_{V_{max}}$ and $P = 0.94$ for $I_{DP,10\%}$. The summary data were as follows: 1) DP elevation reached 10% of APA at an average stimulus strength of $320 \pm 40$ mA/cm$^2$ for both polarities (mean $\pm$ SE, $n = 8$); and 2) the maximum positive $\Delta V_m$ was recorded at an average stimulus strength of $170 \pm 20$ mA/cm$^2$ for cathodal polarity, and maximum negative $\Delta V_m$ was recorded at an average stimulus strength of $240 \pm 30$ mA/cm$^2$ for anodal polarity (means $\pm$ SE, $n = 8$).

**Fig. 2.** A: transmembrane potential ($\Delta V_m$) transients induced by a 20-ms rectangular shock applied during the plateau phase of an action potential (AP). Signals were recorded from a 4 x 4-mm area of the epicardium of the rabbit heart. B: isopotential map of polarizations at the end of the shock.
As in previous studies, observed largest positive and negative values of polarization were about twice smaller than expected for electroporation (100 vs. 300–400 mV) (17, 20). One of the possible explanations proposed previously was the insufficient time resolution of recording systems, i.e., the observer could fail to notice the initial high amplitude transient $V_{m}$ at the onset of rectangular stimuli because this large $V_{m}$ would immediately disappear due to cell membrane electroporation (6, 17). To overcome possible bandwidth limitations, we applied ascending and descending voltage ramps instead of a rectangular pulse stimulus. Strong 20-ms 600 mA/cm$^2$ ramp stimuli induced $V_{m}$ reaching the same largest positive and negative values that were observed during a series of rectangular shocks in all eight experiments.

Figure 6 shows typical $V_{m}$ transients during 20-ms voltage ramps superimposed with the responses to the rectangular pulses. The transient decays start at similar $V_{m}$ levels as with the rectangular pulses despite the fact that the ramp stimulation voltage was still rising. The slow rate of shock voltage increase guaranteed that we could reliably record $V_{m}$ at all moments. This example proves that potential metrological problems (signal undersampling or amplifier bandwidth limitations) cannot explain why the maximum values of $V_{m}$ recorded during electroporating rectangular shocks in previous studies and in our experiments were far less than is assumed to be required for electroporation (17, 20).

To test our hypothesis that our stimuli could electroporate the sarcolemma, we applied strong shocks to the heart perfused with 30 μM PI ($n = 2$). Figure 7, top, shows the initial increase of PI fluorescence after the beginning of perfusion recorded inside the stimulating hole (solid line) and 3 mm outside the hole (dashed line). After a strong cathodal shock (1,600 mA/cm$^2$, 20 ms; arrow in Fig. 7), there was an accelerated accumulation of the fluorophore in the tissue inside the hole but not outside the hole. Similar results were obtained for anodal shocks. The average fluorescence increase during 10 min after the shocks in this experiment was 81 ± 19% inside and 13 ± 1% outside (means ± SD, $n = 2$) the stimulated area. After a single shock was applied, the heart was cryosectioned. At the electroporated area, PI penetrated inside the cells and bound to the nuclei. Figure 7, bottom, shows the fluorescent images made with ×4 and ×40 lens for a 20-μm slice sectioned throughout the stimulated area. The electroporated region is clearly demarcated by the PI-stained nuclei (bright specks in the high-resolution image of dye staining).

To relate the changes in optical potentials to the PI uptake, we dual-stained the hearts ($n = 2$) with PI and the voltage-sensitive dye RH-237. RH-237 and PI fluorescence were collected at the same spot beneath the electrode. Figure 8 shows that two consequent 300 mA/cm$^2$ shocks of both polarities caused neither a diastolic optical potential elevation (black traces) nor PI fluorescence increase (laser trace after black arrow). Two consequent 700 mA/cm$^2$ shocks of both polarities caused DP elevation (gray traces) and the onset of PI fluorescein.
cence increase (laser trace after gray arrow). The same phenomena were observed in a second preparation. After all four stimuli were applied, the heart was cryosectioned. Histological evaluation of the same tissue slice cut through the center of stimulated area (Fig. 8, right) showed that PI staining was localized at the thin layer of cell nuclei near the epicardium in the area adjacent to the electrode, confirming the occurrence of electroporation. Comparing the thickness of the damaged area in Figs. 7 and 8, one can see that lowering stimuli from 1,600 to 700 mA/cm² resulted in a tremendous reduction of the electroporated area depth, from \( \frac{1}{H11011}10 \) mm to 0.2 mm. We did not observe PI uptake in the cryosections where there was no effect of the shock on the PI fluorescence intensity from the epicardium. This means that the electroporation threshold in this experiment was between 700 and 300 mA/cm² when determined by either DP elevation or the PI uptake method.

The similar relatively slow time course of PI uptake was observed in a cell culture (19), where the dye influx continued \( \sim 10 \) min after the application of an electric pulse.

**DISCUSSION**

In the present study, for the first time, in eight whole heart preparations, we detected saturation and subsequent decay of epicardial polarizations during the strong cathodal and anodal shocks applied at the area with a size of several space constants \( [0.8-1.5 \text{ mm in the epicardium (1)}] \). We also found that these effects are accompanied by epicardial postshock DP elevation. The most plausible explanation of these events is electroporation.

The correlation between anodal (negative) \( \Delta V_m \) and diastolic \( \Delta V_m \) elevation was recently reported by Fast and Cheek (8) in myocyte cultures. The same result was shown before by Neunlist and Tung (17) and Cheng et al. (6). Fast and Cheek (8) also suggested that electroporation was a likely mechanism of nonmonotonic \( \Delta V_m \) but, because Lucifer yellow dye uptake was not observed at a shock strength of 50 V/cm, which was above the 30 V/cm threshold of nonmonotonic negative \( \Delta V_m \), this conclusion was not definitive. However, Gillis et al. (11) did observe an accumulation of this dye in the areas close to the anode and cathode after shocks with a field strength of \( \approx 22 \) V/cm in an experimental setup similar to the one used by Fast and Cheek (8), which is puzzling. Interestingly, Fast and Cheek (8) did not observe nonmonotonic \( \Delta V_m \) at the cathodal end of the cell strand even at highest shock strengths. In the present study, we showed PI dye uptake at a shock strength of 700 mA/cm² (35 V/cm), when we detected nonmonotonic \( \Delta V_m \), and no PI uptake at 300 mA/cm² (15 V/cm), when \( \Delta V_m \) was monotonic. We do not have an explanation for the absence of positive \( \Delta V_m \) decay in the Fast and Cheek study (8). The lack of detectable dye uptake in their work could be related to the small exposure time and to the lesser sensitivity of the Lucifer yellow technique. PI has 20- to 30-fold increases in fluorescence after being bound to nucleic acids. Our optical recordings of negative \( \Delta V_m \) responses to high-intensity stimuli are in agreement with results reported by other investigators in strands of cultured myocytes (3, 9), single cells (4), and the frog heart (17) for hyperpolarizing stimuli. The positive \( \Delta V_m \) response in our study was different. We did not observe a plateau or an increase in depolarization transients during
cathodal stimuli for the same stimulus strengths that caused decayed hyperpolarization responses in their studies. Both polarities had the same injury threshold judging by DP elevation, which fits well with Knisley and Grant (15) data showing that cell injury is independent of the intrinsic transmembrane potential. This suggests that electroporation is responsible for saturation and decay of $V_m$.

Recent data (10) showed that the initial positive polarization in virtual cathode areas in wedge preparation is changing to hyperpolarizing responses as stimuli strengths increase to 30 V/cm and above, similar to the behavior of the middle of a single myocyte in the Sharma and Tung study (18). Such observations have been reported previously by Cheng et al. (6) and Zhou et al. (23), who detected hyperpolarization transients near the cathodal shock electrode. If this phenomenon takes place during epicardial stimulation as well, it can explain why depolarization saturation is observed at lower shock current densities than hyperpolarization saturation.

We observed, similar to previous investigators, a smaller maximum hyperpolarization response than could be expected to be high enough for electroporation. Among the possible reasons for this were 1) a “dog bone” virtual polarization near the pacing electrode (17), which could attenuate the response due to optical averaging over areas of opposite polarizations; or 2) insufficient temporal resolution of the optical mapping system (17), which could underestimate the true instantaneous transmembrane voltage produced by a square pulse. Our results for polarization transients recorded during 20-ms ramp waveform stimulation (no temporal resolution limitations) over the 6-mm-diameter area of epicardium (opposite polarization is located 3 mm away from the center recording point) reject such explanations.

![Histological 0.02mm slice](image)

Fig. 7. Uptake of the membrane-impermeable dye propidium iodide after a strong shock. Top: initial increase of propidium iodide fluorescence after the beginning of perfusion recorded inside the stimulating hole and 3 mm outside the hole. After shock application (1,600 mA/cm², 20 ms), there was an accelerated accumulation of the fluorophore in the tissue inside the hole. Bottom: fluorescent images made with ×4 and ×40 lens for a 20-μm slice sectioned throughout the stimulated area. The electroporated region is clearly demarcated by the propidium iodide-stained cell nuclei.

![Transmembrane voltage and Propidium Iodide at the histological slice](image)

Fig. 8. Left: manifestation of electroporation changes in optical potential recordings is associated with an increase of propidium iodide fluorescence under the stimulation electrode. Right: histological images showed a typical pattern of nuclear stain at the thin layer of epicardium at the areas where optical potentials had signs of electroporation.
In our four experiments with PI, we did not detect a PI fluorescence increase during the shock. This suggests that the amount of PI molecules that penetrated through the electroporation holes during the 20-ms stimulus is undetectable in our setup. This also explains why we did not observe a difference in PI uptake for shocks of different polarities despite the charge of the PI molecule. The major amount of PI enters the cells when the external electrical field is already turned off. This is why fluorescence is continuously rising during dye perfusion in our experiments as it did in a cell culture study (19). This means that, in our experiments, electroporated cells were repaired within minutes rather than seconds. We suggest that that DP elevation might be a more sensitive indicator of electroporation than PI uptake because DP elevation can be detected within 1 s after shock application.

Limitations. First measurement of the cellular response directly at the place of stimulation was performed by Neunlist and Tung (17) for a 150–um pipette with a single-channel fiber fluorimeter. In our study, with a 6-mm diameter stimulating hole, we were able to create a larger stimulating field and avoid the possible interference from virtual electrode polarization. However, we cannot exclude that transient decays were in some part related to electrotonic interference from the areas of opposite polarization around the stimulating hole. We performed control experiments with a 14-mm diameter hole and found that the nonmonotonic nature of the responses in the central area was preserved. The use of 2,3-butanedione monoxime could affect the observed transients due to partial ion channel blockage.

In conclusion, changes in the morphology of transmembrane polarization transients during anodal and cathodal shocks from monotonic to nonmonotonic responses are associated with elevation of the resting potential, postshock APA reduction, and PI uptake, implying the occurrence of electroporation. Electroporation changes in transmembrane potential traces are present for hyperpolarized as well as depolarized stimuli of a similar strength.

GRANTS

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REFERENCES