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Physiological Effects of High- and Low-Voltage Pulse Combinations for Gene Electrotransfer in Muscle

Pernille Hojman,¹ Hanne Gissel,² Franck M. Andre,³ Christelle Cournil-Henrionnet,³
Jens Eriksen,¹ Julie Gehl,¹ and Lluís M. Mir³

Abstract

Gene transfer by electroporation is gaining momentum now that high-level, long-term expression of transgenes is being obtained. Several different pulse regimens are efficient, yet little information is available about the physiological muscular response to gene electrotransfer. This paper provides a comprehensive evaluation of the physiological and molecular effects on host tissue after DNA electrotransfer. We have tested several pulse regimens with special emphasis on the pulse combination of a short (100 μ sec) high-voltage (HV) pulse followed by a long low-voltage (LV) pulse used for DNA electrotransfer, comparing it with 8 HV pulses designed to ensure extensive permeabilization of the muscle membrane. Using both mouse and rat skeletal muscle tissue, we investigated cell permeabilization by the ⁵¹Cr-labeled EDTA assay, lactate dehydrogenase release, Na⁺ and Ca²⁺ influx, K⁺ efflux, ATP release, and water content, as well as muscle function both *in vivo* and *ex vivo*, Hsp70 induction, and histology. In all these assays, the HV+LV pulse combination gave rise to minimal disturbance of cell function, in all cases significantly different from results when using 8 HV pulses. The evaluated parameters were normalized after 1 week. The addition of DNA caused significantly more transmembrane exchange, and this may be due to entrance of the DNA through the membrane. In conclusion, this study comprehensively documents the immediate effects of DNA electrotransfer and shows that only slight cell disturbances occur with the HV+LV pulses used for gene transfer. This is highly important, as minimal perturbation of cell physiology is essential for efficient transgene expression.

Introduction

GENE TRANSFER BY ELECTROPORATION *in vivo* offers great potential for the treatment of a large panel of diseases including monogenetic diseases and protein deficiency syndromes, and as well serves a systemic or vaccination purpose. The muscle has proven particularly attractive as target organ for gene electrotransfer, because of high transfection efficacy and its large ability to produce and secrete high levels of transgenes. Moreover, persisting gene expression after gene electrotransfer has been detected for at least 9–12 months in mice (Mir *et al.*, 1998; Muramatsu *et al.*, 2001). In addition, gene electrotransfer to other organs has also proven highly efficient, with prominent examples being the skin for vaccination purposes, and tumors for anticancer treatment (Mir *et al.*, 2005). In fact, the first clinical trials with gene electrotransfer to human malignant tissue and skeletal muscle have been undertaken (Heller and Heller, 2006).

Electroporation (EP) occurs when an external electric field above a critical value (permeabilization threshold) is applied

across the cell membrane. For delivery of small molecules, for example, the cytotoxic bleomycin, trains of short (100 μ sec) high-voltage pulses are typically used (Gothelf *et al.*, 2003). The combination of bleomycin and short electric pulses is known as electrochemotherapy and, after several clinical trials (Belehradek *et al.*, 1993; Marty *et al.*, 2006; Sersa, 2006), it is already being used to treat cutaneous and subcutaneous metastases in clinical practice (Belehradek *et al.*, 1993; Gehl *et al.*, 2006; Sersa, 2006). These short high-voltage (HV) pulses, which induce cell permeabilization *in vivo*, were also used in the first pioneering study of *in vivo* gene electrotransfer (Titomirov *et al.*, 1991). Comparison of trains of HV pulses and trains of identical pulses of 20 to 50 msec duration at about 200 V/cm has shown that the latter give the highest transfection efficiency (Lucas and Heller, 2001), and these have subsequently been widely used for muscle transfection (Mir *et al.*, 2005).

By combining a short (100 μ sec) high-voltage (HV) pulse and one or more long low-voltage (LV) pulses it was demonstrated that the electric pulses fulfill two functions: (1) to

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permeabilize the cell membrane (HV pulses) and (2) to electrophoretically drive the DNA toward the cells (LV pulses) (Bureau *et al.*, 2000; Satkauskas *et al.*, 2002, 2005). Recent developments have shown that combining 1 HV pulse with 1 LV pulse results in highly efficient gene transfer in muscle, skin, liver, and tumor tissue (Hojman *et al.*, 2007b; Andre *et al.*, 2008). Interestingly, when using HV+LV combinations, the highest efficacy was achieved with HV pulses of field strengths close to, or even below, the known reversible permeabilization thresholds in the tissues. Therefore, cells in the tissues exposed to these pulse combinations should be minimally affected by the pulsing.

Until now the effect of electric pulses on the host tissue has been examined by histology, microarray analysis, and blood analyses (Heller *et al.*, 2006; Trollet *et al.*, 2006; Hojman *et al.*, 2007a). Thus a comprehensive physiological evaluation of the effects of electric pulses has been pending. Because muscle is likely the main target for future applications in humans, the muscle is primarily analyzed in this study to evaluate the effects of pulse combinations. Tumors are also important targets; however, the adverse effects of the procedure are less important in this case. We have therefore performed a thorough evaluation of physiological parameters such as ^{51}Cr -labeled EDTA (^{51}Cr -EDTA) uptake, ionic exchange, ATP content, lactate dehydrogenase (LDH) release, force generation, reflex and motor function, and Hsp70 expression in skeletal muscle after electric pulse delivery or gene electrotransfer. We have compared different types of pulses, both electric parameters known to produce high cell electropermeabilization in tissues, and electric pulses associated with gene transfer, in order to be able to determine potential effects of the pulses on the physiological parameters evaluated. Indeed, this study elucidates that combinations of pulses for efficient gene electrotransfer cause only small and transient changes in muscle physiology.

Materials and Methods

Animals and muscle preparation

All animal experiments were conducted in accordance with the recommendations of the European Convention for the Protection of Vertebrate Animals used for Experimentation. Experiments were performed on 7- to 9-week-old female C57BL/6 mice from Taconic (Tornbjerggaard, Denmark) or Janvier (Le Genest St Isle, France), or on 4- to 5-week-old male or female Wistar rats (our own breed, University of Aarhus, Aarhus, Denmark). Animals were maintained in a thermostatted environment under a 12-hr light-to-dark cycle and had free access to food (Altromin pellets; Spezialfutter-Werke, Lage, Germany) and water. The animals were anesthetized 15 min before EP by intraperitoneal injection of tribromoethanol (Royal Veterinary School, Copenhagen, Denmark) for the mice and by subcutaneous injection of Hypnorm (0.4 ml/kg; Janssen Saunderton, Buckinghamshire, UK) and Dormicum (2 mg/kg; Roche, Basel, Switzerland) for the rats. For the Hsp70 experiment the mice were anesthetized with Ketalar (100 mg/kg; Panpharma, Fougères, France) and Rompun (40 mg/kg; Bayer, Puteaux, France). The animals were killed by cervical dislocation, and the rats were then decapitated. Intact tibialis cranialis (TC) or extensor digitorum longus (EDL) muscles with tendons were dissected as previously described (Kohn and Clausen

1971; Chinet *et al.*, 1977). For measurement of ion content muscles were quickly frozen on dry ice and absolute alcohol, whereas muscles for measurement of force and ATP measurement were placed in their appropriate buffer.

Plasmid constructs

The plasmid pHGFP-S65T, encoding the green fluorescent protein (GFP) (Clontech, Palo Alto, CA), was used for most experiments, whereas pEGFP-N1 (Clontech), also encoding GFP, was used for the Hsp70 experiment. DNA preparations of pHGFP-S65T were performed with QIAfilter plasmid maxiprep kits (Qiagen, Hilden, Germany), and pEGFP-N1 was purified with an EndoFree plasmid giga kit (Qiagen, Courtabeuf, France). The concentration and quality of the plasmid preparations were controlled by spectrophotometry. Plasmids were finally dissolved in phosphate-buffered saline (PBS) at a concentration of 0.5 $\mu\text{g}/\mu\text{l}$.

In vivo gene electrotransfer

For mice, 20 μl of plasmid solution was injected intramuscularly along the fibers into the TC muscle, using a 29-gauge insulin syringe. For rats, 10 μl of plasmid solution was injected into the EDL, also with an insulin syringe. Plate electrodes with a 4-mm gap (or a 5-mm gap for the rats) were fitted around the hind legs. Good contact between electrode and skin was ensured by hair removal and the use of electrode gel (Eko-Gel; Camina, Egna, Italy). The electric field was applied with a Cliniporator (IGEA, Carpi, Italy), when applying a combination of a high-voltage (1000 V/cm [applied voltage, 400 V], 100 μsec) pulse followed by a low-voltage (100 V/cm [applied voltage, 40 V], 400 msec) pulse with a 1-sec time lag between the pulses or 8 high-voltage (mice: 1000 V/cm [applied voltage, 400 V], 100 μsec , 1 Hz; rats: 900 V/cm [applied voltage, 450 V], 100 μsec , 1 Hz) pulses; and with a PS-15 Jouan electropulsator (GHT 1281; Jouan, St Herblain, France) when applying 8 pulses of 20 msec (200 V/cm [applied voltage, 80 V] or 240 V/cm [for Hsp70 measurements; applied voltage, 96 V]). The latter pulses were controlled by means of a digital oscilloscope (VC 6025; Hitachi, Tokyo, Japan), whereas the Cliniporator allows online measurement of voltage and current.

Measurement of ^{51}Cr -labeled EDTA in muscle

Mice were given 100 μl containing 5 μg of ^{51}Cr -EDTA (Amersham, Little Chalfont, UK) with a specific activity of 3.7 MBq/ml by intraperitoneal injection. Electric pulses were delivered to the TC muscle 7 min after injection. After 48 hr the mice were killed and the electroporated muscle and the contralateral control muscle were excised, weighed, and γ -counted (Cobra 5002 gamma counter; Packard Instruments, Meriden, CT). The measured net uptake as a result of electroporation was calculated as the measured activity per gram in tissue exposed to electric pulses minus that of the unexposed contralateral muscle in the same mouse. The measured activity was converted to nanomoles of ^{51}Cr -EDTA internalized per gram of muscle.

Determination of water content

Excised muscles were carefully cleared from tendons and the wet weight was determined. The muscles were then

dried to constant weight in a heating cabinet at 60°C and the dry weight was determined. Muscle water content was assessed as the difference between these two weights.

Ion content

After muscle excision the tendons were removed and the muscles were blotted. Wet and dry weights were determined. Then the muscles were soaked overnight in 3 ml of 0.3 M trichloroacetic acid (TCA) to extract Na⁺, K⁺, and Ca²⁺ (Gissel and Clausen, 2000). Ca²⁺ content was determined by atomic absorption spectrophotometry (Solaar AAS, Fisher Scientific UK, Loughborough, UK), using 1.5 ml of the TCA extract mixed with 150 μl of 0.27 M KCl. Muscle extracts were measured against a blank and standards containing 12.5 or 25 μM Ca²⁺. The Na⁺ and K⁺ content of TCA extracts was 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 lactate dehydrogenase

Muscle fiber integrity was examined by measuring the release of LDH into the blood in rats. Blood samples were collected at various times from the tail vein of sedated rats and immediately spun down (9500 × g, 1 min). LDH activity was determined in plasma by measuring the decrease in concentration of NADH by conversion of pyruvate to lactate. Activity was expressed as units per liter. A 40-μl plasma sample was mixed with 1.00 ml of 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-sec intervals for 4 min at 30°C.

Test of function

The treated animals were tested for their ability to seize a grid by placing each animal on the grid and lifting their hindquarters by the tail. Animals with normal muscle function were given the score 0, whereas animals with affected function were awarded 1 point and those that did not seize the grid at all were scored 2. For running, the animals were placed alone in an empty cage. Normal running was given the score 0, whereas animals with a limp were given 1 point and animals that did not place support on the leg were awarded 2 points. A combined score from the two tests was then calculated.

Measurement of force

Contractile force was measured as previously described in detail by Clausen and Everts (1991). In brief, isolated EDL muscles were mounted vertically with their tendons intact on a force displacement transducer (Grass FT03; Grass Technologies, West Warwick, RI) for isometric contractions in thermostatically controlled (30°C) chambers containing standard Krebs–Ringer bicarbonate buffer (pH 7.3) with (in mM) 122.1 NaCl, 25.1 NaHCO₃, 2.8 KCl, 1.2 KH₂PO₄, 1.2 MgSO₄, 1.3 CaCl₂, and 5 D-glucose and gassed continuously with a mixture of 95% O₂ and 5% CO₂. Direct electrical stimulation was delivered via platinum electrodes on either side of the

mid-portion of the muscle. Muscle length was adjusted to optimal length during repeated stimulation with single pulses. Finally, maximum tetanic force was checked using short tetanic contractions induced by supramaximal 1-msec pulses of 10 V at 90 Hz for 0.5 sec.

ATP assay

For determination of ATP content, muscles were blotted, weighed, and extracted overnight by soaking in 1 ml of ice-cold 5% TCA. Supernatant was diluted 1:50 in Tris-acetate buffer, and the pH was calibrated to 7.75. ATP was determined by measuring light production in the ATP-dependent conversion of luciferin to oxyluciferin and light by recombinant luciferase (rL). One hundred microliters of rL per liter of reagent (ENLITEN ATP assay; Promega, Ramcon, Denmark) was added to 100 μl of TCA extract, and light emission was measured with a luminometer (LUMIstar; BMG Biotechnology, Ramcon, Denmark).

Induction of stress gene Hsp70

Total RNA was extracted from muscle by disrupting muscle in 1 ml of RNAlater in a ceramic spheric tube (Bio 101 Systems lysing matrix A tube; Q-Biogene, Illkirch, France) with a high-speed homogenizer followed by TRIzol extraction (Invitrogen, Cergy Pontoise, France). Two micrograms of total RNA was reverse transcribed to cDNA, and polymerase chain reaction (PCR) amplifications were performed with specific primers for mouse Hsp70 (sense, 5'-GGAGGT-CATCTCCTGGCTGGACTC-3'; antisense, 5'-CGCCTGGG-CCCCGAAGCCCCCAGC-3') and mouse glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (sense, 5'-GACAA-CTCACTCAAGATTGTCAG-3'; antisense, 5'-CATTGTCAT-ACCAGGAAATG-3'). For GAPDH amplification 23 cycles of 94°C/45 sec, 56°C/45 sec, and 72°C/45 sec were used, whereas Hsp70 amplification was performed by 27 cycles of 94°C/45 sec, 71°C/45 sec, and 72°C/45 sec. PCR products were analyzed by electrophoresis, and band density was determined from ultraviolet (UV) images, using densitometry analysis software (Scion Image; Scion, Frederick, MD). Hsp70 expression was reported as a ratio of GAPDH (Hsp70/GAPDH).

Histology

Forty-eight hours after treatment muscles were isolated and fixed in 1 ml of formalin buffer or Glyo-Fixx (Shandon, Pittsburgh, PA). After paraffin embedding, transverse sections of 3–5 μm were prepared and stained with hematoxylin and eosin (H&E).

Statistics

Results are given as mean values with the SD. The statistical significance of the difference between EP muscles and untreated contralateral controls was ascertained by Student *t* test for paired observations. To assess the significance of increases in Hsp70 expression, Kruskal-Wallis one-way analysis of variance on ranks followed by all pairwise multiple comparison procedures (Dunn's method) or two-way analysis of variance (ANOVA) followed by all pairwise multiple comparison procedures (Bonferroni *t* test) were applied.

Results

⁵¹Cr-labeled EDTA uptake in permeabilized muscle

The effect of the amplitude of the HV and LV pulses on cell permeabilization was determined by measuring ⁵¹Cr-EDTA uptake in electroporated muscle (Fig. 1A). By increasing the HV pulse (100 μ sec), while maintaining the LV pulse at 80 V/cm for 400 msec, it was shown the permeabilization of the muscle fibers begins at 700–800 V/cm and peaks at 1400 V/cm. At pulse amplitudes higher than 1400 V/cm ⁵¹Cr-EDTA uptake decreases, indicating the beginning of irreversible permeabilization of the cells. The amplitude of the

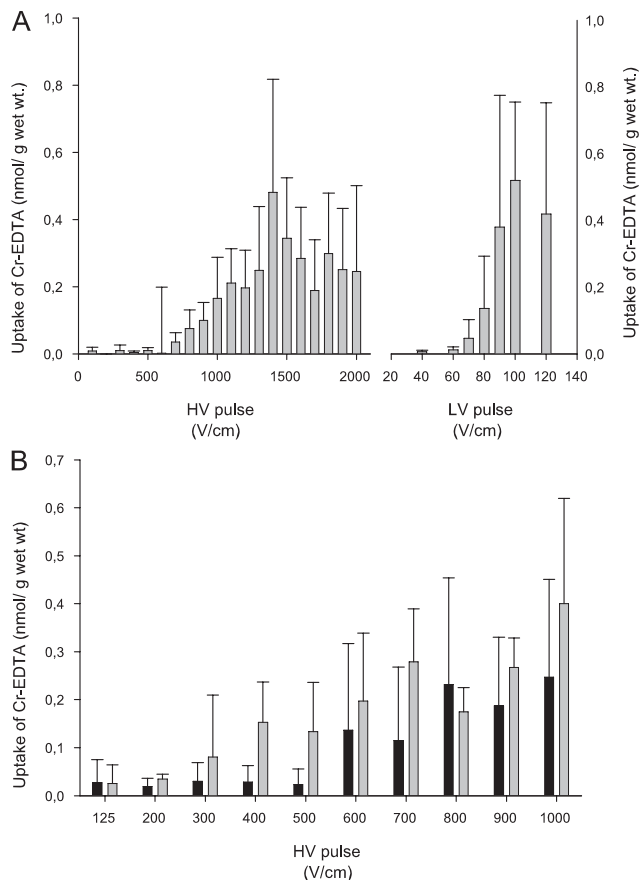


FIG. 1. Effect of electric pulses on ⁵¹Cr-EDTA uptake in mouse tibialis cranialis muscle. Net uptake was calculated as the content of ⁵¹Cr-EDTA in muscle exposed to pulses minus that of the contralateral unexposed muscle and expressed as net internalized ⁵¹Cr-EDTA (nmol/g wet weight). Columns represent the mean net uptake and error bars indicate the SD. **(A)** ⁵¹Cr-EDTA uptake 48 hr after delivery of electric pulses with increasing HV amplitude while keeping LV at 80 V/cm ($n = 5-7$, $p < 0.05$ for all conditions from 800 to 1900 V/cm) or increasing LV amplitude while keeping HV at 800 V/cm ($n = 6$; for values >80 V/cm, $p < 0.05$). For constant LV compared with the first level (125 V/cm), there was significantly higher expression from 700 V/cm and upward ($p < 0.05$). **(B)** ⁵¹Cr-EDTA with or without injection of DNA before pulsing. HV amplitude was varied while LV amplitude was kept constant at 80 V/cm. Solid columns represent EP controls and shaded columns represent uptake in muscles injected with DNA before pulsing.

LV pulse also had a significant effect on ⁵¹Cr-EDTA uptake. Increasing the LV pulse amplitude from 80 to 100 V/cm with a constant HV pulse of 800 V/cm resulted in a 3-fold increase in ⁵¹Cr-EDTA uptake. At LV amplitudes exceeding 100 V/cm, ⁵¹Cr-EDTA uptake started to decrease, also indicating irreversible permeabilization. As shown in Fig. 1B, the presence of DNA during application of the electric pulses increases the uptake of ⁵¹Cr-EDTA. In actuality, a trend toward higher influx of ⁵¹Cr-EDTA for all voltages tested was evident, and there was a statistically significant difference just around the permeabilization threshold ($p < 0.001$ for 400 and 500 V/cm and $p < 0.05$ for 700 V/cm), indicating that the presence of DNA facilitates transmembrane diffusion.

Changes in transmembrane ion flux and LDH release from muscles after gene electrotransfer

During the course of our work, we worked extensively with two combinations of pulse settings: EP 1000/100 (HV: 1000 V/cm, 100 μ sec; LV: 100 V/cm, 400 msec) and EP 800/80 (HV: 800 V/cm, 100 μ sec; LV: 80 V/cm, 400 msec). To evaluate the effects of these different pulse combinations on transmembrane ion flux, mice were killed 4 hr after pulse delivery or gene electrotransfer (DNA injection prior to pulse delivery), and tibialis cranialis (TC) muscles were excised for determination of Na⁺, K⁺, and Ca²⁺ content (Fig. 2). Neither EP 1000/100 nor EP 800/80 caused any significant changes in Na⁺, K⁺, or Ca²⁺ content. However, significant increases in Na⁺ content (Na⁺ content increase from 21.0 ± 1.6 to 30.7 ± 3.9 μ mol of Na⁺ per gram wet weight [$p = 2.8 \times 10^{-5}$, $n = 8$] and to 35.7 ± 5.1 μ mol Na⁺ per gram wet weight [$p = 0.0007$, $n = 8$]) and decreases in K⁺ content (K⁺ content decrease from 107.1 ± 4.4 to 97.3 ± 3.5 μ mol of K⁺ per gram wet weight [$p = 0.0003$, $n = 8$] and to 94.0 ± 4.6 μ mol of K⁺ per gram wet weight [$p = 0.002$, $n = 8$]) were observed when DNA was injected before electropulsing with, respectively, EP 1000/100 and EP 800/80. In actuality, electro-gene therapy (EGT) using EP 1000/100 caused a significantly higher increase in Na⁺ content ($p = 0.045$, $n = 8$) than EGT using EP 800/80. Most interestingly, EP 800/80 alone or in combination with DNA did not induce any significant increases in muscle Ca²⁺ content. EGT using EP 1000/100, on the other hand, led to a significant increase in Ca²⁺ content (6.8 ± 3.0 μ mol of Ca²⁺ per gram wet weight, $p = 0.0036$ vs. control level, $n = 8$, and $p = 0.012$ vs. EGT using EP 800/80 level, $n = 8$). Injection of vehicle or DNA alone did not significantly affect muscle Na⁺, K⁺, and Ca²⁺ content.

The time course of the transmembrane ion flux was determined by excising muscles at the indicated time points. Eight HV pulses (1000 V/cm) resulted in the most significant increase in muscle Na⁺ content from 21.0 ± 1.6 to 129.1 ± 26.7 μ mol of Na⁺ per gram wet weight ($p < 0.01$, $n = 12$), remaining this high for 48 hr, returning to control levels by 1 week (Fig. 3A). HV+LV pulses alone, on the other hand, increased Na⁺ content to 48.3 ± 8.2 μ mol of Na⁺ per gram wet weight 1 hr after EP ($p < 0.01$, $n = 12$), returning to control levels 24 hr after EP. In combination with DNA, the HV+LV pulses resulted in a larger increase in Na⁺ content, peaking at 4 hr (70.7 ± 26.9 μ mol of Na⁺ per gram wet weight, $p < 0.01$, $n = 12$), not returning to basal level until at least 48 hr after EP. DNA injection alone induced a small but significant increase in Na⁺ content lasting 1 hr ($p < 0.01$, $n = 8$). This might be a direct effect of injection of Na⁺-containing vehicle solution.

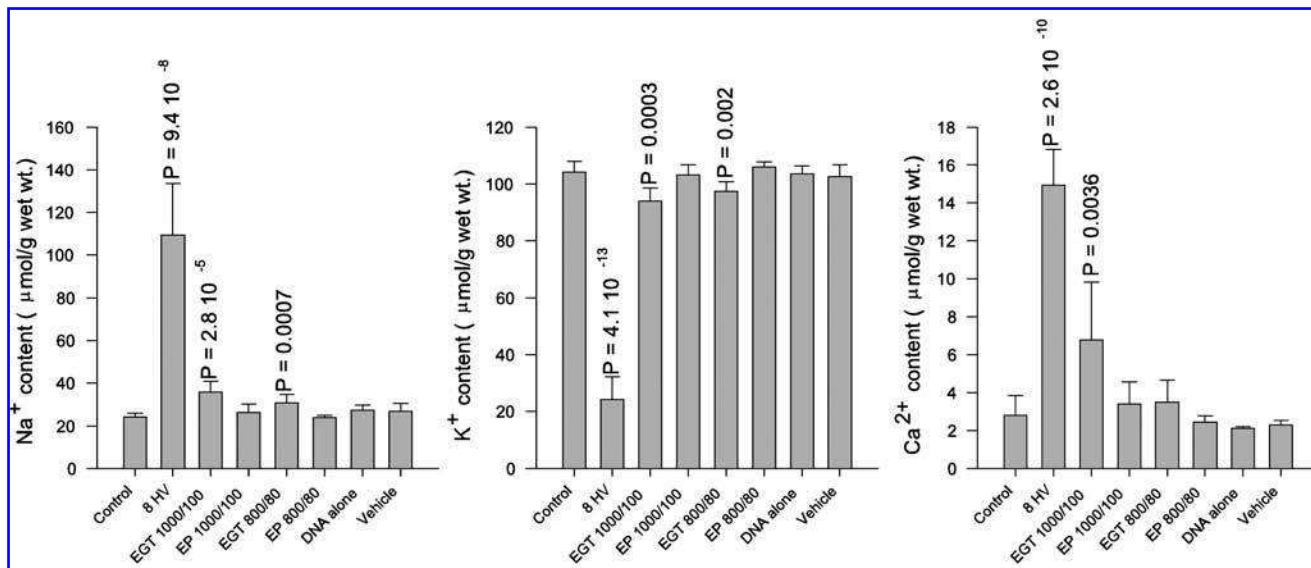


FIG. 2. Effect of HV and LV amplitude on transmembrane ion flux. Four hours after gene electrotransfer muscles were excised; Na⁺, K⁺, and Ca²⁺ were extracted from the muscles in 0.3 M TCA buffer, and determined by flame spectrometry (Na⁺ and K⁺) or atomic absorption spectrophotometry (Ca²⁺). The pulse parameters included the following: 8 HV (100 μ sec, 1000 V/cm, 1 Hz), EGT 1000/100 (DNA + 1 HV [100 μ sec, 1000 V/cm] + 1 LV [400 msec, 100 V/cm]), EP 1000/100 (1 HV [100 μ sec, 1000 V/cm] + 1 LV [400 msec, 100 V/cm]), EGT 800/80 (DNA + 1 HV [100 μ sec, 800 V/cm] + 1 LV [400 msec, 80 V/cm]), EP 800/80 (1 HV [100 μ sec, 800 V/cm] + 1 LV [400 msec, 80 V/cm]), DNA injection alone, and PBS injection alone. Columns represent mean Na⁺, K⁺, and Ca²⁺ content obtained from eight muscles, and are presented with SD values. Groups with significantly different ion content compared with control and tested by *t* test are indicated by the *p* value.

The effects of 8LV+DNA were also tested. These pulses did not give rise to as large changes as 8 HV pulses, but did cause significantly greater uptake of Na⁺ than HV+LV+DNA at all time points except the 5-min time point.

For K⁺ content a similar but reverse pattern was observed (Fig. 3B). Eight HV pulses induced a pronounced reduction in K⁺ content. K⁺ levels dropped from 107.1 \pm 4.4 to 23.3 \pm 10.0 μ mol of K⁺ per gram wet weight within 4 hr after treatment ($p < 0.001$, $n = 12$), staying low for at least 48 hr. DNA injection and HV+LV pulses alone led only to minor decreases in muscle K⁺ content. However, in combination, HV+LV pulses and DNA injection led to a significant drop in K⁺ content to 52.0 \pm 24.5 μ mol of K⁺ per gram wet weight 4 hr after EP ($p < 0.001$, $n = 12$). This was followed by rapid recovery. The Na⁺ response to 8LV+DNA was intermediate between the effects observed with 8 HV pulses and HV+LV+DNA, with the loss of K⁺ being significantly higher than with HV+LV+DNA at all time points.

During measurement of ion content, the water content of muscle was determined from the wet and dry weights, demonstrating that all treated groups showed significant increases in muscle water content (data not shown). The increases were greatest in muscles treated with 8 HV pulses, with the water content increasing from 74 to 84% 48 hr after EP ($p < 0.0001$, $n = 12$), suggesting the formation of edema in the muscles. In the HV+LV+DNA-treated group water content stabilized at about 80%, remaining at this level for 48 hr. HV+LV pulses showed maximal increases of 5% ($p < 0.0001$, $n = 12$) 4 hr after EP, and then returned to the basal level. An initial increase in water content in DNA-injected muscles is expected due to the injection of 20 μ l of vehicle

solution. 8LV+DNA caused the muscle water content to increase to 83% 4 hr after treatment.

LDH activity in plasma is an indirect measurement of loss of cellular integrity. Before EP with 8 HV pulses in rats, the average LDH activity in plasma was determined to be 54 \pm 2 U/liter ($n = 26$). Thirty minutes after EP with 8 HV pulses, LDH activity showed no significant change, but at 60 and 120 min there were significant increases to 57% ($p < 0.005$, $n = 5$) and 111% ($p < 0.005$, $n = 5$), respectively. At 240 min after EP, during which time the animals had started to move around a little after the anesthesia, LDH activity in plasma had further increased to 146 \pm 11 U/liter ($n = 10$). This confirms loss of cellular integrity after treatment with 8 HV pulses. After HV+LV+DNA, the LDH activity in plasma 240 min after treatment was 73.0 \pm 12.5 U/liter compared with a prevalue of 75.0 \pm 17.1 U/liter ($p > 0.05$, $n = 4$). Twenty-four hours after gene electrotransfer LDH activity was 70.2 \pm 8.5 U/liter.

Physiological effects of gene electrotransfer

Reflex and motor functions of treated mice were tested by evaluating their ability to seize a grid and to use their legs to run after EP. Again, 8 HV pulses induced the most pronounced and long-lasting effects ($p < 0.05$, tested against all other groups, $n = 8$). The functional deficit was greatest the first 2 days after EP, not returning to control levels until 8 days after treatment (Fig. 4A). HV+LV pulses, on the other hand, caused only an insignificant ($p > 0.05$, $n = 8$) reduction in muscle function compared with control mice. The effect was exacerbated if DNA was injected before EP, with significant limitations ($p < 0.05$, $n = 8$) compared with con-

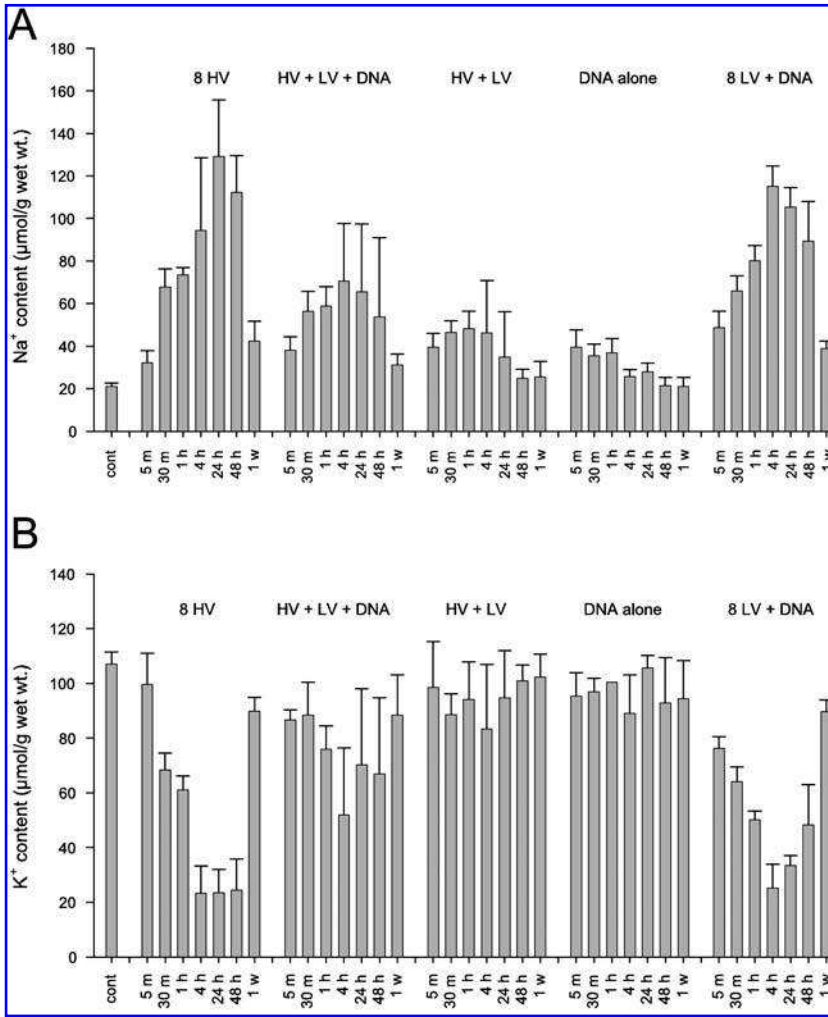


FIG. 3. Time course of Na⁺ and K⁺ content in mouse TC muscles after gene electrotransfer. After electroporation with 8 HV, HV+LV+DNA, HV+LV, injection of DNA, or 8LV+DNA, mice were killed at the indicated time points and Na⁺ and K⁺ content was determined. (A) Columns indicate the mean Na⁺ content obtained from 8 to 12 muscles, along with SD values. The increases in Na⁺ content are significantly different (*p* < 0.01) from the control level for all groups at all time points except for DNA alone at 48 hr and 1 week and 8LV+DNA at 5 min. (B) Mean K⁺ content from 8 to 12 muscles, with SD values. The decreases in K⁺ content are significant (*p* < 0.001) for 8 HV, HV+LV+DNA, and 8LV+DNA treatment at all time points.

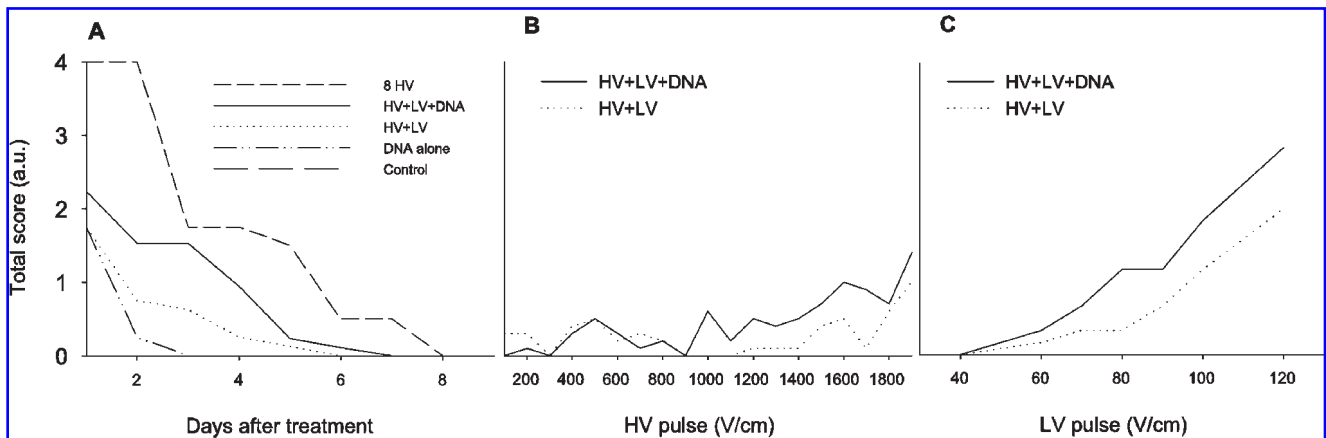


FIG. 4. Effects of gene electrotransfer on reflex and motor function. After treatment mice were tested for reflex and motor capabilities, and were assigned grades (0, normal function; 1, function affected; 2, function gravely affected). The total score (reflex + motor) is depicted for (A) 8 HV (1000 V/cm), HV+LV+DNA (1000 V/cm, 100 µsec + 100 V/cm, 400 msec), HV+LV alone, or DNA alone-treated mice monitored over time (means of eight mice), (B) delivery of electric pulses with increasing HV amplitude, keeping LV at 80 V/cm 24 hr after treatment, and (C) electric pulse delivery with increasing LV amplitude, keeping HV at 800 V/cm 24 hr after treatment.

rol muscles but not compared with HV+LV-treated muscles ($p > 0.05$, $n = 8$). Finally, injection of DNA only had a transitory effect on both seizing and running. The amount of injected DNA did not seem to influence the response (data not shown).

Increasing the amplitude of the HV pulse did not significantly affect reflex and motor functions until the HV pulse exceeded 1800 V/cm (Fig. 4B). The amplitude of the LV pulse, on the other hand, had a pronounced effect on reflex and motor functions. Using LV amplitudes higher than 90 V/cm resulted in significant functional impairments compared with control muscles, with the degree of limitations escalating with increasing LV amplitude (Fig. 4C).

The effects of EP or gene electrotransfer on the force-generating ability of rat extensor digitorum longus (EDL) muscles are shown in Fig. 5. Animals were killed 5, 60, 120, or 240 min after treatment and the tetanic force in isolated EDL muscle was measured; EDL from untreated leg served as control. Five minutes after electroporation by 8 HV, force in the treated muscle was reduced to only $5 \pm 4\%$ ($p = 0.0004$, $n = 4$) of that in the control muscle. At 120 min the force-generating ability had improved somewhat, and 240 min after EP force recovery was $46 \pm 4\%$ ($n = 4$). For EDL muscles treated with HV+LV+DNA, force was reduced to $30.2 \pm 12.4\%$ ($p = 0.016$, $n = 6$) 5 min after EP. Sixty minutes after EP force-generating ability had recovered to $55.7 \pm 13.6\%$ ($n = 4$) and at 240 min recovery was complete (force-generating ability, $100.01 \pm 11.4\%$; $n = 6$).

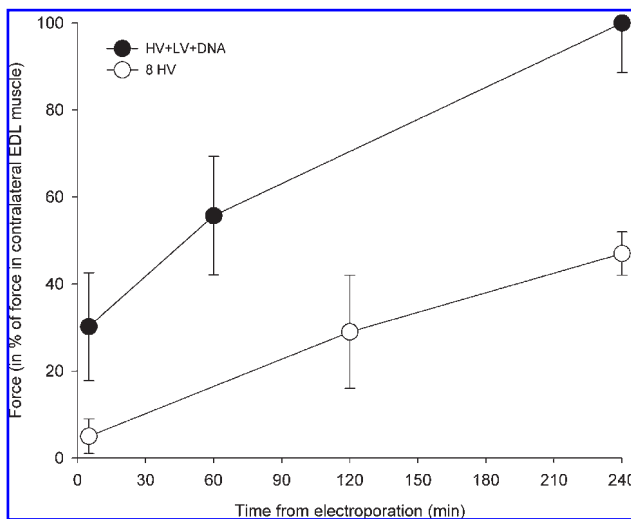


FIG. 5. Time course of force recovery in rat EDL muscle after gene electrotransfer *in vivo*. The right hind leg of rats was electroporated, using 8 HV pulses with a field strength of 900 V/cm, or electrotransferred, using HV+LV pulses (1000 V/cm, 100 μ sec + 100 V/cm, 400 msec). The left hind leg served as control. At the times indicated the animals were killed and EDL muscles were excised and placed in a force displacement transducer. Force was measured with 90-Hz pulse trains for a duration of 0.5 sec. Force exerted by the treated muscle was recorded and related to force in the control muscle. Mean values are given (error bars denote the SD); $n = 3$ or 4 muscles.

Changes in cellular homeostasis characterized through changes in ATP and Ca^{2+} content

ATP is essential for restoring membrane integrity and ion homeostasis. As seen in Fig. 6, the ATP content dropped immediately from 2.79 ± 0.069 to 2.02 ± 0.87 μ mol of ATP per gram wet weight after HV+LV pulsation ($p < 0.01$, $n = 8$) and to 2.17 ± 0.31 μ mol of ATP per gram wet weight after DNA injection ($p < 0.001$, $n = 8$). After 4 hr, the ATP levels were restored. In combination, DNA injection and HV+LV pulses also induced a decrease in ATP content to 1.74 ± 0.75 μ mol of ATP per gram wet weight ($p < 0.001$, $n = 8$), and the ATP content was not restored 48 hr after treatment. After 8 HV pulses, ATP levels dropped dramatically and reached a minimum after 4 hr at 0.23 ± 0.04 μ M ATP per gram wet weight ($p < 0.001$, $n = 8$), indicating irreversible membrane permeabilization and possible loss of cellular viability. Because ATP content is expressed relative to muscle wet weight, injection of 20 μ l of DNA vehicle solution may cause a relative decrease in ATP content. Likewise, formation of edema in the tissue may also cause relative decreases in ATP content.

In line with the measurements of Na^+ and K^+ content in mouse TC muscles, the largest increases in total muscle Ca^{2+} content occurred after 8 HV pulses, peaking at 17.0 μ mol of Ca^{2+} per gram wet weight 24 hr after EP ($p < 0.001$, $n = 12$) (Fig. 6). Muscles treated with HV+LV+DNA showed similar, yet smaller, increases in Ca^{2+} content, peaking at 8.5 μ mol of Ca^{2+} per gram wet weight 24 hr after treatment ($p < 0.001$, $n = 12$). HV+LV pulses resulted in initial increases in muscle Ca^{2+} content that were similar to those observed for HV+LV+DNA and 8 HV; however, in contrast to HV+LV+DNA and 8 HV, Ca^{2+} content after HV+LV treatment seemed to stabilize and normalize at this point (30 min). DNA injection alone did not induce any significant changes in Ca^{2+} content. The Ca^{2+} content was normalized after 1 week. 8LV+DNA gave rise to a greater uptake of Ca^{2+} compared with HV+LV+DNA at all time points except 5 min, 30 min, and 1 week. The increases were not as large, however, as those observed with 8 HV pulses.

Hsp70 expression after gene electrotransfer

Induction of the stress indicator Hsp70 in muscle fibers at various time points after gene electrotransfer was determined. The HV+LV pulse combination was compared with 8 pulses of 20 msec duration (240 V/cm), a pulse combination also used for gene transfer but presumably less damaging than 8 HV pulses. The HV+LV pulses led to an increase in Hsp70 mRNA level, peaking at 24 hr ($p < 0.05$, Dunn's method), and returning to normal after 48 hr ($p > 0.05$, Dunn's method) (Fig. 7). Interestingly, splitting the LV pulse into eight 50-msec pulses induced a significantly lower level of Hsp70 mRNA (ratio, 0.86) at 24 hr than with the 400-msec LV pulse (ratio, 2.12). The eight 20-msec pulses led to a significantly greater increase in Hsp70 mRNA levels than the HV+LV pulses, peaking at a ratio of 3.45 forty-eight hours after treatment ($p < 0.001$, Bonferroni t test). The presence of DNA did not have any significant effect on Hsp70 mRNA level (data not shown).

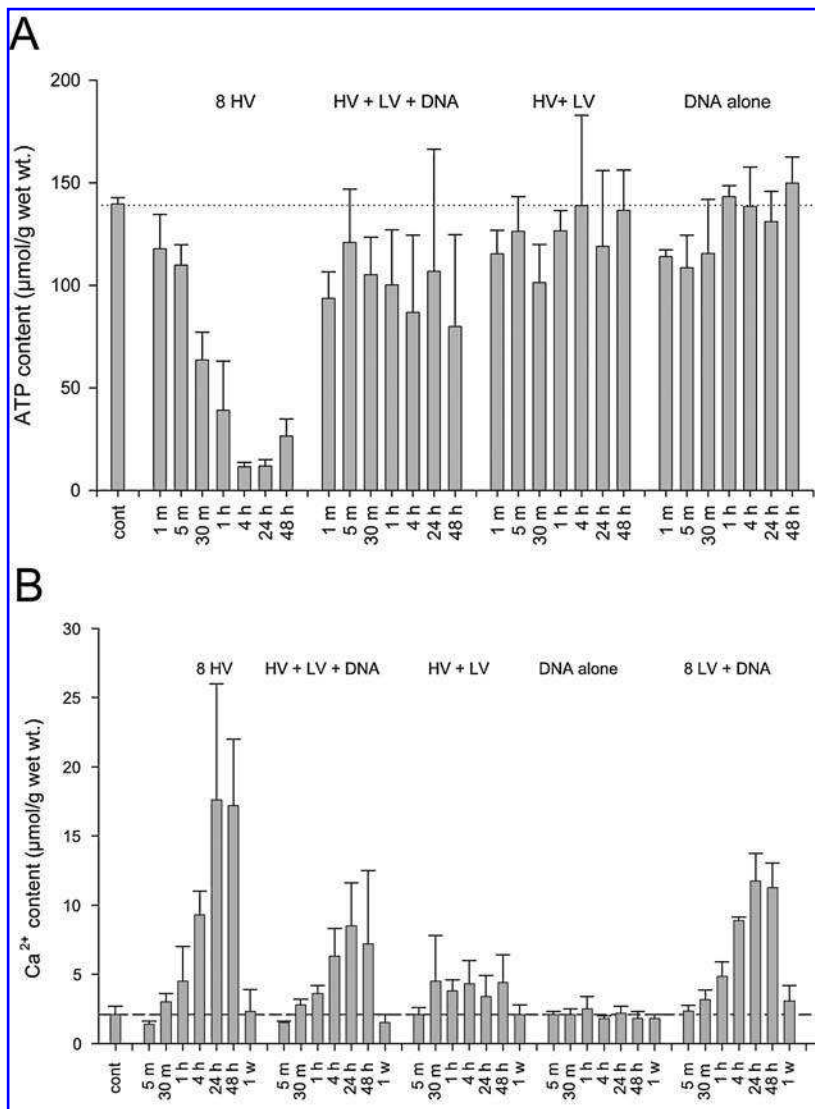


FIG. 6. Time course of changes in ATP and Ca^{2+} content in electroporated murine muscles. After electroporation with 8 HV, HV+LV+DNA, HV+LV, injection of DNA, or 8LV+DNA, mice were killed at the indicated time points. TC muscles were excised and Ca^{2+} and ATP were extracted in 0.3 M TCA buffer. (A) ATP content was determined by bioluminescence in muscle extracts from muscles excised at 1 min, 5 min, 30 min, 1 hr, 4 hr, 24 hr, and 48 hr. Mean values from 4 to 8 muscles (error bars denote SD values) are depicted. Eight HV induced a significant decrease ($p < 0.001$) in ATP content at all time points. HV+LV+DNA also induced significant decreases ($p < 0.001$) in ATP content at most time points except for 5 min and 24 hr. HV+LV treatment led to a significant fall ($p < 0.01$) at 1 and 30 min, whereas DNA alone significantly ($p < 0.001$) decreased ATP content at 1, 5, and 30 min. (B) Ca^{2+} content was determined by atomic absorption in muscle extracts from muscles excised at 5 min, 30 min, 1 hr, 4 hr, 24 hr, 48 hr, and 7 days. Mean values from 8 to 12 muscles (error bars denote SD values) are depicted. The dashed line represents the control level. For 8 HV, HV+LV+DNA, and HV+LV treatment the Ca^{2+} content is significantly different ($p < 0.001$) from the control level at all time points except at 1 week, whereas DNA injection alone did not induce any significant changes. For 8LV+DNA values were significant at all time points except 5 and 30 min and 1 week.

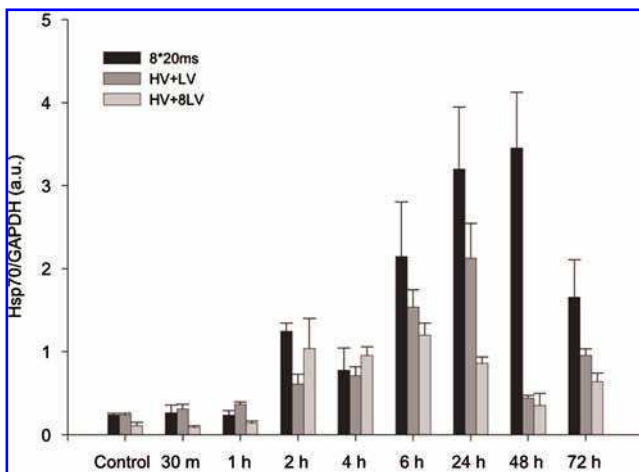


FIG. 7. Expression of Hsp70 after muscle electroporation. After electroporation with 1 HV and 8 LV (50 msec) (light gray columns), 1 HV + 1 LV (400 msec) (darker gray columns), or 8 HV (20 msec, 1 Hz, 240 V/cm) (solid columns), muscles were excised at various time points and the Hsp70 mRNA level was determined by semiquantitative RT-PCR.

Histological changes after gene electrotransfer

Forty-eight hours after gene electrotransfer, mice were killed and TC muscles were excised for histological analysis. Eight HV pulses induced substantial cell death as observed by necrotic cells with blebs as well as mononuclear infiltration (Fig. 8). HV+LV+DNA also induced some cell damage and mononuclear infiltration, whereas no histological changes were detected after HV+LV pulses alone or DNA injection.

Discussion

In this study, we have focused on muscle as an indicator of the direct physiological effects of gene electrotransfer with the HV+LV pulse combination. Muscle has several advantages that make it an attractive model tissue. First, highly efficient long-lasting transfection can be obtained in muscle. Second, several reliable methods for assessing permeabilization are available including ^{51}Cr -EDTA uptake, uptake of Ca^{2+} , changes in Na^{+} and K^{+} content, and LDH release from muscle. Third, evaluation of muscle function is accessible through determination of reflex and motor function and

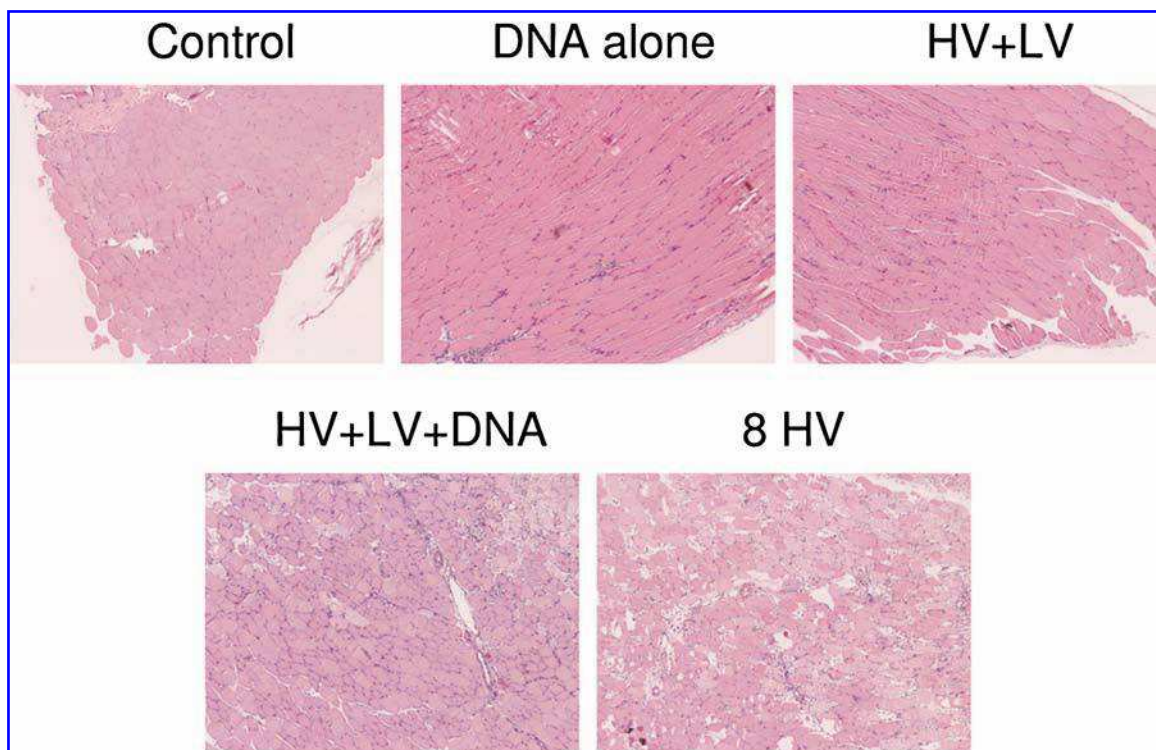


FIG. 8. Histological changes in muscle after gene electrotransfer. Forty-eight hours after treatment muscles were excised and fixed for histological analysis. The panels show representative pictures of control muscle and muscle treated with DNA alone, HV+LV, HV+LV+DNA, or 8 HV ($n = 4$). The slides were stained with hematoxylin and eosin.

force generation capacity. Several markers of stress including ATP loss, acute loss of force, Ca^{2+} influx, and up-regulation of Hsp70 are easily quantifiable. And finally, changes in morphology are easy to appreciate because of the homogeneous tissue structure of muscle.

HV+LV pulses and membrane permeabilization

^{51}Cr -EDTA uptake confirms that the common parameters of pulse combinations used for gene electrotransfer to skeletal muscle (1 HV [100 μsec , 800 V/cm] + 1 LV [400 msec, 80 V/cm]) result only in low-level muscle fiber permeabilization (Fig. 1). The EGT parameters tested in the present study (1 HV [100 μsec , 1000 V/cm] + 1 LV [400 msec, 100 V/cm]) caused more permeabilization of muscle fibers. Surprisingly, the amplitude of the LV pulses also affected fiber permeabilization. (It is possible that the HV pulse destabilized the membrane so that the LV pulses were able to affect permeabilization.) The degree of permeabilization using the control pulses (trains of 8 HV pulses or trains of 8 LV pulses) has been demonstrated in earlier studies (Gehl and Mir, 1999; Gehl *et al.*, 1999; Mir *et al.*, 1999). The levels of permeabilization obtained with the HV+LV pulses were lower than those reached with either 8 HV of 1000 V/cm (Gehl *et al.*, 1999) or 8 LV of 20 msec and 240 V/cm (Gehl and Mir, 1999).

All tests performed in the present study using trains of high-voltage pulses demonstrated that the level of cell permeabilization induced by these pulses results in perturbations of the physiological parameters measured. However, perturbations caused by EP 800/80 pulses were low and even when performing EGT with EP 1000/100 pulses, only

moderate perturbations were observed and the time of return to normal levels was shorter as in the case with the 8 LV pulses, and in particular the trains of high-voltage pulses (8 HV).

The permeabilization detected by ^{51}Cr -EDTA uptake was confirmed by measurements of changes in Na^+ , K^+ , and Ca^{2+} content. HV+LV pulses induced small transient changes in Na^+ , K^+ , and Ca^{2+} content, showing that membrane integrity rapidly recovered and that normal ion distribution reestablished. It is puzzling that, in two groups injected with DNA (HV+LV+DNA and DNA), the correlation between Na^+ and K^+ was lost at the later time points, with the K^+ levels too low compared to the Na^+ levels. Because this discrepancy was observed only in groups injected with DNA, it might be explained as a cytotoxic effect of DNA on the host tissue, which might affect the Na^+/K^+ pump, leading to loss of intracellular K^+ (Sejersted and Sjøgaard, 2000).

During membrane recovery, Na^+ and K^+ gradients are normally restored by the Na^+/K^+ pump, which is a prerequisite for normal muscle function (Clausen and Gissel, 2005). Functional testing showed rapid recovery of muscle function when HV+LV pulses were delivered. Indeed, determination of muscle force and ATP measurements showed complete recovery in 4 hr. Moreover, Ca^{2+} content was not affected by exposure to HV+LV pulses.

One concern could be that the rather large injection volume (approximately 40% of muscle volume) could affect the Na^+ , K^+ , and Ca^{2+} content of muscle. Figure 3 shows that there are temporary changes in Na^+ and K^+ content, but by 4 hr this is normalized. Ca^{2+} content is not affected at all.

Because Na^+ , K^+ , and Ca^{2+} are measured in whole muscle, in order for changes to occur the ions must be supplied or removed by circulation. Ca^{2+} keeps increasing as long as the permeability of the membrane is increased, and changes in Na^+ and K^+ represent fluxes through the membrane (either due to increased permeability or muscle activity) countered by pumping of the Na^+/K^+ pump. Extracellular edema may increase Na^+ content and decrease K^+ content, which could be the case here. By 1 week Ca^{2+} content had normalized, suggesting that membrane integrity had recovered completely. If the technique is used on a larger species the injection volume-to-muscle volume ratio would be much smaller than is the case here, and even though there may be local changes similar to those observed in this study around the site of injection, this would not necessarily affect the whole muscle.

Another parameter of cellular stress is the induction of heat shock proteins, including Hsp70. Heat shock proteins play a protective role as molecular chaperones facilitating protein folding and intracellular assembly of protein, and protect cells from oxidative damage. In this study we found that the induction of Hsp70 is highly dependent on the pulse type and amplitude, indicating that there is a significant difference in the degree of cellular stress induced by the various pulses.

The effect of transfection using 8 LV pulses (8LV+DNA) was tested on the basis of a few chosen parameters (Ca^{2+} , Na^+ , K^+ , and water content) and in all cases disturbances by this paradigm were greater than those observed when using HV+LV pulses for transfection (HV+LV+DNA). However, it is important to note that we did not do any optimization of the 8 LV pulses, and thus the disturbances could possibly be reduced by using other pulse parameters.

Effect of the presence of DNA at the time of pulse delivery

Our data show that the presence of DNA at the time of pulse delivery affects several of the physiological parameters analyzed. We observed greater changes in ^{51}Cr -EDTA uptake; muscle Na^+ , K^+ , and Ca^{2+} content; impairment in the functional tests; morphological changes; and decreases in ATP content when injecting DNA before the pulses.

DNA is, of course, needed to obtain gene electrotransfer. Comparisons between transfection efficiencies of the HV+LV pulse combinations and the 8 LV pulses have shown that there is no difference between the efficiency of the two pulse regimens (Andre *et al.*, 2008). The train of HV pulses, on the other hand, has proven far less efficient for gene transfer, probably because of the vast muscle damage induced by these pulses (Mir *et al.*, 1999; Lucas and Heller, 2001).

In vitro studies have shown that during pulsation DNA is absorbed in spots at the membrane facing the cathode (Golzio *et al.*, 2002). When DNA is inserted in these membrane spots, it becomes inaccessible to DNases. Moreover, reverse polar electric fields are not capable of releasing the DNA from the membrane (Golzio *et al.*, 2002), indicating that there is a tight association between the DNA and the membrane, facilitated by the pulses. During its passage across the membrane, DNA might create consequential membrane permeabilizations that can explain the increased changes in ^{51}Cr -EDTA uptake; Na^+ , K^+ , and Ca^{2+} content; and func-

tional impairments observed when injecting DNA before the gene electrotransfer pulses. This effect has already been described *in vitro*, where the uptake of the small nonpermeant molecule Lucifer Yellow was increased when DNA was present (Sukharev *et al.*, 1992). In a similar manner, another study has shown that the presence of DNA increases the current across a membrane during electric pulsing (Spasova *et al.*, 1994).

Low level of permeabilization ensures cell homeostasis

Two important parameters for maintaining muscle fitness after treatment are Ca^{2+} content and ATP level. ATP plays a key role in gene electrotransfer by controlling intracellular trafficking of plasmid DNA to the nucleus as well as being mandatory for cell membrane resealing and restoration of ion balance, processes essential for cell survival (Rols *et al.*, 1998). Therefore, the transfection efficiency is highly dependent on the intracellular ATP level. HV+LV pulses gave rise to minor loss of ATP, which was restored within 60 min. This also confirmed rapid resealing of the muscle fibers after HV+LV delivery. Indeed, in addition to the direct loss of ATP through the permeabilized membrane, vast amounts of ATP are required to restore both membrane integrity (Rols *et al.*, 1998) and the imbalances in ion content due to the free diffusion across the membrane. The remaining ATP stores might be exhausted because of the amount of energy used to clear Ca^{2+} to the sarcoplasmic reticulum and mitochondria if resealing of muscle fibers did not occur rapidly.

Detrimental effects occur if intracellular Ca^{2+} homeostasis is lost and the muscle experiences cellular Ca^{2+} overload. A vicious cycle of continuous degradation and persistent influx of Ca^{2+} could be activated (Armstrong, 1990; Gissel and Clausen, 2001). The electrochemical gradient for Ca^{2+} across the cell membrane is so large that even small increases in the permeability of the membrane may result in a large influx of Ca^{2+} . Despite the great capacity of muscle cells for clearing Ca^{2+} into the sarcoplasmic reticulum and the mitochondria, an increased influx of Ca^{2+} has been shown to lead to muscle cell damage (Jackson *et al.*, 1984; Jones *et al.*, 1984; Duan *et al.*, 1990; Gissel and Clausen, 2003).

The relatively small changes found in ATP and Ca^{2+} content after HV+LV combinations may explain the rapid recovery in the functional tests. Indeed, a prerequisite for using muscle as the target organ for gene transfer is that muscle function is not impaired by the transfer. In this study we tested both reflex and motor functions and the force generation capacity. It is noteworthy that the HV and LV field strengths at which high levels of gene expression were obtained did not cause serious functional impairments. It is also noteworthy that the functional testing indicated that functional impairment is in fact highly dependent on the LV pulse amplitude, and increasing the LV amplitude above 90 V/cm induced significant impairments. However, keeping the LV amplitude at lower strengths showed that observed deficits were transient, whereas transgene expression lasted for months.

Conclusion

For gene electrotransfer to occur, changes in a membrane structure (such as membrane hydration and rotation of phospholipid heads as suggested by molecular dynamics model-

ing) must be induced. However, these changes should not result in perturbation of the cell, which is paramount for preserving muscle morphology and function, and is an important prerequisite for efficient gene transfer.

In general, electropermeabilization of any tissue largely depends on the strength of the electric field. In a previous study permeabilization and gene transfer efficacy were compared, showing that expression is optimal during reversible permeabilization, whereas when the first cell is irreversibly permeabilized, gene expression drops dramatically (Gehl *et al.*, 2002).

In this study we have demonstrated that the HV+LV pulse combination, which can be used for efficient gene electrotransfer, induces only small and transient changes in cell physiology. This study focuses on the short-term effects of gene electrotransfer; and in fact we observed, more or less, a normalization of all evaluated parameters within 1 week of gene electrotransfer. However, an evaluation of the long-term effects of gene electrotransfer and in particular the expression of transgenic genes is still warranted. Still, this is the first time to our knowledge that the physiological effects of gene electrotransfer have been so comprehensively evaluated.

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Author Disclosure Statement

Lluis M. Mir is a beneficiary of patents licensed for use in the Cliniporator, produced by IGEA (Carpi, Italy).

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