

The DC electrical-field-induced Ca^{2+} response and growth stimulation of multicellular tumor spheroids are mediated by ATP release and purinergic receptor stimulation

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Summary

It has been demonstrated that adenosine 5'-triphosphate (ATP) is actively secreted by cells, thereby eliciting Ca^{2+} -dependent signal transduction cascades in an autocrine and paracrine manner. In the present study the effects of direct current (DC) electrical fields on ATP release, the intracellular Ca^{2+} concentration $[\text{Ca}^{2+}]_i$ and growth of multicellular prostate tumor spheroids were investigated. Treatment of multicellular tumor spheroids by a single DC electrical field pulse with a field strength of 750 Vm^{-1} for 60 seconds resulted in a transient Ca^{2+} response, activation of c-Fos and growth stimulation. The initial $[\text{Ca}^{2+}]_i$ signal was elicited at the anode-facing side of the spheroid and spread with a velocity of approximately $12 \mu\text{m}$ per second across the spheroid surface. The electrical-field-evoked Ca^{2+} response as well as c-Fos activation and growth stimulation of tumor spheroids were inhibited by pretreatment with the anion channel blockers NPPB,

niflumic acid and tamoxifen. Furthermore, the Ca^{2+} response elicited by electrical field treatment was abolished following purinergic receptor desensitivation by repetitive treatment of tumor spheroids with ATP and pretreatment with the purinergic receptor antagonist suramin as well as with apyrase. Electrical field treatment of tumor spheroids resulted in release of ATP into the supernatant as evaluated by luciferin/luciferase bioluminescence. ATP release was efficiently inhibited in the presence of anion channel blockers. Our data suggest that electrical field treatment of multicellular tumor spheroids results in ATP release, which concomitantly activates purinergic receptors, elicits a Ca^{2+} wave spreading through the tumor spheroid tissue and stimulates tumor growth.

Key words: Electric field, Multicellular tumor spheroid, ATP release, Anion channel, Intracellular calcium

Introduction

Different responses of biological systems towards exogenous electromagnetic fields (EMFs) have been reported in recent years. These include cytoskeletal reorganization (Cho et al., 1996), cell surface receptor redistribution (Cho et al., 1994), as well as changes in intracellular Ca^{2+} (Pessina et al., 2001; Cho et al., 1999; Ihrig et al., 1997; Onuma and Hui, 1988; Wartenberg et al., 1997) and intracellular levels of reactive oxygen species (ROS) (Wartenberg et al., 1997; Rosenspire et al., 2001). The molecular and biochemical responses of cells towards EMFs may underly observations on the cellular level which have recently been attributed to exogenous EMFs, for example, the galvanotropic migration and orientation of cells (Fang et al., 1998; Zhang et al., 2000a; Farboud et al., 2000; Nuccitelli et al., 1993; Zhao et al., 1999a; Zhao et al., 1999b; Zhao et al., 1996), the electrical-field-induced growth stimulation of tumor cells (Wartenberg et al., 1997; Sauer et al., 1997), the enhancement of cardiomyogenic differentiation of embryonic stem cells (Sauer et al., 1999a) and the beneficial effects of EMF fields in the support of bone (Ito and Shirai, 2001; Mammi et al., 1993; Chang et al., 1991) and wound (Goldman and Pollack, 1996; Dindar et al., 1993) healing. Besides the well documented effects of EMFs on cellular

homeostasis, the occurrence of endogenous electrical fields has been reported for a number of vertebrate embryos, such as *Xenopus* (Robinson and Stump, 1984), chicken (Jaffe and Stern, 1979; Hotary and Robinson, 1990) and mouse (Wiley and Nuccitelli, 1986; Nuccitelli and Wiley, 1985), and has been discussed to regulate pattern formation during embryogenesis as well as organogenesis.

In the present study we report on the underlying mechanisms of change in intracellular Ca^{2+} and the subsequent growth stimulation of multicellular tumor spheroids, which are well established model systems for avascular micrometastases (Sutherland, 1988; Acker et al., 1987; Mueller-Klieser et al., 1986) with significant endogenous drug resistance (Wartenberg et al., 2000; Wartenberg et al., 1998; Olive and Durand, 1994; Olive et al., 1997). Changes in intracellular Ca^{2+} following EMF treatment of cells have been reported previously and have been attributed mainly to transmembrane Ca^{2+} influx through voltage-gated Ca^{2+} channels, and/or activation of stretch-activated cation channels which, upon opening, permit the influx of cations including Ca^{2+} (Cho et al., 1999; Ihrig et al., 1997; Onuma and Hui, 1988). As a further mechanism of intracellular Ca^{2+} elevation in response to EMF fields the stimulation of cell membrane receptors with subsequent

activation of phospholipase C, generation of inositol triphosphate and Ca^{2+} release from intracellular stores has been discussed (Eichwald and Kaiser, 1995; Eichwald and Kaiser, 1993). In previous studies, we have demonstrated that DC electrical fields transiently elevate intracellular Ca^{2+} in multicellular prostate tumor spheroids by a mechanism involving an elevation of intracellular ROS (Wartenberg et al., 1997). This elevation of intracellular Ca^{2+} was mediated by Ca^{2+} release from intracellular stores and resulted in growth stimulation of multicellular tumor spheroids. The data of the present study indicate that electrical field treatment of multicellular tumor spheroids results in the release of intracellular ATP via anion channels to the extracellular compartment, which then may activate purinergic receptors and elicit a transient Ca^{2+} response. ATP release has been reported to occur in a variety of preparations after mechanical stretch (Mitchell, 2001; Mitchell et al., 1998; Cotrina et al., 1998; Sabirov et al., 2001; Verderio and Matteoli, 2001; Ostrom et al., 2001) and, in prostate cancer cells grown in monolayer culture, it has been demonstrated by us to elicit a Ca^{2+} wave propagating radially from the site of mechanical perturbation (Sauer et al., 2000). It is demonstrated that a comparable mechanism may be involved in the elevation of intracellular Ca^{2+} and growth stimulation following treatment of multicellular tumor spheroids with DC electrical fields.

Materials and Methods

Materials

5-nitro-2-(3-phenylpropylamino)-benzoate (NPPB) was obtained from Calbiochem (Bad Soden, Germany). Tamoxifen, niflumic acid, suramin, and apyrase were purchased from Sigma (Deisenhofen, Germany).

Culture technique of multicellular tumor spheroids

The human androgen-insensitive prostate cancer cell line DU-145 was used for the experiments. Monolayers were cultivated in 25 cm² tissue culture flasks (Greiner, Solingen, Germany) in 5% CO₂-humidified air at 37°C. The monolayer cultures were trypsinized and replated once a week. Cell culture medium was Ham's F-10 medium (Invitrogen, Fernwald, Germany) supplemented with 10% fetal calf serum (Invitrogen), 100 IU/ml penicillin, and 100 µg/ml streptomycin (Invitrogen). Spheroids were grown from single cells seeded in siliconized 250 ml spinner flasks (Integra Biosciences, Fernwald, Germany) at a cell density of 1×10⁵ cells/ml. The spinner flask medium (175 ml) was stirred at 25 rpm, using a stirrer system (Cell Spin, Integra Biosciences) and partly changed every day. For the experiments, tumor spheroids with a diameter of 100±50 µm were used.

Electrical field treatment and confocal laser scanning microscopy

Electrical field pulses were applied to multicellular tumor spheroids under the optical control (fluorescence/transmission mode) of an inverted confocal laser scanning microscope (LSM 410; Zeiss, Jena, Germany) using a 25× oil immersion objective, numerical aperture 0.8 (Zeiss, Neofluar). Processing of images (512×512 pixels, 8 bit) was carried out using the Time-software facilities of the confocal setup. Full-frame images were acquired and stored automatically at 2 second time intervals to a 16-megabyte video memory of the confocal setup. Each series of images was scaled between pixel intensity 0 (background fluorescence) and 255 (maximum fluorescence in that

series). The minimum, maximum, mean, standard deviation and integrated sum of the pixel values in a region of interest (selected using an overlay mask) were written to a data file and routinely exported for further analysis to the commercially available Sigma Plot (Jandel Scientific, Erkrath, Germany) graphic software.

For electropulse experiments, multicellular tumor spheroids were suspended in a low-ionic content *N*-2-hydroxyethylpiperazine-*N'*-2-ethane-sulfonic acid (Hepes) (5 mM, pH 7.2)-buffered 'pulsing medium' that contained 255 mM sucrose, 1 mM CaCl₂ and 1 mM MgCl₂, and had a conductivity of 500 µS cm⁻¹. They were then placed in an incubation chamber between stainless steel electrodes with an electrode area of 0.4 cm² and an electrode distance of 0.2 cm. The electrodes were connected to a custom-made voltage generator, which gave square electric pulses. Voltage pulses of a field strength of 750 V m⁻¹ (1.5 V at the electrodes) and a duration of 60 seconds were applied to multicellular tumor spheroids. The total current in the chamber was 1.5 mA. The magnetic flux density in the proximity of the tumor spheroids was calculated to 0.079 µT, which is below the average laboratory noise level for low-frequency EMFs (~9.5 µT) (Cameron et al., 1993). In control experiments without tumor spheroids we ensured that no water hydrolysis, ROS production, or pH and temperature shifts occurred in the pulsing chamber during the duration of the experiments.

Ca²⁺ imaging during electrical field exposure

[Ca²⁺]_i was monitored using the fluorescent dye fluo-3,AM (Molecular Probes, Eugene, OR). Multicellular tumor spheroids were loaded for 45 minutes in F-10 cell culture medium with 10 µM fluo-3,AM dissolved in dimethyl sulfoxide (final concentration 0.1%) and PluronicTM (Molecular Probes; final concentration <0.025%). After loading, the tumor spheroids were rinsed in 'pulsing buffer' and placed in the experimental chamber, and then electropulses were applied. For fluorescence excitation, the 488 nm line of an argon ion laser of the confocal setup was used. Emission was recorded by the use of a 515 nm longpass filter. Because fluo-3 does not permit use of ratio measurements to determine absolute free Ca²⁺ levels, data are presented in arbitrary units as percentage of fluorescence variation (F/F₀) with respect to the resting level F₀.

Antibody staining

The polyclonal rabbit c-fos (AB-2) antibody was obtained from Calbiochem (Bad Soden, Germany) and was used in a dilution of 1:100. Multicellular tumor spheroids were fixed in ice-cold methanol-acetone for 60 minutes at -20°C, washed with phosphate-buffered saline (PBS) plus 0.1% Triton X-100 and blocked against unspecific binding in 10% nonfat milk powder for 60 minutes. Incubation with the primary antibody was performed for 60 minutes. As secondary antibody, a Cy3-labelled goat anti-rabbit IgG (H+L) antibody (Jackson ImmunoResearch Laboratories, West Grove, PA) (concentration 1.2 mg ml⁻¹) was used at a dilution of 1:100.

Bioluminescence experiments

ATP release from multicellular tumor spheroids was determined using a luciferin-luciferase assay (Sigma, Deisenhofen, Germany) in a chemiluminescence apparatus (Bioluminescence Analyzer XP2000, SKAN AG, Basel, Switzerland) under dim light. For data sampling the output of the photomultiplier tube of the setup was connected to a multimeter (Votcraft M-3610D, Conrad electronics, Hirschau, Germany) and a Tandon 286/N personal computer (Tandon, Moorpark, CA). An aliquot of approximately 50 tumor spheroids was washed three times in 'pulsing buffer' and treated with DC electrical fields. Subsequently, 100 µl of the supernatant was removed and pressure injected via a light-tight access into a 3 ml glass cuvette containing the luciferase cocktail consisting of 50 µl of the ATP assay

mix and 1.5 ml ATP assay mix dilution buffer (Sigma). In control experiments the background chemiluminescence signal from supernatants of tumor spheroids that were not treated with electrical fields was analyzed and set to 100%. For the experiments with anion channel inhibitors, cells were preincubated for 20 minutes in 'pulsing buffer' that was supplemented with the respective inhibitor. We have previously assured that none of the applied anion channel inhibitors interfered in the applied concentrations with the activity of luciferase enzyme activity (Sauer et al., 2000).

Statistical analysis

Data are given as mean values \pm standard deviation, with n denoting the number of experiments. Student's t -test for unpaired data was applied as appropriate. A value of $P < 0.05$ was considered significant.

Results

Ca²⁺ response of multicellular tumor spheroids during treatment with DC electrical fields

We have previously shown that treatment of multicellular tumor spheroids with a single electrical field pulse raised

intracellular [Ca²⁺]_i owing to release of Ca²⁺ from intracellular stores (Wartenberg et al., 1997). In the present study a closer inspection of the Ca²⁺ response revealed that treatment of multicellular tumor spheroids with a single electrical field pulse with a field strength of 750 Vm⁻¹ for 60 seconds elicited a Ca²⁺ wave arising at the anode-facing side of the tumor spheroid and spreading with a velocity of approximately 12 μ m per second towards the cathode-facing side (Fig. 1A,B; $n=10$). The Ca²⁺ response could be elicited repetitively; however, a reduced amplitude was observed with repetitive field treatment (Fig. 1C; $n=3$).

Involvement of purinergic receptor stimulation in the electrical-field-induced Ca²⁺ response

The Ca²⁺ response elicited by DC electrical field treatment of multicellular tumor spheroids may arise from the stimulation of purinergic receptors. Various subtypes of purinergic receptors have been demonstrated to be present in DU-145 tumor spheroids (Janssens and Boeynaems, 2001; Sauer et al., 2001). To investigate this issue tumor spheroids were

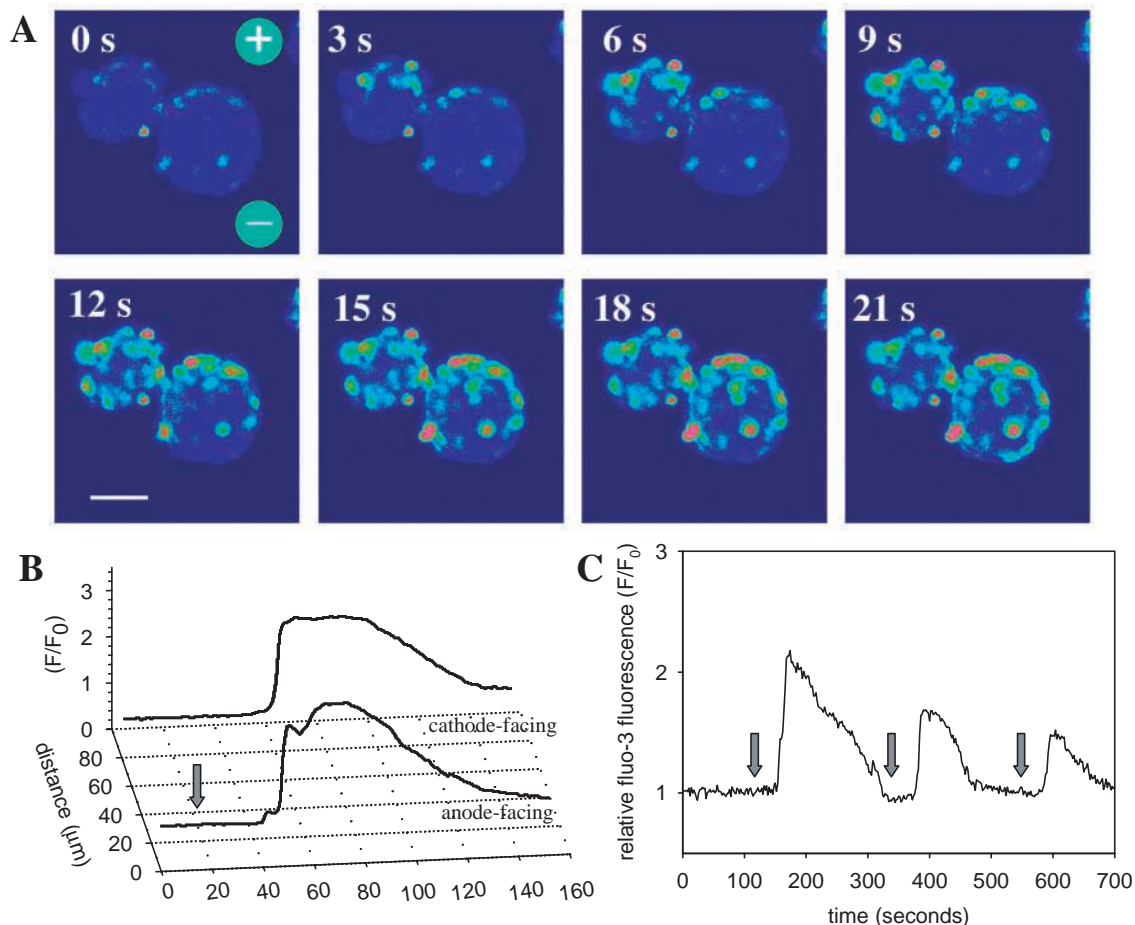


Fig. 1. The electrical-field-induced Ca²⁺ response in multicellular prostate tumor spheroids. (A) Image gallery of representative multicellular tumor spheroids treated with a single electrical field pulse (750 Vm⁻¹, 60 seconds). Images were recorded from the start point of the Ca²⁺ response, which occurred approximately 40 seconds after the onset of the electrical field. Tumor spheroids were loaded with the fluorescent Ca²⁺ indicator fluo-3. The Ca²⁺ response started at the anode-facing side and propagated towards the cathode-facing side. Images were recorded in 3 second intervals. Bar, 50 μ m. (B) Representative tracings of the electrical-field-treated Ca²⁺ response recorded in single cells at the anode- as well as the cathode-facing side of the tumor spheroid. (C) Ca²⁺ responses in single cells of multicellular tumor spheroids after repetitive treatment with electrical field pulses (750 Vm⁻¹, 60 seconds). Note that repetitive treatment with the electrical field decreased the amplitude as well as the duration of the Ca²⁺ response.

repetitively treated with 10 μM ATP and subsequently with a single electrical field pulse (750 Vm^{-1} for 60 seconds). Addition of ATP to the incubation medium resulted in a transient elevation of $[\text{Ca}^{2+}]_i$ (Fig. 2A). Repetitive treatment of tumor spheroids with ATP resulted in a decline of the amplitude of the Ca^{2+} response. Subsequent treatment with a DC electrical field pulse failed to raise $[\text{Ca}^{2+}]_i$, presumably owing to an interference of the signal transduction of purinergic receptors with the signalling cascade elicited by the electrical field pulse (Fig. 2A; $n=3$). To yield further information on the involvement of purinergic receptors in the electrical-field-evoked $[\text{Ca}^{2+}]_i$ response, tumor spheroids were pretreated for 60 minutes with the purinergic receptor antagonist suramin (300 μM ; $n=4$; Fig. 2B) or for 30 minutes with 2 U/ml apyrase ($n=3$; Fig. 2C), which scavenges extracellular ATP. Under both experimental conditions the $[\text{Ca}^{2+}]_i$ transient in response to electrical field treatment was

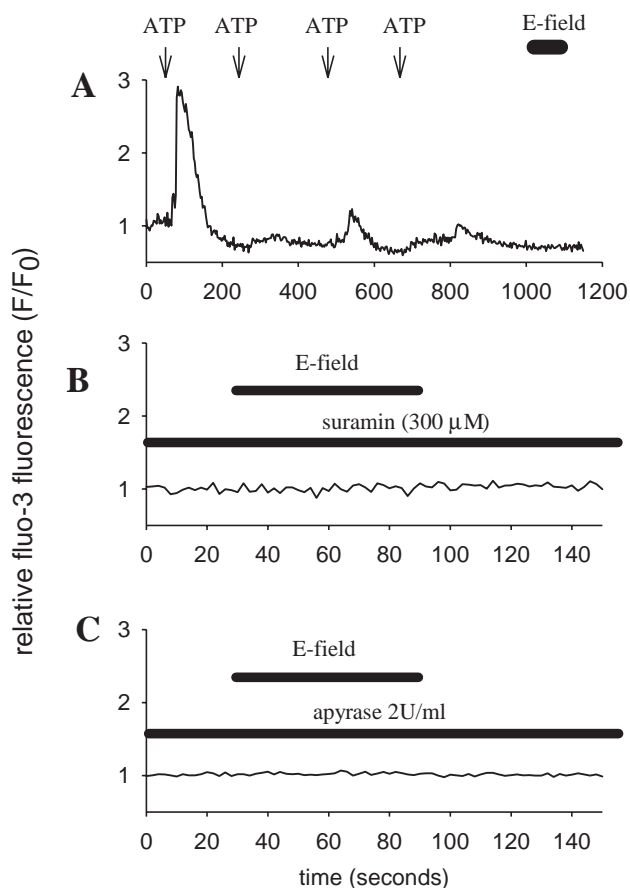


Fig. 2. Involvement of purinergic receptor stimulation in the electrical-field-induced Ca^{2+} response. (A) Tumor spheroids were repetitively treated with 10 μM ATP, which elicited a Ca^{2+} response with declining amplitude during repetitive application of ATP. Subsequent application of an electrical field (750 Vm^{-1} , 60 seconds) failed to raise $[\text{Ca}^{2+}]_i$, indicating interference with ATP-mediated signaling pathways. (B) Pretreatment of tumor spheroids for 60 minutes with 300 μM suramin, which inhibits purinergic receptor activation, blunted the electrical-field-induced Ca^{2+} response. (C) Inhibition of Ca^{2+} elevation was likewise achieved after incubation for 30 minutes with the ATP scavenger apyrase (2 U/ml). The tumor spheroids were treated with electrical field during the time indicated by the horizontal solid bar.

totally abolished, indicating that stimulation of purinergic receptors as well as ATP in the extracellular medium is a prerequisite for electrical-field-induced Ca^{2+} signaling.

ATP release upon treatment of multicellular tumor spheroids with DC electrical fields

In a recent study we have demonstrated that DU-145 cancer cells grown in monolayer culture release ATP via anion channels upon mechanical stimulation (Sauer et al., 2000). Since similar signal transduction cascades may prevail in Ca^{2+} signaling upon electrical field treatment, ATP release from tumor spheroids was investigated by the use of luciferase-based bioluminescence (Fig. 3). It was demonstrated that within 1 minute after electrical field treatment of multicellular tumor spheroids with a single electrical field pulse, ATP bioluminescence increased to $424 \pm 72\%$ ($n=9$) compared with the untreated control (set to 100%). Pretreatment of tumor spheroids for 20 minutes with the anion channel inhibitors tamoxifen (50 μM ; $n=4$), niflumic acid (200 μM ; $n=4$), and NPPB (50 μM ; $n=4$) significantly reduced electrical-field-evoked bioluminescence to $121 \pm 45\%$, $145 \pm 64\%$, and $150 \pm 22\%$, respectively, indicating that ATP may be released to the supernatant via anion channels.

Inhibition of the electrical-field-evoked Ca^{2+} response by anion channel inhibitors

We assumed that the elevation of $[\text{Ca}^{2+}]_i$ following treatment of tumor spheroids with a DC electrical field pulse may be caused by ATP release through anion channels and subsequent stimulation of purinergic receptors. To verify this assumption $[\text{Ca}^{2+}]_i$ changes upon electrical field treatment were recorded in the presence of anion channel inhibitors. As shown in Fig. 4, preincubation of tumor spheroids for 60 minutes with either tamoxifen (50 μM ; $n=10$), niflumic acid (200 μM ; $n=9$) or NPPB (50 μM ; $n=7$) totally abolished the Ca^{2+} response, indicating that ATP released via anion channels elicits the Ca^{2+}

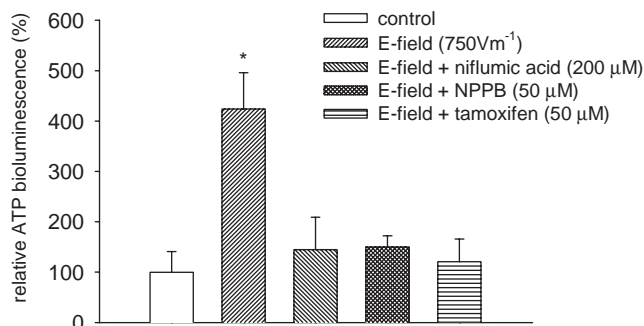


Fig. 3. ATP release upon treatment of multicellular tumor spheroids with DC electrical fields. ATP release after electrical field treatment (750 Vm^{-1} , 60 seconds) was evaluated by luciferin/luciferase-based bioluminescence. Between 30 and 50 tumor spheroids were treated with an electrical field pulse and subsequently a 150 μl aliquot of the supernatant was analyzed in the bioluminescence apparatus. Pretreatment for 30 minutes with the anion channel inhibitors niflumic acid (200 μM), NPPB (50 μM) and tamoxifen (50 μM) significantly inhibited the electrical-field-induced ATP release, indicating that ATP efflux occurs via anion channels.

response during electrical field treatment. Comparable results were achieved with the anion channel inhibitor DIDS (data not shown), which has also been shown to antagonize purinergic receptor stimulation (Zhang et al., 2000b).

Inhibition of DC electrical-field-induced c-Fos induction and growth stimulation of multicellular tumor spheroids by suramin and anion channel antagonists

Treatment of multicellular prostate tumor spheroids with DC electrical fields accelerates tumor growth by a mechanism involving a transient elevation of $[Ca^{2+}]_i$ (Sauer et al., 1997; Wartenberg et al., 1997). Consequently it should be expected that inhibition of the electrical-field-induced Ca^{2+} response blunts the stimulation of tumor growth. To investigate this issue tumor spheroids were treated with a single electrical field pulse, and tumor growth was investigated 6 days after electrical

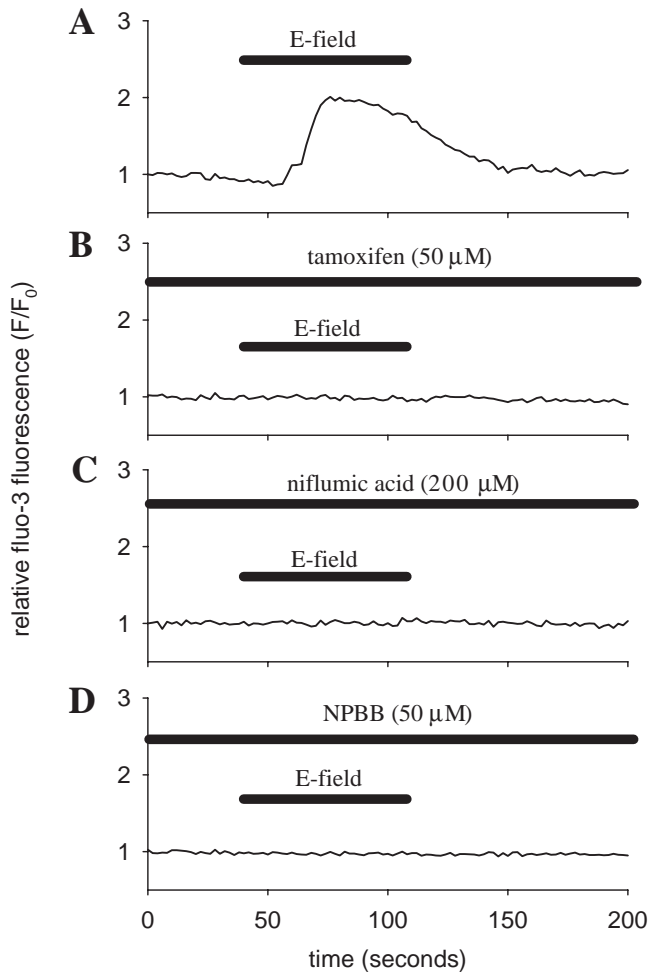


Fig. 4. Inhibition of the electrical-field-evoked Ca^{2+} response by anion channel inhibitors. Multicellular prostate tumor spheroids were pretreated for 60 minutes with the anion channel inhibitors tamoxifen (50 μ M) (B), niflumic acid (200 μ M) (C), and NPPB (50 μ M) (D). Under these experimental conditions the electrical-field-induced $[Ca^{2+}]_i$ response (A) was totally inhibited. Representative tracings recorded from single cells in multicellular tumor spheroids. The tumor spheroids were treated with electrical field during the time indicated by the horizontal solid bar.

field treatment (Fig. 5A). Furthermore, the protein levels of the growth-associated immediate early response gene *c-fos* were evaluated 1 hour after electrical field treatment (Fig. 6). To exclude that the applied anion channel inhibitors exerted toxic side effects, tumor spheroids were pretreated for 30 minutes with niflumic acid (200 μ M), tamoxifen (50 μ M) or NPPB (50 μ M), and subsequently 10 μ M ATP was added, which has been demonstrated to stimulate tumor spheroid growth (Sauer et al., 2001). After a further 30 minutes the medium was completely exchanged, and tumor spheroid growth was evaluated after 24

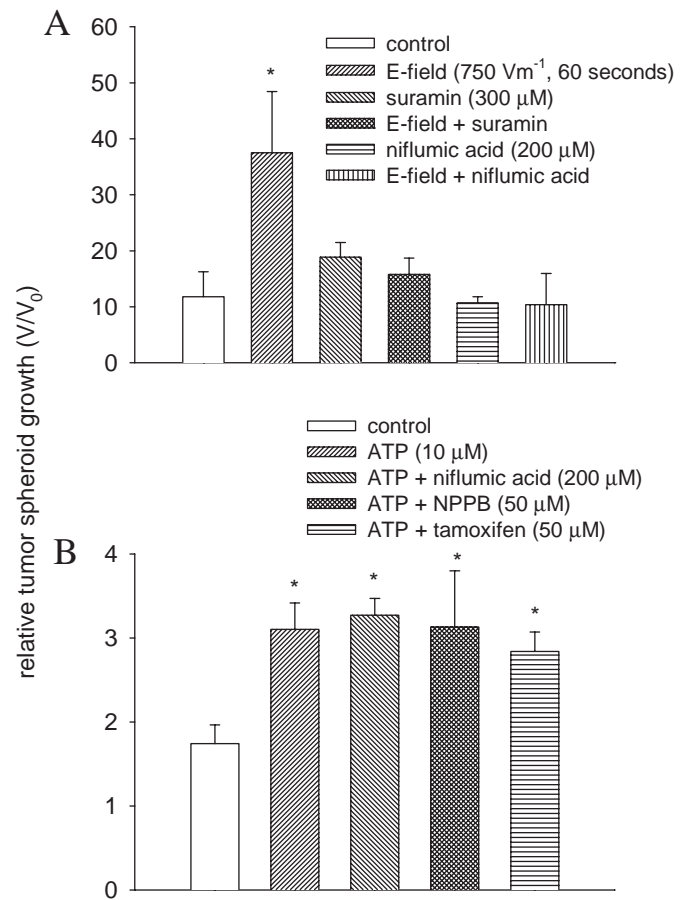


Fig. 5. Inhibition of DC electrical-field-induced growth stimulation of multicellular tumor spheroids by suramin and the anion channel antagonist niflumic acid (A), and effects of anion channel blockers on tumor spheroid growth induced by exogenous ATP (B). (A) Multicellular tumor spheroids were treated with a single electrical field pulse (750 Vm^{-1} , 60 seconds) in the absence (control) or presence of either suramin (300 μ M) or the anion channel inhibitor niflumic acid (200 μ M). Subsequently they were immersed in cell culture medium in the absence of the compounds and cultivated for a further 6 days. Tumor spheroid volumes were determined immediately after electrical field treatment and after 6 days. (B) Tumor spheroids were treated with niflumic acid (200 μ M), tamoxifen (50 μ M) or NPPB (50 μ M). After 30 minutes of incubation 10 μ M ATP was added and tumor spheroids were incubated for a further 30 minutes. Subsequently, tumor spheroids were washed and tumor spheroid growth was evaluated after 24 hours. Tumor spheroid growth is presented as relative volume increase V/V_0 where V_0 is the spheroid volume at the beginning of the experiment and V is the spheroid volume at the end of the experiment.

hours (Fig. 5B). Electrical field treatment of tumor spheroids accelerated tumor growth ($n=6$), which resulted in a 38 ± 11 -fold (V/V_0) increase in tumor spheroid volume in the electrical-field-treated sample compared with the untreated control, which exerted a 12 ± 4 -fold volume (V/V_0) increase during the 6 days of experimental observation (Fig. 5A). When $10\ \mu\text{M}$ ATP were exogenously added to tumor spheroids a significant growth stimulation was observed that was unchanged in the presence of anion channel inhibitors (Fig. 5B; $n=3$), excluding that the applied anion channel blockers exerted toxic effects on the tumor spheroids. Incubation of tumor spheroids for 1 hour with anion channel inhibitors alone did not impair tumor

spheroid growth (data not shown). Furthermore, a pronounced increase in the expression of c-Fos protein ($n=3$) with a maximum effect after 1 hour was obvious (Fig. 6A,B). The growth stimulation observed upon electrical field treatment could be efficiently inhibited when tumor spheroids were preincubated for 60 minutes with the anion channel inhibitor niflumic acid ($n=3$), as well as with the purinergic receptor antagonist suramin (Fig. 5A; $n=3$). Additionally, the elevation of c-Fos protein was totally abolished in the presence of suramin, as well as after preincubation with the anion channel inhibitors niflumic acid, tamoxifen and NPPB (Fig. 6A,B; $n=3$). Hence it is concluded that the increased c-Fos expression and growth stimulation of multicellular tumor spheroids by DC electrical field treatment requires the stimulation of purinergic receptors by ATP released to the extracellular space via anion channels.

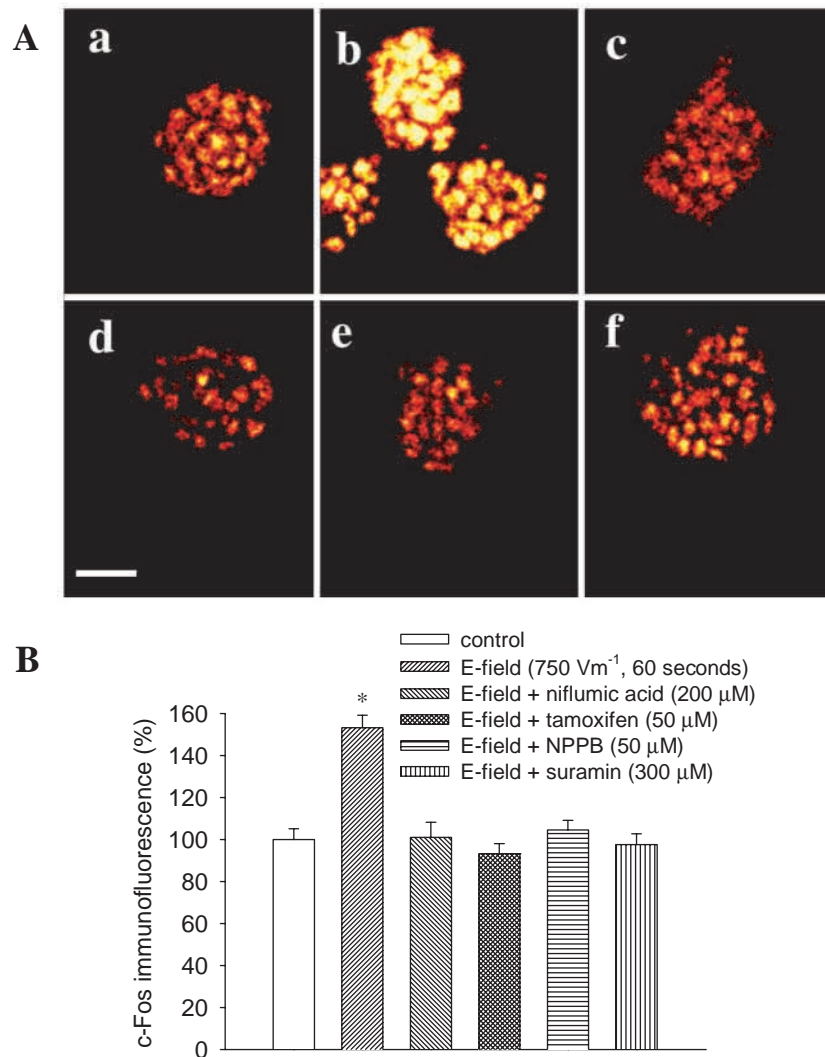


Fig. 6. Inhibition of DC electrical-field-induced c-Fos induction in multicellular tumor spheroids by suramin and anion channel antagonists. (A) Representative tumor spheroids immunolabeled with an antibody directed against Fos protein. Multicellular tumor spheroids remained untreated (control) (Aa) or were treated with a single electrical field pulse ($750\ \text{Vm}^{-1}$, 60 seconds) in the absence (Ab) or presence of either suramin ($300\ \mu\text{M}$) (Ac) or the anion channel inhibitors niflumic acid ($200\ \mu\text{M}$) (Ad), tamoxifen ($50\ \mu\text{M}$) (Ae), and NPPB ($50\ \mu\text{M}$) (Af). They were subsequently immersed in cell culture medium in the absence of the compounds and fixed after 1 hour of incubation. Bar, $75\ \mu\text{m}$. (B) Quantitative evaluation of Fos immunofluorescence in control and electrical-field-treated samples. Note that in the presence of suramin or anion channel blockers the induction of c-Fos following electrical field treatment was significantly inhibited.

Discussion

Active ATP release has been demonstrated previously to occur in a number of cell types. However, the mechanism of ATP release and the physiological role of released extracellular ATP are still scarcely defined. The present study was undertaken to characterize transient Ca^{2+} responses that arise when multicellular prostate tumor spheroids are treated with DC electrical fields. The measurements were undertaken with tumor spheroids immersed in a low-ionic medium, which was chosen in order to reduce the magnetic field component of the EMF field to the laboratory noise level (Cameron et al., 1993). Furthermore, it was excluded that under the applied experimental conditions neither reversible nor irreversible membrane permeabilization occurred (Sauer et al., 1999b). It was observed that a single electrical field pulse with a field strength of $750\ \text{Vm}^{-1}$ and a duration of 60 seconds elicited a transient increase in $[\text{Ca}^{2+}]_i$ occurring approximately 30–40 seconds after the onset of the electrical field. Interestingly, it was observed that the elevation of $[\text{Ca}^{2+}]_i$ occurred at the anode-facing side of the tumor spheroids and spread with a velocity of about $12\ \mu\text{m}$ per second to the cathode-facing pole of the tumor spheroid. Comparable observations have been recently achieved with prostate cancer cells of the androgen-dependent cell line LNCaP (Perret et al., 1999). We have previously shown that the anode-facing side of multicellular tumor spheroids is hyperpolarized during the electrical field whereas the cathode-facing side is depolarized (Sauer et al., 1997). The polarization of the tumor spheroid should – in the absence of voltage-operated Ca^{2+} channels – result in an increase in the driving force for Ca^{2+} ions at the anode-facing side and a decrease at the cathode-facing side (Robinson, 1985). Alternatively, the observation that the

Ca²⁺ signal starts at the anode-facing side of the tumor spheroids may be explained by the electrophoretic movement of ATP in the electric field, which is in the range of 60 μm per second, and results in a higher concentration of ATP at the anode side of the spheroid. Since intracellular coupling via gap junctions is absent in prostate tumor cells of the DU-145 cell line grown in monolayer culture (Sauer et al., 2000), it was assumed that the Ca²⁺ wave spreading across the tumor spheroids surface was directed through an extracellular signaling pathway.

Extracellular pathways for the propagation of Ca²⁺ waves have been reported for several preparations including basophil leukemic cells (Osipchuk and Cahalan, 1992), hepatocytes (Schlosser et al., 1996), ciliary epithelial cells (Homolya et al., 2000) and osteoblastic cell lines (Jorgensen et al., 1997). It was demonstrated that these extracellular pathways of Ca²⁺ wave propagation were based on activation of purinergic receptors of the P2Y class that activate phospholipase C, resulting in the generation of Ins(1,4,5)P₃ and intracellular Ca²⁺ release from Ins(1,4,5)P₃-sensitive Ca²⁺ stores. It was shown that mechanical stimulation of cells resulted in release of ATP stored inside the cell to the extracellular space, which subsequently stimulated purinergic receptors in an autocrine and paracrine manner and elicited intracellular Ca²⁺ responses. Since it was recently demonstrated by our group that comparable mechanisms of ATP release were existent in prostate cancer cells of the DU-145 cell line (Sauer et al., 2000), we tested whether electrical DC field treatment increased ATP in the extracellular medium. Indeed we found that within approximately 1 minute after electrical field treatment substantial amounts of ATP could be detected in the supernatant of electrical-field-treated tumor spheroids. It has been recently demonstrated by us that under hypotonic conditions DU-145 prostate cancer cells grown in monolayer culture release approximately 1.6 pmol ATP per 10⁵ cells, which is sufficient to raise an intracellular (Ca²⁺)_i response (Sauer et al., 2000). In the present study the role of released ATP in the electrical-field-induced Ca²⁺ response was investigated by preincubation of tumor spheroids with the purinergic receptor antagonist suramin (1 hour) and the ATP scavenger apyrase (30 minutes). Both experimental conditions totally abolished the ATP-induced [Ca²⁺]_i transient, which indicates that ATP in the extracellular medium and purinergic receptor stimulation are prerequisites for electrical-field-induced Ca²⁺ signaling.

To investigate the release mechanisms for intracellular ATP, tumor spheroids were preincubated with antagonists of anion channels that significantly inhibited ATP release. ATP release through anion channels has been described for a variety of preparations (Mitchell, 2001; Mitchell et al., 1998; Cotrina et al., 1998; Sabirov et al., 2001) and their relationship to the cystic fibrosis transmembrane conductance regulator (CFTR) has been critically discussed (Schwiebert, 2001). Recently it has been shown that CFTR may not by itself release ATP but may regulate the activity of a separate anion channel (Braunstein et al., 2001). Anion channels with less selectivity for chloride versus other halides or larger anions are prime candidates for putative ATP channels. These include the outwardly rectifying Cl⁻ channel (ORCC) as well as plasma membrane forms of the voltage-dependent anion channel (VDAC). Recently, an ATP-conducting anion channel that was

activated under hyperpolarizing conditions was characterized in *Xenopus* oocytes. During hyperpolarizing pulses the permeability of this channel was more than 4000 times higher for ATP than that for Cl⁻ (Bodas et al., 2000).

Furthermore, it has been demonstrated that the multidrug resistance transporter P-glycoprotein-associated Cl⁻ channel, which belongs to the ATP-binding cassette (ABC) transporter superfamily, may regulate ATP release channels (Roman et al., 2001). We have previously shown that multicellular tumor spheroids of different origin, including spheroids of the DU-145 cell line, express intrinsic P-glycoprotein with the development of quiescent cell areas in the depth of the tissue. The size class of tumor spheroids (diameter 100±50 μm) used in the present study express low, but detectable, levels of intrinsic P-glycoprotein that could serve as a mediator for ATP release.

The physiological function of ATP release to the extracellular medium is still a matter of debate. It has been argued that, in ciliary epithelial cells of the eye, released ATP may modulate aqueous humor flow by autocrine and paracrine mechanisms within the two cell layers of this epithelium (Mitchell et al., 1998). In liver cells (Wang et al., 1996; Feranchak et al., 2000) and biliary epithelial cells (Roman et al., 1999), recovery from swelling is mediated by an autocrine pathway involving conductive release of ATP. In endometrial (Chan et al., 1997), intestinal (Merlin et al., 1994), and epidymal epithelial cells (Chan et al., 1995), regulation of Cl⁻ secretion is mediated by extracellular ATP. Recently, it has been demonstrated that ATP released constitutively from Madin-Darby canine kidney (MDCK), COS-7 and HEK-293 cells modulates phosphatidylinositol signaling and turnover as well as cAMP production. It was assumed that autocrine and paracrine ATP signaling occurs constantly in the extracellular milieu and may establish a 'set point' for multiple signal transduction pathways or signaling molecules (Insel et al., 2001; Ostrom et al., 2000). In the tumor spheroid model used in the present study it is demonstrated that electrical field treatment with a single electrical field pulse increased c-Fos expression and accelerated tumor growth. c-Fos activation in response to EMF fields has been previously reported in HeLa cells that were transiently transfected with plasmids containing upstream regulating regions of *c-fos* (Rao and Henderson, 1996). The activation of *c-fos* was shown to be sensitive to the presence of extracellular Ca²⁺ (Karabakhtsian et al., 1994). In the present study, inhibiting ATP release by pretreatment with anion channel blockers and antagonizing activation of purinergic receptors by suramin significantly inhibited c-Fos elevation and growth stimulation after electrical field treatment of multicellular tumor spheroids. This clearly indicates that the observed effect of the electrical field on the acceleration of tumor growth was caused by ATP release and purinergic receptor stimulation.

The mitogenic effect of ATP has been shown for several preparations including prostate cancer cells (Wartenberg et al., 1999; Sauer et al., 2001), smooth muscle cells (Wang et al., 1992; Erlinge, 1998), ovarian tumor cells (Popper and Batra, 1993), renal proximal tubule cells (Paller et al., 1998), and mesangial cells (Schulze-Lohoff et al., 1992). By contrast, EMF fields have been repeatedly reported to accelerate tumor cell growth in vitro and, although contradictory data exist (Gurney and van Wijngaarden, 1999; van Wijngaarden et al.,

2001; Jahn, 2000), epidemiological studies have suggested that longterm exposure to EMF fields increase the incidence of several cancers (Robinson et al., 1999; Loomis et al., 1998; Bianchi et al., 2000; Villeneuve et al., 2000; Caplan et al., 2000). The signal transduction pathways underlying the growth-stimulating and tumor-promoting effects of EMF fields are far from being sufficiently investigated. Hence, the data of the present study performed with the complex 3D neoplastic tissue of multicellular tumor spheroids may have extended impact on our understanding of how electrical fields promote either benign or neoplastic prostate tumor growth.

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