

## Full-length article

**Inhibitory effect of Ca<sup>2+</sup> on *in vivo* gene transfer by electroporation<sup>1</sup>**Yong-gang ZHAO<sup>2</sup>, Hui-li LU<sup>2</sup>, Jin-liang PENG<sup>2</sup>, Yu-hong XU<sup>2,3,4</sup><sup>2</sup>School of Life Science and Biotechnology and <sup>3</sup>School of Pharmacy, Shanghai Jiaotong University, Shanghai 200240, China**Key words**

gene therapy; electroporation; gene transfer; calcium ion

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**Abstract**

**Aim:** To investigate the specific effects of Ca<sup>2+</sup> on transgene expression during electroporation-mediated gene transfer in mice. **Methods:** Skeletal muscle and skin were subjected to *in vivo* electroporation with a luciferase reporter plasmid, with or without Ca<sup>2+</sup> and various other ions. **Results:** For *in vivo* electroporation, the presence of just 10 mmol/L Ca<sup>2+</sup> in the DNA solution drastically reduced the resulting transgene expression, to less than 5% of control values. Only Ca<sup>2+</sup>, not other ions, caused inhibition, and the effect was not tissue specific. More surprisingly, even when Ca<sup>2+</sup> ions were delivered by electroporation before or after DNA administration, similar effects were still observed. **Conclusion:** The inhibitory effect of Ca<sup>2+</sup> on *in vivo* gene transfer by electroporation is specific, ie, the inhibitory effect may be related to the cell membrane properties after electroporation and the subsequent resealing event.

**Introduction**

Electroporation is a commonly used gene delivery method with relatively high efficiency *in vivo*<sup>[1]</sup>. The method has been employed to transfect genes into various tissue types, including skeletal muscle, liver, skin, cornea, vasculature, and tumor tissues<sup>[2]</sup>. High transfection efficiencies can be achieved relative to other nonviral delivery methods<sup>[3]</sup>. It is possible that the electroporation delivery process can be further optimized to achieve higher transfection efficiency by varying the electric pulse parameters, the plasmid DNA concentration, the ionic strength, or adding helper molecules such as polymers<sup>[4–6]</sup>. In particular, it is known that ion composition and concentration in the DNA solution affects the efficiency of electroporation-mediated transfection. Lee *et al* examined the effect of a wide range of ionic strengths, and found that half saline (71 mmol/L) was best for electroporation in skeletal muscles<sup>[7]</sup>. In another study, Ca<sup>2+</sup> was identified as an important ionic component for electroporation; transgene expressions were shown to be highest when Ca<sup>2+</sup> concentrations were in the range 20–100 mmol/L<sup>[8]</sup>.

We reported here our findings using a different experimental protocol, which also suggest the importance of Ca<sup>2+</sup> in *in vivo* electroporation, but the effect we observed was quite the opposite of that observed in the previous study.

We found that the presence of Ca<sup>2+</sup> in DNA solution significantly inhibited transgene expression after electroporation. By comparing the effects of Ca<sup>2+</sup> and various other ions at various concentrations in various experimental settings, we believe that the inhibitory effect of Ca<sup>2+</sup> in electro-gene transfer is a real and important phenomenon that has not been discussed before. An in-depth understanding of such a phenomenon may provide some insights into the fundamental mechanism of *in vivo* electro-gene transfer.

**Materials and methods**

**Plasmid and reagents** The luciferase-encoding plasmid under the control of a SV40 promoter and a SV40 enhancer in the pGL3 backbone (pGL3-Control Vector) was bought from Promega (Madison, USA). Plasmid batches were all purified using the Qiagen GIGA kit. CaCl<sub>2</sub> and all other reagents were analytical grade and were purchased from Shanghai Chemical Reagent Co and Siji Reagent Co (Shanghai, China).

**Animals and animal experiment protocols** Female 4- to 6-week-old BALB/c mice were purchased from Fudan University Animal Facility (Shanghai, China). At least 6 animals were included in each experimental group. The animal experiment protocol in the present study was designed in accordance with the regulations of the Chinese Animal Welfare

Agency. Before the experiments, mice were anesthetized using 40 mg/mL chloral hydrate at 1% volume relative to body mass. After complete sedation, the gastrocnemius muscle or ventral skin was shaved in each mouse and the mice were given injections of 20  $\mu\text{L}$  0.1  $\mu\text{g}/\mu\text{L}$  plasmid DNA in various ionic solutions, followed by electroporation treatment using an electric pulse generator made in our laboratory<sup>[9]</sup>. The electrodes (two silver acupuncture needles, 3 mm apart, each 1 cm long, and 0.3 mm in diameter) were inserted into the muscle or skin covering the site of injection, and 650 ms square wave pulses were delivered at 1 s interval. The field strength was set at 200 V/cm. Transgene expression was usually assayed after 2 d as described below.

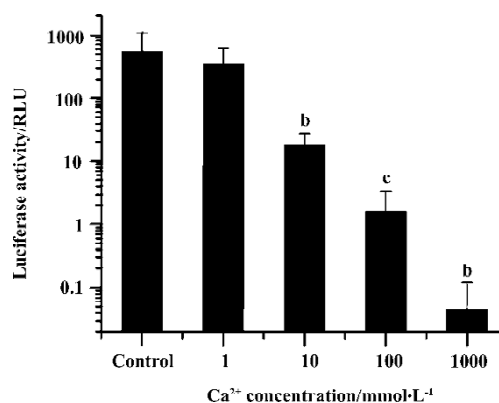
**Luciferase assay** The mice were killed and the gastrocnemius muscles or ventral skin samples were collected for examination. For the luciferase activity assay, tissues were homogenized in 1 mL Promega lysis reagent using a Beadbeater (Cole Pharma, USA). The homogenates were then centrifuged at 2000 $\times$ g for 6 min and the supernates were assayed using a TD20-20 luminometer (Turner BioSystems, CA), by integration of the light produced during 10 s, starting after the addition of 50  $\mu\text{L}$  of luciferase assay substrate (Promega) to 10  $\mu\text{L}$  supernatant.

**Statistical analysis** Unless otherwise stated, we tested the significance of the differences between individual groups using the one-tailed Student's *t*-test for unpaired values. For statistical comparisons of several groups we used one-way ANOVA. In the figures we report luciferase activity as mean relative light unit (RLU)  $\pm$ SD.

## Results

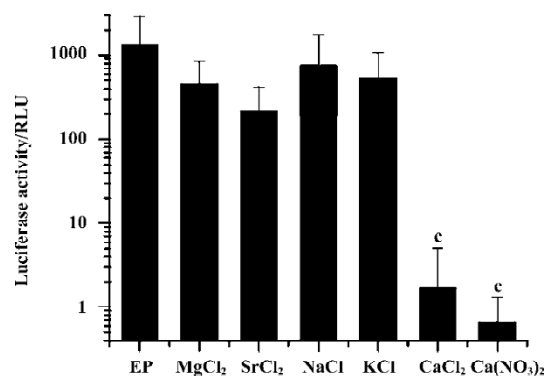
We examined the effect of different  $\text{Ca}^{2+}$  concentrations in the plasmid DNA solution on luciferase expression after electroporation delivery (Figure 1). With only 10 mmol/L of  $\text{Ca}^{2+}$  included in the injection medium, transgene expression was reduced to only 2% of the value without  $\text{Ca}^{2+}$  ( $P < 0.05$ , *t*-test). Further increasing the  $\text{Ca}^{2+}$  concentration to 100 mmol/L and 1 mol/L resulted in even lower gene expression. The experiment was repeated a few times using  $\text{CaCl}_2$  from different sources to avoid any effects of potential chemical impurities, but the inhibitory effect was always large and always reproducible.

To test whether other ions have similar inhibitory effects, we added  $\text{Mg}^{2+}$ ,  $\text{Sr}^{2+}$ ,  $\text{Na}^+$ , or  $\text{K}^+$  into the DNA solution instead of  $\text{Ca}^{2+}$  at comparable ionic concentrations and assayed for transgene expression after electroporation. Only  $\text{Ca}^{2+}$  had a profound inhibitory effect; while all other cations had no effect. In addition, the substitution of the anion  $\text{Cl}^-$



**Figure 1.** Luciferase activity after electroporation-mediated intramuscular DNA delivery with different  $\text{Ca}^{2+}$  concentrations. Data are mean  $\pm$  SD. <sup>b</sup> $P < 0.05$  vs control, <sup>c</sup> $P < 0.01$  vs  $\text{Ca}^{2+}$  10 mmol/L, <sup>b</sup> $P < 0.05$  vs  $\text{Ca}^{2+}$  100 mmol/L.

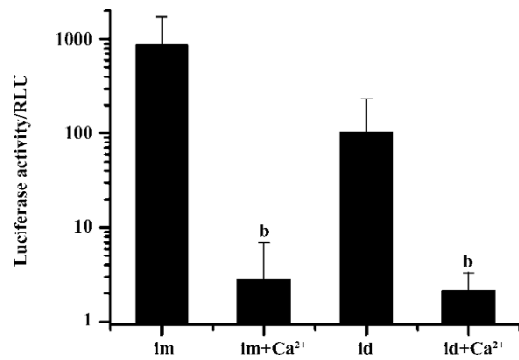
with  $\text{NO}_3^-$  in  $\text{Ca}^{2+}$ -containing solutions did not alter the inhibitory effect (Figure 2).



**Figure 2.** Luciferase activity after electroporation-mediated intramuscular DNA delivery with 10 mmol/L of various ionic salts. Mean  $\pm$  SD. <sup>c</sup> $P < 0.01$  vs electroporation (EP).

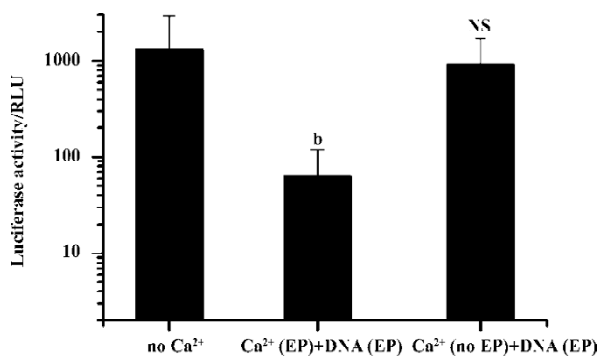
Because the  $\text{Ca}^{2+}$  ion plays an important role in the physiological function of muscles, we wondered if the inhibitory effect was unique to muscle tissues. Therefore, we examined the effect of  $\text{Ca}^{2+}$  in another tissue type: skin. A mere 5 mmol/L of  $\text{CaCl}_2$  resulted in an approximately 50-fold reduction in luciferase expression in skin after electroporation (Figure 3).

We further examined the effects on transgene expression when the  $\text{Ca}^{2+}$ -containing buffer was administered separately before or after plasmid DNA injection and electroporation. When the  $\text{Ca}^{2+}$ -containing buffer was administered by simple needle injection 10 min before the plasmid DNA injection and electroporation, no inhibitory effect was observed. But when the  $\text{Ca}^{2+}$ -containing buffer was injected and immedi-



**Figure 3.** Luciferase activity after electroporation-mediated intramuscular or intradermal DNA delivery with or without Ca<sup>2+</sup>. im, intramuscular injection without Ca<sup>2+</sup>; im+Ca<sup>2+</sup>, intramuscular injection with 5 mmol/L Ca<sup>2+</sup>; id, intradermal injection without Ca<sup>2+</sup>; id+Ca<sup>2+</sup>, intradermal injection with 5 mmol/L Ca<sup>2+</sup>. Mean±SD. <sup>b</sup>P<0.05 vs im. <sup>c</sup>P<0.05 vs id.

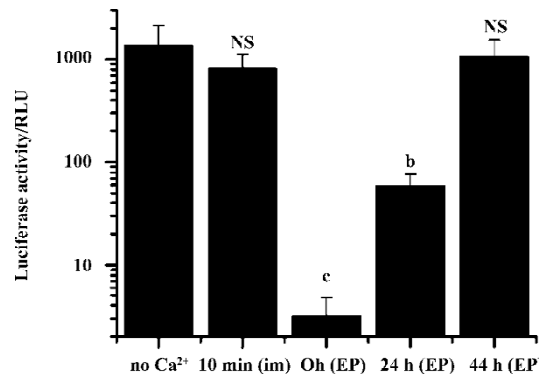
ately followed by an electroporation treatment, and 10 min later the DNA solution was injected and electroporated, the resulting transgene expression was greatly reduced (Figure 4).



**Figure 4.** Luciferase activity when Ca<sup>2+</sup>-containing buffers were administered with or without electroporation before intramuscular DNA injection. no Ca<sup>2+</sup>, intramuscular electroporation-mediated DNA delivery without Ca<sup>2+</sup>; Ca (no EP)+DNA (EP), intramuscular injection of 20 μL Ca<sup>2+</sup> 10 mmol/L solution, and 10 min later followed by electroporation-mediated intramuscular DNA delivery; Ca<sup>2+</sup> (EP) +DNA (EP), electroporation-mediated intramuscular delivery of 20 μL 10 mmol/L Ca<sup>2+</sup> solution, and 10 min later followed by electroporation-mediated intramuscular DNA delivery. Mean±SD. <sup>b</sup>P<0.05 vs no Ca<sup>2+</sup> group.

Simple intramuscular needle injection of Ca<sup>2+</sup>-containing buffer 10 min after plasmid DNA injection and electroporation had no inhibitory effect. But again, if the Ca<sup>2+</sup>-containing buffers were delivered accompanied by an electroporation treatment, transgene expression was greatly reduced. Even when Ca<sup>2+</sup> administration with electroporation was delayed

for more than 24 h, the inhibitory effect on transgene expression was still evident (Figure 5).



**Figure 5.** Luciferase activity when Ca<sup>2+</sup> solutions (20 μL 10 mmol/L Ca<sup>2+</sup>) were administered with or without electroporation at various times after electroporation-mediated intramuscular DNA delivery. no Ca: electroporation-mediated intramuscular DNA delivery without Ca<sup>2+</sup> in solution; 10 min (im), Ca<sup>2+</sup> solution was administered by simple intramuscular injection 10 min after electroporation-mediated plasmid DNA delivery; 0 h, Ca<sup>2+</sup> solution was administered with electroporation-mediated DNA delivery; 24 h, Ca<sup>2+</sup> solution was administered by electroporation 24 h after electroporation-mediated DNA delivery; 44 h, Ca<sup>2+</sup> solution was administered by electroporation 44 h after electroporation-mediated DNA delivery. Mean±SD. <sup>a</sup>P>0.05, <sup>b</sup>P<0.05, <sup>c</sup>P<0.01 vs no Ca group.

**Discussion**

Ca<sup>2+</sup> is one of the most common divalent cations found in biological fluids. For muscles, the extracellular calcium concentration is estimated to be approximately 2 mmol/L<sup>[10]</sup>. In the present study, the addition of only 10 mmol/L Ca<sup>2+</sup> to the DNA solution (which would be quickly diluted after injection) greatly interfered with transgene delivery and expression, not only in muscles, but also in skin. The higher the Ca<sup>2+</sup> concentration was, the lower the transgene expression. Interestingly, other studies on *in vitro* electroporation for cell transfection concluded that Ca<sup>2+</sup> is indispensable in the transfection medium to maintain appropriate ion concentrations<sup>[11]</sup>. But the results of the present study show that the situation is completely different *in vivo*.

The mechanism responsible for this inhibitory effect *in vivo* is unclear. It is possible that calcium might inhibit the activity of luciferase, so to determine whether this was the case, we added 1–5 mol/L of Ca<sup>2+</sup> ions into a standard solution of luciferase, but no decrease in luciferase activity was observed (data not shown). Therefore, calcium does not affect the activity of luciferase. Another possibility is that the

presence of  $\text{Ca}^{2+}$  ions affects the ionic strength as well as the conductivity of the plasmid DNA solution, and therefore alters the movement of DNA molecules in the tissue and into cells. In a recent study by Lee and coworkers, it was suggested that ionic strength was the determining parameter of the effect of  $\text{Ca}^{2+}$  on gene transfer. But data from the present study indicated that the inhibitory effect was  $\text{Ca}^{2+}$  specific<sup>[7]</sup>: none of  $\text{K}^+$ ,  $\text{Na}^+$ ,  $\text{Mg}^{2+}$ , or  $\text{Sr}^{2+}$  at similar ionic strengths had any inhibitory effect. A similar argument could also be used to rule out the possibility that the divalent  $\text{Ca}^{2+}$  interacted with plasmid DNA via a charge interaction, which altered the stability and physical properties of DNA molecules. In addition, by injecting  $\text{Ca}^{2+}$  separately from the DNA solution, we also minimized the interaction between  $\text{Ca}^{2+}$  ions and plasmid DNA, but the inhibitory effect was still evident.

The data gathered during the separate injection experiment were quite striking. We found that  $\text{Ca}^{2+}$ -containing buffers could be delivered as late as 24 h after delivery of the plasmid to cause the inhibitory effect. Another important observation is that the inhibitory effect was only evident when  $\text{Ca}^{2+}$  was also delivered by electroporation, suggesting that  $\text{Ca}^{2+}$  possibly needs to enter the cells in order to exert its inhibitory effect. When delivered by simple needle injection,  $\text{Ca}^{2+}$  ions would be mostly present in the extracellular space, so the inhibitory effect would be limited.

The electroporation process would cause cell membrane disruption, which would allow  $\text{Ca}^{2+}$  influx into the cells to result in damage to the muscle cells<sup>[12]</sup>. During our experiments, we inspected the damage caused by electroporation with or without  $\text{Ca}^{2+}$ . There was significant damage in the muscle fibers caused by electroporation. However, there was no noticeable difference in the amount of damage with or without the addition of  $\text{Ca}^{2+}$ . However, it is still possible that the  $\text{Ca}^{2+}$  caused damage that was not detected by us.

Another possibility is that  $\text{Ca}^{2+}$  influx exerted an effect on cell membrane stability. A recent report suggested that the  $\text{Ca}^{2+}$  influx may initiate a membrane resealing mechanism<sup>[13]</sup>. In that study, cell behaviors were studied after ultrasound treatment, which is supposed to cause pores in the cell membrane. It was found that resealing of the cell membrane required extracellular  $\text{Ca}^{2+}$  to enter cells through the pores. So in our experiments, it is possible that the administration of different concentrations of  $\text{Ca}^{2+}$  ions effectively resulted in changes in cell membrane permeability to DNA molecules. The pores in the cell membrane caused by electro-pulses resealed quickly after  $\text{Ca}^{2+}$  influx to limit plasmid DNA entrance into the cells. Such a theory would also explain the importance of extracellular  $\text{Ca}^{2+}$  in *in vitro* electroporation

mediums, because *in vitro* electroporation conditions are harsher and are thought to result in more cell deaths. However, the exact mechanism for  $\text{Ca}^{2+}$  influx-induced cell membrane pore resealing is not clear, and requires further investigation.

In summary, we hope that the present study will attract more attention on the complexity of optimizing *in vivo* gene transfection by electroporation. Further studies to investigate the details of these effects will eventually build up a clearer picture of the underlying mechanism of this promising technology.

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