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Electrophoretic Component of Electric Pulses Determines the Efficacy of *In Vivo* DNA Electrotransfer

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ABSTRACT

Efficient DNA electrotransfer can be achieved with combinations of short high-voltage (HV) and long low-voltage (LV) pulses that cover two effects of the pulses, namely, target cell electropermeabilization and DNA electrophoresis within the tissue. Because HV and LV can be delivered with a lag up to 3000 sec between them, we considered that it was possible to analyze separately the respective importance of the two types of effects of the electric fields on DNA electrotransfer efficiency. The tibialis cranialis muscles of C57BL/6 mice were injected with plasmid DNA encoding luciferase or green fluorescent protein and then exposed to various combinations of HV and LV pulses. DNA electrotransfer efficacy was determined by measuring luciferase activity in the treated muscles. We found that for effective DNA electrotransfer into skeletal muscles the HV pulse is prerequisite; however, its number and duration do not significantly affect electrotransfer efficacy. DNA electrotransfer efficacy is dependent mainly on the parameters of the LV pulse(s). We report that different LV number, LV individual duration, and LV strength can be used, provided the total duration and field strength result in convenient electrophoretic transport of DNA toward and/or across a permeabilized membrane.

INTRODUCTION

ELECTRICALLY MEDIATED GENE TRANSFER, also termed DNA electrotransfer or electrogene therapy, has gained real interest as it is one of the most effective methods of *in vivo* nonviral gene transfer (André and Mir, 2004). The method has been shown to be effective in electrotransferring plasmid DNA to various tissues: muscles (Aihara and Miyazaki, 1998; Mir *et al*., 1998a, 1999), liver (Heller *et al*., 1996; Suzuki *et al*., 1998), skin (Titomirov *et al*., 1991; Zhang *et al*., 1996), tumors (Heller *et al*., 2000; Wells *et al*., 2000; Heller and Coppola, 2002), mouse testis (Muramatsu *et al*., 1997, 1998), and so on (André and Mir, 2004).

The mechanisms by which electric pulses mediate DNA transfer into target cells are not well understood. Nevertheless, there is common agreement that for improved DNA transfer into tissue, cells in that tissue must be permeabilized. Such permeabilization can be achieved using simple runs of short square-wave electric pulses (in the range of 100 μsec) (Mir *et al*., 1991b; Gehl *et al*., 1999; Miklavčič *et al*., 2000). This kind of pulse has been widely used for the local delivery of nonpermeant anticancer drugs (such as bleomycin or cisplatin) in a form of treatment termed "antitumor electrochemotherapy" (Mir *et al*., 1991a, 1998b; Glass *et al*., 1997; Sersa *et al*., 1998; Rodriguez *et al*., 2002). Indeed, the delivery to tumors of, for example, eight pulses of 1300 V/cm and 100 μsec either *in vitro* or *in vivo* is sufficient to induce transient rearrangements of the cell membrane that allow nonpermeant anticancer molecules such as bleomycin to enter the cell by diffusion and to fully exert their cytotoxic activity (Mir *et al*., 1991b; Poddevin *et al*., 1991; Gehl *et al*., 1998).

These short permeabilizing electric pulses have also been shown to increase the transfer of plasmid DNA into several tissue types (Heller *et al*., 1996, 2000). However, another type of square-wave electric pulse was applied to muscles (Aihara and Miyazaki, 1998; Mir *et al*., 1999), tumors (Rols *et al*., 1998), liver (Suzuki *et al*., 1998), and some other tissues (André and Mir, 2004), and was found to be more effective for DNA electrotransfer (Mir *et al*., 1999; Heller *et al*., 2000). These pulses usually are of lower voltage but much longer duration (in the

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range of tens of milliseconds) (Aihara and Miyazaki, 1998; Rols *et al*., 1998; Mir *et al*., 1999; Bettan *et al*., 2000; Matsumoto *et al*., 2001). It is assumed that this type of pulse mediates DNA transfer into cells by inducing two distinct effects that include cell permeabilization (like the short pulses) and DNA electrophoretic migration during the delivery of the electric field (Klenchin *et al*., 1991; Sukharev *et al*., 1992; Neumann *et al*., 1996; Mir *et al*., 1999; Golzio *et al*., 2002). The double role of the electric pulses in *in vivo* DNA electrotransfer was demonstrated by using combinations of electric pulses consisting of high-voltage, short pulses (or HVs; e.g., 800 V/cm and 100 μsec) followed by low-voltage, long pulses (or LVs; e.g., 80 V/cm and 100 msec) (Bureau *et al*., 2000; Šatkauskas *et al*., 2002). In a previous study we found that these HV and LV pulses can be separated by various lag times between the HV and LV pulses without significant loss in transfection efficiency. These lag times ranged up to 300 sec for a combination of one HV and one LV, and up to 3000 sec for a combination of one HV and four LV (Šatkauskas *et al*., 2002).

Taking into account these lag times between the HV and LV pulses, we thought it possible to characterize separately the respective importance of the two effects of the electric fields—electropermeabilization and electrophoresis—on DNA electrotransfer efficacy.

MATERIALS AND METHODS

Plasmid DNA

We used plasmid pXL3031 (pCMV-Luc+) containing the cytomegalovirus promoter (nucleotides 229–890 of pcDNA3; Invitrogen, Carlsbad, CA) inserted upstream of the sequence for *luc*, encoding a modified cytosolic wild-type firefly luciferase (Soubrier *et al*., 1999). We prepared plasmid DNA according to the usual procedures (Ausubel *et al*., 1994). Alternatively, we also used plasmid pEGFP-N1 (BD Biosciences Clontech, Saint Quentin Yvelines, France), featuring the gene encoding green fluorescent protein (GFP) under the control of the CMV promoter and prepared in phosphate-buffered saline (PBS; GIBCO/Invitrogen, Cergy-Pontoise, France) with an EndoFree Plasmid Giga kit (Qiagen, Courtabeuf, France).

Animals

For all experimental procedures we anesthetized female, 7- to 9-week-old C57BL/6 mice by intraperitoneal administration of the anesthetics ketamine (Ketalar, 100 mg/kg; Panpharma, Fougères, France) and xylazine (Rompun, 40 mg/kg; Bayer, Puteaux, France). Before performing the experiments subject legs were shaved with an electric shaver. At least 10 muscles (5 mice) were included in each experimental group for luciferase determinations. In the case of the GFP qualitative data, four muscles were used for each experimental condition.

DNA injection

For the luciferase experiments, we injected 3 μg of plasmid DNA prepared in 30 μl of 0.9% NaCl. In most of our experiments (see Figs. 1–3) we supplemented the DNA solution with heparin (120 IU/ml; Laboratoires Leo, Saint Quentin en Yvelines, France; 1 mg of the heparin [MW 10,000–12,000] corresponded to approximately 137 IU). We injected the DNA into tibialis cranialis muscles, using a Hamilton syringe with a 26-gauge needle. Because the quality of injection of the plasmid may affect transfection efficacy, all injections within a given experiment were performed by the same well-trained investigator. For GFP experiments, 4 μg in 20 μl of PBS was injected into each treated tibialis cranialis muscle, always in the absence of heparin.

DNA electrotransfer

HV and LV pulse combinations were generated by a device consisting of square-wave electropulsator (PS-15; Jouan, St. Herblain, France) and a microprocessor-driven switch/function generator built at the Faculty of Electrical Engineering at the University of Ljubljana (Ljubljana, Slovenia). The device allowed for precise control of every electrical parameter of HV and LV pulse combinations (Šatkauskas *et al*., 2002).

HV and LV pulse combinations were delivered soon (40 $\pm$ 15 sec) after intramuscular DNA injection. In all the experiments we fixed the lag between HV and LV to 1 sec. For pulse delivery to the muscles we used stainless plate electrodes 4.4 mm apart. The 1-cm plates encompassed the whole leg of each mouse. To ensure good contact between the tibialis cranialis muscle of the exposed leg and the plates of the electrodes a conductive gel was used. Electric field values (in volts per centimeter) are always expressed in terms of the ratio of the voltage applied (volts) to the distance between the electrodes (centimeters).

For the GFP experiments the pulse combinations were delivered with a Cliniporator (IGEA, Carpi, Modena, Italy) generator and electrodes (5 mm apart) from the same company.

Luciferase activity measurement

We killed the mice 2 days after DNA electrotransfer. We removed and homogenized the muscles (net weight, approximately 60 mg) in 1 ml of cell culture lysis reagent solution (10 ml of cell culture lysis reagent; Promega, Charbonnières, France), diluted with 40 ml of distilled water and supplemented with one protease inhibitor cocktail tablet (Roche, Mannheim, Germany). After centrifugation at 12,000 rpm for 10 min at 4°C, we assessed the luciferase activity in 10 μl of the supernatant, using a Wallac Victor^2 luminometer (PerkinElmer Life and Analytical Sciences, Boston, MA), by integration of the light produced over 1 sec, starting after the addition of 50 μl of luciferase assay substrate (Promega) to the muscle lysate. We collected the results from the luminometer in relative light units (RLU). Calibration with purified firefly luciferase protein showed that 10^6 RLU corresponds to approximately 70 ng of expressed luciferase. We expressed the final results as picograms of luciferase per muscle.

GFP fluorescence observations

We killed the mice 3 days after the injection of pEGFP-N1 plasmid and observed the transfected tissue with an MZ12 fluorescence stereomicroscope with a GFP Plus filter set (excitation filter, 480/40 nm; dichroic mirror, 505 nm LP; barrier filter, 510 nm LP) (Leica, Rueil-Malmaison, France). After removal of the leg skin, pictures were taken with a digital cooled

color camera (AxioCam HRc; Zeiss, Le Pecq, France), and quantification of GFP expression was made by software (AxioVision Light Edition, release 4.1.1.0; Zeiss) integration of the light detected by the camera. Pictures were taken either at a constant exposure time (100 msec) or at a variable exposure time, that is, allowing the camera to adjust the exposure time to acquire an equivalent amount of light from picture to picture. Each experimental condition was repeated four times (four muscles treated). Quantitative analysis was done by determining the mean density of the green color in these images, using a relative scale with 256 levels of intensity.

Statistical analysis

For statistical comparison of several groups we used the two-tailed Student *t* test for unpaired values. In the figures we reported luciferase expression data as means ± SD.

RESULTS

In the luciferase experiments, because of the high sensitivity of the measurements, we injected a solution of plasmid DNA supplemented with low amounts of heparin (120 IU/ml). Heparin at this dose causes a large decrease in the spontaneous uptake of DNA by the muscle but does not significantly impair the efficacy of DNA electrotransfer into the muscle fibers (Šatkauskas *et al*., 2001). Therefore, the respective contributions of HV and LV pulses to the efficiency of DNA electrotransfer can be analyzed more precisely in the presence of heparin. In addition, we fixed the lag time between HV and LV pulse(s) to 1 sec.

Influence of HV pulse duration and number

To analyze the role of the electropermeabilizing (HV) pulses we used LV pulses giving the best level of gene expression according to previous data (Šatkauskas *et al*., 2002). Therefore we fixed the LV component parameters to four LVs of 80 V/cm and 100-msec duration, with a delay between pulses of 1 sec.

We tried to improve muscle permeabilization by increasing either the number (from one to eight) or the duration (from 100 to 500 μsec) of the HV pulses. As shown in Fig. 1, neither an increase in HV duration, nor an increase in HV pulse number, significantly enhanced muscle transfection.

Influence of LV pulse number

As a consequence of the results shown in Fig. 1, we always used a single HV pulse of 800 V/cm and 100 μsec to analyze the role of the LV component. First, we examined the influence of the number of LVs. We fixed LV pulse strength at 80 V/cm, with a duration of 100 msec, and the delay between LVs at 1 sec. Luciferase expression markedly increased when we increased LV number from one to four (Fig. 2A). Consistent with previous data (Šatkauskas *et al*., 2002), with four LVs the luciferase expression was 10 times higher than with one LV. No further significant increase was observed with a larger number (six or eight) of LV pulses (Fig. 2A).

Subsequent experiments on the influence of pulse number on gene transfer efficacy were performed with 50-msec LV(s) (Fig. 2B). We observed the same trend as in the case of 100-msec LV(s) (Fig. 2A). In both cases the beginning of the plateau in luciferase gene expression started at a total pulse duration of 400 msec. Again, no further significant increase was observed with increased number (12 or 16) of LV pulses. These data also suggested that similar levels of DNA electrotransfer and gene expression may be achieved with different combinations of LV pulse number and duration. To test this hypothesis, we used four different combinations of number (n) and duration (d) of LVs, such that the product $n \times d$ was constant and equal to 400 msec (Fig. 2C). A tendency to a progressive decrease in luciferase gene expression with the concomitant decrease in individual pulse duration and increase in pulse number was found (Fig. 2C). For instance, HV and LV combinations using one LV of 400 msec resulted in about two times higher luciferase gene expression compared with eight LVs of 50 msec ($p < 0.001$).

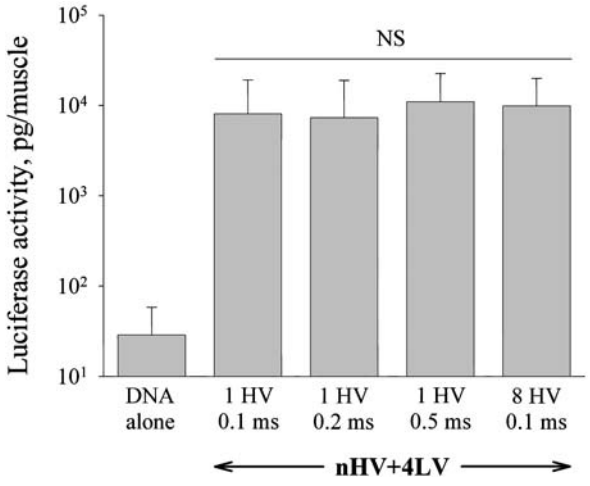

FIG. 1. Luciferase expression after DNA electrotransfer based on combinations of one or eight HV pulses (800 V/cm; 0.1, 0.2, or 0.5 msec) and four LV pulses (80 V/cm and 100 msec) (nHV+4LV pulse combination). Data are presented as means ± SD. The significance of differences between each of the nHV+4LV groups was calculated by *t* tests; NS, not significant. Each column represents results from at least 18 muscles treated in two experiments.

Influence of LV pulse strength

Figure 3 shows the dependence of DNA electrotransfer and expression on the electric field strength of the LV pulses. We examined LV pulse strengths ranging from 20 to 100 V/cm. At low electric field strengths (20 and 40 V/cm), luciferase expression was not significantly increased ($p > 0.05$) in comparison with that achieved with the HV pulse alone. At 60 V/cm LV pulses started to play a significant role and luciferase expression was already more than 100 times higher than that obtained by HV pulse alone. We obtained the highest values of luciferase expression with LVs of 100 V/cm (Fig. 3).

Combination of LV pulse strength and duration

In this set of experiments, we aimed to analyze whether the decrease in efficacy when using LVs of lower field strength

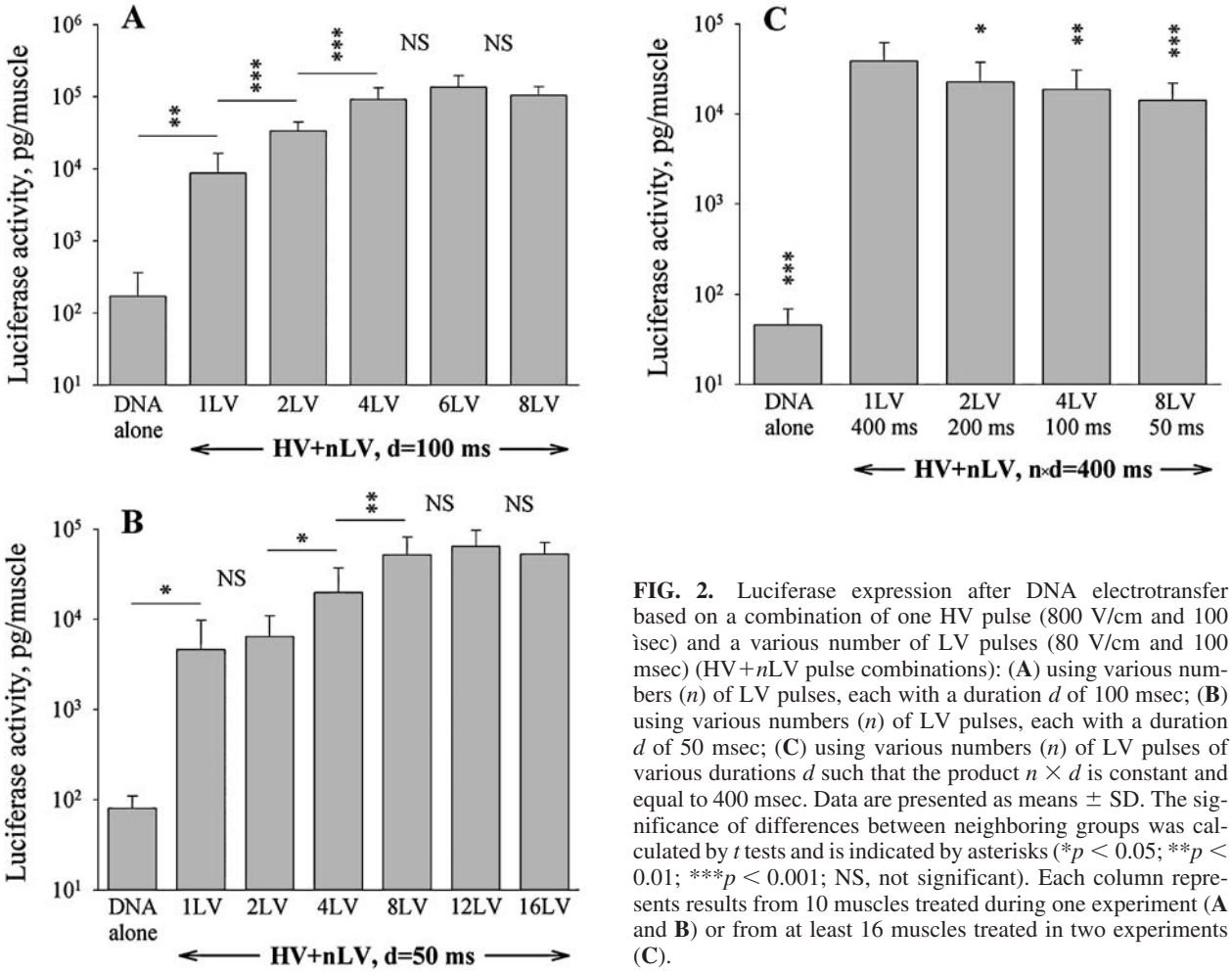

FIG. 2. Luciferase expression after DNA electrotransfer based on a combination of one HV pulse (800 V/cm and 100 ìsec) and a various number of LV pulses (80 V/cm and 100 msec) (HV+*n*LV pulse combinations): (**A**) using various numbers (*n*) of LV pulses, each with a duration *d* of 100 msec; (**B**) using various numbers (*n*) of LV pulses, each with a duration *d* of 50 msec; (**C**) using various numbers (*n*) of LV pulses of various durations *d* such that the product $n \times d$ is constant and equal to 400 msec. Data are presented as means ± SD. The significance of differences between neighboring groups was calculated by *t* tests and is indicated by asterisks ($*p < 0.05$; $**p < 0.01$; $***p < 0.001$; NS, not significant). Each column represents results from 10 muscles treated during one experiment (**A** and **B**) or from at least 16 muscles treated in two experiments (**C**).

(e.g., 60 V/cm, as in Fig. 3) could be counterbalanced by the use of longer pulses. We thus compared several combinations of one HV and four LVs, using LVs of either 60 or 80 V/cm and individual pulse durations between 100 and 800 msec (Fig. 4).

With one HV and four LVs at a pulse strength of 80 V/cm we obtained similar luciferase expression at the three LV pulse durations tested (100, 200, and 400 msec) (Fig. 4). With one HV and four LVs at a pulse strength of 60 V/cm and a pulse duration of 400 msec we obtained a result similar to those achieved under the previously tested conditions at 80 V/cm, whereas at a longer pulse duration (800 msec), luciferase expression was significantly higher with respect to the 80-V/cm, 100-msec pulses (Fig. 4).

GFP fluorescence observations

The distribution and intensity of the fluorescence within muscles after electrotransfer of the GFP gene were qualitatively and semiquantitatively measured with a fluorescence stereo microscope (Fig. 5). For the electrotransfer of the GFP gene we used one HV of 100 μsec and 800 V/cm followed after a 1-sec delay by one 400-msec LV pulse of either 60, 80, or 100 V/cm. Pictures were taken either at a constant exposure time (100 msec; Fig. 5A–C) or at a variable exposure time, that is, allowing the camera to adjust the exposure time to acquire an equivalent amount of light from picture to picture (Fig. 5D–F). These pictures represent the images observed in four muscles for each experimental condition. Two series of pictures are reported to show the reproducibility of the results as well as the large increase in fluorescence with the increase in field strength of the LV pulses (Fig. 5A–C). Quantitative analysis of the mean density of the green color in these images sustains the qualitative data: at a relative scale with 256 levels of intensity, levels 41 (left muscle) and 33 (right muscle) were reached at 60 V/cm (Fig. 5A), whereas levels 111 and 89 were reached at 80 V/cm (Fig. 5B) and levels 138 and 127 were reached at 100 V/cm (Fig. 5C). These pictures also show that the volume of transfected (fluorescent) muscle is similar whatever the LV field strength (Fig. 5D–F). These data suggest that the overall increase in fluorescence results from an increase in the fluorescence of each fiber, and not from an increase in the volume (and thus the number) of muscle fibers susceptible to fluorescence.

DISCUSSION

Using combinations of HV and LV pulses, we previously showed that *in vivo* DNA electrotransfer is a multistep process

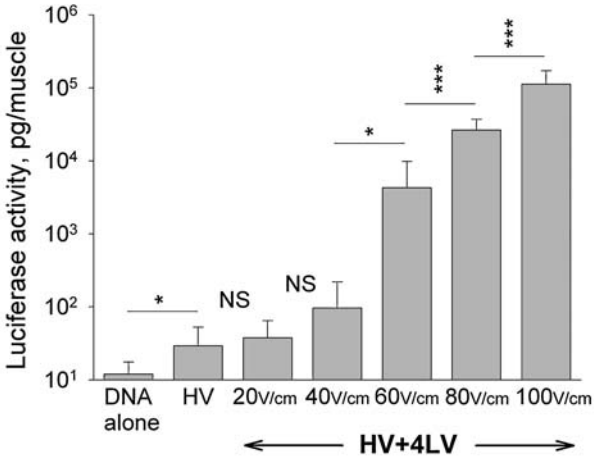

FIG. 3. Luciferase expression after DNA electrotransfer based on a combination of one HV pulse (800 V/cm and 100 μsec) and four 100-msec LV pulses as a function of the strength of the LV pulses. Data are presented as means ± SD. The significance of differences between neighboring groups was calculated by *t* tests and is indicated by asterisks ($*p < 0.05$; $***p < 0.001$; NS, not significant). Each column represents results from 10 muscles treated in one experiment.

that includes DNA distribution, cell permeabilization, and DNA electrophoresis (Šatkauskas *et al*., 2002). We found that the role of the HV pulse was limited mainly to cell permeabilization, whereas LV pulses had a direct effect on DNA, probably DNA electrophoresis. However, the interplay between HV and LV pulses, and how the parameters of HV and LV pulses influenced DNA electrotransfer, were still unclear. In elegant *in vitro* experiments using 1, 2, and 3% agarose gels and pulses similar to our LV pulses, Zaharoff and Yuan (2004) analyzed DNA electromobility: they showed that, using pulses of 10 to 99 msec at 100 to 400 V/cm (comparable to our LV pulses), plasmids were transported over distances longer, by two to three orders of magnitude, than those achieved with pulses of 99 μsec at 2.0 kV/cm (comparable to our HV pulses). We discuss here both old and new data on the influence of HV and LV pulses on DNA electrotransfer efficacy in light of these *in vitro* data.

It was known that one 100-μsec HV pulse alone is not sufficient for efficient DNA electrotransfer (Šatkauskas *et al*., 2002); this could be due to insufficient electrophoretic transport of DNA into the tissue. Interestingly, the data reported here show that neither HV pulse duration nor the number of HV pulses had an effect on DNA electrotransfer efficiency. Because eight HVs permeabilize muscle to a significantly greater extent than one HV (Bureau *et al*., 2000), it seems possible that the achievement of optimal permeabilization by the HV is not critical for effective DNA electrotransfer, at least under the experimental conditions in which LV pulses are optimal or close to optimal (e.g., four LVs at 80 V/cm and 100 msec). Thus muscle must be permeabilized to some extent but not necessarily to the optimal level. This is also supported by our previous studies on the kinetics of membrane resealing after the permeabilization of muscle with one HV of 800 V/cm and 100 μsec: even though the level of muscle permeabilization after one HV pulse significantly decreased after 300 sec, high and similar levels of DNA electrotransfer and gene expression were still reached with combinations of HV and LVs in which the four LVs were delivered even 3000 sec after the HV (Šatkauskas *et al*., 2002). Nevertheless, HV delivery is prerequisite, because the reverse order of the pulses, that is, an LV plus HV sequence, resulted in levels of DNA expression as low as those obtained by the delivery of one HV pulse alone (Šatkauskas *et al*., 2002).

Contrary to the HV pulse, DNA electrotransfer efficacy is highly dependent on the electrical parameters of the LV pulses. For individual LV durations of 100 or 50 msec (Fig. 2A and B), DNA electrotransfer efficacy increased with the number and/or individual duration of the LV pulses until a plateau was reached, when the total duration of the LVs reached 400 msec. It is interesting to note that, with respect to total LV pulse duration, patterns of luciferase expression for both individual LV durations were similar (Fig. 2A and B). Because electrophoretic transport (and thus migration distance) in a given medium and at a given voltage depends mainly on the duration of the applied electric field, the results in Fig. 2A and B argue in favor of a true electrophoretic role for the LV pulses. The importance of the electrophoretic effects on DNA electrotransfer efficacy can be explained by the fact that DNA must overcome physical barriers in the interstitial space before achieving close contact with the permeabilized plasma membrane and crossing it. Electrophoretic transport should thus ensure DNA interaction with the permeabilized cell membrane, which explains why the efficiency of DNA electrophoresis governs the efficacy of DNA electrotransfer.

When comparing transfection efficacy levels produced by an identical total duration of LV pulses, but using four different durations for the individual LV pulses, significantly higher levels of gene expression were achieved with the longest individual LV pulses (Fig. 2C). At first glance, this does not seem compatible with the electrophoretic effect of LV pulses suggested previously. However, "compatibility" with a pure electrophoretic effect can

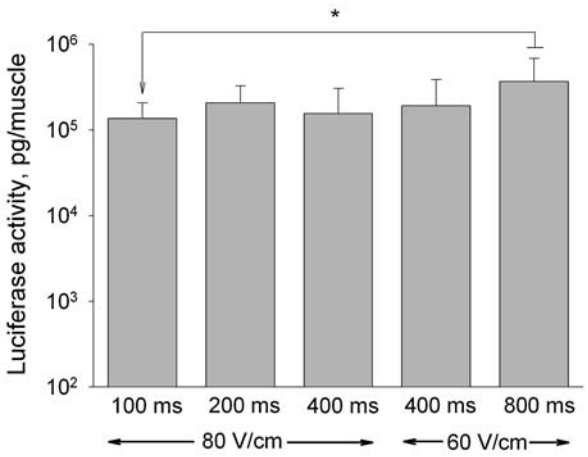

FIG. 4. Luciferase expression after DNA electrotransfer by combinations of one HV pulse (800 V/cm, 100 μsec) and four LV pulses as a function of LV pulse strength and duration. Data are presented as means ± SD. A statistically significant difference, calculated by *t* tests, was found only between the 60-V/cm, 800-msec group and the 80-V/cm, 100-msec group, and is indicated by an asterisk ($*p < 0.05$). Each column represents results from 10 muscles treated in one experiment.

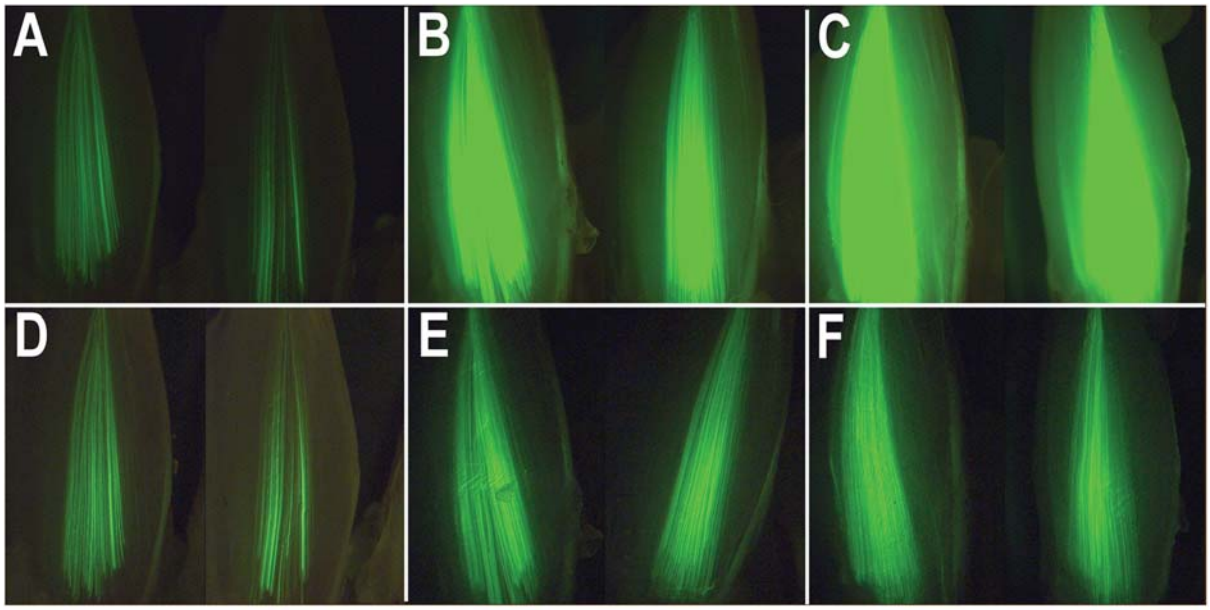

FIG. 5. GFP expression in tibialis cranialis muscles after DNA electrotransfer by combinations of one HV pulse (800 V/cm, 100 μsec) and one LV pulse of 400 msec and either 60, 80, or 100 V/cm. (**A** and **D**) LV of 60 V/cm; (**B** and **E**) LV of 80 V/cm; (**C** and **F**) LV of 100 V/cm. (**A**–**C**) Camera exposure time was fixed at 100 msec. (**D**) Exposure times were 243 msec (left muscle) and 392 msec (right muscle). (**E**) Exposure times were 36 msec (left muscle) and 55 msec (right muscle). (**F**) Exposure time was 23 msec for both muscles. Representative images of four muscles treated under each of the experimental conditions are shown.

be shown by taking into account the *in vitro* results of Zaharoff and Yuan (2004) on the mobility in agarose gels of DNA molecules exposed to pulse durations similar to those of the LV pulses described here. The agarose gels used by these authors are supposed to mimic interstitial barriers in biological tissues. They found that the dependence of plasmid electromobility on pulse duration was not linear and displayed a sigmoid shape: at shorter durations electromobility is low, whereas with longer durations it increases, reaching a plateau at 50 msec in 1% agarose gels, and at higher pulse duration (80–100 msec) in more concentrated gels (2–3% agarose). Indeed, for a plasmid to move through the narrow passages in agarose gels, random coiled DNA should elongate in the direction of motion and shrunk in the perpendicular direction (Zaharoff and Yuan, 2004). Then, if a long LV pulse is substituted by multiple shorter LV pulses, separated by 1 sec, the elongated plasmid relaxes between the pulses. The consecutive LV pulses must again align the plasmid along the direction of the electric field. This alignment takes time and therefore electrophoresis of the plasmid is not as efficient as in the case of a single but long pulse. Moreover, because tissue structure is much more complex than an agarose gel, electromobility of the plasmid should still be more dependent on pulse duration.

Plasmid DNA electrophoresis in tissues is further supported by another study by Zaharoff *et al*. (2002) showing that in tumors, electric pulses similar to the LV pulses used here can actually produce the electrophoretic migration of DNA over distances of about 1 μm. Thus the LV pulses should be sufficient to bring the DNA from the bulk of the injected liquid into close contact with the permeabilized plasma membrane of the muscle cell and/or to contribute to translocation of the DNA through the permeabilized membrane. In addition to pure electrophoretic effects, it cannot be excluded that LV pulses might increase the myofiber permeabilization created by the HV pulse (in particular for the longest and more intense LV pulses). This might contribute to enhanced electrotransfer efficacy. However, the results of Fig. 1 clearly show that the level of cell permeabilization is not of primary importance. Moreover, the results of Fig. 2 strongly argue in favor of the hypothesis that efficacy of gene electrotransfer is governed mainly by the electrophoretic forces of the LV pulses.

When we tested various LV pulse field strengths (Fig. 3), the same low level of luciferase expression was achieved with LV pulses up to 40 V/cm than with the HV pulse alone (equivalent to the delivery of an LV at 0 V/cm). Low transfection efficiency using LV pulses of 20 or 40 V/cm may be explained by the absence of an "electrophoretic field" in the muscle, caused by the voltage drop across the skin. Indeed, even if skin is electropermeabilized, it still remains highly nonconductive, provoking a substantial voltage drop (data not shown). Thus, on the one hand, it is possible that with LVs of 20 or 40 V/cm the electrophoretic force in the muscle tissue was negligible and, on the other hand, that an increase in LV pulse amplitude from 60 to 100 V/cm resulted in a progressive increase in luciferase expression (Fig. 3), according to the basic rules of electrophoresis. Pulses of 60 V/cm and 20 to 83 msec in duration do not contribute to muscle fiber permeabilization (Gehl and Mir, 1999; Bureau *et al*., 2000), even if they are delivered after one 100-μsec HV (Mir *et al*., in preparation). Thus, using pulses of 60 V/cm, only the electrophoretic effects of such pulses should be observed. Interestingly, the data reported in Fig. 4 demonstrate that long-enough pulses (400 msec) at 60 V/cm result in luciferase expression similar to that achieved with the 80-V/cm pulses, and that much longer pulses (800 msec) result in still higher luciferase expression. So, the decreased electrotransfer efficacy with the 60-V/cm LVs (at a du-

ration of 100 msec) can be fully compensated by an increase in pulse duration, which is in clear agreement with electrophoresis principles.

As expected from the results with the luciferase gene, the increase in field strength of the LVs resulted in an increase in GFP fluorescence (Fig. 5). Qualitative and quantitative data clearly showed that this increase in fluorescence resulted from the greater fluorescence of each fiber; the volume of tissue affected by the electrotransfer remained the same. This is in agreement with the previous assignment of roles of the HV and LV pulses. The experiments reported in Fig. 5D–F show that the same volume of tissue was affected by the DNA electrotransfer, which was expected because the same HV pulse was used under the three experimental conditions. Within the volume of tissue affected by the HV pulse, the fluorescence of the individual fibers increased with an increase in the strength of the LVs from 60 to 100 V/cm (Fig. 5A–C). The increase in electrophoretic effect should bring more plasmid molecules in contact with the electropermeabilized membrane and, consequently, more plasmid molecules should be able to cross the plasma membrane, resulting in the observed enhancement of the efficacy of DNA electrotransfer.

The electrophoretic effect of LV pulses may contribute to increased transfection efficacy in several ways. First, they may induce sufficient DNA electrophoresis to bring plasmid DNA from the bulk of the interstitium into contact with permeabilized membranes. Second, when the plasmid molecules are already in contact with permeabilized membranes, the electrophoretic force of LV pulses may facilitate translocation of the plasmid molecules into the cells.

In conclusion, consistent with previous *in vitro* reports and our previous *in vivo* work, the present study confirms that efficient electrogene transfer is based on at least two distinct effects exerted by the electric pulses: cell permeabilization and DNA electrophoresis. The results of the current study highlight the importance of *in vivo* DNA electrophoresis in electrotransfer efficacy. We demonstrate that, provided some muscle permeabilization is achieved by the prerequisite HV pulse, DNA electrotransfer efficiency is governed by the electrophoretic effect of the LV pulse(s). These results provide new avenues for further optimization of *in vivo* electrogene therapy.

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