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ORIGINAL RESEARCH PAPER

Efficient electroporation of peptides into adherent cells: investigation of the role of mechano-growth factor in chondrocyte culture

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Abstract Peptide therapeutics are of increasing interest due to their biological specificity. We used a simple technique to study the efficacy of inducing peptides into adherent chondrocytes by transiently permeabilizing the membrane with electric pulses (in situ electroporation). Mechano-growth factor (MGF) was selected as a model peptide. FITC-labeled MGF was added to cultures of adherent primary chondrocytes grown on ITO coated glass slides. Cells were subjected to 3–9 pulses of 175–275 V and evaluated by flow cytometry. Under optimal conditions, an electroporation efficiency of close to 50% could be achieved. This technique can be used to study the functional domains of intracellular peptides, peptide inhibition of signal transduction and intracrine-mediated effects of peptides in adherent cells.

Keywords Chondrocytes · In situ electroporation · Indium tin oxide · Mechano-growth factor · Peptide therapeutics

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Introduction

Biologic drugs, including peptides, antibodies, antisense RNA and siRNA, represent one of the fastest growing segments of the pharmaceutical industry. Their administration is challenging due to their poor stability and the difficulty in penetrating the cell and nuclear membrane. These drugs, however, are appealing due to their unsurpassed biological specificity and activity as well as minimal cytotoxicity. Although there have been many recent advances in peptide transport mechanisms including modification of molecules with cell permeable peptides (Torchilin et al. 2001; Dom et al. 2003) and the development of peptide nanostructures, there is still a great need for fundamental studies in which *unmodified* peptides can be easily introduced into adherent cells and their nuclei to study their mechanism of action. Electroporation is a well established, versatile technique which transiently permeabilizes the cell membrane, allowing entry of peptides, siRNA and DNA vectors. This technique is available in multiple commercial formats, for in vivo applications (Swartz et al. 2001) and has been amenable to technological advances and specializations including in situ electroporation of adherent cells (Brownell et al. 1997; Firth et al. 1997), electroporation on electronic chips (Yamauchi et al. 2004), and even single cell electroporation arrays (Khine et al. 2007). In situ electroporation has been a particularly relevant advance for the study of connective tissue cells, as it is not necessary to disrupt their focal adhesions before

treatment. The goal of this study was to optimize the efficiency of in situ electroporation-induced peptide entry into large populations of mammalian primary cells grown in adhesion culture.

Mechano-growth factor (MGF) was selected as a model peptide for these studies because of its putative intracrine mode of action. MGF is a splice variant of insulin-like growth factor 1 (IGF-1) (Barton 2006) whose expression is regulated by dynamic stretch of muscle tissue. Biologically, MGF renews the myogenic satellite (stem) pool, thus allowing for subsequent rounds of muscle repair and hypertrophy (Yang and Goldspink 2002; Hill and Goldspink 2003; Goldspink 2005). Evidence is accumulating to suggest that MGF is one of the key trophic agents which protect many tissues in the body from mechanical-damage and degeneration by inducing proliferation of stem cells and repair of over-strained or challenged tissues (Dluzniewska et al. 2005; Carpenter et al. 2007). MGF is thought to exert its effect independently of the IGF-1 receptor and, in accordance with a nuclear localization sequence present in its unique *C*-terminal domain, transfection studies reveal that it localizes to the nucleus (Goldspink 2005). We hypothesized that inducing the direct entry of MGF into cells/nuclei, could be an important system in which to study the biological activity of this peptide. In situ electroporation was used to deliver pulses of direct current to adherent chondrocytes and the uptake of peptide was confirmed by fluorescence microscopy and flow cytometry.

Materials and methods

In situ electroporation device

The electroporation device used has been described in the literature (Brownell et al. 1997; Raptis and Firth 2008) and consists of a stainless steel electrode connected to a pulse generator, an indium/tin oxide (ITO)-coated glass slide, and a polydimethylsiloxan (PDMS) frame (32 × 10 × 0.74 mm) (Fig. 1, right). The PDMS frame was pressed onto the ITO-coated glass slide (50 × 50 mm, UQG Optics Ltd) resulting in a water-tight chamber into which cells were seeded and culture media could be held. The voltage source was a function generator (SRS DS345 Synthesized Function Generator) supplying DC from 0.2–3 V. This signal was amplified 1250-fold by a high-voltage amplifier and divided down by a resistive voltage divider network (Fig. 1, left). This system was used in conjunction with a capacitive discharge circuit to produce pulses. The current traveled from the positive electrode, through the culture media, cells and ITO glass to the negative electrode. Initial investigations to determine the optimal electroporation parameters were performed using Lucifer yellow staining, a strongly fluorescent dye which normally does not pass the cell membrane. Furthermore, a scratch in the ITO-coated provided a non-electroporated control area within the same glass slide.

Mechano growth factor (MGF) peptide

The 25 amino acid *C*-terminal MGF peptide (YQPP STNKNTKSQRRKGSTFEEHKC) from GenScript, Inc. was end-labeled with *N*-terminal fluorescein isothiocyanate (FITC) and had a purity 90.6%. Immediately prior to experiments, the peptide was dissolved in calcium-free DMEM (US Biological) at 0.1 mg/ml. In order to determine if other peptides translocated to the nucleus, FITC-labelled insulin (Sigma) was also studied using the same electroporation parameters as MGF.

Cell culture and electroporation

Primary chondrocytes were isolated from the knees of 6 months old calves using a collagenase and pronase digestion (Wong et al. 2001) and cultured in DMEM + GlutaMax media (Invitrogen) supplemented with 10% (v/v) fetal bovine serum. Passaged cells were released with trypsin–EDTA and between 150,000 and 300,000 cells were seeded onto the PDMS frame. The glass slide was placed inside a Petri dish and incubated for 24 h. Prior to electroporation, the medium was aspirated and the cells gently washed with calcium-free and serum-free DMEM. This culture medium was removed and the electrode was placed on top of the cells, resting on the PDMS frame. A small hole (about 1 mm^2) between each of the two lower corners of the anode (facing the cathode) and the PDMS frame allowed the injection of the electroporation medium (300 µl). The electrode was connected to the capacitive discharge circuit and the voltage was applied. To identify the optimal conditions for electroporation of the peptide, the voltage was varied from 175 to 275 V and

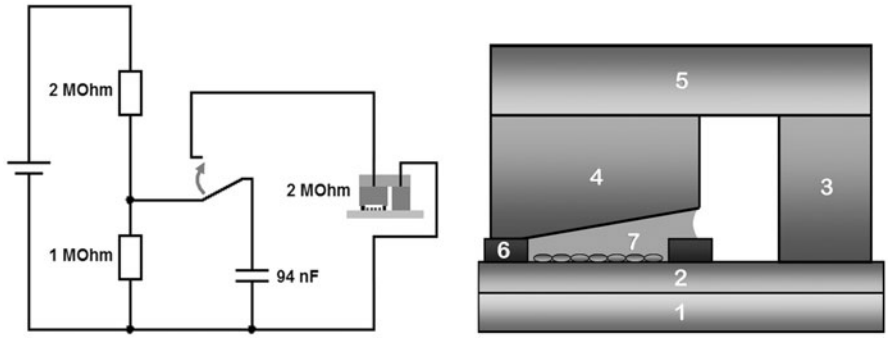

Fig. 1 *Left*: Electrical circuit. In charge mode, the divider network (2, 1 MOhm) divides the input voltage by three and charges the capacitance (94 nF) over the switch. When the switch is thrown, the system changes to discharge mode and the capacitance is discharged over the load (electrode-cell-slide complex, ~2 MOhm). *Right*: In situ electroporation device modified from Raptis and colleagues: *1* glass slide, *2* ITO layer, *3* negative electrode, *4* positive electrode, *5* holder, *6* PDMS frame, *7* adherent cells with electroporation medium

3–9 pulses were applied. After electroporation, the electrode was removed and the cells were incubated in calcium-free media at room temperature for 2 min to allow the pores to seal. The cells were washed two times with calcium free DMEM and the cell fluorescence was examined with a fluorescence microscope.

Flow cytometry

Flow cytometry acquisition and analysis of the electroporated chondrocytes were performed on a FACSAria (BD Biosciences) with a blue (488 nm) solid state laser (530/30 and 502LP filter) to determine the percentage of MGF–FITC containing cells. The cells were trypsinized (800 µl, 25% trypsin/EDTA, Gibco) and centrifuged. The cell pellet was resuspended in 500 ml PBS buffer and filtered through a 50 µm CellTrics filter (Partec). A negative control sample was used which was treated in the same way, except the MGF solution was replaced by calcium-free DMEM. Mean fluorescence intensity (MFI) was calculated based on the photon flux/time of each sorted cell, and the start of the gate was chosen as the point where the highest fluorescence intensity occured in the negative sample. The ratio of MFI between each sample and the negative control was calculated using FlowJo software (ver. 8.8.7).

Results

Fluorescence microscopy revealed that nearly 100% of the cells had incorporated Lucifer yellow upon electroporation (Fig. 2a), whereas ~30% of the cells had taken up the MGF–FITC peptide (Fig. 2d) under the same conditions. No fluorescence was detected in the non-electroporated controls which were incubated with MGF–FITC (Fig. 2b) and the morphology of the cells was not visibly effected by the electroporation treatment (Fig. 2c). For the Lucifer yellow, MGF and insulin (not shown), the fluorescence was strongest in the nuclei of the cells, indicating the molecules had passed through the cell and nuclear membranes.

Figure 3 describes the flow cytometry results showing the dependence of fluorescence intensity factor on cell density and the applied voltage. With three pulses, the highest fluorescence intensity factor (7.2) was with 150,000 cells 225 V, with the scatter plot showing a clear delineation of this population of cells based on their fluorescence intensity (Fig. 3). In experiments in which the pulse number was varied, the highest percentage of fluorescing cells was with six pulses (46%).

Figure 4 shows the increased incidence of fluorescing cells as well as their higher fluorescence intensity after electroporation with increasing pulse number. The shift in the intensity curve demonstrated that cells exhibited greater fluorescent intensity with six pulses. Applications of nine pulses decreased the cell viability reflecting a "burning" of the cells on the ITO glass slides. The flow cytometry revealed only 5.6% cells were positively labelled in non-electroporated controls, indicating that the peptide entry was largely mediated by membrane electropermeabilization.

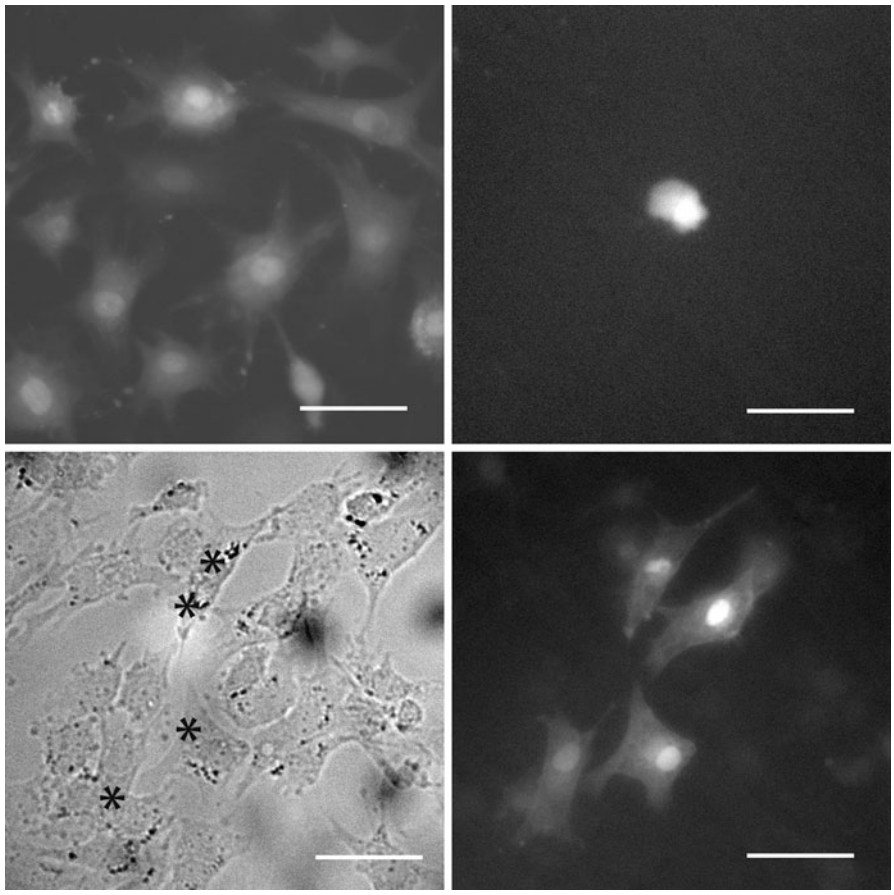

Fig. 2 **a** Adherent chondrocytes electroporated with Lucifer yellow at optimal conditions (225 V, 150,000 cells, 3 pulses). **b** Cells incubated with MGF–FITC in the absence of electroporation showing no fluorescence. Bright field (**c**) and fluorescence (**d**) image of cells electroporated in the presence of MGF–FITC. *Stars* in (**c**) mark the four cells in (**d**) showing uptake of MGF–FITC. *Scale bar* is 50 microns

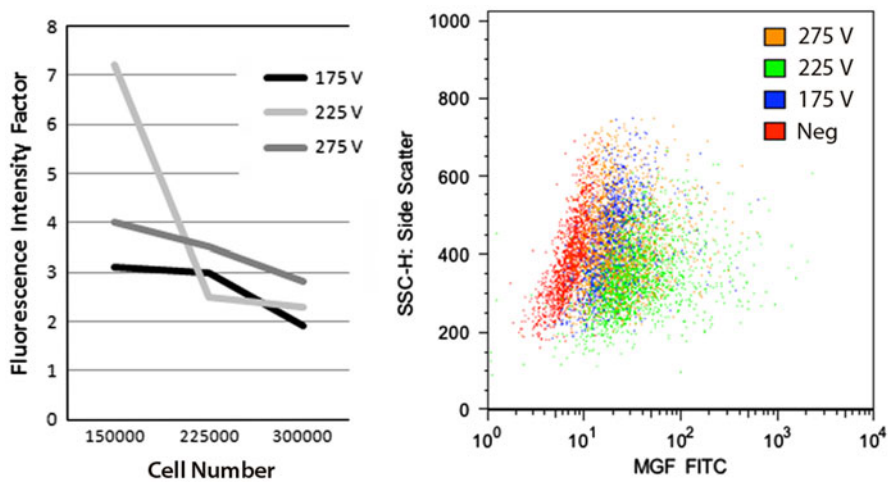

Fig. 3 *Left*: Fluorescence intensity factor versus cell number for the three voltages studied. *Right*: Scatter plot of fluorescence versus side scatter (150,000 cells/well) for electroporation with MGF–FITC at 275 V (*orange*), 225 V (*green*), 175 V (*blue*) and with serum-free DMEM (*red*). Number of sorted cells were 2580, 3030, 2610 and 2445 respectively

Discussion

In situ electroporation is a simple, effective method for introducing peptides into large numbers of adherent cells. One major advantage of this technique over conventional electroporation is that it is not necessary to release the cells from their substrate using proteolytic digestion, a process that damages cells and disrupts their native cell–cell and cell–substrate adhesions. Maintaining functional connection with the

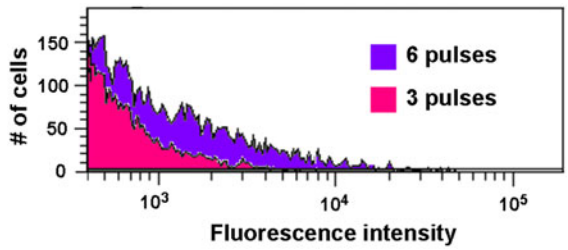

Fig. 4 Effect of 3 and 6 pulses on fluorescence intensity and number of cells uptaking MGF–FITC investigated by (electroporated at 225 V, 150,000 cells)

substrate and other cells is important given recent evidence showing that cytoskeletal disruption inhibits normal biochemical signaling via the nucleus (Nelson and Nusse 2004). Normally, adherent cells, while in suspension, might not be sensitive to the immediate effects of introduced biologically active peptides. Furthermore, cytoplasmic-nuclear traffiking requires cytoskeletal integrity. Finally, regions of electroporated and non-electroporated cells can be examined in adjacent regions depending on the patterning of the conductive substrate.

In our system the optimal conditions for electroporation was at 225 V, with six pulses and 150,000 cells/well. One possible reason for the lower electroporation efficiency above 150,000 cells/well could be that the current flows through a greater number of cells per given area of substrate, thereby effectively reducing the potential that each cell experiences. Lower than 225 V, it is likely that fewer cells reach the threshold value for membrane permeabilization. The decrease in the number of fluorescent cells at higher voltages (275 V) could be related to the fact that higher voltages might damage cells. It is important to note, that these conditions have been optimized for one cell type and the optimal parameters for other cell types might differ.

Further development of the in situ electroporation technique described here will focus on the transfection of plasmid DNA and siRNA. Yamauchi et al. (2004, 2005) used layer-by-layer assembly of poly(ethyleneimine) and plasmid DNA to coat ITO-electrodes and could obtain temporal spatial control of plasmid vector expression upon application of electric pulses. For a spatially specific parallel transfer of multiple genes, they further developed a small interfering RNA (siRNA) microarray (Fujimoto et al. 2008). This system uses the base-pairing properties of nuclei acids by adding labeled RNA molecules to single DNA spots resulting in spatially-specific gene silencing (Fujimoto et al. 2006).

The results of this study illustrate the versatility of using in situ electroporation to investigate diverse biological processes. Here we show that peptides, which typically do not enter the cytosol (MGF), can be induced to do so using in situ electroporation. Other peptides, such as insulin, readily enter the nucleus (Goldfine et al. 1977) both with and without electroporation. Our initial results and those of others suggest that in situ electroporation of DNA is useful for the spatial and temporal control of protein expression, or silencing of targeted genes.

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