Effects of varying pulse parameters on ion homeostasis, cellular integrity, and force following electroporation of rat muscle in vivo

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Gissel H. Effects of varying pulse parameters on ion homeostasis, cellular integrity, and force following electroporation of rat muscle in vivo. Am J Physiol Regul Integr Comp Physiol 298: R918–R929, 2010. First published January 27, 2010; doi:10.1152/ajpregu.00692.2009.—Electroporation is a technique used in vitro, ex vivo, and in vivo to permeabilize cell membranes. The effect on the tissue describes a continuum ranging from mild perturbations to massive tissue damage. Thus care should be taken when choosing pulses for a given application. Here the effects of electroporation paradigms ranging from severe to very gentle permeabilization were investigated on soleus, mainly composed of slow-twitch fibers, and extensor digitorum longus (EDL) and tibialis anterior (TA), almost exclusively composed of fast-twitch fibers. Five key physiological parameters were studied: force, muscle tibialis anterior (TA), almost exclusively composed of fast-twitch fibers, and extensor digitorum longus (EDL) and soleus; fiber type

Electroporation (EP) is a technique used in vitro, ex vivo, and in vivo to permeabilize cell membranes. The effect on the tissue describes a continuum ranging from mild perturbations used in, e.g., DNA electrotransfer to massive tissue damage used in, e.g., electrochemotherapy. The permeabilization of the membrane may be transient, with the membrane resealing spontaneously within a few minutes, or it may be of a more permanent nature, showing no spontaneous resealing (stable permeabilization). The effect on the tissue varies depending on the extent and the degree of the permeabilization as well as the nature of the pores. At lower field strengths permeabilization is primarily reversible, allowing the otherwise nonpermeant ions and molecules to enter the cell in the minutes immediately after EP, but with spontaneous resealing of the membrane in the following minutes (11). However, when the field strength exceeds a certain level stable permeabilization starts to occur (11), and the membrane does not spontaneously reseal. Here cellular membrane repair mechanisms must be activated in order to reseal the membrane.

The cell membrane separates the interior of the cell from the outer environment. Because of a selective permeability of the membrane as well as the presence of transport mechanisms in the membrane many ions and substitutes show gradients across the cellular membrane. The gradients for Na+, K+, and Cl− across the membrane give rise to the membrane potential, important for many cellular functions. Ca2+ acts as an important signaling molecule, and intracellular free Ca2+ concentration ([Ca2+]i) is under tight cellular control and normally kept at submicromolar levels. Since the concentration of free Ca2+ in plasma is ∼1.3 mM, a very large gradient for Ca2+ exists, pointing into the cell. The existence of this very large gradient is possible because of a very low permeability of the membrane for Ca2+ as well as Ca2+-transporting mechanisms within the cell assisting in clearing the cytoplasm for Ca2+.

If the permeability of the membrane is increased, a large influx of Na+ and Ca2+ would be expected as well as a large efflux of K+ and cellular constituents such as ATP and the intracellular enzyme lactate dehydrogenase (LDH). The rundown of the Na+ and K+ gradients leads to an almost complete depolarization of the membrane potential and a loss of force-generating ability (6). Loss of excitability may also occur as a direct consequence of changes in membrane structural integrity. In situations with reversible permeabilization membrane integrity is restored and the Na+ and K+ gradients and the membrane potential may be recovered by activity of the Na+-K+ pump, leading to recovery of force-generating ability (4, 6). The influx of Ca2+ may lead to muscle Ca2+ overload. Despite the large capacity of the muscle cells for clearing Ca2+ into the sarcoplasmic reticulum (SR) and the mitochondria, an increased influx of Ca2+ has repeatedly been shown to lead to muscle cell damage (8, 15, 17, 18). In isolated rat skeletal muscle EP markedly increased the influx of Ca2+, and this led to the release of the intracellular enzyme LDH (6, 15). Ca2+ has also repeatedly been suggested as a causative factor in defibrillation damage, due to EP of the myocardium and thoracic muscle, following repetitive defibrillation of the heart (1, 19, 20, 32, 33). Finally, loss of ATP during permeabilization may impair the ability of the cell to repair damage to the membrane and thus decrease the chance for cell survival.

Experiments with isolated skeletal muscle cells and intact skeletal muscle tissue indicate that the fields produced in victims of electric shock are sufficient to cause EP of the cellular membrane (2, 3, 21) and that EP, and not local heating, may be the cause of the major part of the tissue damage observed in some cases of electrical injury (2).
EFFECTS OF ELECTROPORATION OF SKELETAL MUSCLE IN VIVO

There is a lot of knowledge of how EP affects cells in vitro but little information on how muscle tissue is affected in vivo. This information very important when deciding which pulses to use for a given application. This study describes how muscle tissue integrity and function are affected with a range of pulses. The main focus was on eight pulses of 100-μs duration. In addition, pulses relevant for DNA electrotransfer, a high-voltage/low-voltage paradigm consisting of one high-voltage pulse (1,000 V/cm) of 100-μs duration followed by one low-voltage pulse (100 V/cm) of 400-ms duration, as well as an eight low-voltage paradigm consisting of eight pulses (150 V/cm) of 20-ms duration, have been included.

Three different muscles were studied, tibialis anterior (TA) and extensor digitorum longus (EDL), muscles almost exclusively composed of fast-twitch fibers, and soleus, a muscle mainly composed of slow-twitch fibers (23). Important differences exist between the different fiber types (e.g., size, oxidative capacity, and Ca²⁺ storage capacity), which may have an impact on how well exposure to electrical pulses and EP is tolerated.

METHODS

Animals

Four-week-old female and male Wistar rats (own breed) weighing 65–70 g were used in all experiments. The animals were kept in a thermostated environment (21°C) with constant day length (12 h). Animals had free access to food and water. All handling and use of animals complied with Danish Animal Welfare Regulations and American Physiological Society principles for the care and use of animals. Permission for the experiments was obtained from the Danish Animal Welfare Committee. The animals were anesthetized 15 min before EP by a subcutaneous injection of Hypnorm (0.4 ml/kg) and Dormicum/midazolam (2 mg/kg). The animals were kept under a heating lamp to maintain body temperature. The initial dose kept the animals sedated for at least 120 min. At 240 min the animals started moving around a little, and they were carefully monitored for signs of discomfort. However, in no instance was this observed. All animals were killed by cervical dislocation followed by decapitation.

Electroporation

The electric pulses were supplied by a Cliniporator (IGEA, Carpi, Italy) using either eight pulses with a fixed pulse duration of 100 μs (8HV) or 20 ms (8MV) delivered at a frequency of 1 Hz or one short high-voltage pulse (HV, 1,000 V/cm, 100 μs) followed by one low-voltage pulse (LV, 100 V/cm, 400 ms) (HVLV). Parallel plate electrodes with a 6-mm gap between electrodes were used (1 cm wide, 1 mm thick).

The applied electric field was varied from 0 to 1,200 V/cm. The electrodes were placed on each side of the leg across the tibia as close to the knee joint as possible. The electrode was placed so that the electrical field was approximately perpendicular to the muscle fibers in all three muscles (Fig. 1). Good contact between electrode and skin was ensured by removal of hairs and the use of electrode paste. Electrodes were placed around both legs; in some experiments only one leg was electroporated while the other served as a control, whereas in others both legs were electroporated. At the times indicated the animals were killed, and TA as well as intact soleus and EDL muscles were dissected out.

Muscle Weights

After excision the muscles were weighed [wet weight (WW)]; then they were dried at 60°C until constant weight, and dry weight (DW) was determined.

Determination of Ion Contents

After weighing, the muscles were soaked overnight in 3 ml of 0.3 M TCA to extract Na⁺, K⁺, and Ca²⁺. Previous studies showed that this procedure was as efficient in extraction of ions as homogenization and subsequent centrifugation of the TCA extract (5, 13). Ca²⁺ content was determined by atomic absorption spectrophotometry (Solaar AAS, Thermo) using 1.5 ml of the TCA extract mixed with 150 μl of 0.27 M KCl. The muscle extracts were measured against a blank and standards containing 12.5 or 25 μM Ca²⁺ and the same amount of TCA and KCl as the muscle extracts. Na⁺ and K⁺ contents of the TCA extracts were determined with a Radiometer FLM3 flame photometer (Copenhagen, Denmark) with lithium as internal standard. For each 0.5-ml sample of the TCA extract, 1.5 ml of 5 mM LiCl and 0.5 ml of 0.3 M TCA were added.

Determination of LDH Activity

Blood samples were collected at various times from the tail vein. Immediately after collection, the samples were centrifuged (9,500 g, 3 min), and plasma was isolated and frozen for later analysis. LDH activity was determined by measuring the decrease in the concentration of the substrate NADH by conversion of pyruvate to lactate and expressed as units per liter of plasma (U/l; 1 unit being the amount of enzyme that catalyzes the utilization of 1 μmol of substrate/min). A 40-μl plasma sample was mixed with 1.00 ml of a phosphate buffer (0.1 M K₂HPO₄ titrated with KH₂PO₄ to pH 7.0) containing NADH (0.3 mM) and pyruvate (0.8 mM), and the absorbance of NADH was measured at 340 nm at 30-s intervals for 4 min at 30°C.
experiments have shown that the increase in plasma LDH activity is associated with EP. All tissue between the electrodes may be permeabilized, and thus LDH may not exclusively arise from muscle tissue. However, since muscle constitutes most of the tissue between the electrodes and has a high content of LDH we expect the major part of the LDH to arise from muscle tissue.

**Force Measurements**

After EP, the animals were killed at the times given and intact soleus and/or EDL muscles were excised. The muscles were mounted for isometric contractions on a muscle holder with platinum wire electrodes on either side of the central part of the muscle. One end was connected to a force displacement transducer. The muscles were kept in a standard Krebs-Ringer bicarbonate buffer (pH 7.3) containing (in mM) 122.1 NaCl, 25.1 NaHCO3, 2.8 KCl, 1.2 KH2PO4, 1.2 MgSO4, 1.3 CaCl2, and 5 D-glucose. Incubation took place at 30°C. The buffer was gassed continuously with a mixture of 95% O2 and 5% CO2. With the use of single pulses muscle length was adjusted to optimal length for twitch force development. Force was checked with short tetanic contractions. Soleus muscles were exposed to 60 Hz (0.2-ms pulses, 400 ms) pulse (HVLV); or 8 pulses with a duration of 20 ms and a field strength of 150 V/cm (8MV).

The other hind leg served as control. Sixty minutes after EP the animals were killed, the muscles excised, and total Ca2+ content determined. Filled bars, soleus; open bars, EDL. Mean values with bars denoting SD are given; n = 3–19 muscles. Significance of the difference between electroporated muscles and paired nonelectroporated controls was assessed with a paired t-test. *P < 0.05, **P < 0.01, ***P < 0.001.

**Results**

8HV Pulses: Effects of Applied Voltage

Ca2+. Permeabilization of the membrane will lead to an influx of Ca2+. Figure 2 shows the Ca2+ content of muscles, measured 60 min after EP, as a function of increasing field strength. At 600 V/cm muscle Ca2+ content starts to increase in both soleus and EDL, increasing further as field strength is increased. At 1,200 V/cm the increase in Ca2+ content was highly significant, with 4.5- and a 7.6-fold increases in soleus and EDL, respectively.

In a few experiments Ca2+ content in TA was determined. At 1,200 V/cm Ca2+ content in TA from the electroporated leg was 9.35 μmol/g wet wt (SD 1.24) compared with 1.40 μmol/g wet wt (SD 0.08) in the control leg (P < 0.001, n = 4 vs. 4).

8MV pulses resulted in a significant increase in muscle Ca2+ content in both EDL and soleus (P = 0.021 and P = 0.005, respectively), while no significant increases in muscle Ca2+ content were observed in soleus, EDL, or TA 60 min after EP using HVLV pulses.

Na+ and K+. Permeabilization is also expected to lead to an influx of Na+ and an efflux of K+. As shown in Fig. 3A, EP at 600 V/cm and above induced a significant increase in the Na+ content of soleus and EDL measured 60 min after EP. In soleus the Na+ content reached a maximum increase of 65% at 1,200 V/cm [to 56.7 μmol/g wet wt (SD 16)], and in EDL the maximum increase in Na+ content was 270% [to 100.2 μmol/g wet wt (SD 8.6)]. Na+ content in TA electroporated at 1,200 V/cm was 108.3 μmol/g wet wt (SD 11.8) compared with 20.6 μmol/g wet wt (SD 0.6) in the controls (P < 0.001, n = 4 vs. 4). 8MV and HVLV both gave rise to significant increases in Na+ content in both soleus and EDL. In soleus the increase was significantly larger with 8 MV than with HVLV (P < 0.001).

In EDL loss of K+ was observed from 600 V/cm, while in soleus nothing significant was observed until a field strength of 1,200 V/cm, at which a small but significant decrease (8%) in K+ content was observed. In TA K+ content was 22.3 μmol/g wet wt (SD 5.4) after EP at 1,200 V/cm, compared with 113.9 μmol/g wet wt (SD 15.9) in the controls (P < 0.001, n = 4 vs. 4).

8MV caused a significant decrease in K+ content in soleus, while HVLV caused no significant changes in K+ content in soleus or EDL.

Since the results from 8MV pulses were similar but not quite as good as those obtained with HVLV, further studies were performed with the HVLV paradigm alone.
Changes Over Time Following EP

To investigate how the muscles recover from EP, a series of experiments were performed in which muscle function, ion homeostasis, and integrity were followed over time. Depending on the EP paradigm, varying degrees of recovery would be expected.

Force recovery. It was shown above that muscle ion content is affected by permeabilization. However, if permeabilization is followed by resealing the Na\(^+/\)H\(^+\) pump will be able to recover the Na\(^+\)/H\(^+\) and K\(^+\)/H\(^+\) gradients and possibly muscle function. To investigate this, a second series of experiments was performed in which the animals were killed at 5 min, 4 h, 24 h, and 2 wk after EP in order to follow recovery of force in EDL and soleus (Fig. 4). Tetanic force was tested, and muscles from the untreated leg served as control.

Five minutes after EP at 1,200 V/cm no force could be generated in either EDL or soleus. At 4 h EDL had recovered to 16% (SD 14) ([n](n=3)), while soleus was at 24% (SD 6) ([n](n=3)). At 24 h there was a large scatter in the recovery data. In EDL recovery ranged from 7% to 73% with an average value of 24% (SD 16) ([n](n=16)), while in soleus it ranged between 9% and 90% with an average value of 39% (SD 25) ([n](n=6)). By 2 wk force recovery was complete in both muscles (Fig. 4 A).

Five minutes after EP at 900 V/cm tetanic force was 13% (SD 7) in EDL and 9% (SD 3) ([n](n=3)) in soleus. Four hours after EP force recovery was 50% (SD 9) ([n](n=7)) in EDL and 79% (SD 6) ([n](n=3)) in soleus. Again, a large scatter was observed at 24 h. In EDL force recovery ranged from 11% to 73% with an average value of 42% (SD 21) ([n](n=8)), and in soleus it ranged between 20% and 104% with an average value of 66% (SD 30) ([n](n=6)). By 2 wk force recovery was complete in both EDL and soleus ([n](n=4)) (Fig. 4B).

EP using HVLV had a much smaller effect on the force-generating ability of the muscles. Five minutes after EP average force production in EDL was 60% (SD 22) ([n](n=5)), and in soleus it was 82% (SD 10) ([n](n=5)). At 4 h force recovery was complete in both EDL and soleus ([n](n=3)). Since force had recovered to control levels by 4 h and remained at control level at 24 h, force at 2 wk was not tested.

Ca\(^{2+}\). Resealing and EP-activated damage processes determine the fate of the muscle. If membrane integrity is recovered rapidly, minor changes in ion homeostasis are expected; however, if resealing is slow, cells may suffer Ca\(^{2+}\) overload and Ca\(^{2+}\)-activated membrane damage giving rise to further disturbance in muscle ion content. In this series of experiments animals were again treated with either 8HV pulses (at 900 or 1,200 V/cm) or HVLV. Soleus, EDL, and in some instances TA were harvested, and total muscle Ca\(^{2+}\) content was determined by atomic absorption (Fig. 5).

As can be seen from Fig. 5A, EP at 1,200 V/cm resulted in a very large increase in muscle Ca\(^{2+}\) content, in particular in EDL. The highest average value was obtained at 12 h (23.1 μmol/g wet wt (SD 6.0), [n]=3). At 24 h the Ca\(^{2+}\) content in TA was 13.3 μmol/g wet wt (SD 1.6) ([n]=6). In soleus the highest value was measured at 4 h (7.3 μmol/g wet wt (SD 1.9), [n]=16). In soleus Ca\(^{2+}\) content had
Fig. 4. Force recovery in rat EDL and soleus muscle following EP in vivo. One hind leg was electroporated either with 8HV pulses at 1,200 V/cm (A) or 900 V/cm (B) or with HL HV pulses (C). The other hind leg served as control. Animals were killed 5 min, 4 h, 24 h, or 2 wk (w) after electroporation, and force was tested as described in METHODS. *, EDL; †, soleus. Mean values with bars denoting SD are given; n = 3–16. Significance of the difference between electroporated muscles and paired nonelectroporated controls was assessed with a paired t-test. **P < 0.01, ***P < 0.001.

Fig. 5. Ca²⁺ content in rat EDL, TA, and soleus muscle after EP in vivo. One hind leg was electroporated with 8HV pulses at either 1,200 V/cm (A) or 900 V/cm (B) or with HL HV pulses (C). The other hind leg served as control. At the times indicated the animals were killed and total muscle Ca²⁺ content was determined. Filled symbols, electroporated muscles; open symbols, untreated controls; circles, EDL; squares, TA; triangles, soleus. Mean values with bars denoting SD are given; n = 3–20.
efflux from 24 to 96 h. By 96 h muscle Na\(^+\) content was still significantly increased (P < 0.05, n = 4 vs. 18), whereas the K\(^+\) content was not significantly different from the control group. EDL and TA showed even larger changes in Na\(^+\) and K\(^+\). At 240 min after EP Na\(^+\) content was 103 \(\mu\)mol/g wet wt (SD 11) in EDL and 108 \(\mu\)mol/g wet wt (SD 12) in TA [compared with 24 \(\mu\)mol/g wet wt (SD 2) and 20 \(\mu\)mol/g wet wt (SD 1) in control EDL and TA, respectively]. Comparable decreases were observed in K\(^+\) content. By 96 h recovery of Na\(^+\) was 75% and 74% in EDL and TA, respectively, with a comparable recovery in K\(^+\) (69% and 67% for EDL and TA, respectively); however, values were still significantly different from control (P < 0.001, n = 4 vs. 18).

As shown in Fig. 7, C and D, the changes in Na\(^+\) and K\(^+\) as a result of EP using 900 V/cm were initially almost as large as those observed with 1,200 V/cm for EDL and TA. The highest Na\(^+\) values were obtained 4 h after EP [91.7 \(\mu\)mol/g wet wt (SD 6.5) and 88.7 \(\mu\)mol/g wet wt (SD 3.3) in EDL and TA, respectively]. In soleus the changes were less pronounced; here the largest increase in Na\(^+\) content was obtained 15 min after EP [51.4 \(\mu\)mol/g wet wt (SD 2.8)]. However, again in contrast to 1,200 V/cm, a large recovery was observed for EDL and TA at 24 h (87% and 65%, respectively). In soleus recovery was 60%–30 min after EP. By 96 h values were no longer significantly different from the control.

The reductions in K\(^+\) content corresponded very well to the increases in Na\(^+\) content. Here recovery was complete by 24 h. HVLV pulses only gave rise to minor changes in muscle Na\(^+\) and K\(^+\) content (significant values indicated in insets), and by 6 h values were normalized (Fig. 7, E and F).

Fig. 7A the flux of Na\(^+\) across the membrane following EP at 1,200 V/cm was calculated and is presented in Fig. 8. As with the Ca\(^{2+}\) flux there is a large influx the first 15 min after EP. In EDL the influx continues at 30 min, whereas in soleus a large net efflux is observed. Soleus gradually disposes of the excess Na\(^+\), and by 96 h muscle Na\(^+\) content is close to normal [40 \(\mu\)mol/g wet wt (SD 4) (n = 18) vs. 34 \(\mu\)mol/g wet wt (SD 4) (n = 4)]. In EDL there is a small net efflux from 24 h on; however, the efflux is not large enough to clear all the excess Na\(^+\), and by 96 h muscle Na\(^+\) content is still elevated [46 \(\mu\)mol/g wet wt (SD 4) (n = 18) compared with 23 \(\mu\)mol/g wet wt (SD 1) (n = 4)].

LDH. As a marker of an increased permeability of the membrane, caused by either permeabilization during the pulses or as a result of tissue damage, the activity of the enzyme LDH was measured in plasma. Again the animals either received 8HV pulses at 900 or 1,200 V/cm or were exposed to one HV and one LV pulse. Both legs were electroporated.

Figure 9A shows the LDH activity in plasma from rats exposed to 8HV pulses at 1,200 V/cm. One hour after electroporation there was a large increase in plasma LDH activity [903 U/l (SD 111) (n = 5) vs. 73 U/l (SD 35) (n = 3)], and four hours after EP the activity was even higher [1,204 U/l (SD 68) (n = 5) vs. 62 U/l (SD 20) (n = 3)]. By 24 h activity was reduced but still significantly higher in the electroporated animals compared with the control animals. By 48 h plasma LDH activity was normalized.

EP with 900 V/cm also gave rise to large increases in LDH activity [912 U/l (SD 52), n = 5], but not quite as high as with 1,200 V/cm. Again the activity was slightly but still significantly elevated at 24 h but normalized by 48 h (Fig. 9B).

LDH activity in plasma was also determined from rats exposed to 8HV pulses using 600 or 300 V/cm. Four hours after EP a significant increase in LDH activity [204 U/l (SD 88)]
(n = 3) vs. 61 U/l (SD 6) (n = 3)] was observed in plasma from rats exposed to 600 V/cm; 300 V/cm did not give rise to significant increases in plasma LDH activity.

HVLV pulses did not give rise to large increases in plasma LDH activity. However, a small but significant increase is observed 24 h after EP [87 U/l (SD 15), n = 7] (Fig. 9C).

Muscle weight. Soleus and EDL muscles are excised intact; therefore if one leg is electroporated and the other serves as control, it is possible to compare muscle weights (WW and DW). Muscle DW gives information regarding muscle mass, while the relation between muscle WW and DW gives information regarding the water content of the muscle and thus edema.

Figure 10 shows WW and DW in EDL and soleus muscle exposed to HV pulses using 1,200 V/cm. As can be seen from Fig. 10 there were large changes in muscle weight following EP. Initially WW of the muscles were increased, suggesting the formation of edema. In EDL EP also led to a decrease in

Fig. 7. Na⁺ and K⁺ content in rat EDL, TA, and soleus muscle after EP in vivo. One hind leg was electroporated with HV pulses at either 1,200 V/cm (A and B) or 900 V/cm (C and D) or with HVLV pulses (E and F). The other hind leg served as control. At the times indicated the animals were killed and total muscle Na⁺ and K⁺ content was determined. Filled symbols, electroporated muscles; open symbols, untreated controls; circles, EDL; squares, TA; triangles, soleus. Mean values with bars denoting SD are given; n = 4–18 muscles. Insets: Na⁺ or K⁺ content the first 4 or 6 h. Significant differences (E and F): *P < 0.05.
muscle DW that was significant from 24 h after EP, and 96 h after EP muscle DW was reduced to 49% of control. In soleus the changes were less drastic. The only significant difference was DW, which was slightly reduced by 7 days. By 14 days muscle weights had returned to control.

With exposure to 8HV using 900 V/cm, no significant changes were observed in DW and WW in EDL and soleus measured at 24 h, 96 h, and 14 days.

**DISCUSSION**

This study shows that by varying the pulse parameters it is possible to achieve gentle permeabilization, as in the case with the HVLV pulse combination, or efficient permeabilization lasting at least 15 min, judged from the ion flux calculations using 8HV pulses.

Not surprisingly, 1,200 V/cm gave rise to larger (Ca\(^{2+}\) content) or longer-lasting (Na\(^{+}\) and K\(^{+}\) contents) changes than 900 V/cm. However, ion contents were largely recovered by 96 h at both field strengths. Soleus is less affected than EDL or TA, possibly because of the difference in fiber type composition. Of the two DNA electrotransfer paradigms HVLV seemed to cause less disturbance to the tissue. This was also the conclusion in a recently published study (16) in which it was shown that DNA electrotransfer using HVLV caused less disturbance in Na\(^{+}\), K\(^{+}\), and Ca\(^{2+}\) content than DNA electrotransfer using 8MV.

**EP Threshold**

From the results it is clear that a threshold for the effect of EP on ion contents and force exists. A field strength of 300 V/cm caused no change in muscle ion content, whereas field strengths of 600 V/cm and above gave rise to increases in total muscle Ca\(^{2+}\) and Na\(^{+}\) contents and a decrease in K\(^{+}\) content, as well as increases in plasma LDH activity. Taken together the data show that permeabilization of the membrane occurred at field strengths of 600 V/cm and above. This value is well in agreement with earlier in vivo studies on mice in which a...
permeabilization threshold of 530 ± 30 V/cm was obtained in skeletal muscle based on measurements of the uptake of the extracellular marker $^{51}$Cr-EDTA, using an identical EP procedure (12).

Force

Immediately after EP using 8HV pulses the membrane potential of the muscle cells is reduced to less than $-20$ mV (6). This is due to the massive increase in the permeability of the membrane and the ensuing flux of Na$^{+}$ and K$^{+}$ and explains the almost complete loss of force observed in the minutes after EP both in this study and previously (6), due to loss of excitability of the muscle fibers. Depending on the severity of EP the muscle cells may spontaneously reseal and the membrane potential is recovered. In isolated soleus muscles a recovery of the membrane potential was observed that correlated with the force recovery (6). With the HVLV pulses a complete recovery of force is observed within 4 h, suggesting resealing of the membrane (supported by data on Na$^{+}$ and K$^{+}$ contents) and recovery of the membrane potential.

With 8HV pulses a longer-lasting depression of force is observed. Initially membrane depolarization (predicted from the large fluxes of Na$^{+}$ and K$^{+}$) is the explanation for the loss of force. However, with this more severe kind of EP three things can occur, which possibly represent a continuum: 1) immediate cell death, 2) delayed cell death as a result of damages incurred due to permeabilization, or 3) very late membrane resealing that the cells survive. Since the force exerted by the muscle is the combined effort from all the viable fibers, damage to muscle fibers will lead to a reduction in force. Destruction of fibers will eventually be observed as a reduction in the DW of the muscle. Muscles exposed to 900 V/cm showed a relatively large recovery of force at 4 h but no further improvement at 24 h. Muscle DW does not indicate any significant loss of muscle mass, so the force deficit may represent a fraction of viable but nonexcitable fibers (supported by the observation that Na$^{+}$ and K$^{+}$ contents are not normalized at this time). However, by 14 days force is normalized. Muscles exposed to 1,200 V/cm only show a small force recovery at 4 h, with a small improvement at 24 h. Analysis of DW shows that EDL suffers a dramatic decrease in DW (to 49% of control at 96 h), suggesting that a large part of the fibers have been lost. This is supported by very high Na$^{+}$ values suggesting the presence of damaged fibers with a Na$^{+}$ content like the extracellular space (147 µmol/g wet wt). In soleus no drastic changes in DW are observed despite a large force deficit; again this may represent a fraction of viable but nonexcitable fibers. Both soleus and EDL muscles are normalized with regard to DW and force by 14 days.

In general the recovery of force seems to occur faster and more completely in soleus than in EDL (see discussion below).

Soleus vs. EDL

In all instances the changes observed in the present study were larger in EDL and TA than in soleus, suggesting a greater degree of damage to the EDL and TA muscles. This greater resistance to EP in soleus has also been observed in isolated muscles electroporated in vitro. These studies showed that an 80% reduction in force was achieved in EDL when exposed to 350 V/cm, whereas in soleus the field strength needed to be increased to 500 V/cm in order to achieve the same reduction in force (6). The reason for this difference between the two

![Fig. 10. Wet and dry weights of rat EDL and soleus after electroporation using 8HV pulses at 1,200 V/cm. Animals were anesthetized, and the right leg was electroporated. At the times given the animal was killed and intact EDL and soleus muscles were excised. Wet weight of the muscles was determined, and after drying overnight at 60°C dry weight of the muscles was determined. Muscle weights were normalized to weight of the contralateral control muscle, and significant differences were ascertained with a paired t-test. Open columns, wet weight; gray columns, dry weight. Mean values with bars denoting SD are given; n = 3–4. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.](http://ajpregu.physiology.org/)

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muscles is not entirely clear, but fiber type composition seems a likely candidate. In a 4-wk-old rat the soleus contains ~60% slow-twitch fibers (type 1), whereas >90% of the fibers in EDL and TA are fast-twitch fibers (approximately divided equally between types 2A and 2B) (23).

Many things differ between the fiber types. One major difference is oxidative capacity. In a recent study it was shown in adult rat muscle that the mitochondrial density volume was approximately twofold higher in soleus than in white gastrocnemius (a muscle of fiber composition similar to EDL and TA) (27). Thus even when age-related changes in fiber type composition are taken into account, the soleus muscle in this study has a larger oxidative capacity than EDL and TA. It has been shown that muscle cells have a large potential for repairing membrane leakage (24), and any damage is likely to be repaired quickly. Studies on Chinese hamster ovary (CHO) cells showed that resealing in the first few minutes after EP is not dependent on ATP, but cell survival in the hours following EP is strongly dependent on the ATP level of the cells (29). Thus the higher oxidative capacity of the soleus may improve cell survival following permeabilization.

Another difference between fast-twitch and slow-twitch fibers is the Ca\(^{2+}\)-pumping capacity into the SR and the degree of saturation of the SR. In fast-twitch fibers the concentration of Ca\(^{2+}\)-ATPase (determined by Ca\(^{2+}\)-dependent phosphorylation) is approximately six times that of slow-twitch fibers (9). At resting conditions the SR of slow-twitch fibers is fully saturated with Ca\(^{2+}\), and the fibers do not take up more Ca\(^{2+}\) when Ca\(^{2+}\) is increased. SR of fast-twitch fibers, on the other hand, is only one-third saturated with Ca\(^{2+}\). Thus when exposed to increased Ca\(^{2+}\), Ca\(^{2+}\) content of the fiber may increase up to 3 mM (equivalent to 3 \(\mu\)mol/g wet wt) (10). Thus in EDL and TA increases in Ca\(^{2+}\) content of up to 3 \(\mu\)mol/g wet wt can be stored in SR, whereas in soleus only SR in the fraction of fast-twitch fibers will accumulate Ca\(^{2+}\).

Further storage of Ca\(^{2+}\) will most likely occur in the mitochondria. Ca\(^{2+}\) uptake into mitochondria uses the mitochondrial membrane potential as driving force, while uptake into SR depends on the Ca\(^{2+}\)-ATPase and thus use of ATP. Therefore a Ca\(^{2+}\) overload situation in EDL could lead to a rapid depletion of ATP due to Ca\(^{2+}\) pumping into the SR, whereas in soleus it would result in uptake into the mitochondria with no direct energy expenditure [unless excess Ca\(^{2+}\) loading leads to opening of the permeability transition pore (PTP); see below].

Since the radius of the cell is of importance for EP (larger cells are more easily electroporated than smaller cells) (25), fiber size would be expected to be of importance.

In a carefully conducted study Maltin et al. (23) found that there was no major difference in fiber cross-sectional area between soleus, EDL, and TA from 4-wk old rats. Thus the tolerance of soleus to EP must be attributed to the other physiological differences.

**Cellular Integrity**

On the basis of findings of this study and the findings of others, a sequence of events is proposed as shown in Fig. 11. EP transiently increases the permeability of the membrane, allowing an influx of Na\(^{+}\) and Ca\(^{2+}\) and an efflux of K\(^+\) and possibly release of ATP and LDH. The breakdown of the Na\(^{+}\) and K\(^{+}\) gradients results in loss of excitability and loss of force.

The pores created by EP are expected to show spontaneous resealing. Studies on mouse muscle exposed to EP in vivo using 8HV pulses (at 1,200 V/cm) showed that 63% of the pores created by EP were resealed after ~9 min (11). Resealing would allow the Na\(^{+}\)-K\(^{+}\) pump to restore the Na\(^{+}\) and K\(^{+}\) gradients more efficiently. In soleus the largest increase in Na\(^{+}\) content following 8HV pulses at 1,200 V/cm was observed 15 min after EP, where Na\(^{+}\) content was increased by 156%. By 30 min a large recovery had occurred, with Na\(^{+}\) content now being only 71% above control. In EDL, however, the increase in Na\(^{+}\) content was largest at 30 min (286%), and by 60 min it was still increased by 284%. This suggests that in soleus resealing occurs relatively rapidly, allowing a large recovery within the first 30 min. In EDL, however, and TA for that matter, almost no recovery of Na\(^{+}\) content is observed. This lack of recovery may represent damage/lack of resealing to a large fraction of the muscle fibers in the EDL and TA muscle. In EDL muscle Ca\(^{2+}\) content keeps increasing for 12 h, suggestive of an ongoing damage process.

Incomplete resealing of the membrane may lead to muscle Ca\(^{2+}\) overload. Ca\(^{2+}\) may be stored in the SR and the mitochondria. Low ATP levels could possibly affect the clearance of Ca\(^{2+}\) from the cytoplasm by the Ca\(^{2+}\)-ATPases in the SR.
and plasma membrane, leading to loss of cellular Ca\(^{2+}\) homestasis (i.e., increased [Ca\(^{2+}\)]).

Increased [Ca\(^{2+}\)], could lead to activation of Ca\(^{2+}\)-sensitive proteases and lipases that may result in further breakdown of the sarcolemma, causing additional Ca\(^{2+}\) uptake and LDH release. On top of that, production of reactive oxygen species may also be stimulated (26, 31), leading to peroxidation of membrane lipids again increasing the permeability of the membrane. This may activate a vicious cycle of continuous degradation and further influx of Ca\(^{2+}\) (14). Ca\(^{2+}\) is also stored in the mitochondria. This is important since Ca\(^{2+}\) loading of the matrix is the most important and obligatory trigger for PTP opening. PTP opening will result in massive release of Ca\(^{2+}\) from the mitochondria, collapse of the mitochondrial membrane potential, and ATP hydrolysis. Although multiple destructive processes are activated by Ca\(^{2+}\), lethal outcomes are determined largely by Ca\(^{2+}\)-induced mitochondrial permeability transition leading to cell death by apoptosis or necrosis (7).

After 8HV pulses LDH activity in the plasma showed a large increase 60 min after EP, increasing further at 4 h. It has been suggested that lymphatic transport may be the predominant pathway by which enzymes released by muscle cells reach the intravascular compartment (22). Studies have shown that lymph flow from muscle of sedated rats is low (3 \(\mu\)L/g tissue \(1\cdot h^{-1}\)) (28), and since the animals are sedated and do not move around the first 2 h after EP, a certain delay would be expected before an increased LDH activity could be observed in plasma. The half-life of the muscle-specific isoform of LDH in plasma (LD-5) is 30 min (30). EP alone may induce some release of LDH to the blood, but the progressive increase in LDH activity from 60 to 240 min suggests that an ongoing damage process is occurring.

**Perspectives and Significance**

This study shows that electric field strength is of utmost importance for the degree and duration of permeabilization of muscle cells exposed to EP in vivo. If cell damage is the objective (e.g., for ablation in cancer therapy) 8HV pulses at high field strengths (1,200 V/cm) effectively permeabilize cells, causing large disturbances in ion homeostasis. This could be combined with addition of drugs (e.g., bleomycin) to enhance the damage process. On the other hand, for victims of electrical shock strategies for resealing of the membrane and recovery of ATP and ion homeostasis would be important. For the introduction of drugs and molecules lower field strengths should be used; 600 V/cm causes permeabilization with relatively little tissue damage. Finally, if transfer of DNA is the objective as in gene therapy the HVLV combination would be recommended.

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**DISCLOSURES**

No conflicts of interest are declared by the author(s).

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