

Electroporation-mediated gene transfer in cardiac tissue

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Abstract Delivery of genes or macromolecules to cardiovascular tissues provides new therapeutic opportunities for the treatment of many acquired and inherited diseases. To investigate electroporation as a delivery method in cardiac tissue, embryonic chick hearts were studied for uptake of propidium iodide (PI) or DNA encoding either green fluorescent protein (GFP) or luciferase following electrical shock. PI uptake increased monotonically from 6% of heart tissue after 3 shocks to 77% with 12 shocks. GFP and luciferase expression varied in proportion to shock number, with detectable levels in all electrically treated hearts. Thus, electroporation promotes uptake of PI and DNA in cardiac tissue, suggesting further application of this method for therapeutic genes.

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Key words: Electroporation; Gene transfer; Green fluorescent protein; Luciferase; Embryonic chick heart

1. Introduction

A critical step in gene therapy is the efficient transfection of target cells or tissues [1]. The development of gene transfer methods such as viral delivery systems [2,3], calcium phosphate precipitation [4,5], lipofection [6,7], direct DNA injection [8,9], and microparticle bombardment [10], has led to areas of intensive study. While certain advantages exist for each of these techniques, limitations such as anatomical constraints, low transfection efficiencies, non-specific targeting, inconsistent reproducibility, safety concerns and cellular toxicity may restrict their applicability to target tissues [11]. Electroporation may provide an alternate or adjunctive method of tissue-targeted gene transfer which avoids some of these limitations.

Electroporation is a technique involving the application of short duration, high intensity electric field pulses to cells or tissue [12,13]. The electrical stimulus causes membrane destabilization and the subsequent formation of nanometer-sized pores. In this permeabilized state, the membrane can allow passage of DNA, enzymes, antibodies and other macromolecules into the cell. Electroporation holds potential not only in gene therapy, but also in other areas such as transdermal drug delivery and enhanced chemotherapy [13].

Electroporation is commonly used for *in vitro* gene transfection of cell lines [14,15] and primary cultures [16,17], but limited work has been reported in tissue. In one study, electroporation-mediated gene transfer was demonstrated in rat

brain tumor tissue [18]. Plasmid DNA was injected intraarterially immediately following electroporation of the tissue. Three days after shock treatment, expression of the lacZ gene or the human monocyte chemoattractant protein-1 (MCP-1) gene was detected in electroporated tumor tissue between the two electrodes but not in adjacent tissue. Electroporation has also been used as a tissue-targeted method of gene delivery in rat liver tissue [19]. This study showed that the transfer of genetic markers β -galactosidase (β -gal) and luciferase resulted in maximal expression at 48 h, with about 30–40% of the electroporated cells expressing β -gal, and luciferase activities reaching peak levels of ~ 2500 pg/mg of tissue. In another study, electroporation of early chicken embryos was compared to two other transfection methods: microparticle bombardment and lipofection [10]. Of the three transfection techniques, electroporation yielded the strongest intensity of gene expression and extended to the largest area of the embryo.

Cardiac tissue may be particularly amenable to permeation by electrical shocks, by virtue of its property as an electrical syncytium. Using stage 18 embryonic chick hearts as a cardiac model, we investigated the application of pulsed electrical shock as a method of effective gene delivery. Three different indicators of electroporation, propidium iodide (PI) and expression vectors for green fluorescent protein (GFP) and luciferase, were used to analyze the transfer of macromolecules into the tissue.

2. Materials and methods

Chick embryos were removed from 3–4-day eggs (Truslow Farms, Chestertown, MD, USA) through a hole made in the top of each egg. The membranous covering was removed, and the vessels severed. The embryo was placed on a sterile, dark background under a dissecting microscope. Each heart (stage 18) was carefully excised and transferred to a dish containing OptiMEM (Life Technologies, Gaithersburg, MD, USA).

2.1. Electroporation experiments

Hearts were individually placed between two aluminum electrodes, 0.4 cm apart, in a modified electroporation cuvette mounted on a glass coverslip (Fig. 1A), and subjected to a series of 200 V/cm electrical stimuli from a commercial stimulator (Model S44, Grass Instrument Company, Quincy, MA, USA). Varying numbers of 10-ms pulses were applied 10 s apart and monitored with a digital storage oscilloscope (Hitachi VC-6025, Tokyo, Japan). To avoid an accumulation of charge at the electrodes, the shock polarity was reversed after each shock. Hearts were subjected to one of the following three protocols.

2.1.1. Propidium iodide (PI). Hearts were bathed in culture medium containing 75 μ M propidium iodide (PI), a membrane-impermeant fluorescent probe for nucleic acids. Each heart was subjected to a shock triplet, a set of three 10-ms, 200-V/cm shocks applied 10 s apart (Fig. 1B, inset). Each triplet was applied at 5-min intervals until a cumulative total of 15 shocks (5 triplets) was achieved (Fig. 1B). Using a 510–560-nm excitation filter and a 590-nm emission filter,

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fluorescent photomicrographs were taken with an automatic photomicrographic system (model PM-10ADS, Olympus, Lake Success, NY, USA) on color slide film (Ektachrome P1600, Eastman Kodak, Rochester, NY, USA) immediately prior to and following the application of each shock triplet. Video images were obtained with a slide scanner (Coolscan LS-10, Nikon, Garden City, NY, USA) at a resolution of 205 pixels/inch and transferred into a desktop computer (Macintosh Quadra 650, Apple Computer, Cupertino, CA, USA). The level of PI introduced into each heart was quantified with commercial imaging software (Adobe Photoshop, Adobe Systems, San Jose, CA, USA). Each heart image was manually outlined to determine the total, two-dimensional area, and the red channel intensity of this area was analyzed. Histograms were created to depict the number of pixels at each pixel intensity level. The percentage of pixels fluorescing above 50% of the maximum intensity level achieved in a given image was calculated. This highly fluorescent area represented the uptake of PI into the tissue. The hearts were cultured at 37°C in a 5% CO₂ incubator for at least three days to determine tissue adherence to the culture surface and contractility, indicators of tissue viability.

2.1.2. GFP expression vector. Hearts were placed in 100 µl of OptiMEM containing plasmid DNA (50 µg/ml) with pEGFP, encoding CMV-driven GFP (Clontech Laboratories, Palo Alto, CA, USA). This plasmid is a GFP variant encoding a protein that fluoresces about 35 times more intensely than the wild-type GFP [20]. Hearts were subjected to 0–5 shock triplets and cultured on a collagen-based gel (described below). Hearts were photographed 1–2 days following shock treatment, and the green channel intensity of the digitized video images was analyzed for GFP expression. Only one group of shocks was applied to each heart, followed by a 1–2-day lag period to allow for protein expression. Since the total number of shocks differed for each heart, the images could not be normalized to one another based on a common cumulative total. Instead, based on the pixel intensity histograms, levels greater than 30 (a level exceeding normal baseline levels for unshocked hearts) were chosen to represent the area of the heart expressing GFP. Hearts not used for tissue sectioning were maintained in culture for up to three weeks to demonstrate tissue viability.

Following whole-heart analysis, serial sections were sliced from hearts subjected to 6 and 12 shocks. Hearts were placed in frozen tissue embedding media (Histoprep, Fisher Scientific, Pittsburgh, PA, USA) and fresh frozen in 2-methylbutane chilled to the temperature of liquid N₂. The frozen tissue block was placed in a motorized cryostat (Microm HM 500, Carl Zeiss, Thornwood, NY, USA) for 1–2 h to allow the temperature of the block to equilibrate to the temperature of the cryostat. Serial cryostat sections (16 µm) were sliced and placed on slides (Superfrost Plus, Curtin Matheson Scientific, Houston, TX, USA). Sections were photographed and images analyzed for GFP expression. Slides were wrapped in foil and refrigerated.

2.1.3. Luciferase. Plasmid DNA (65 µg/ml) encoding firefly luciferase (pRE-luciferase) with a Rous sarcoma virus (RSV) promoter/enhancer was introduced into hearts as in the GFP experiments. Hearts were subjected to either 0, 2 or 4 shock triplets and cultured for three days on a collagen gel (described below). Each heart was transferred to an Eppendorf tube, washed in phosphate-buffered saline solution (PBS) and placed in 100 µl reporter lysis buffer (Promega, Madison, WI, USA) for 20 min. The tissue was dissociated with an ultrasonicator until it was completely dissolved. Twenty microliters of each sample were added to 50 µl of substrate and assayed for luciferase activity in a luminometer (Monolight 2010, Analytical Luminescence Laboratory, Ann Arbor, MI, USA). Total protein content for each sample was determined (DC Protein Assay, Bio-Rad Laboratories, Hercules, CA, USA), and absorbances were read at 750 nm on a UV visible recording spectrophotometer (model UV-160, Shimadzu, Japan). Results were normalized to yield luciferase activity in light units (LU) per mg protein. Mean luciferase activities were reported for each shock number and statistically compared using the Wilcoxon rank sums test.

2.2. Adenovirus-GFP studies

Hearts were placed in 500 µl of OptiMEM containing varying concentrations of adenovirus-GFP. Three hearts were placed in each well, containing either 10⁶, 10⁷, 10⁸ or 10⁹ transduction units/ml of adenovirus-GFP and incubated at 37°C for 1 h. Hearts were washed 3 times

in fresh OptiMEM and cultured for 48 h on a collagen-based gel (described below). Hearts were then photographed, and the green channel intensity of the digitized video images was analyzed for GFP expression.

2.3. Collagen gel preparation

In a sterile 15-ml conical tube, 4.75 ml of type I rat tail collagen (Collaborative Biomedical Products, Bedford, MA, USA) was mixed with an equal amount of water and placed on ice. In another tube, 1.4 ml of 10× M199, 1.4 ml of 0.14 M NaOH and 2 µl of both penicillin-streptomycin and fungizone (Life Technologies) were mixed. The two tubes were combined and mixed thoroughly, and ~0.4 ml of solution was quickly pipetted into each well of a 24-well tissue culture plate. The plate was incubated at 37°C in a 5% CO₂ incubator for approximately 1 h. Each gel was rinsed 3 times with 1× M199 and 3 times with 1× OptiMEM (Life Technologies). One final layer of OptiMEM was applied to the gels, and the plate was stored in an incubator until needed.

3. Results

3.1. Propidium iodide uptake is directly related to the extent of electroporation

Embryonic hearts were individually placed in PI solution and subjected to electrical shock treatment. The fluorescent photomicrographs in Fig. 2 show the progression of PI uptake in one heart for increasing numbers of shocks. A control heart (panel A) was photographed prior to the application of electrical shock. There was no fluorescence above a pixel intensity of 128, indicating no uptake of PI. Panels B and C show data from the same heart after the application of 3 and 5 shock triplets, respectively. The significant increase in pixel intensity correlates with the increase in shock number and indicates an increase in the concentration of PI in the tissue. Hearts were cultured for two days following the electroporation procedure and were found to maintain their ability to anchor to a culture surface and spontaneously contract. This suggests that

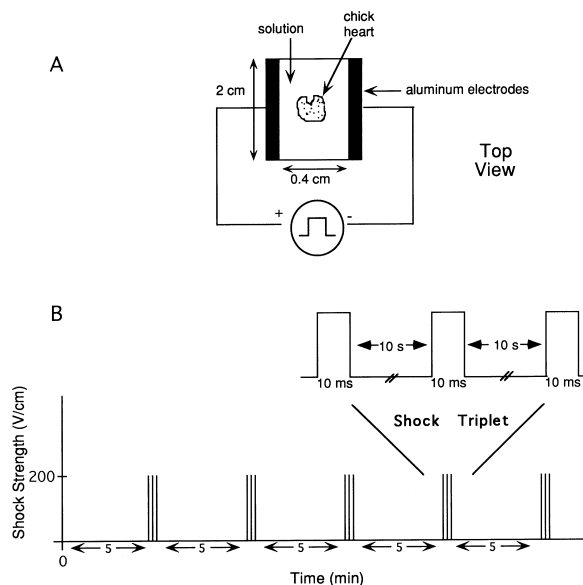


Fig. 1. Hearts were placed between two aluminum electrodes in solution containing PI or plasmid DNA encoding either GFP or luciferase (panel A). Hearts were subjected to a series of shock triplets, sets of three 10-ms, 200-V/cm shocks applied 10 s apart (panel B, inset). For PI experiments, each heart was subjected to a total of 5 shock triplets, at 5-min intervals (panel B). Hearts in DNA solution were subjected to 0–5 shock triplets in succession without the 5-min inter-shock interval.

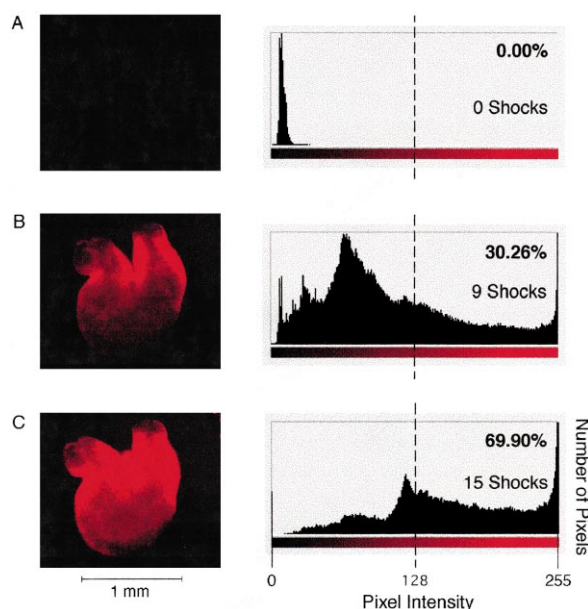


Fig. 2. Fluorescent heart images show a shock-dependent increase in PI uptake within the same heart. The images were photographed before exposure to electrical shock (panel A) and immediately following the 9th and 15th shock (panels B and C). The histograms depict the pixel percentage fluorescing above an intensity level of 128, which represents the uptake of PI into the tissue.

although PI binds nucleic acids in both viable and non-viable cells, most of the tissue was viable.

To determine when the PI was entering the cells, the hearts were photographed immediately prior to and following each shock triplet. The results are plotted in Fig. 3, as PI fluorescence vs. time. Arrows represent the application of each shock triplet, at 5-min intervals. At lower shock numbers, the slope of the line during the shock application (indicated by the arrows) was found to be greater than the slope during the 5-min inter-shock interval. This indicates that the fluorescence increase occurred primarily at the time of shock in comparison to the 5-min inter-shock interval. However, this effect diminished as higher shock numbers were attained. The total uptake of PI by the tissue leveled off at 15 shocks.

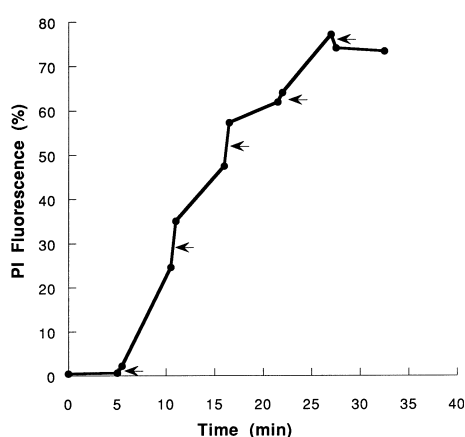


Fig. 3. Line graph of PI fluorescence vs. time. Arrows represent the application of a shock triplet, applied at 5-min intervals until a total of 15 shocks was achieved. Fluorescence intensities were averaged from 3 different experiments.

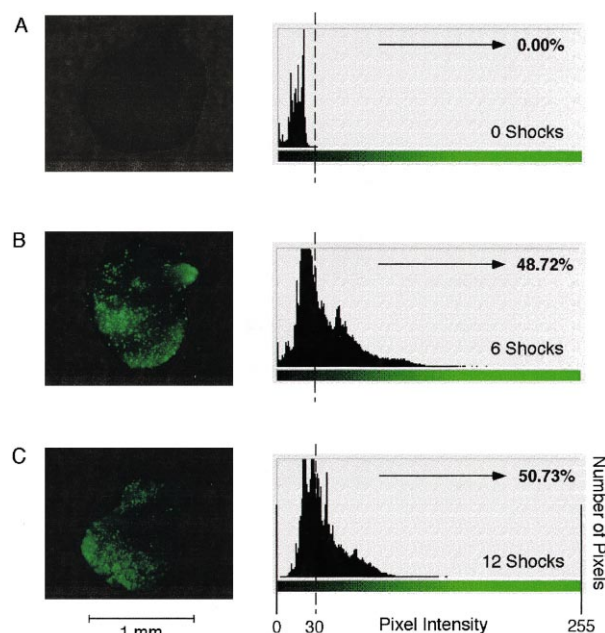


Fig. 4. Fluorescent images of GFP expression in electroporated hearts, photographed 48 h after shock treatment. Photos were digitized and the green channel intensity of the images analyzed. Histograms depict the pixel percentage fluorescing above an intensity level of 30, which represents the area of the heart expressing GFP.

3.2. Electroporation facilitates uptake of marker gene DNA

Fluorescent images of GFP expression in hearts 48 h after electroporation are shown in Fig. 4. GFP offers an advantage over PI in that it conclusively demonstrates cell viability following electroporation. As depicted by the histograms, the percentage of each heart image which fluoresced above a pixel intensity level of 30 is representative of the area of the heart expressing GFP. Hearts exposed to GFP DNA in the absence of electrical shock did not express detectable levels of GFP (panel A), whereas hearts subjected to 2 and 4 shock triplets (panels B and C, respectively) expressed GFP in relation to shock number.

Serial sections of hearts subjected to electrical shock revealed the penetrance of GFP expression into the tissue (Fig. 5). Panel A is a brightfield image of the serial section, and panel B is the corresponding fluorescent image. It is clear that gene expression in hearts subjected to electroporation is not limited to surface cells and is able to penetrate within the tissue.

To compare electroporation with another gene transfer method, adenovirus-GFP studies were performed in the same model. The mean percentage of pixels fluorescing above a level of 30 was calculated for hearts subjected to 6 or 12 electrical shocks and for hearts exposed to varying concentrations of adenovirus-GFP (Fig. 6). Electroporation was approximately equivalent to an adenovirus dose of 10^6 transduction units but was less effective than a 10^7 dosage. Higher concentrations of adenovirus saturated the total number of cells and transfected the entire tissue.

Luciferase activity in hearts subjected to 0, 2 and 4 shock triplets is shown in Fig. 7. The analysis of luciferase levels enables the gene expression in electroporated hearts to be quantified per mg total protein. The results indicate an increase in mean luciferase activity with an increase in the num-

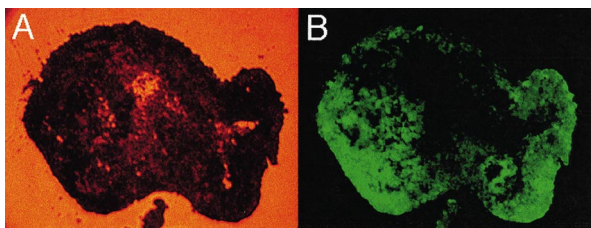


Fig. 5. Images of a 16- μ m thick tissue slice, from a heart subjected to 12 electrical shocks. Hearts were sliced 48 h after electroporation. Panel A is the brightfield image and panel B is GFP expression in the corresponding fluorescent image. Images were analyzed as in Fig. 4.

ber of applied shocks. No detectable levels of luciferase activity were observed in control hearts, whereas hearts subjected to 6 shocks expressed a mean luciferase activity level of $8.2 \pm 3.8 \times 10^6$ LU/mg protein (mean \pm S.E.M.), which increased to $12.9 \pm 5.3 \times 10^6$ LU/mg protein in hearts subjected to 12 shocks. Luciferase levels in electroporated hearts were statistically different than those in control hearts ($P < 0.05$); however, there was no statistical difference between hearts subjected to 6 and 12 shocks ($P < 0.05$).

4. Discussion

To investigate electroporation as a method of gene transfer in cardiac tissue, we electrically shocked chick embryonic hearts in a bath containing one of three markers. The first marker studied, propidium iodide, offered the advantage of an immediate response with membrane permeabilization [13]. The absence of a delay for PI visualization also meant that the increase in fluorescence could be monitored with time and with increasing numbers of shocks within the same heart. We found that the fluorescence levels increased monotonically with greater numbers of pulses until reaching a plateau at

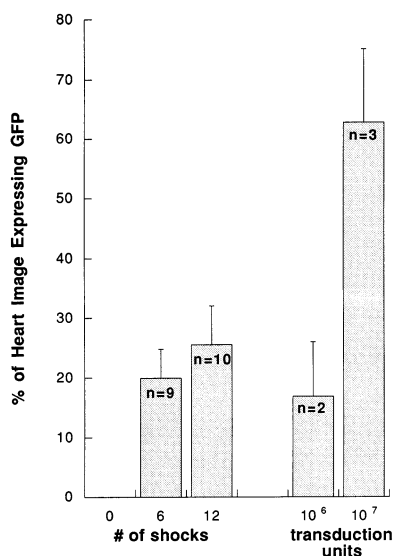


Fig. 6. Comparison of electroporation with adenoviral-mediated transduction. The percentage of heart tissue expressing GFP in hearts subjected to electroporation or adenovirus-GFP was determined by measuring the percentage of pixels fluorescing above an intensity level of 30. Images were analyzed as in Fig. 4. Bars represent means \pm S.E.M.

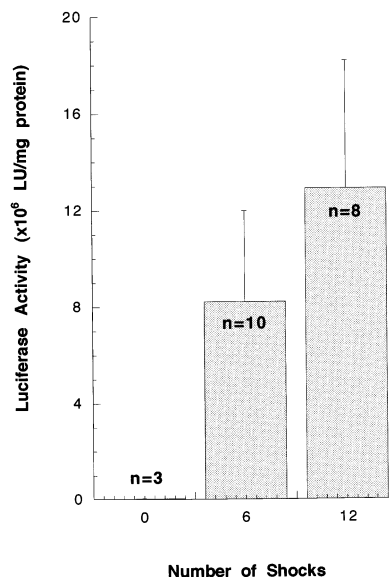


Fig. 7. Bar graph of luciferase activity relative to shock number. Hearts were assayed for luciferase activity 3 days after shock treatment. Results were normalized and mean luciferase values \pm S.E.M. were reported for each shock number. Data from electroporated vs. control hearts were statistically different, with $P < 0.05$ (Wilcoxon rank sum test).

12 shocks (Fig. 3). Entry of PI into the heart primarily occurred during the shock application. The total, cumulative PI fluorescence was observed in up to 75% of the total area of the heart. A limitation of this approach was the inability to distinguish the exact proportion of PI fluorescent cells that remained viable after the shock. However, hearts maintained their ability to anchor to a culture surface and continued to spontaneously contract in culture, indicating the tissue as a whole was healthy.

The second marker, plasmid DNA with pEGFP, was studied in whole heart and serial tissue sections. GFP expression was observed in all hearts subjected to electrical shock, whereas no expression was observed in unshocked, control hearts (Fig. 4). By varying the plane of focus, GFP expression was visible throughout the wall of the heart. To gain insight into the depth of marker penetration, our GFP analysis was extended to study 16- μ m serial sections of the tissue. GFP expression was observed in all tissue slices well below the surface of the tissue (Fig. 5).

The final marker protein studied, luciferase, allowed a more precise quantification of marker gene expression in relation to the whole tissue. Luciferase expression in electroporated hearts increased with increasing numbers of shocks (Fig. 7), reaching a level of $12.9 \pm 5.3 \times 10^6$ LU/mg protein with 12 shocks.

4.1. Relative efficiency of electroporation as a gene transfer method

To our knowledge, there have been no reports in cardiac tissue using the same reporter genes with which we can compare electroporation as a method of transduction. Thus, we performed additional experiments utilizing adenovirus as the transduction method. Electroporation was found to be as approximately equivalent to adenovirus-GFP at a concentration of 10^6 transduction units/ml but not as effective when com-

pared to higher viral concentrations. However, electroporation is advantageous in that it has *in vivo* possibility as a tissue-specific gene transfer technique through the judicious placement of electrodes, and it does not suffer from the same safety concerns that accompany viral-mediated transduction techniques.

In other related studies, four non-viral transfection methods were studied in primary cultures of neonatal cardiac myocytes to determine the most effective method of overexpressing *c-myc* and *c-fos* proto-oncogenes [16]. The transfection efficiencies of the cardiac myocytes for calcium phosphate precipitation, DEAE-dextran treatment, liposome-mediated transfection and electroporation were determined by using the bacterial chloramphenicol acetyltransferase (CAT) reporter gene. CAT expression levels were undetectable in cells transfected by calcium phosphate precipitation and DEAE-dextran treatment. CAT expression levels as high as 51.9 ± 0.6 pmol/mg protein/h were detected using the liposome-mediated transfection method, but were significantly less than those obtained with electroporation, which were as high as 103.3 ± 0.3 pmol/mg protein/h. Thus, electroporation was determined to be the optimal gene delivery method. Electroporation has also been compared in early chicken embryos to two other non-viral transfection techniques: microparticle bombardment and lipofection [10]. Using *lacZ* as a reporter gene, that study showed the intensity of gene expression to be greatest in embryos transfected by electroporation. The expression also extended throughout a larger area of the embryo when electroporation was used. The three transfection methods were also compared in mouse testis, and electroporation again proved to be the best gene delivery method.

4.2. Targeted gene transfer

There are several promising therapeutic applications of cardiac gene transfer, including the treatment and prevention of both inherited and acquired cardiovascular diseases [11,21]. Targeting of gene transfer in rat cardiac tissue has been reported using intramuscular injection of plasmid DNA [8]. Limited success was achieved utilizing this technique alone as β -gal expression was limited to a small area of the heart, and the fraction of myocytes expressing the recombinant protein was minimal. The inability to target a large area of the tissue and the relatively low transfection rate could potentially be improved if this technique were used in conjunction with electroporation. This combination was examined in a study measuring luciferase and β -gal expression in rat liver tissue [19]. Hepatic DNA injection followed by electrical shock provided a higher transfection efficiency than DNA injection alone. Another study examined *in vivo* targeted gene transfer in rat brain tumors using electroporation followed by intra-arterial plasmid DNA injection [18]. Human MCP-1 cDNA was transferred and expressed in shocked tumor tissue for at least 3 weeks. These studies suggest a possible future opportunity for *in vivo* targeted gene transfer in cardiac tissue by utilizing a combination of electroporation and direct DNA injection.

In the present study we demonstrate that electroporation is a technique capable of reversibly permeabilizing cardiac cellular membranes, allowing for introduction of macromolecules and plasmid DNA into the cell. Variables such as electrical parameters of the applied shock, DNA concentrations and age of the heart were not tested but may play a role in the efficacy of transfection. Electroporation-mediated gene transfer may be especially valuable in augmenting delivery of macromolecules not only to heart tissue but also to other electrically coupled tissues containing gap junctions, such as smooth muscle, liver and vascular endothelial tissue. Such tissues develop larger transmembrane potentials and hence lower effective electroporation thresholds than tissues without gap junctions.

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