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ARTICLE

Mechanisms of *in Vivo* DNA Electrotransfer: Respective Contributions of Cell Electropermeabilization and DNA Electrophoresis

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Efficient cell electrotransfection can be achieved using combinations of high-voltage (HV; 800 V/cm, 100 μs) and low-voltage (LV; 80 V/cm, 100 ms) pulses. We have developed equipment allowing the generation of various HV and LV combinations with precise control of the lag between the HV and LV pulses. We injected luciferase-encoding DNA in skeletal muscle, before or after pulse delivery, and measured luciferase expression after various pulse combinations. In parallel, we determined permeabilization levels using uptake of ^{51}Cr-labeled EDTA. High voltage alone resulted in a high level of muscle permeabilization for 300 seconds, but very low DNA transfer. Combinations of one HV pulse followed by one or four LV pulses did not prolong the high permeabilization level, but resulted in a large increase in DNA transfer for lags up to 100 seconds in the case of one HV + one LV and up to 3000 seconds in the case of one HV + four LV. DNA expression also reached similar levels when we injected the DNA between the HV and LV pulses. We conclude that the role of the HV pulse is limited to muscle cell permeabilization and that the LV pulses have a direct effect on DNA. *In vivo* DNA electrotransfer is thus a multistep process that includes DNA distribution, muscle permeabilization, and DNA electrophoresis.

Key Words: gene therapy, electrogenetherapy, DNA electrotransfer, electropermeabilization, electroporation, electric pulses, nonviral gene therapy, muscle, naked DNA, plasmid DNA

INTRODUCTION

Cell electrotransfection, or the transfer of DNA into living cells using electric fields, has been known since the pioneering report of Neumann and colleagues [1]. Because of its ease of application and its efficiency, use of the method of DNA electrotransfer rapidly expanded and has become a routine technique for introducing foreign genes into bacterial, yeast, plant, and animal cells *in vitro* [2–5]. The high efficiency of DNA transfer into living cells led to *in vivo* experiments that genetically transform tissues and organs.

Particular attention has been devoted to *in vivo* experiments for gene electrotransfer into skeletal muscles [6–10]. DNA is injected intramuscularly in relatively high volumes of a physiological solution that allows distribution of DNA through the tissue by convection. Electric pulses are then applied, generally using external electrodes. Potential applications include the treatment of myopathies [11] and vaccination [12]. The postmitotic nature of myofibers and the high vascularization of the muscles make DNA electrotransfer in the muscle particularly promising to achieve the secretion of therapeutic proteins [13–16].

It is generally accepted that the main action of the electric pulses in DNA electrotransfer is cell permeabilization. However, it has also been suggested that the electric fields applied could have other modes of action, such as a direct effect on DNA molecules. Indeed, DNA must be present in the cell suspension [17] or in the tissue during the delivery of the electric field [10]. DNA must also be located close to the cell membranes, in that divalent ions like Ca^{2+}, which abolish electrostatic repulsion between DNA and the cell membrane, greatly enhance DNA electrotransfer efficacy [18]. It has also been suggested that the negative charge of DNA might lead to its electrophoretic movement by electric forces, thereby bringing the DNA in contact with the cell membrane and facilitating its transfer. This hypothesis has

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also been advanced in the case of *Escherichia coli in vitro* transfection [19].

Such an electrophoretic component of DNA electrotransfer has been shown *in vitro* by an experiment in which transfection efficiency on cells in monolayer on a porous film was found to vary depending on whether the electric field applied had a polarity inducing DNA electrophoresis toward the cells or away from the cells [20]. Moreover, that transfection efficiency decreased in conjunction with an increase in medium viscosity or a decrease of the effective charge of DNA also indicated there was active transport of the DNA [20]. Finally, a technique combining two types of pulses (a high-voltage, short-duration pulse, HV, and a low-voltage, long-duration pulse, LV) developed by Sukharev and colleagues [17] was used to separate two different effects of the electric field and to sustain the hypothesis that there are two active components in the delivered electric pulses: cell electropermeabilization and electrophoretic forces exerted on the DNA. Experiments such as these demonstrated the importance of the lag between HV and LV pulses: the shorter the lag between HV and LV pulses, the higher the transfection efficiency obtained. These results were initially explained by the fact that the resealing of the cell membrane from the permeabilized state starts as soon as the electric pulse is turned off [17]. Furthermore, an extremely low transfection efficacy resulted when the DNA was added to the cell suspension after the HV pulse but before the LV pulse, leading to the suggestion that the presence of DNA during the first pulse is also necessary for successful transfection [17].

The hypothesis that electric pulses have electropermeabilizing and electrophoretic components was substantiated by *in vivo* experiments using a similar two-type pulse technique [7]. A combination of one HV and four LV pulses was shown to be almost as efficient as the standard optimal pulses (eight pulses, 200 V/cm, 20 ms, 1 Hz for the skeletal muscle) previously defined [10]. However, because a manual switch was used to connect the HV and LV pulse generators to the electrodes, the lag between HV and LV pulses was poorly controlled and longer than 1 second. Therefore, it was impossible to precisely analyze the respective contributions from each type of pulse.

The objective of this study was to investigate the importance of the lag between the HV and LV pulses on DNA electrotransfer *in vivo*, using a custom-made automatic switch allowing precise control of the duration of this lag. This work led to an analysis of the mechanisms involved in DNA electrotransfer *in vivo* and to a clear description of the roles (cell permeabilization and DNA electrophoresis) of each component of the HV and LV pulse combination.

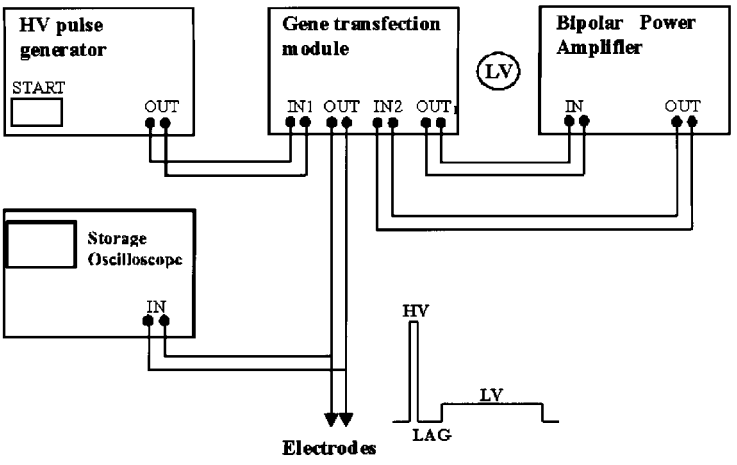

FIG. 1. Block diagram of the gene transfection system that we designed and built for the generation of combinations of HV and LV pulses.

RESULTS

Pulse Delivery Equipment

The custom-made electronic device for the experiments reported here consists of a commercially available high-voltage pulse generator with an external trigger that was used to deliver the desired HV pulse. The HV pulse reaches the electrodes through a gene transfection module specifically designed and manufactured to control the sequence of pulses and to generate the signals that are then amplified by a commercially available amplifier to constitute the LV pulses. The gene transfection module allows one to program the number of HV pulses, the duration of the lag between the HV and the (first) LV pulse, the number of LV pulses, the voltage amplitude (and thus the field strength, depending on the distance between the electrodes), and the duration of the LV pulses, as well as the lag between two consecutive LV pulses. When the HV pulse or pulses flow through the gene transfection module, the pulse counter counts down until it reaches the zero value that triggers the generation of the lag and the delivery of the LV pulse(s). Consequently, after the programming and the activation of the gene transfection module, the entire sequence of pulses is under the control of the "start" button on the HV generator, directly or through a pedal connected to the external trigger (Fig. 1).

DNA Electrotransfer

We carried out the initial studies using one HV square pulse of 100 μs at 800 V/cm and one LV square pulse of 100 ms at 80 V/cm separated by a lag ranging from 5 ms to 1000 seconds (Fig. 2).

We obtained little variation in luciferase expression for experiments with a lag between the pulses comprising from 300 ms to 100 seconds. With shorter lags of 5 ms or

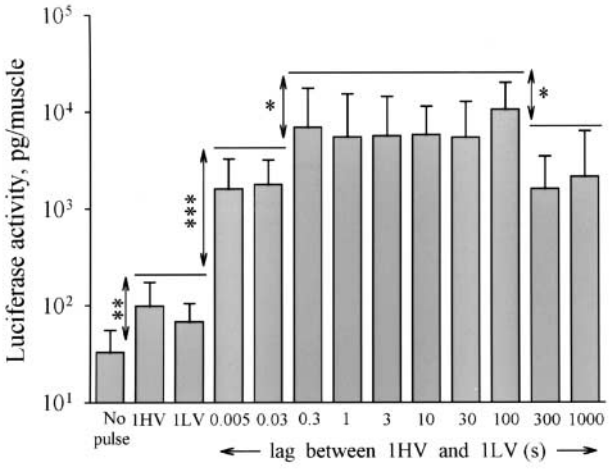

FIG. 2. Luciferase expression after DNA electrotransfer using one HV pulse (100 μs, 800 V/cm) and one LV pulse (100 ms, 80 V/cm) as a function of the lag between the HV and the LV pulses. We injected 3 μg of DNA (in 30 μl of NaCl 0.9%, supplemented with 3.6 IU of heparin) into the muscles. Soon (45 ± 15 seconds) after the injection, we pulsed the muscles with either one HV or one LV or combination of one HV + one LV pulses, with a lag between the pulses comprising between 5 ms and 1000 seconds. Data are presented as mean ± SD. For the lags comprising from 300 ms to 100 seconds, the groups are not statistically different (one-way ANOVA). If we include the groups with the lags of 5 and 30 ms from one side and/or the groups with the lags of 300 and 1000 seconds from other side, the groups are statistically different (* $P < 0.05$; one-way ANOVA). Statistical difference between other groups was tested using Student's *t*-test (** $P < 0.01$; *** $P < 0.001$).

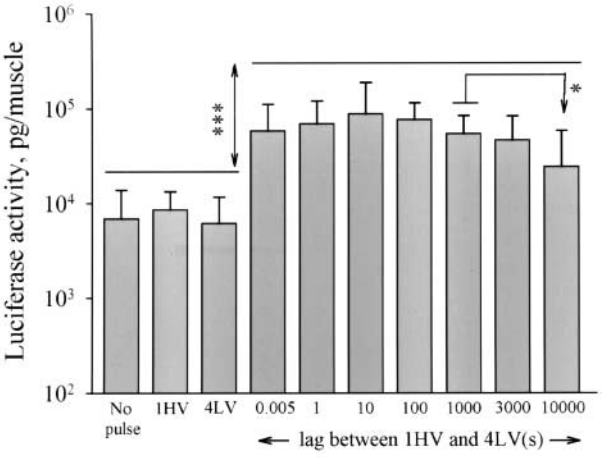

FIG. 3. Luciferase expression after DNA electrotransfer using one HV pulse and four LV pulses as a function of the lag between the HV and the LV pulses. We injected 3 μg of DNA (in 30 μl of NaCl 0.9%) into the muscles. Soon (45 ± 15 seconds) after the injection, we pulsed the muscles with either one HV or four LV or a combination of one HV + four LV pulses, with a lag between the pulses comprising between 5 ms and 10,000 seconds. Data are presented as mean ± SD. All 1HV + 4LV groups are not statistically different (one-way ANOVA). If we include the groups marked as "No pulse," "1HV," and "4LV," the group differences approach statistical significance (*** $P < 0.001$; one-way ANOVA). "1HV + 4LV 10,000 s" is statistically different (* $P < 0.05$; *t*-test) from the other 1HV + 4LV groups except for "1HV + 4LV 3,000 s."

30 ms (5 ms was the shortest lag that the pulse delivery equipment could generate), luciferase expression was at least three times lower. With longer lags, 300 seconds or 1000 seconds, we also observed a net decrease in DNA expression (Fig. 2).

The levels of luciferase expression using one HV pulse and one LV pulse were lower than those previously reported using one HV and four LV pulses of 83 ms [7]. Therefore, we also studied the influence of the lag in a combination of one HV + four LV pulses—that is, under more efficient conditions (Fig. 3).

High luciferase expression in the muscles was obtained using lags ranging from 5 ms to 3000 seconds with insignificant variation (Fig. 3). With longer lags, luciferase expression started to decline. When we set the lag to 10,000 seconds, luciferase expression was significantly ($P < 0.05$; *t*-test) lower than with the lags up to 3000 seconds. Nevertheless, even with this long lag, gene expression was significantly higher ($P < 0.001$; *t*-test) than when we used the HV pulse alone or with only the four LV pulses (Fig. 3).

Comparison of the results obtained with one HV + one LV and one HV + four LV revealed that gene transfer efficacy was one order of magnitude higher after the delivery of one HV + four LV pulses. Moreover, we detected a high plateau level of luciferase expression for longer lags. Indeed, the significant decline from the plateau value with the one HV + four LV pulses combination appeared when we used a lag of 10,000 seconds, whereas we already observed a significant decrease at 300 seconds lag in the case of the one HV + one LV combination (Figs. 2 and 3).

Determination of Muscle Permeabilization

We investigated muscle permeabilization levels as a function of the time after the HV pulse delivery. For this purpose, we injected ^{51}Cr-EDTA into the muscles at various times after the delivery of the HV pulse alone. We measured the radioactivity still present in the muscles 24 hours later (Fig. 4).

Muscle permeabilization declined with an increase of the ^{51}Cr-EDTA injection time after HV pulse delivery. However, this decrease was not statistically significant during the first 300 seconds. Moreover, our results showed that muscle permeabilization was long-lived and that uptake of ^{51}Cr-EDTA injected 50 minutes (3000 seconds) after HV pulse was still significantly higher ($P < 0.05$; *t*-test) than that detected in the absence of any pulse (Fig. 4).

We also determined muscle permeabilization after the delivery of various combinations of HV and LV pulses, as a function of the lag between these two types of pulses (Fig. 5, one HV + one LV, and Fig. 6, one HV + four LV). The combination of one HV + one LV pulses, with the lags up to 300 seconds, gave rise to a somewhat higher uptake

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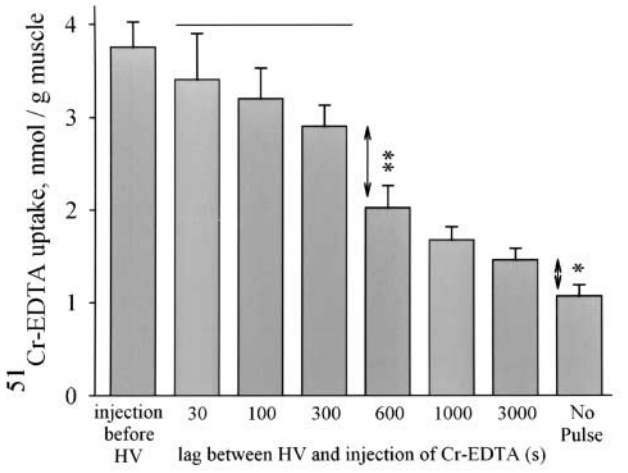

FIG. 4. Muscle permeabilization after the HV pulse. We injected ^{51}Cr-EDTA either before or at various times after the HV pulse. Data are presented as mean ± SEM. Even if a clear tendency to cell resealing is seen, the permeabilization levels before and at 30, 100, and 300 seconds after HV pulse are not statistically different ($P > 0.05$; *t*-test). For longer lags we found statistical differences with the permeabilization measured when ^{51}Cr-EDTA was injected before the HV pulse (*$P < 0.05$; **$P < 0.01$; *t*-test).

of ^{51}Cr-EDTA than the HV pulse alone ($P < 0.05$ for 1, 100, 300 seconds; *t*-test). At longer lags of 1000 seconds and 3000 seconds, ^{51}Cr-EDTA uptake sharply decreased and was lower than the uptake achieved after the HV pulse alone (Fig. 5). Similarly, the combination of one HV + four LV pulses gave rise to higher uptake ($P < 0.05$ for 1 second; $P < 0.001$ for 5 ms, 10, 100, 300 seconds; *t*-test) of ^{51}Cr-EDTA than HV alone, only for the lags up to 300 seconds. At longer lags, starting from 1000 seconds, ^{51}Cr-EDTA uptake decreased to levels similar to those in the case of one HV + one LV (Fig. 6).

With only four LV pulses we were able to slightly permeabilize the muscles ($P < 0.01$; *t*-test), whereas we achieved no effect when using only one LV pulse (no statistically significant difference between no pulse and one single LV pulse, that is, $P > 0.05$; *t*-test). On the contrary, the single HV pulse did permeabilize muscle tissue, and ^{51}Cr-EDTA uptake was as much as twofold or more higher than it was without any pulse or with only four LV pulses ($P < 0.001$; Figs. 5 and 6).

Interpulse DNA Injection

The observation that lags of as long as 100 seconds did not result in a reduction of DNA electrotransfer efficacy led us to investigate the specific contributions of HV and LV pulses using interpulse DNA injections, that is, injections done just after the "permeabilizing" HV pulse and before the LV pulse. We used a lag of 100 seconds between the HV and LV pulses, having determined that such a lag was sufficient to assure a reproducible experimental procedure. The results showed that luciferase expression did not change significantly whether we injected the DNA before the HV and LV pulses or in between them (Fig. 7). Reversing the order of the pulses resulted in a reduction of two orders of magnitude in luciferase expression, down to a level similar to that obtained with the HV pulse alone. DNA injection after the combination of HV and LV pulses resulted in a reduced luciferase expression similar to that obtained with only the LV pulse (Fig. 7).

DISCUSSION

Some earlier insights into the mechanism of DNA electrotransfer had been obtained *in vitro* as well as *in vivo* using two-pulse techniques [7,17]. Both reports concluded that electric pulses mediate DNA internalization by exerting two different effects: cell membrane permeabilization and DNA electrophoresis. We used the same approach to further characterize the effects of the electric pulses on the DNA delivery into the muscle cells *in vivo*. Several technical improvements we made allowed us to study the respective specific contributions of electropermeabilizing (HV) and electrophoretic (LV) pulses, and to emphasize new aspects of the mechanisms of DNA electrotransfer using combinations of HV and LV pulses.

First, our recent finding that polyanionic heparin inhibits naked DNA uptake by the muscle cells *in vivo* [21] enabled us to separate the expression of spontaneously uptaken DNA from that attributable to the electric pulses. This finding in turn led to the possibility of simplifying the combination of the two types of pulses to a sequence of one HV and one LV (noting that without the inhibition with heparin, expression of spontaneously uptaken DNA

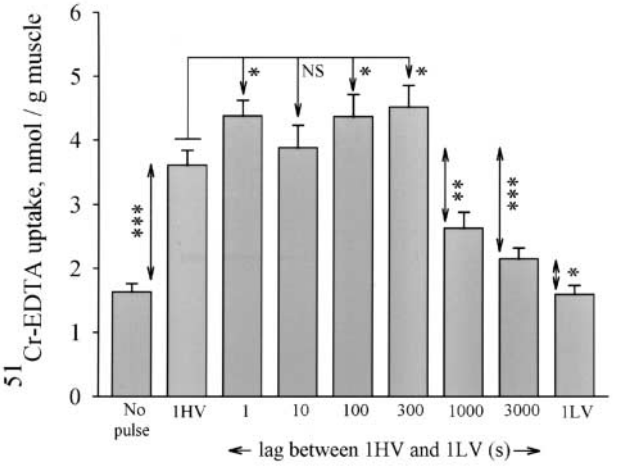

FIG. 5. Muscle permeabilization after one HV pulse alone or after a combination of one HV + one LV pulses as a function of the lag between the pulses. We injected the ^{51}Cr-EDTA 45 ± 15 seconds after the delivery of the LV pulse. As a control, ^{51}Cr-EDTA injection after one LV alone is also reported. Data are presented as mean ± SEM (NS, not significant; *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; *t*-test).

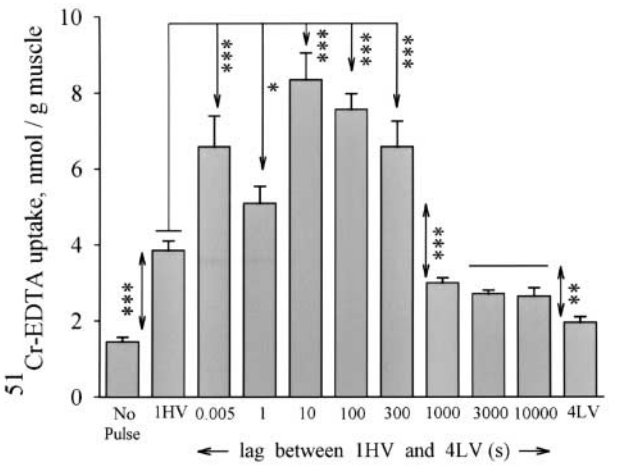

FIG. 6. Muscle permeabilization after one HV pulse alone or after a combination of one HV + four LV pulses as a function of the lag between the pulses. We injected the ^{51}Cr-EDTA 45 ± 15 seconds after the delivery of the last LV pulse. As a control, ^{51}Cr-EDTA injection after four LV alone is also reported. Data are presented as mean ± SEM (*P < 0.05; **P < 0.01; ***P < 0.001; t-test).

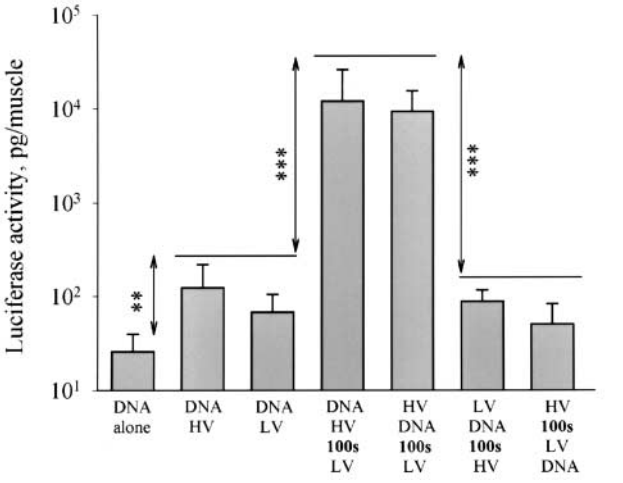

FIG. 7. Luciferase expression after DNA electrotransfer using one HV pulse and one LV pulse and various combinations of pulse delivery and DNA injection. We injected the DNA (in 30 μl of NaCl 0.9% supplemented with 3.6 IU of heparin) into the muscles before, after, or in between the HV and the LV pulses, according to the sequence shown below the corresponding histogram. When we used a combination of pulses, the lag between the pulses was 100 s. Data are presented as mean ± SD (**P < 0.01; ***P < 0.001; t-test).

reaches a level close to that obtained after DNA electrotransfer using pulse combinations of one HV + one LV). Second, we improved the method of ^{51}Cr-EDTA uptake [22] by greatly increasing its sensitivity: measuring muscle radioactivity after a time lapse of 24 hours after the injection of the ^{51}Cr-EDTA allowed a more extensive washout of the radioactive molecules from the blood and from the muscle extracellular spaces, permitting us to determine more specifically the actual accumulation of radioactivity in the muscles due to ^{51}Cr-EDTA internalization into the reversibly electropermeabilized myofibers. Third, we developed equipment that allowed precise control of the lags between the HV and LV pulses, making it possible to separate accurately the HV and LV pulses and to analyze separately their respective contributions (Fig. 1).

Initial DNA electrotransfer experiments with pulse combinations of one HV and one LV pulse revealed that similar and almost constant levels of luciferase expression can be achieved with lags between the pulses ranging from 300 ms to 100 seconds (Fig. 2). The result was somewhat unexpected because in previous *in vitro* studies using similar combinations of two types of pulses, a decrease in transfection efficacy was found with the increase of the lag [17]. Contrary to that, we found in our *in vivo* study a significant decrease of luciferase expression with the shortest lags of 5 and 30 ms (Fig. 2). This lower value cannot be attributed to an insufficiency in the time necessary to achieve a permeabilized state, because *in vitro* [23–25] and *in vivo* (David Cukjati, D.M., and L.M.M., unpublished data) studies estimated that structural rearrangements require only a few tens of microseconds.

Moreover, the results using a pulse combination of one HV and four LV showed no significant decrease in DNA expression using the shortest 5-ms and 30-ms lags (Fig. 3). We will discuss below these apparently contradictory observations. With the one HV + four LV combination, we found a plateau in transfection efficacy for lags ranging between 5 ms and 3000 seconds. The decline from the DNA expression plateau started at 10,000 seconds with one HV + four LV pulses, although we already observed a significant difference from plateau value at the lag of 300 seconds with one HV + one LV (Figs. 2 and 3 and Table 1).

To gain insight into the reasons for the existence of these plateaus in luciferase expression, as well as the other differences between the one HV + one LV and one HV + four LV combinations, we determined muscle permeabilization by means of ^{51}Cr-EDTA uptake using the various combinations of HV and LV pulses (Figs. 4–6 and Table 1).

It was interesting to note that muscle permeabilization achieved with the HV pulse was long-lived. Muscle permeabilization was high (without significant difference) up to 300 seconds after the HV pulse with, nevertheless, continuous and progressive cell resealing for more than 3000 seconds (Fig. 4). With both the one HV + one LV and one HV + four LV combinations we observed a high permeabilization with lags not exceeding 300 seconds (Figs. 4–6 and Table 1). Nevertheless, despite these similarities in the muscle permeabilization, the transfection efficiencies with various combinations of pulses (one HV + one LV and one HV + four LV) differed substantially. These results therefore suggest a major role of LV pulses in transfection efficacy.

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TABLE 1: A comparison of tissue permeabilization and gene expression levels and their duration as a function of the combination of HV and LV pulses applied

	High level of permeabilization	High level of gene expression
HV pulse alone	Until 300 s after HV	No large improvement
HV + 1LV	Until lags of 300 s after HV	Improvement for lags up to 100 s
HV + 4LV	Until lags of 300 s after HV	Improvement for lags up to 3000 s

According to previous hypotheses [7,17,19] and to the theory of Neumann and colleagues [18,26], LV pulses can contribute to the electrophoretic transport of DNA toward the cell membranes, which would facilitate DNA interaction with the permeabilized membrane and its subsequent internalization into the electropermeabilized cell. The data reported in Table 1 seem to sustain the existence of this role of the electric pulses, demonstrating moreover that the electrophoretic role of the electric pulses is critical in transfection efficacy.

First of all, a single LV pulse appeared to have no effect on cell electropermeabilization. The transfection of the cells in this case was also negligible. However, if the LV pulse followed the HV pulse, it resulted in a large increase in transfection efficacy. Thus, an LV pulse should have mainly a direct effect on DNA. Second, the number of LV pulses was critical in transfection efficacy: the larger the electrophoretic component after a HV pulse was applied, the greater the efficacy of DNA transfer obtainable. This was also demonstrated by the observation that one HV pulse followed by four LV pulses resulted in 10 times higher luciferase expression than one HV pulse followed by only one LV pulse (Figs. 2 and 3). Table 1 also shows that the electrophoretic component was so important that even if permeabilization is not optimal (for example, for lags > 300 seconds and < 3000 seconds), efficiency is maintained if the electrophoretic component remains intense (four LV). Nevertheless, LV pulses alone (even four) did not result in efficient DNA transfer, demonstrating the necessity of permeabilization.

To definitively demonstrate the interaction of the two different effects of HV and LV pulses in DNA electrotransfer according to the mechanism described above, we investigated DNA electrotransfer using interpulse DNA injections, that is, injections done just after the HV pulse, but before the LV pulse. We found that luciferase expression was not significantly affected by the timing of DNA injection: before the one HV and one LV pulses or in between the two (Fig. 7). We obtained a similar result using one HV + four LV (data not shown). This indicates that DNA must be present after tissue permeabilization by HV pulse and before LlV pulses delivery to ensure DNA electrotransfer efficacy by LV pulses, but the presence of DNA is not required before the "permeabilizing" HV pulse.

The efficacy of DNA electrotransfer using an interpulse DNA injection helps to explain the differences observed in electrotransfer efficacy with the very short time lags of 5 and 30 ms (that is, with one HV + one LV, efficacy was lower than for longer lags, whereas there was no such decrease with the one HV + four LV combinations). First, it is necessary to recall that muscle tissue electropermeabilization affects muscle cells as well as other cells in the volume of the tissue exposed to the electric pulses. DNA injected in a given volume of saline before or after the HV pulse is distributed in the tissue by convection (that is, by means of the liquid in which DNA is diluted). The electropermeabilization achieved by the HV pulse could thus overcome tissue barriers to permeabilization, allowing a better redistribution of the solution (NaCl 0.9%) containing the DNA within the muscle tissue. Moreover, the muscle contractions stimulated by the pulses could in themselves facilitate the redistribution of the solution in the cases in which DNA was injected before HV delivery. The importance of the access of plasmid to the muscle fibers has already been demonstrated: an improved plasmid distribution increased DNA expression both in the case of naked DNA injections [27] and in that of DNA electrotransfer [28]. In these reports, improved DNA distribution was achieved by means of the preinjection (before the DNA injection) of either a sucrose solution that forced the generation of spaces between muscle fibers [27], or hyaluronidase, which breaks down components of the extracellular matrix and provides some permeability of the connective tissues without total disruption [28].

In our case, the permeabilization achieved by the HV pulse, and perhaps the concomitant muscle contraction, could facilitate DNA redistribution before the one LV or four LV pulses push the DNA toward and/or inside the cells. It may thus be hypothesized that with one LV pulse delivered very shortly after the HV pulse (at 5- or 30-ms lags in our experiments), DNA does not have enough time to redistribute within the tissue and the efficacy of transfer is not maximized (plateau level). With four LV pulses this expression decrease at 5 ms was not observed because the second LV pulse was delivered at 1105 ms (lag of 5 ms + LV of 100 ms + interval of 1000 ms) after the HV, thus at a time at which redistribution had already been achieved as shown in the experiments involving one single LV pulse. This plausible explanation of the observations reported here reinforces our conclusions that the HV pulse affects the cells (membrane permeabilization), but does not directly affect DNA transfer, thus highlighting the important electrophoretic role of the LV pulse in the efficacy of DNA electrotransfer.

Our results demonstrate that *in vivo* DNA transfer with electric pulses is a process that includes injection and

distribution of the DNA in the tissue, cell permeabilization (HV pulse), a probable improvement in DNA distribution in the permeabilized tissue, and DNA transfer facilitated by DNA electrophoresis in the tissue (LV pulse).

MATERIALS AND METHODS

Plasmid DNA. We used the plasmid pXL 3031 (pCMV-Luc+) containing the cytomegalovirus promoter (nt 229–890 of pcDNA3, Invitrogen) inserted upstream of the coding sequence of the modified cytosolic *luc*+ gene coding for firefly luciferase [29]. We prepared the plasmid DNA using the usual procedures [30]. As a general rule, at least 80% of the plasmid molecules were supercoiled.

Animals. For all experimental procedures we anesthetized female, 7- to 9-week-old, C57BL/6 mice by the i.p. administration of the anesthetics: ketamine (100 mg/kg; Ketalar, Panpharma, France) and xylazine (40 mg/kg; Rompun, Bayer, France). Preceding the experiments, we shaved the legs using an electric shaver. At least 10 muscles were included in each experimental group.

DNA injection. We injected 3 μg of plasmid DNA prepared in 30 μl of 0.9% NaCl. In the electrotransfer experiments involving one HV and only one LV (Figs. 2 and 7), we supplemented the DNA solution with polyanionic heparin 120 IU/ml (Laboratoires Leo, Saint Quentin en Yvelines, France; 1 mg heparin (MW 10–12 kDa) corresponded to ~ 137 IU) to analyze with more precision the effects of the electric pulses. Indeed, we had already shown that this amount of heparin was able to block spontaneous DNA uptake by muscle cells *in vivo*, without modifying DNA electrotransfer efficacy [21]. In the experiments involving one HV and four LV (Fig. 3), the increase in DNA uptake was larger and it was not necessary to avoid the spontaneous DNA uptake using heparin. We injected the DNA into tibial cranial muscles using a Hamilton syringe with a 26-gauge needle.

DNA electrotransfer. To generate the HV pulse (1 pulse, 800 V/cm, 100 μs) we used a square-wave electropulsator PS-15 (Jouan, St Herblain, France). The LV pulses (one or four pulses of 80 V/cm and 100 ms, delivered at 1-second intervals) were generated by a gene transfection module (a microprocessor-driven switch/function generator) built at the University of Ljubljana (Faculty of Electrical Engineering, Slovenia) and amplified by a power amplifier (Kepco Inc., Flushing, NY). The module also allowed for precise control of the lag between HV and LV pulses, ranging from 5 ms to 10 seconds or more.

Muscles were pulsed soon (40 ± 15 seconds) after intramuscular DNA injection. Depending on the protocol, we used various lags between HV and LV pulses ranging from 5 ms to 10,000 seconds. When interpulse DNA injections were performed, we fixed a lag between HV and LV to 100 seconds. In such cases we injected the DNA as soon as possible (20 ± 5 seconds) after the HV pulse.

For pulse delivery to the muscles we used two opposing stainless-steel plate electrodes, of 1 cm width and 0.5 mm thickness, maintained 4.4 mm apart by an insulating holder. The electrodes encompassed the whole leg of the mouse. We applied conductive gel to assure a good contact between the shaved leg skin and the electrodes.

Luciferase activity measurement. Two days after DNA electrotransfer, the mice were killed and the muscles removed and homogenized in 1 ml cell culture lysis reagent solution (10 ml cell culture lysis reagent (Promega Charbonnières, France), diluted with 40 ml distilled water and supplemented with one tablet of protease inhibitor cocktail (Boehringer Mannheim, Mannheim, Germany)). After centrifugation at 12,000 rpm for 10 minutes at 4°C, we assessed the luciferase activity on 10 μl of the supernatant, using a Walac Victor^2 luminometer, by integration of the light produced during 1 second, 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 ~ 70 ng of expressed luciferase. We expressed the final results as picograms of luciferase per muscle.

Measurements of ^{51}Cr-EDTA muscular uptake. For the evaluation of the muscle permeabilization generated by the electric pulses, we used a described method [22]. We injected 30 μl ^{51}Cr-EDTA (Amersham, UK; specific activity 3.7 MBq/ml) diluted in 0.9% NaCl (1:1) into both tibial cranial muscles using a Hamilton syringe. Specific pulse sequences, as defined by the protocol, were applied to muscles before or after the injection. Mice were killed 24 hours after ^{51}Cr-EDTA injections and the radioactivity of treated muscles was measured on a Cobra 5002 Packard γ-counter (Packard Instrument Company, Meridien, CT). We expressed the final results as nanomoles of ^{51}Cr-EDTA uptake per gram of muscle.

Statistical analysis. Unless otherwise stated, we tested the significance of the differences between the individual groups using the one-tailed Student's *t*-test for unpaired values. For statistical comparison of several groups we used the one-way ANOVA. In the figures we report luciferase expression data as mean ± SD, and we express ^{51}Cr-EDTA uptake as mean ± SEM.

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