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# Effects of Electrical Fields on Cardiomyocyte Differentiation of Embryonic Stem Cells

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**Abstract** The effects of electromagnetic fields (EMFs) on the differentiation of cardiomyocytes in embryoid bodies derived from pluripotent embryonic stem (ES) cells were investigated. A single direct current (DC) field pulse was applied to 4-day-old embryoid bodies. The electrical field induced a hyperpolarization of the anode-facing side of embryoid bodies and a depolarization at the cathode-facing side. Significant effects of a single electrical field pulse applied for 90 s on cardiomyocyte differentiation were achieved with field strengths of 250 and 500 V/m, which increased both the number of embryoid bodies differentiating beating foci of cardiomyocytes and the size of the beating foci. The 500-V/m electrical field increased intracellular reactive oxygen species (ROS), but not $[Ca^{2+}]_i$ and activated nuclear factor kappa B (NF-κB). A comparable increase in the number of beating embryoid bodies was achieved by an incubation for 1 h with $H_2O_2$ (1–10 nM), indicating that the electrical field effect was transduced via the intracellular generation of ROS. Because the radical scavengers dehydroascorbate and pyrrolidinedithiocarbamate (APDC) and the NF-κB antagonist N-tosyl-L-phenylalanine chloromethyl ketone (TPCK) inhibited cardiac differentiation, we assume that ROS and NF-κB may play a role in early cardiac development. J. Cell. Biochem. 75:710–723, 1999. 

**Key words:** electromagnetic field; reactive oxygen species; embryoid body; nuclear factor κB

There is increasing experimental evidence that externally applied electromagnetic fields (EMFs) exert various effects on embryonic cells and impair normal embryonic development [Robinson, 1985; Cameron et al., 1993; Jaffe and Nuccitelli, 1977]. By contrast, it has been discussed for years that, during embryo development, not only diffusible chemical gradients, but also endogenous electrical fields, may determine the emergence of spatial patterns [Nuccitelli, 1992].

Currents arise by unidirectional ion transport in epithelial cell layers and generate an essentially DC voltage across the layer [Jaffe and Nuccitelli, 1977]. Hence, currents will flow through tissues and organs enclosed by polarized epithelia and will consequently lead to the formation of an electrical field. During early vertebrate embryogenesis the morula develops to a blastula and the embryo gets organized into a simple epithelium, the blastoderm. In later stages of development, all three germ layers form epithelia that could give rise to endogenous EMFs of variable strengths as a result of unidirectional transport processes. In *Xenopus* [Robinson and Stump, 1984; Hotary and Robinson, 1994] and chicken embryos [Jaffe and Stern, 1979], the blastopore has been shown to be a site of strong outward ionic current; it has been suggested that the transembryonic current, which evokes EMFs, may guide migrating cells by galvanotaxis. In 4-day-old chicken embryos a significant intraembryonic electrical field with a field strength of 20 V/m has been demonstrated (Hotary and Robinson, 1990). Large currents with a current density of approximately 20 $\mu A/cm^2$ have been reported to leave the primitive streak in mouse embryos [Winkel and Nuccitelli, 1987]. These observations suggest that endogenous electrical fields play an important role during normal embryogenesis and indicate that disturbance of the endogenous field by environmental electrical fields may result in abnormal embryonic development.

In the present study, the effects of an externally applied electrical field on the differentiation pattern of embryoid bodies grown from

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pluripotent embryonic stem (ES) cells were studied. ES cells resemble cells of the inner cell mass and primitive ectoderm. When grown in methylcellulose-supplemented suspension culture to spherical embryoid bodies with a volume of approximately 0.03 $mm^3$, they recapitulate many aspects of embryogenesis. These include formation of postimplantation embryonic tissues such as embryonic endoderm, ectoderm, and mesoderm with the potential of hematopoiesis [Wiles, 1993], cardiogenesis [Maltsev et al., 1994], neurogenesis [Strübing et al., 1997], and angiogenesis [Vittet et al., 1996; Wartenberg et al., 1998]. We applied a single electrical field pulse in a range of 100–500 V/m and a duration of 90 s, which is in the order of magnitude of endogenous "physiological" DC electrical fields that naturally occur in animal tissues (10–200 V/m) [Nuccitelli, 1992] on 4-day-old undifferentiated embryoid bodies. At field strengths of 250 and 500 V/m a marked enhancement of cardiomyocyte differentiation was observed subsequent to plating of embryoid bodies 3 days after the electrical field treatment. We further tested whether the electrical field activated the transcription factor NF-κB, which was previously demonstrated to be induced by ROS and to initiate the transcription of a variety of genes involved in inflammation, immune response, lymphoid differentiation, growth control, and development [Sun and Oberley, 1996; Beauparlant and Hiscott, 1996]. Because the externally applied electrical field pulse induced changes in the intracellular redox state, an endogenous electrical field may act via modulation of the intracellular redox state level and activation of redox-regulated transcription factors (e.g., NF-κB), which in turn induce mesodermal development and, in particular, cardiogenesis.

## MATERIALS AND METHODS

### Cell Culture

The permanent embryonic stem cell line CCE [Robertson et al., 1986], cultivated in the undifferentiated state on primary cultures of mouse embryonic fibroblasts, was used throughout the study. Cells were cultivated on feeder layers in Dulbecco's modified Eagle's medium (Gibco-BRL, Life Technologies, Germany) supplemented with 15% fetal calf serum (FCS) (Boehringer Mannheim, Germany), L-glutamine (2 mM) (Gibco-BRL), β-mercaptoethanol (final concentration $5 \times 10^{-5}$ M) (Sigma Chemical Co., Deisenhofen, Germany), nonessential amino acids (NAA; Gibco-BRL; stock solution diluted 1:100), 100 U/ml of penicillin, 0.1 mg/ml of streptomycin (Gibco-BRL) and leukemia inhibitory factor (LIF) (10 μg/ml) in a humidified atmosphere containing 5% $CO_2$. For differentiation, single embryonic stem cells at a cell density of 300–600 cells/ml were cultivated into the spherical three-dimensional tissue of embryoid bodies in Iscoves's modified Dulbecco's medium (IMDM) (Gibco) containing 0.9% methylcellulose (Methocel MC, Fluka, Germany), 20% FCS, and supplemented with the same additives as described above. Electrical fields were applied to 4-day-old embryoid bodies. Subsequent to electrical field treatment control and treated samples were further cultivated in methylcellulose-supplemented IMDM containing 1 mM L-glutamine and $2.5 \times 10^{-5}$ M β-mercaptoethanol. After 7 days in suspension culture embryoid bodies were plated into 24-well multiwell plates (Falcon) containing IMDM devoid of methylcellulose. Beating outgrowing embryoid bodies were counted from day 1 after plating (7 + 1) to day 6 after plating (7 + 6). Experiments were performed with embryoid bodies of the age ranging from 2 to 13 days.

### Electrical Field Treatment and Confocal Laser Scanning Microscopy

Electrical field pulses were applied to embryoid bodies under the optical control (transmission mode) of an inverted confocal laser scanning microscope (LSM 410, Zeiss, Jena, Germany) using either a 10× objective N.A. 0.5 or an oil-immersion 25× objective N.A. 0.80 (Neofluar, Zeiss, Jena, Germany). Embryoid bodies were suspended in a low ionic content "pulsing buffer," which contained (in mM) sucrose 255, $CaCl_2$ 1, $MgCl_2$ 1, and HEPES 5 (pH 7.2) and had a conductivity of 500 μS/cm. They were then placed in an incubation chamber between stainless steel (V4A) electrodes with an electrode area of 0.4 $cm^2$ and an electrode distance of 0.2 cm. The electrodes were connected to a custom made electropulser generating square-wave electric pulses. A single electrical field pulse with field strengths of 100, 250, and 500 V/m and a duration of 90 s, unless otherwise indicated, was applied to embryoid bodies and monitored by an oscilloscope. A field strength of 500 V/m resulted in a total current

in the chamber of 1 mA, corresponding to a current density of 2.5 mA/cm$^2$. The magnetic flux density in the proximity of the embryoid bodies was calculated to 0.079 μT, which is below the average laboratory noise level for low-frequency EMFs (≃0.5 μT) [Cameron et al., 1993]. Before conducting the experiments, we ensured that no water electrolysis, pH, or temperature shifts occurred in the pulsing chamber. Furthermore, we excluded by luminol-dependent chemiluminescence [Wymann et al., 1987] that $H_2O_2$ was generated in the pulsing chamber during the experiment. The untreated control sample of embyroid bodies was incubated in the pulsing chamber filled with pulsing buffer for the same time period as the treated sample. After the experiment electrical field-treated and control embryoid bodies were placed in 8.5-cm bacteriological Petri dishes containing IMDM medium and cultivated as described above.

### Imaging of $[Ca^{2+}]_i$ and Cardiomyocyte Contractions

$[Ca^{2+}]_i$ was monitored using the fluorescent dye fluo-3 acetoxymethylester (Molecular Probes, Eugene, OR). Embryoid bodies were loaded for 45 min in IMDM cell culture medium with 10 μM fluo-3, AM, dissolved in dimethylsulfoxide (DMSO) (final concentration 0.1%) and pluronic™ F-127 (Molecular Probes) (final concentration <0.025%). After loading, the embryoid bodies were rinsed three times either in E1-buffer containing (in mM) NaCl 135, KCl 5.4, $CaCl_2$ 1.8, $MgCl_2$ 1, glucose 10, HEPES 10 (pH 7.4 at 37°C) or in pulsing buffer and placed in the experimental chamber. Determinations of $[Ca^{2+}]_i$ changes during electrical field treatment were performed in pulsing buffer, whereas measurements on $[Ca^{2+}]_i$ spikes and contractions of cardiomyocytes were performed in E1-buffer. For fluorescence excitation the 488-nm line of an argon ion laser of the confocal setup was used. Emission was recorded with a 515-nm longpass filter. To determine contractions of cardiomyocytes, transmission images and fluorescence images were recorded simultaneously using the overlay option of the confocal setup. Contractions of cardiomyocytes caused changes in light diffraction in the transmission image and were monitored as deviations from the basal gray level. One full frame image (256 × 256 pixels) was captured every 0.365 s. Data are presented in arbitrary units as percentage of fluorescence or transmission graylevel variation F with respect to the resting level $F_0$.

### Imaging of Membrane Potential During Electrical Field Treatment

The membrane potential-sensitive dye di-8-ANNEPS (Molecular Probes, Eugene, OR) was used to monitor electrical field-induced membrane potential changes in embryoid bodies. A membrane potential change of 100 mV results in a fluorescence change of 8–10% [Gross et al., 1986]. Embryoid bodies were incubated with di-8-ANNEPS (dissolved in ethanol, final concentration 1μM) in E1 buffer for 30 min and subsequently washed twice. Excitation was performed using the 543-nm line of a helium-neon laser of the confocal setup. Emission was recorded using a longpass LP 590-nm filter set.

### Determination of the Intracellular Redox State

Intracellular redox state levels were measured using the fluorescent dye dichlorofluorescein diacetate (DCFH-DA) (Molecular Probes), a nonpolar compound that is converted into a nonfluorescent polar derivative (DCFH) by cellular esterases after incorporation into cells. The membrane-impermeable DCFH is rapidly oxidized to the highly fluorescent 2′,7′-dichlorofluorescein (DCF) in the presence of intracellular ROS [Frenkel and Gleichauf, 1991].

For electrical field experiments, embryoid bodies were incubated in IMDM medium containing 20 μM DCFH-DA for 30 min at room temperature. Embryoid bodies were then washed twice in IMDM medium and once in "pulsing medium" and transferred into the experimental chamber. For fluorescence excitation, the 488-nm line of the argon ion laser was used. Fluorescence emission recording was performed with a longpass LP515 nm filter set. Full frame images (512 × 512 pixels) were taken every 5 s. DCF fluorescence was evaluated in 5,000-μm$^2$ regions of interest (ROIs) in the center of embryoid bodies. Data are presented in arbitrary units as percentage of fluorescence variation F with respect to the resting level $F_0$.

### Immunohistochemistry

Immunohistochemistry was performed with whole mount embryoid bodies. In brief, embryoid bodies were washed three times in phos-

phate-buffered saline (PBS) and subsequently fixed in ice-cold 7:3 methanol/acetone for 60 min. After washing for three times with PBS (pH 7.4) containing 0.1% Triton X-100 (PBST), embryoid bodies were incubated for 1 h in PBS containing 10% milk powder, to block against unspecific binding. Incubation with primary antibodies was performed for 1 h in PBS containing 10% milk powder. After incubation with the primary antibody, embryoid bodies were washed three times with PBST and staining with secondary antibodies was performed in PBS containing 10% milk powder. The primary antibodies used were: monoclonal anti α-cardiac myosin heavy chain (gift from Dr. A.M. Wobus, IPK Gatersleben, Germany) used undiluted; monoclonal anti-α-actinin (sarcomeric), clone number EA-53 (Sigma, Deisenhofen, Germany) used in a concentration of 10 µg/ml; monoclonal antitropomyosin (sarcomeric), clone number CH1 (Sigma), used in a concentration of 30 µg/ml; monoclonal anti-α-actin (sarcomeric), clone 5C5, used undiluted; monoclonal anticardiac Troponin T (Serotec, Camon, Germany), used in a 1:200 dilution; monoclonal anti-NF-κB (recognizing an independent epitope at the C-terminus of p65) (Pharmingen, San Diego, CA), used at a concentration of 1:20. As secondary antibody a Cy5™-conjugated rabbit anti-mouse IgG (H + L) (Dianova, Germany) was used in a 1:150 dilution. Excitation was performed using the 633-nm band of a He-Ne laser of the confocal setup. Emission was recorded using a longpass LP 655 nm filter set.

### Statistical Analysis

Data are given as mean values ±SEM, with n denoting the number of experiments performed with different embryoid body cultures. In each experiment at least 30 embryoid bodies were examined. Student's *t*-test for unpaired data was applied as appropriate. A value of $P < 0.05$ was considered significant.

## RESULTS

### Effects of DC Electrical Fields on Membrane Potential of Embryoid Bodies

The membrane potential changes in embryoid bodies during electrical field treatment were evaluated by staining cell membranes with the potential sensitive dye di-8-ANNEPS. As shown in Figure 1, treatment of 4-day-old embryoid bodies with an electrical field pulse (field strength 2,000 V/m, duration 8 s) resulted in a marked change in the relative fluorescence of di-8-ANNEPS (Fig. 1A) (n = 3). Depolarization at the cathode-facing side due to opposing external DC field and cell membrane potential vectors resulted in a drop in fluorescence. At the anode-facing side of the embryoid body the electrical field vectors evoked by both cell membrane potential and external DC field acted in the same direction [Sauer et al., 1997], which resulted in a hyperpolarization and a fluorescence increase (Fig. 1B).

### Effects of DC Electrical Fields on $[Ca^{2+}]_i$ of Embryoid Bodies

We have recently shown that growth stimulation of multicellular tumor spheroids with a 500-V/m electrical field was accompanied by a transient rise of $[Ca^{2+}]_i$ owing to $Ca^{2+}$ release from intracellular stores [Wartenberg et al., 1997]. To investigate whether electrical fields led to $[Ca^{2+}]_i$ changes, 4-day-old embryoid bodies were loaded with the fluorescent $Ca^{2+}$ indicator fluo-3 and $[Ca^{2+}]_i$ changes were monitored during treatment of embryoid bodies with electrical fields of different field strengths. As shown in Figure 2, field strengths of 500 and 1,000 V/m were without effect on $[Ca^{2+}]_i$. When a field strength of 2,000 V/m was applied, a transient rise of $[Ca^{2+}]_i$ was observed which declined during electrical field treatment below basal $[Ca^{2+}]_i$. Our data suggest that the $[Ca^{2+}]_i$ response was not attributable to tissue injuries, which should result in a long-lasting sustained rise in $[Ca^{2+}]_i$.

### Cardiomyocyte Differentiation in Embryoid Bodies Grown From ES Cells of the CCE Cell Line

When embryoid bodies were plated after 6–8 days in suspension culture, spontaneously beating foci of cardiomyocytes appeared in outgrowing embryoid bodies. The beating foci showed rhythmic $[Ca^{2+}]_i$ elevations that were time locked with the contractions (n = 3) (Fig. 3). As shown in Figure 4, the optimum day of plating for cardiomyocyte differentiation was day 7 (n = 5). Two days after plating (7 + 2), 12.5 ± 1% of embryoid bodies showed beating foci. The percentage of beating embryoid bodies increased to 26.5 ± 2%, 43 ± 3%, 59 ± 4% and 60 ± 4% on day 7 + 3, 7 + 4, 7 + 5 and 7 + 6, respectively. When embryoid bodies were plated after 5 days in suspension culture no cardiomyocyte differentiation occurred (data not shown).

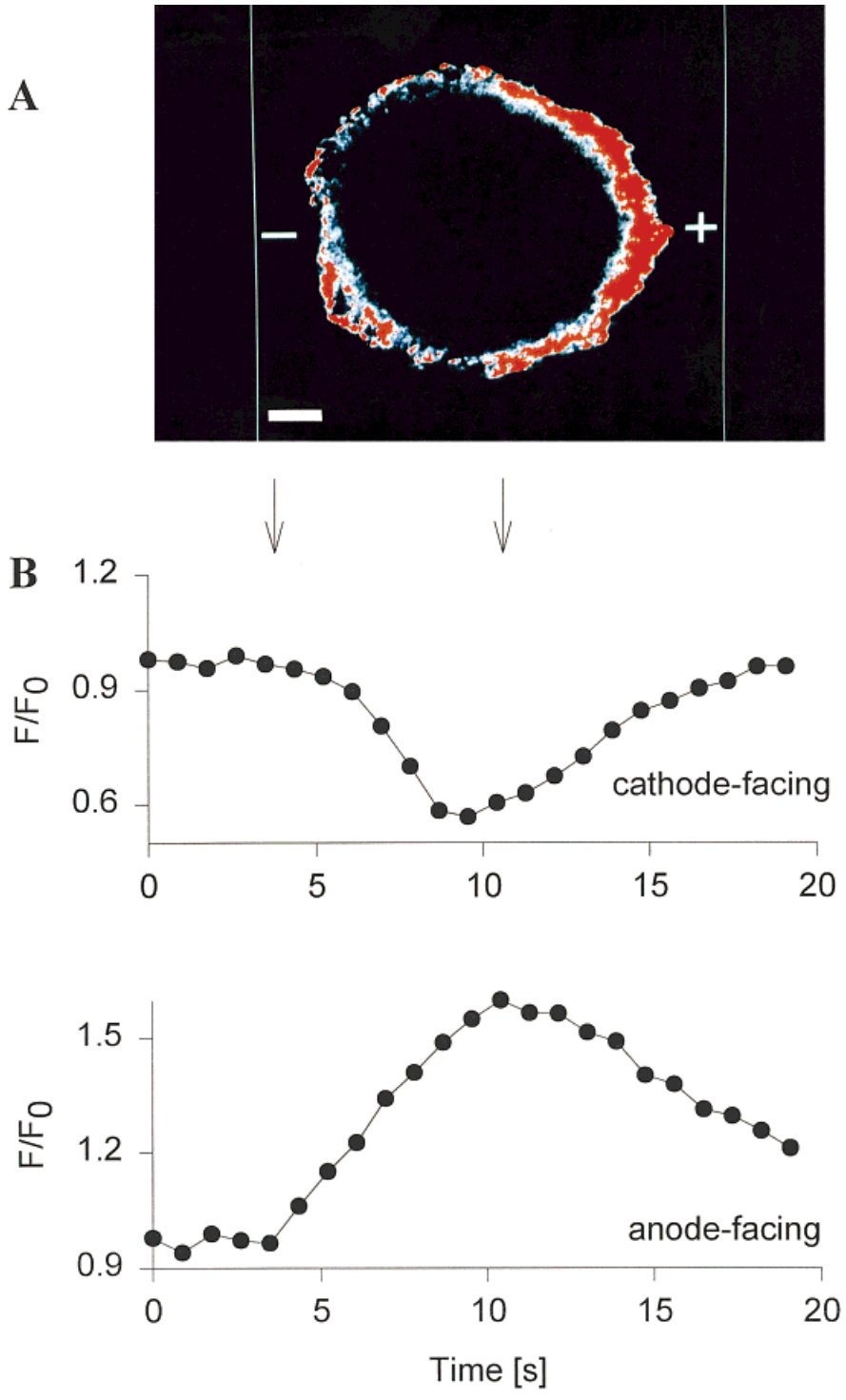


**Fig. 1.** Changes in membrane potential attributable to an external electrical field. **A**: Representative embryoid body during electrical field treatment. The peripheral cell layers were stained with the membrane potentential sensitive dye di-8-ANNEPS. The focus is in the equatorial plane of the embryoid body (false colors). Scale bar = 50 μm. **B:** Time course of membrane potential during electrical field treatment. An electrical field of 2,000 V/m was applied during the time period indicated by arrows. Depolarization at the cathode-facing side results in a fluorescence drop, whereas at the anode-facing side a fluorescence increase was observed. Data are presented in arbitrary units as relative fluorescence variation $F/F_0$ with respect to the resting level $F_0$ (representative tracings).

### Effects of DC Electrical Field Treatment on Cardiomyocyte Differentiation in Embryoid Bodies

To evaluate whether DC electrical fields influence cardiomyocyte differentiation, 4-day-old embryoid bodies were treated with a single DC electrical field pulse with a field strength of 100, 250, and 500 V/m and a duration of 90 s. We have previously shown that the maximum field strength of 500 V/m applied in the present study to investigate electrical field effects on cardiomyogenesis is below the threshold of reversible and irreversible membrane breakdown, stimulates the growth of multicellular

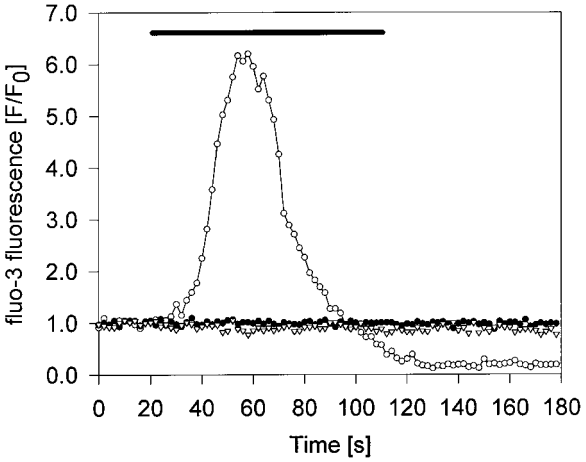


**Fig. 2.** Changes of $[Ca^{2+}]_i$ during treatment of embryoid bodies with electrical fields of different field strength. Undifferentiated 4-day-old embryoid bodies were treated for 90 s with electrical fields of 500 V/m (●), 1,000 V/m (▽), and 2,000 V/m (○). The solid line indicates the duration of electrical field treatment. Representative tracings.

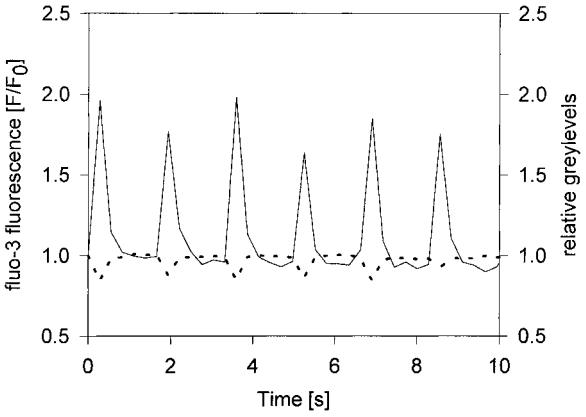


**Fig. 3.** $[Ca^{2+}]_i$ spikes and contractions of cardiomyocytes differentiated from ES cells in embryoid bodies. Fluo-3 fluorescence (solid line) and contraction (transmission) (dashed line) was simultaneously recorded. Measurements were performed with whole-mount 7 + 3-day-old embryoid bodies. Representative tracings.

tumor spheroids, and induces c-fos expression [Sauer et al., 1997; Wartenberg et al., 1997].

Treatment of embryoid bodies with a single electrical field pulse of 100, 250, and 500 V/m stimulated cardiomyogenesis (Fig. 5A–C), but not growth (data not shown), which resulted in an increased number of beating embryoid bodies when the electrical field pulse was applied on 4-day-old embryoid bodies, plating was performed 3 days later (i.e., at day 7 of suspension culture), and beating embryoid bodies were counted 2 days after plating (day 7 + 2) (Fig. 5A). Under these experimental conditions, the number of beating embryoid bodies (in %)

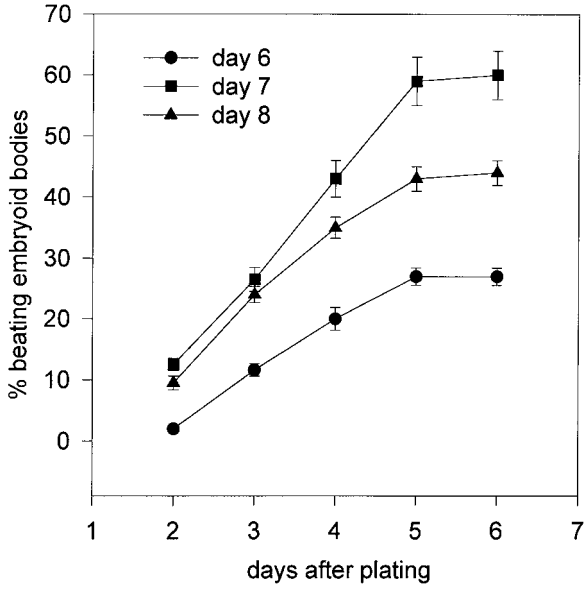


**Fig. 4.** Development of beating foci of cardiomyocytes in embryoid bodies with respect to the time of plating. Note that the optimum day of plating for the differentiation of cardiomyocytes was day 7. Plating on days 6 and 8 resulted in a significantly reduced percentage of beating embryoid bodies. Data are means ±SEM.

amounted to 12.3 ± 2, 17.6 ± 0.3, 19 ± 1, and 32 ± 3 for control, and electrical field treatment with 100 V/m (90 s), 250 V/m (90 s) and 500 V/m (90 s), respectively (n = 3, for each experimental condition). In pilot control experiments, embryoid bodies were either incubated in the pulsing chamber in the absence of an electrical field or were incubated in pulsing buffer previously treated with a 500-V/m electrical field for 90 s. Under these conditions, cardiomyocyte differentiation was not significantly different from control (data not shown), indicating that the observed effects were not due to chemical alterations of the incubation medium upon electrical field treatment. The time course of formation of beating foci was investigated using a field strength of 500 V/m (90 s) (Fig. 5B). The number of beating embryoid bodies in the electrical field-treated sample was significantly elevated, as compared with control with 10.4 ± 1.4%, 31.6 ± 2.7%, 43.3 ± 1.7%, and 58.7 ± 4.3% on days 7 + 1, 7 + 2, 7 + 3, and 7 + 4, respectively (n = 5). In the control sample, the number of beating embryoid bodies amounted to 3 ± 1% (day 7 + 1), 8.8 ± 1.4% (day 7 + 2), 21 ± 2% (day 7 + 3), and 36 ± 4.3% (day 7 + 4) (n = 5). On day 5 after plating, beating activity was not significantly different in the electrical field-treated and untreated sample with 66 ± 2.5% and 61 ± 3%, respectively. To determine whether

the electrical field (500 V/m, 90 s) exerted an effect on the size of beating foci, 50 treated and untreated embryoid bodies were immunostained for α-cardiac myosin heavy chain on day 7 + 2, whereas 28 treated and untreated embryoid bodies were investigated on day 7 + 5. Our data demonstrate that on both day 7 + 2 and day 7 + 5, the mean area of beating foci was significantly increased in the electrical field-treated sample, as compared with control (Fig. 5C). On day 7 + 2, the mean area of cardiomyocytes amounted to 0.14 ± 0.015 $mm^2$ and 0.029 ± 0.004 $mm^2$ in electrical field-treated and control embryoid bodies. On day 7 + 5, the mean areas in the electrical field-treated sample remained unchanged with 0.14 ± 0.012 $mm^2$, whereas in the control sample the area of cardiomyocytes increased to 0.047 ± 0.068 $mm^2$. These data indicate that electrical field treatment of embryoid bodies enhanced the time course of cardiomyocyte differentiation and resulted in an increased area within the embryoid body covered with cardiomyocytes. The beating frequency was not altered in control and DC-field-treated embryoid bodies (data not shown).

The immunohistochemical characterization of cardiomyocytes differentiated in embryoid bodies is shown in Figure 6. Beating foci exhibited positive immunostaining for sarcomeric α-actin, myosin heavy chain (not shown), tropomyosin, sarcomeric α-actinin and cardiac Troponin T (not shown). In nonbeating embryoid bodies (day 7 + 2 to day 7 + 6), either cardiomyocytes were absent, or only small foci of cardiomyocytes comprising few cells were observed (data not shown).

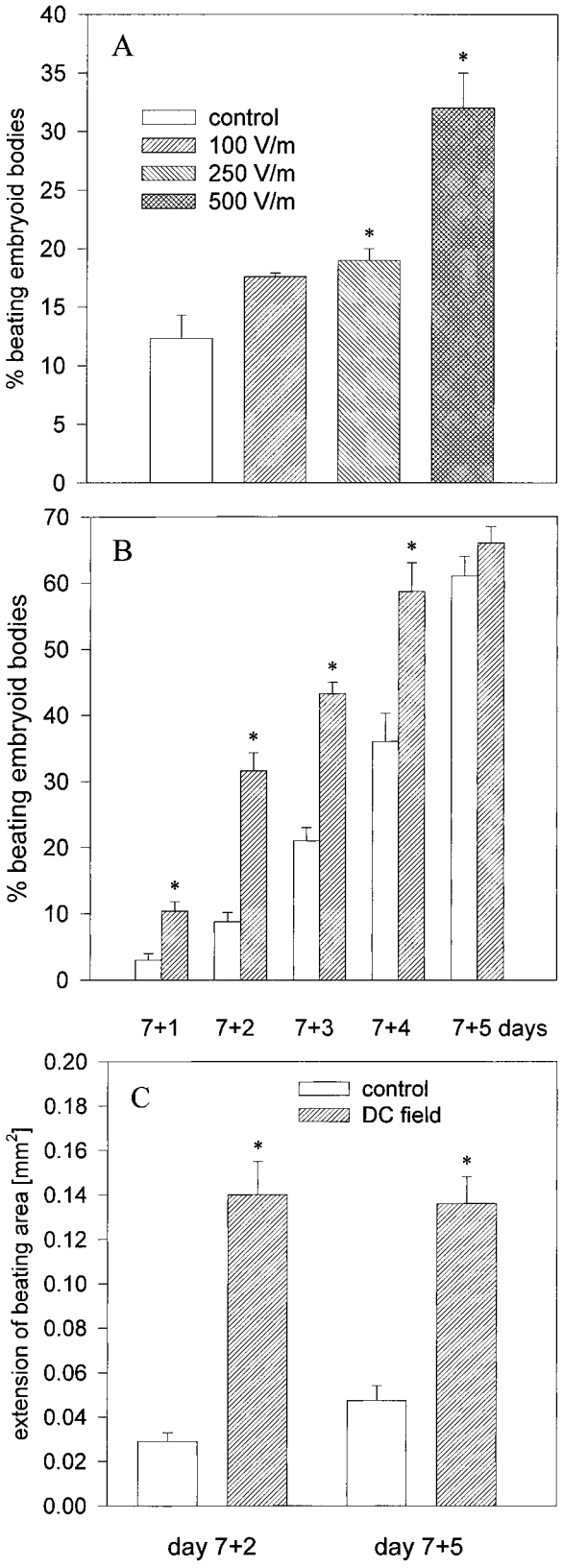


**Fig. 5.** Cardiomyocyte differentiation in embryoid bodies treated with electrical fields. **A:** Effect of electrical fields of different field strengths on the development of beating embryoid bodies; 4-day-old embryoid bodies were treated with a single electrical field pulse of either 100, 250, or 500 V/m for 90 s and were plated 3 days later. Beating activity was evaluated 2 days after plating (day 7 + 2). **B:** Time course of the development of beating embryoid bodies treated with a single electrical field pulse of 500 V/m for 90 s. Open bars, untreated control. Hatched bars, electrical field-treated sample. About 30 embryoid bodies in each of three independent experiments were used for the determination of each data point. Data are means ±SEM. **C:** Extension of beating areas of cardiomyocytes in electrical field (500 V/m, 90 s)-treated and control embryoid bodies 2 days (day 7 + 2) and 5 days (day 7 + 5) after plating. Immunostaining of cardiomyocytes was performed using an antibody directed against cardiac myosin heavy chain. Areas were evaluated using the image analysis software of the confocal setup. Data are means ±SEM. *$P < 0.05$, significantly different from control.

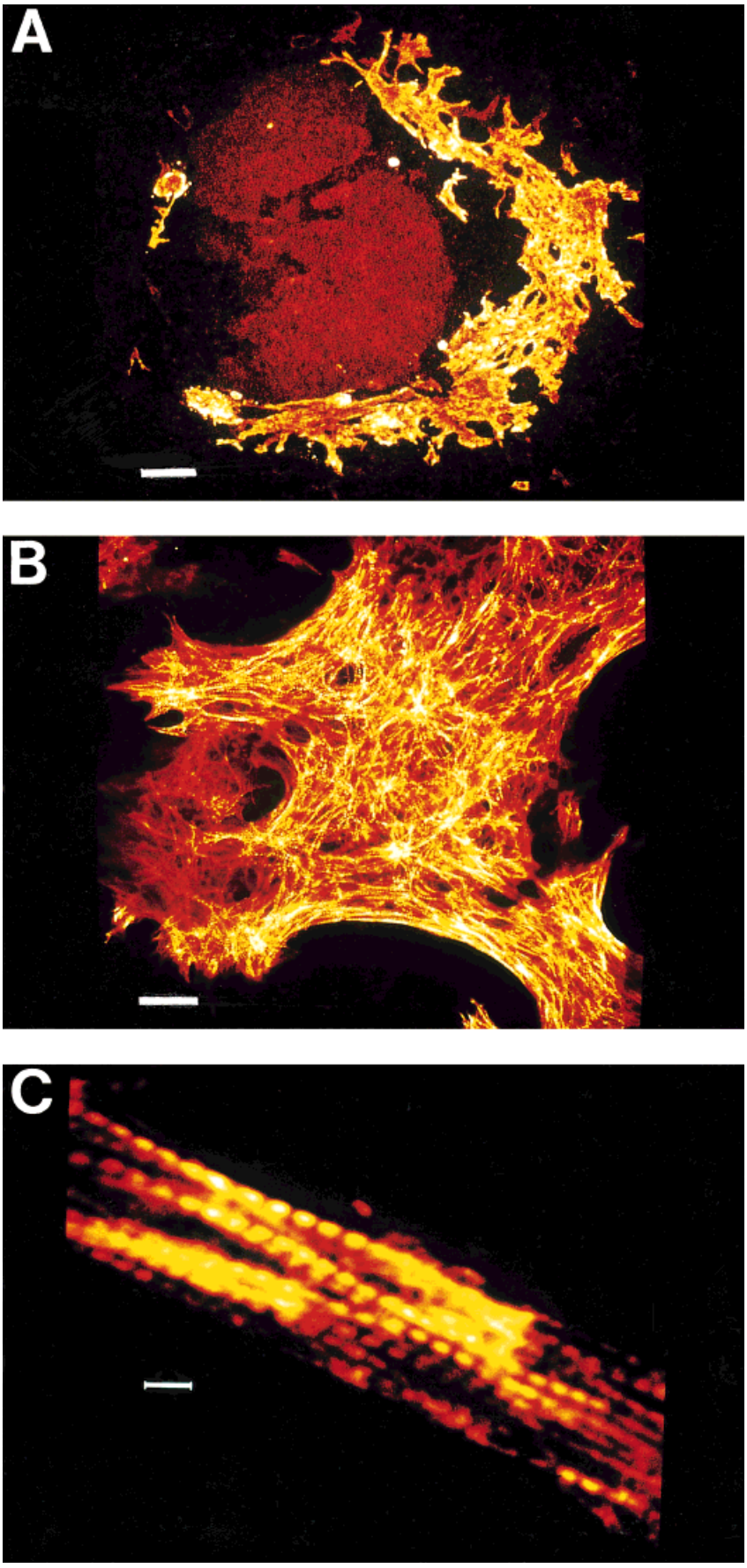


**Fig. 6.** Immunohistochemistry of electrical field-treated (500 V/m, 90 s) representative embryoid bodies 4 days after plating. **A:** Sarcomeric α-actin staining. Scale bar = 240 μm. **B:** Sarcomeric α-actinin staining. Scale bar = 60 μm. **C:** Tropomyosin staining. Scale bar = 5 μm.

### DC Electrical Field Treatment Raises ROS in Embryoid Bodies

To evaluate the physiological basis for the effect of the electrical field (500 V/m, 90 s) on cardiomyocyte differentiation, 4-day-old embryoid bodies were loaded with DCFH-DA, a sensitive indicator for intracellular ROS. After electrical field treatment, the fluorescence of oxidized DCF was evaluated using confocal laser scanning microscopy. As shown in Figure 7A, electrical field treatment raised intracellular ROS during and after the field pulse (n = 4). The increase in ROS was observed for at least 20 min. The DCF-derived fluorescence increase was totally abolished when embryoid bodies were preincubated for 3 h with 2.5 mM dehydroascorbate (n = 3), a potent free radical scavenger. To determine the amount of ROS produced as a consequence of the electropulse, embryoid bodies were incubated for 15 min with different concentrations of $H_2O_2$ ranging from 1 nM to 1 µM (n = 3). Figure 7B shows that the change in intracellular redox state of electrical field-treated embryoid bodies 20 min after electropulsing was equivalent to the increase of fluorescence observed after the addition of 1–10 nM $H_2O_2$ to the incubation medium.

### Effects of External $H_2O_2$ on Cardiomyocyte Differentiation

To evaluate whether ROS production during electrical field treatment was sufficient to enhance cardiomyocyte differentiation, 4-day-old embryoid bodies were incubated for 2 h with different concentrations of $H_2O_2$ in a range of 0.1 nM to 1 µM. As shown in Figure 8, incubation with 1 to 10 nM $H_2O_2$ significantly increased the percentage of embryoid bodies containing beating foci of cardiomyocytes at day 7 + 2 as compared with control (n = 4). The most prominent effect was observed with a $H_2O_2$ concentration of 1 nM, which enhanced cardiomyocyte differentiation comparably to the effect observed after electrical field treatment. A $H_2O_2$ concentration of 1 µM depressed cardiomyocyte differentiation as compared with control.

### DC Electrical Field Treatment of Embryoid Bodies Activates NF-κB

ROS have been previously shown to activate transcription factors such as NF-κB, which regulate the transcription of a variety of genes,

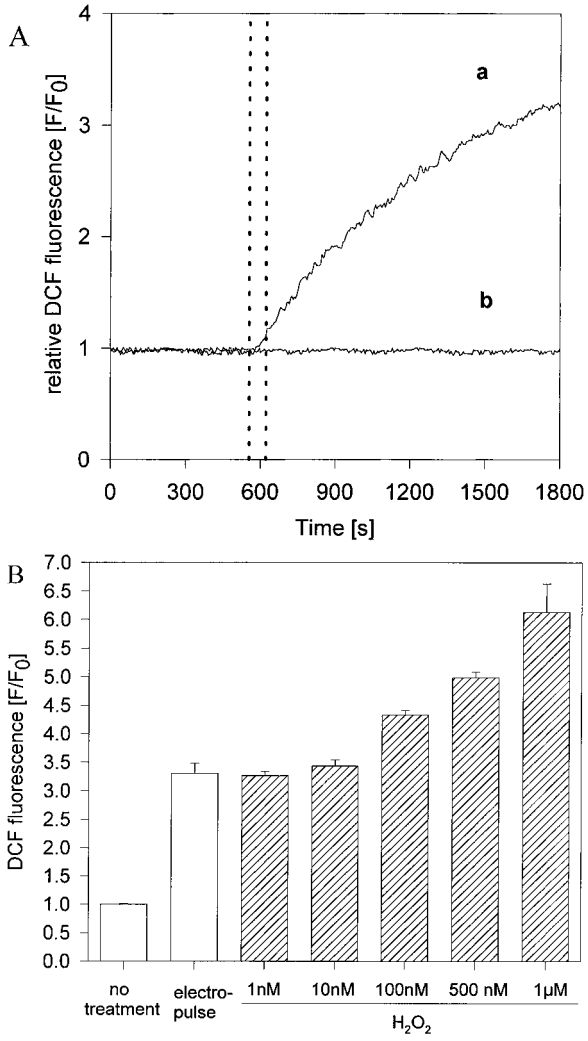


**Fig. 7.** Generation of reaction oxygen species (ROS) in embryoid bodies attributable to electrical field (500-V/m, 90-s) treatment. **A:** Normalized dichlorofluorescein (DCF) fluorescence under control conditions (a) and effects of the radical scavenger dehydroascorbate (2.5 mM) (b). **B:** Changes in intracellular redox state of 4-day-old embryoid bodies after electrical field treatment. Embryoid bodies were either electrical field treated (open bar) or incubated for 10 min with different concentrations of $H_2O_2$ (hatched bars). About 30 embryoid bodies in each of three independent experiments were used for the determination of each data point. Data are means ±SEM.

including genes for cytokines that may play a role in cardiac development [May and Ghosh, 1997]. To assess whether electrical field treatment activates NF-κB, 4-day-old embryoid bodies were treated with a single electrical field pulse (500 V/m) and translocation of the p65 subunit of activated NF-κB to the nucleus was investigated by immunohistochemistry in whole-mount embryoid bodies. Figure 9 shows that, in the untreated sample, p65 immunofluorescence

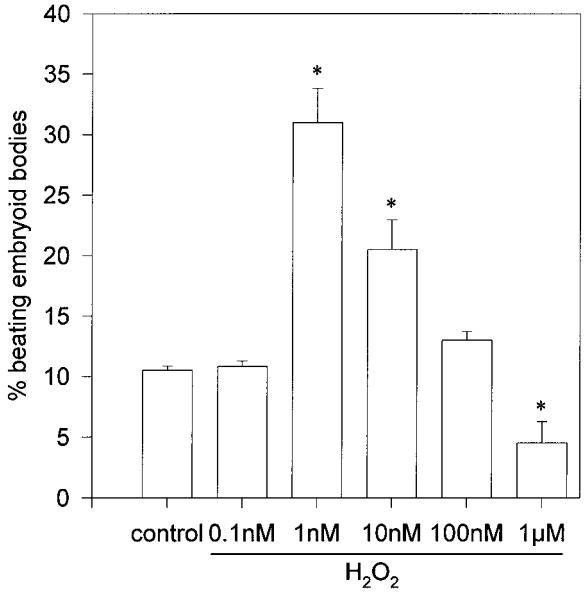


**Fig. 8.** Development of beating embryoid bodies after treatment on day 4 for 1 h with different concentrations of $H_2O_2$. Beating and nonbeating embryoid bodies were counted on day 7 + 2 after plating. About 30 embryoid bodies in each of four independent experiments were used for the determination of each data point. Data are means ±SEM. $^*P < 0.05$, significantly different from control.

was distributed in the cytoplasm but excluded from the nucleus. By contrast, electrical field treatment of embryoid bodies resulted in an intensive staining of cell nuclei 120 min after electrical field exposure, indicating activation of NF-κB and translocation of p65 to the nucleus.

### Effects of Free Radical Scavengers and Inhibition of NF-κB on Cardiomyocyte Differentiation in Embryoid Bodies

If cardiomyocyte differentiation in embryoid bodies is related to ROS production and NF-κB activation after electrical field treatment, an inhibition of ROS production and NF-κB activation should abolish cardiomyocyte differentiation. Therefore, embryoid bodies were incubated during electrical field treatment with dehydroascorbate (2.5 mM), which is known as a potent radical scavenger, APDC (20 µM), which is known as both a radical scavenger and an inhibitor of NF-κB [Lee et al., 1997; Schreck et al., 1992], and TPCK (50 nM), which acts as an inhibitor of NF-κB by preventing the proteolytic degradation of I-κB, the cytoplasmic inhibitor of NF-κB [Rovin et al., 1995]. Figure 10 shows that incubation of 4-day-old untreated and electrical field-treated embryoid bodies with dehydroascorbate, APDC, and TPCK during and following electrical field treatment (n = 3) significantly inhibited cardiomyocyte differentiation, which indicates that ROS may be involved in cardiac commitment.

## DISCUSSION

The present study demonstrates for the first time that electrical fields affect cardiomyocyte differentiation in ES cells grown in the multicellular tissue of embryoid bodies, leading to an increased percentage of embryoid bodies differentiating beating foci of cardiomyocytes subsequent to electrical field treatment. In parallel, the size of the contractile areas increased.

Although it has been reported that electrical fields display effects on embryonic cells derived from a variety of organisms [Robinson, 1985], the biophysical and biochemical means by which the electrical field exerts its effects on the cells are not well described. The most likely target of the electrical field is the cell membrane. We have previously shown that the cell membranes of multicellular cancer spheroids are depolarized at the cathode-facing side of the spheroid, whereas a hyperpolarization was observed at the anode-facing side [Sauer et al., 1997]. Similar results were obtained in embryoid bodies. According to Bernhardt and Pauly [1973], it was calculated that an electrical field with a field strength of 500 V/m resulted in a membrane potential change of approximately 75 mV when embryoid bodies with a diameter of 200 µm were treated. In our experiments with multicellular cancer spheroids, we unequivocally demonstrated that the electrical field resulted in the generation of intracellular ROS, which in turn released $Ca^{2+}$ from intracellular stores. The observed enhanced growth kinetics of multicellular cancer spheroids due to electrical field treatment was strictly $Ca^{2+}$ dependent [Wartenberg et al., 1997].

In corroboration with the experiments on multicellular cancer spheroids, ROS were likewise generated in embryoid bodies after electrical field treatment. The ROS generation evaluated by DCF fluorescence was equivalent to the fluorescence signal observed with 1–10 nM externally added $H_2O_2$ and therefore about 10 times lower than the ROS generated in multicellular cancer spheroids [Wartenberg et al., 1997]. This may explain why $[Ca^{2+}]_i$ remained unchanged when embryoid bodies were treated with a field

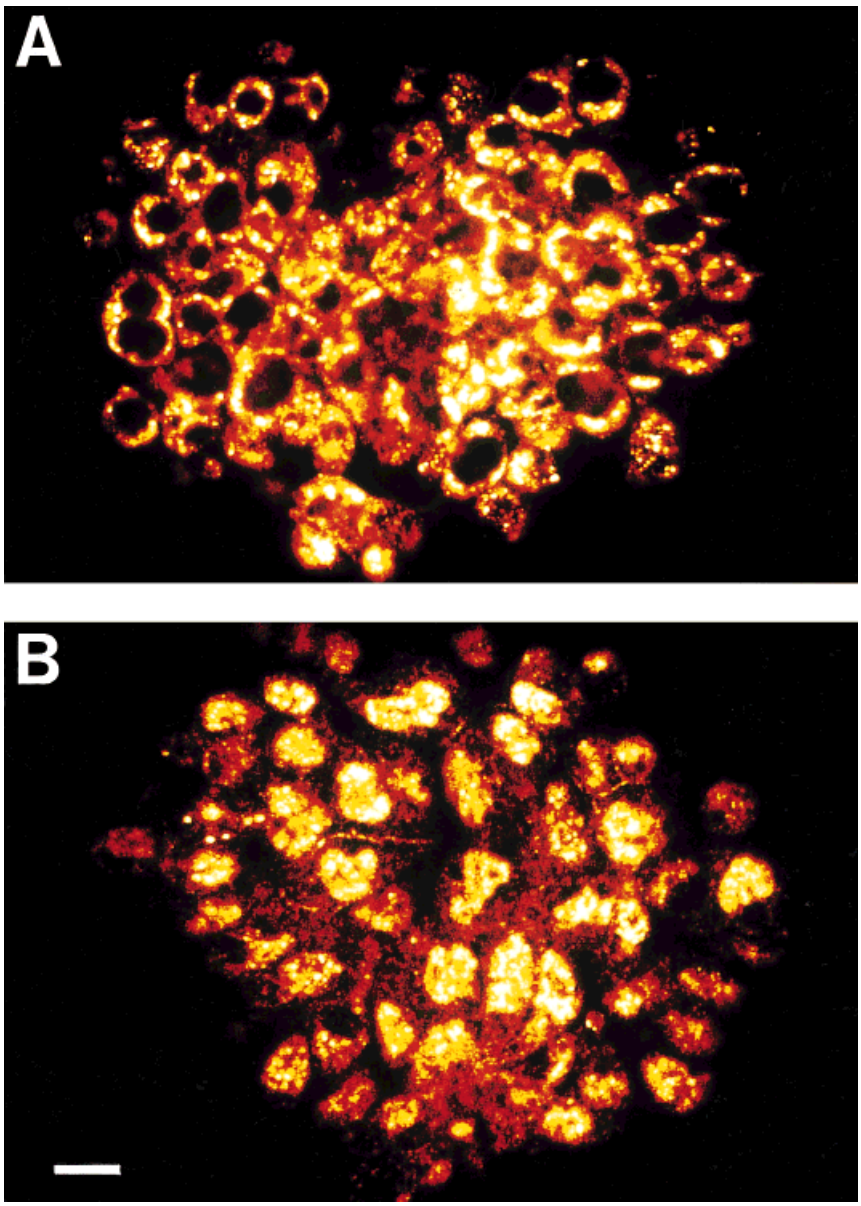


**Fig. 9.** Activation of NF-κB after electrical field (500-V/m, 90-s) treatment of representative embryoid bodies. Control **(A)** and electrical-field-treated **(B)** whole-mount 4-day-old embryoid bodies were fixed after a 120-min postincubation period and stained with an antibody directed against the p65 subunit of NF-κB. The focal plane is in the peripheral cell layers of embryoid bodies. Note that after activation the labeled p65 subunit is translocated to the cell nucleus. Scale bar = 10 μm.

strength of 500 V/m, which was effective for the enhancement of cardiomyocyte differentiation. Obviously, the ROS generated during electrical field treatment were the crucial biochemical effectors of the observed enhancement of cardiomyocyte differentiation, since in the absence of an applied electrical field, exogenously added $H_2O_2$ in the same nanomolar concentration range as was generated during electrical field treatment yielded comparable results.

It should be mentioned that a single electropulse with a duration of 90 s, rather than a long-term exposure, was sufficient to enhance cardiomyocyte differentiation, indicating that cell orientation or migration toward the cathode and anode, as previously reported [Jaffe and Poo, 1979; Erickson and Nuccitelli, 1984; Sulik et al., 1992] is not involved. The electropulse led to elevated levels of intracellular ROS for at least 20 min. In line with this observation of a brief elevation of ROS, a single treatment for 1 h with exogenous $H_2O_2$ increased the number of beating embryoid bodies after plating. Long-term exposure (>2 h) to either electrical fields or nanomolar concentrations of $H_2O_2$ resulted in cytotoxic effects (data not shown).

Transcription factors like AP-1 and NF-κB have been implicated in the inducible expression of a variety of genes in response to oxidative stress [Remacle et al., 1995]. NF-κB is

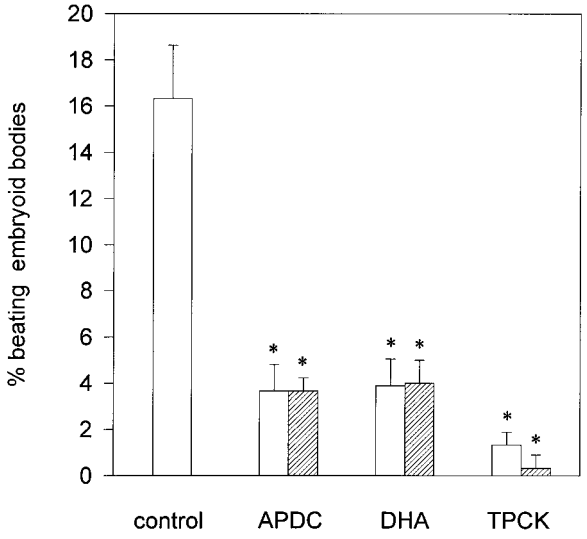


**Fig. 10.** Effects of radical scavengers and inhibitors of NF-κB on cardiomyocyte differentiation in embryoid bodies. Electrical field (500-V/m, 90-s)-treated (hatched bars) and control (open bars) embryoid bodies were incubated from day 4 of embryoid body development on with either 20 μM APDC, 2.5 mM DHA or 50 nM TPCK. About 30 embryoid bodies in each of three independent experiments were used for the determination of each data point. Beating activity was evaluated at day 7 + 3. Data are means ±SEM. $^*P < 0.05$, significantly different from control.

normally present in the cytoplasm in an inactive form through its association with the inhibitory subunit I-κB. A variety of recent studies have shown that the activation of NF-κB by release of I-κB is under the control of a redox-regulated mechanism in which ROS play a central role [Baeuerle et al., 1996]. In a recent study, activation of a number of transcription factors including NF-κB as a consequence of treatment of several cell lines with a single electrical field pulse was demonstrated [Pazmany et al., 1995]. The data in the present study show an activation of NF-κB in embryoid bodies upon electrical field treatment, as the p65 subunit of the transcription factor was effectively translocated into the nucleus. To investigate whether inhibition of NF-κB activation would impair cardiomyocyte differentiation, embryoid bodies were incubated during and after electrical field treatment with known antagonists of NF-κB. The same was done with the untreated control sample. Our data show that inhibition of NF-κB in the electrical field-treated and likewise in the untreated control sample inhibited cardiomyocyte differentiation, although under the applied conditions neither of the agents used was cytotoxic (data not shown). This indicates that NF-κB activation plays a specific role for cardiac cell differentiation.

Involvement of NF-κB in embryonic pattern formation is evident at least for *Drosophila* embryogenesis, where NF-κB homologue dorsal acts as a morphogen that determines the size and fate of regions along the dorsoventral axis and activates the mesoderm determinant twist [Jiang et al., 1991; Govind et al., 1992]. Experimental evidence for a direct involvement of NF-κB in mammalian pattern formation and cardiomyogenesis has not yet been provided. However, at least two lines of evidence point toward a possible indirect or direct role of NF-κB on cardiac development. First, it has been demonstrated that NF-κB induces the transcription of the gene coding for interleukin-6 (IL-6) [Chauhan et al., 1996]. IL-6 belongs to a pleiotropic family of cytokines with overlapping biological functions that also includes cardiotrophin-1 (CT-1), a cytokine that has been suggested to play an autocrine role during cardiac chamber growth and morphogenesis by promoting the survival and proliferation of immature myocytes [Sheng et al., 1996]. CT-1 acts via the same gp130-dependent signaling pathway used by several members of the IL-6 family [Wollert and Chien, 1997]. This may indicate that either CT-1 expression is under the control of NF-κB or Il-6 mimics the action of CT-1. Second, it has recently been shown that the NF-κB subunit p65 can act as an accessory protein for the serum response factor (SRF), suggesting that NF-κB may participate in the regulation of serum response element (SRE)-dependent promoters [Franzoso et al., 1996] which are present in a number of myogenic specified genes [Chen and Schwartz, 1997].

According to the free radical theory of development, discrete changes in antioxidant defenses and parameters in oxidation may direct the initiation of certain developmental events [Allen and Balin, 1989]. Gradients in endogenous electrical fields may bring on redox gradients. By these means, membrane potential and ROS gradients could induce germ layer-specific gene activation and formation of morphogenic patterns. It was recently shown that postimplantation murine embryos have the capability to generate and release ROS [Gagioti et al., 1995]. In ES cells, the small heat shock protein hsp27 induced by oxidative stress [Huot et al., 1995] has been reported to be transiently upregulated during the early stages of ES cell differ-

entiation [Mehlen et al., 1997]. A role of the stress-activated kinase p38 in myocardial cell growth and cardiac-specific gene expression has been recently suggested [Zechner et al., 1997]. These findings indicate that during early embryonic development the embryo is in a microenvironment of an elevated redox state which may be downregulated in later stages and may lead to germ layer specific gene activation.

The currents driven by DC voltage differences across epithelial cells are large when the cells are less tightly apposed, leading to a lower resistance to current flow between them [Nuccitelli, 1992]. We recently have shown that within 8 days of embryoid body development the peripheral cell layers of embryoid bodies differentiate from loosely packed cell aggregates to transporting epithelial layers of visceral and parietal endoderm [Sauer et al., 1998]. During this process of epithelial layer formation, internal electrical fields should arise in embryoid bodies. These endogenous electrical fields may modulate the intracellular redox state, influence enzyme function, and induce signal transduction pathways resulting in the commitment of the cardiac cell lineage.

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