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Low electric fields induce ligand-independent activation of EGF receptor and ERK via electrochemical elevation of H^+ and ROS concentrations

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ABSTRACT

Physiological electric fields are involved in many biological processes and known to elicit their effects during long exposures ranging from a few hours to days. Following exposure to electric fields of physiological amplitude, epidermal growth factor receptor (EGFR) was demonstrated to be redistributed and upregulated with further intracellular signaling such as the MAPK signaling cascade. In our study we demonstrated EGFR activation and signaling induced by short train of pulsed low electric field (LEF) (10 V/cm, pulse-width 180 μs, 500 Hz, 2 min) in serum-free medium, following 24-hour starvation, and in the absence of exogenous EGF ligand, suggesting a ligand-independent pathway for EGFR activation. This ligandless activation was further confirmed by using neutralizing antibodies (LA1) that block the EGFR ligand-binding site. EGFR activation was found to be EGFR kinase dependent, yet with no dimerization following exposure to LEF. ERK activation was found to be mainly a result of EGFR downstream signaling though it partially occurred via EGFR-independent way. We demonstrate that reactive oxygen species and especially decrease in pH generated during exposure to LEF are involved in EGFR ligandless activation. We propose a possible mechanism for the LEF-induced EGFR ligand-independent activation and show activation of other receptor tyrosine kinases following exposure to LEF.

1. Introduction

Physiological electric fields (EFs) are known to be in the range of up to several volts per cm and are associated with development, physiology, regeneration and pathology, and various biological systems have been reported to respond to endogenous and exogenous EFs. Voltage gradients which are present for several hours or even days were found to be involved in cell division, directional cell migration, wound healing and cell differentiation (for review see [1,2]).

External low DC EFs in the range of 1–30 V/cm applied to adherent cells, induced lateral electrophoretic displacements of charged membrane proteins and lipids, thereby resulting in segregation of these components at the cell surface [3–5]. One of the receptors studied in relation to EF is the epidermal growth factor receptor (EGFR) which plays a pivotal role in normal and pathological cellular functions. EFs of physiological strength activate several signaling pathways, including EGF receptors, MAPK, ERK, Src and PI3K signaling (for a review see [6]). The EGF-receptors on corneal epithelial cells [7], on fibroblasts [8] and on keratinocytes [9] were redistributed by a physiological applied EF and accumulated cathodally. EF-induced upregulation and redistribution of EGFR (and colocalization with actin) towards the cathodal side of corneal cells did not occur in serum-free medium [7]. EGFRs accumulated cathodally with membrane lipids and the asymmetry of EGFRs also induced asymmetric intracellular signaling through MAPK signaling cascade. Increased activation of phosphorylated ERK1/2 was demonstrated predominantly on the cathode side [10].

Physiological EFs elicit their biological effects only during long exposure ranging from a few hours to days. As short exposures are preferable for electric based therapeutic treatments, we examined the possibility of affecting EGFR activation and signaling by short exposures to pulsed EF with amplitudes higher than physiological ones.

In this study we demonstrate direct EGFR activation and signaling in COS5-7 and HaCaT cells, induced by short train of pulsed low EFs (LEF) (10 V/cm, pulse-width 180 μs, 500 Hz, for 2 min). The activation takes place in serum-free medium following 24-hour starvation and in the absence of exogenous EGF ligand, suggesting a ligand-independent activation pathway for EGFR. We found that this activation is EGFR-kinase dependent and attributed to electrochemical products formed in the solution during electric stimulation. We also propose a general possible mechanism for LEF-induced EGFR activation and demonstrate tyrosine phosphorylation of other receptor tyrosine kinases (RTKs) following exposure to LEF.

Abbreviations: EF(s), Electric field(s); LEF, Low electric fields; ROS, Reactive oxygen species; H_2DCF-DA, Dichlorodihydrofluorescein diacetate; DHA, Dehydroascorbic acid; RTK, Receptor tyrosine kinase; PTPs, Protein tyrosine phosphatases; DC, Direct current; FITC, Fluorescein isothiocyanate; HBSS, Hank's buffered salt solution; BSA, Bovine serum albumin; TBHP, *tert*-Butyl hydroperoxide; BS^3, Bis(sulfosuccinimidyl) suberate; DMEM, Dulbecco's modified Eagles medium; FCS, Fetal calf serum; PI, Propidium iodide; HRP, Horseradish peroxidase; PS, Phospholipid phosphatidylserine; PIP_2, Phosphatidylinositol 4,5-bisphosphate; MTT, 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

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2. Materials and methods

2.1. Antibodies and chemicals

Phospho-EGF receptor (Tyr1173) (53A5) rabbit mAb was purchased from Cell Signaling (Boston, MA). Monoclonal anti-phosphotyrosine clone PY20 and neutralizing antibodies (monoclonal anti-EGFR antibodies, clone LA1) were purchased from Upstate Biotechnology (Lake Placid, NY). Rabbit polyclonal anti-EGFR (1005), rabbit polyclonal ERK 2 (C-14), mouse monoclonal anti-EGFR (528):sc-120 and FITC-conjugated donkey anti-mouse IgG were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Monoclonal anti MAPK, activated (diphosphorylated ERK-1&2) and anti-actin were purchased from Sigma-Aldrich. Horseradish peroxidase (HRP)-conjugated anti-mouse and anti-rabbit secondary antibodies for Western blot were purchased from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA, USA). Tyrphostin AG1478 was purchased from Calbiochem. EGF, H_2DCF-DA, DHA, *tert*-butyl hydroperoxide (TBHP) were purchased from Sigma-Aldrich. Bis(sulfosuccinimidyl) suberate (BS^3) was obtained from Pierce.

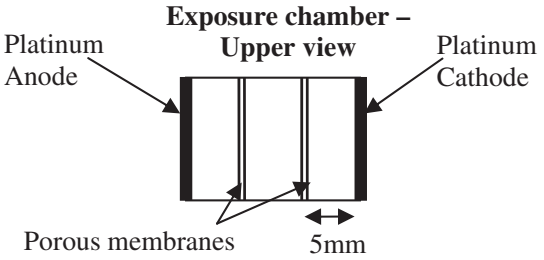
2.2. Cell culture

COS5-7 (fibroblast-like cells), African green monkey kidney derived from CV-1 subclone of COS5-7 and HaCaT cells (human keratinocyte cell line, a gift from Prof. N. E. Fusenig, German Cancer Research Center, Heidelberg, Germany) were cultured in Dulbecco's modified Eagles medium (DMEM) supplemented with L-glutamine (2 mM), 10% fetal calf serum (FCS) and 0.05% PSN solution (penicillin 10,000 units/ml, streptomycin 10 mg/ml and nystatin 1250 units/ml). All cells were maintained in monolayer culture and grown to confluence in 75 cm^2 tissue culture flasks (Corning, NY) at 37°, in 95% air and 5% CO_2. Cells were harvested by trypsinization (0.25% trypsin and 0.05% EDTA) for 3 min at 37 °C and trypsin inhibitor (Sigma-Aldrich) was subsequently added. Since trypsin is a proteolytic enzyme, we verified that the trypsinization process did not lead to EGFR receptor cleavage (data not shown). Cells were then washed twice by centrifugation (5 min, 210 ×g; RT6000D, Sorvall, Asheville, NC). Cells were resuspended (~1.5×10^6 cells/ml) in LEF exposure medium (serum-free DMEM without phenol red, supplemented with 25 mM Hepes), or in HBSS where indicated. All culture media, antibiotics, trypsin and FCS were purchased from Biological Industries (Beit Haemek, Israel). For all experiments, cells were starved for 24 h in serum-free DMEM. Where indicated, cells were incubated with EGF (15 ng/ml) (Sigma-Aldrich) in serum-free DMEM at room temperature for 5 min.

2.3. Exposure of cells to pulsed electric fields and pH changes

Exposure of cells to a train of low intensity unipolar rectangular voltage pulses was carried out by employing an electric pulse generator (Grass S88 Stimulator). The exposure was performed in a plastic cuvette by placing 0.5 ml of cell suspension (~1×10^6 cells/ml) between two parallel platinum electrodes separated by 0.5 cm, yielding a uniform EF. Where indicated, cells were electrified in a three-compartment chamber as shown in Scheme 1; a rectangular chamber made of polystyrene, 15 mm in length, 10 mm in width and two platinum electrodes on each side 0.5 cm^2 in area. The chamber was divided into three compartments (anode, central and cathode) by two porous membranes (polyethersulfone, 0.8 μm pores, 200 μm depth), allowing free passage of charges and solutes but preventing cells from transfer from one compartment to another. The two porous membranes possessed very low electrical resistivity, thereby not altering the EF strength which was equal for each of the compartments. The volume of cell suspension (~1×10^6 cells/ml) added to each compartment was 300 μl.

The EF parameters were monitored on-line by recording voltage and current on an oscilloscope. Cells were exposed to a train of 20 V/cm or 10 V/cm of 180 μs-width pulses at frequency of 500 Hz, for total exposure time of 1 min for 20 V/cm and 2 min for 10 V/cm. All experiments were performed after cells were starved in serum-free DMEM for 24 h.



Scheme 1. The three-compartment chamber.

In selected experiments, cells were pre-incubated with the EGFR kinase inhibitor, AG1478 (Calbiochem), in a concentration of 2 μM as specified in the figure legend, for 1.5 h at 37 °C prior to treatment. For evaluating the involvement of pH changes in EGFR phosphorylation, cells were exposed to LEF 10 V/cm for 2 min in HBSS supplemented with different concentrations of Hepes buffer with initial pH of 7.4, as indicated in the appropriate legend. For direct extra-cellular acidification, HBSS + 10 mM MES was titered in advance (prior to suspending the cells) to the indicated pH values by adding HCl directly to the buffer. Cells were then resuspended for 1 min in the acidic buffer. Following exposure to the above mentioned stimuli, cells were diluted 5-fold with DMEM + 25 mM Hepes at pH 7.4 before Western Blotting.

The measurement of the medium's pH in the three compartment chamber was carried out shortly after termination of exposure to LEF of 10 V/cm for 2 min both by a pH electrode (SevenEasy, Mettler) and using a universal paper indicator (Riedel-de Haën) yielded, pH $\leq$ 5.5 in the anode compartment, pH $\approx$ 7.5 in the central compartment and pH $\geq$ 10 in the cathode one.

2.4. Detection of cell viability

Cell viability was detected using propidium iodide (PI) exclusion test — after exposure of cells to LEF, cells were centrifuged, washed and incubated with 15 μM PI for 5 min before evaluation. Sample analysis was carried out by flow cytometry (488 nm excitation and 580 nm emission) and analysis of data was performed using WINMDI flow cytometry application software. In addition, cell viability was examined by MTT and Neutral Red uptake assays using Elisa Reader as described previously [65].

2.5. Total protein measurement

Total protein measurement was performed after cells were washed, centrifuged and lysed using RIPA buffer (Pierce). Protein content in lysates was measured by BCA protein detection kit (Pierce) according to the manufacturer's instructions.

2.6. Western blotting

After exposure to the different stimuli, cells were collected into microcentrifuge tubes. Cells were then centrifuged and washed with cold PBS, centrifuged again and lysed using RIPA buffer (Pierce) supplemented with Protease Inhibitor Cocktail 1:100 (Calbiochem), 0.1 mM PMSF and 1 mM orthovanadate for 30 min on ice. Lysates were clarified at 14,000 rpm in an Eppendorf centrifuge for 15 min at 4° in order to pellet the cell debris. The supernatant was transferred to a new tube and protein concentrations in lysates were measured using BCA protein detection kit (Pierce) according to the manufacturer's instructions. Then, a sample buffer (×4 containing 0.5% bromophenol

blue, 62 mM Tris–HCl 6.8, 20% glycerol, 2% SDS, 5% β-mercaptoethanol) was added to the samples which were then boiled for 5 min. Equal amounts of protein samples were subjected to SDS-PAGE gels (9%) for separation and transferred to nitrocellulose membranes. The membranes were blocked at room temperature for 1 h in TTBS + 1%BSA (TTBS contains Tris–HCl 20 mM pH 7.6, NaCl 0.137 M, Tween 0.05%) and immunoblotted using the specific primary antibodies indicated in the figures. The primary antibodies were used diluted in the blocking buffer according to the following specification: anti-pTyr1173 1:800, anti-EGFR (1005) 1:800, anti-pERK1/2 1:10,000, anti-ERK2 1:1000 and anti-actin 1:1500. The membranes were incubated with the primary antibodies for 1 h at room temperature except for anti-pTyr1173 which required overnight incubation at 4 °C. Proteins were detected using HRP-conjugated secondary antibodies (diluted 1:5000–1:20,000 in TTBS + 0.1% BSA) and ECL Western blotting detection reagents (Pierce) according to the manufacturer's instructions.

2.7. Statistical analysis

Quantification of blots was performed using software for densitometric evaluation TINA version 2.0. For each group, the ratio between phospho-protein and total protein was calculated. To further minimize variances between different experiments in the series, the values obtained following treatments were normalized to the values obtained for the respective unexposed (control) group. Data represented in bar-charts are expressed as mean of the normalized-to-control ratios (fold of induction) ± SD, from 3 or more independent experiments. Two-tailed one-sample t-test was performed for comparison of treatments with control (ratio of 1). P-value <0.05 was considered to be statistically significant. Repeated measures analysis of variance (ANOVA) was implemented in order to investigate the effect of buffer capacity on EGFR and ERK phosphorylation using SPSS 15.0.1 statistical package.

Some experiments performed only in duplicates are represented in charts by columns containing ranges instead of error bars.

2.8. Measurement and neutralization of ROS formation

Changes in ROS level were determined using H_2DCF-DA probe (Sigma-Aldrich) by flow cytometry (FACSort, Becton Dickinson, San Jose, CA) employing 488-nm argon laser excitation. The probe turns into green fluorescent product (488 nm excitation and 530 nm emission) upon oxidation by ROS. Cells harvested as described above were resuspended in low-glucose (1 mg/ml) DMEM supplemented with 5% FCS and pre-loaded with 10 μM H_2DCF-DA at 37 °C, in 95% air and 5% CO_2 for 1.5 h. At the end of the incubation, cells were washed with DMEM supplemented with 25 mM Hepes before exposure to LEF. For neutralizing ROS, cells were pre-loaded with 1 mM dihydro-ascorbic acid (DHA) similarly to the preloading protocol of H_2DCF-DA. As a positive control for oxidation, cells were incubated with 1 mM *tert-butylhydroperoxide* (TBHP) for 5 min. Following treatment, cells were washed once in DMEM with 5% FCS and placed on ice before analysis by flow cytometry. Data analysis was performed using WINMDI flow cytometry application software.

2.9. Dimerization assay

After different treatments, cells were washed with cold PBS and incubated with the membrane impermeable cross linker BS^3 (final concentration — 2 mM) dissolved in PBS pH 8.0 for 30 min on ice and for additional 30 min at room temperature. Reaction was quenched by 1 M Tris, pH 7.5 (final concentration — 20 mM) for 15 min at room temperature. The cells were then washed twice with PBS and prepared as described above for Western blotting before analysis by 5% SDS-PAGE and immunoblotting with rabbit polyclonal anti-EGFR (1005) followed by a secondary antibody linked to HRP.

2.10. Activation of human phospho-receptor tyrosine kinases

HaCaT Cells were exposed to LEF in suspension as described above and subjected to Human Phospho-RTK Array Kit (catalog number ARY001; R&D systems). This array was used for parallel determination of the relative phosphorylation level of 42 different RTKs. The array eliminates the need for numerous immunoprecipitations and/or Western Blots. Level of phosphorylation is assessed using HRP-conjugated pan phosphor-tyrosine antibody followed by chemiluminescent detection. Samples were prepared and protocol was performed according to the manufacturer's instructions.

3. Results

3.1. Low electric fields induce EGF receptor and ERK phosphorylation both in HaCaT cells and in COS5-7 cells

We examined whether tyrosine phosphorylation of EGF receptor increased in response to exposure of serum-starved COS5-7 and HaCaT cells to a train of pulsed LEF (180 μs pulse duration, 500 Hz frequency, 10 V/cm EF strength) for 2 min. Cell lysates were resolved by SDS-PAGE and blotted with anti-p-EGFR antibody which recognizes only site-specific tyrosine-phosphorylated EGFR (Tyr-1173, which is an autophosphorylated site). Immediately after exposure there is a significant increase in EGFR phosphorylation level by ~10.3 and ~13.3 fold relative to unexposed cells, in both HaCaT and COS5-7 (Fig. 1A) cells, respectively. However, 15 min following termination of exposure this elevated level of tyrosine phosphorylation decreases down to 4.3-fold and 6.7-fold for HaCaT and COS5-7, respectively. Yet, EGF (15 ng/ml) produces a dramatic increase in EGFR phosphorylation (50.1-fold and 30.8-fold for HaCaT and COS5-7, respectively) when using this ligand as a positive control. In addition, exposure to LEF also causes ERK phosphorylation in both cell lines. In order to examine whether the LEF induced EGFR phosphorylation level can be further elevated we exposed COS5-7 cells to higher EF strength of 20 V/cm for 1 min (where the rest of electric parameters were identical) (Fig. 1B). Indeed, we obtained a higher level of EGFR tyrosine phosphorylation. However, the exposure to LEF of 20 V/cm for 1 min results in decreased cell viability by 35% relative to unexposed cells ($p = 0.0018$ by one-sample t-test) using both PI exclusion test and total protein assay. In contrast, viability following exposure to 10 V/cm for 2 min is not significantly different from control cells ($p = 0.11$ by one-sample t-test) (Fig. 1C). Viability was also examined by MTT and Neutral Red assays. Exposure to 10 V/cm yielded viability of 97% (MTT) and 100% (Neutral Red) relative to unexposed control. However, exposure to 20 V/cm resulted in viability of 40% (MTT) and 38% (Neutral Red) relative to unexposed control. Despite the higher level of EGFR phosphorylation upon exposure to 20 V/cm, we continued studying the effects of LEF possessing strength of 10 V/cm in order to ensure that the signal obtained originated only from live cells.

3.2. LEF-induced EGFR phosphorylation is ligand-independent but depends on its kinase activity

3.2.1. LEF-induced EGFR phosphorylation is ligand-independent

It is important to note that all experiments were performed with serum-starved cells, in the absence of exogenous EGF, implying that phosphorylation of EGFR, induced by LEF, is ligand-independent. However, it has been demonstrated in previous studies that transactivation of EGFR can occur due to a release of membrane-anchored EGFR ligands, an event mediated by activation of transmembrane metalloproteinases [11–15]. We therefore wanted to verify whether LEF-induced activation of EGFR proceeds through a truly ligand-independent pathway by using neutralizing antibodies (monoclonal anti-EGFR antibodies, clone LA1, Upstate Biotechnology) that effectively block the ligand-binding site of EGFR. As depicted in

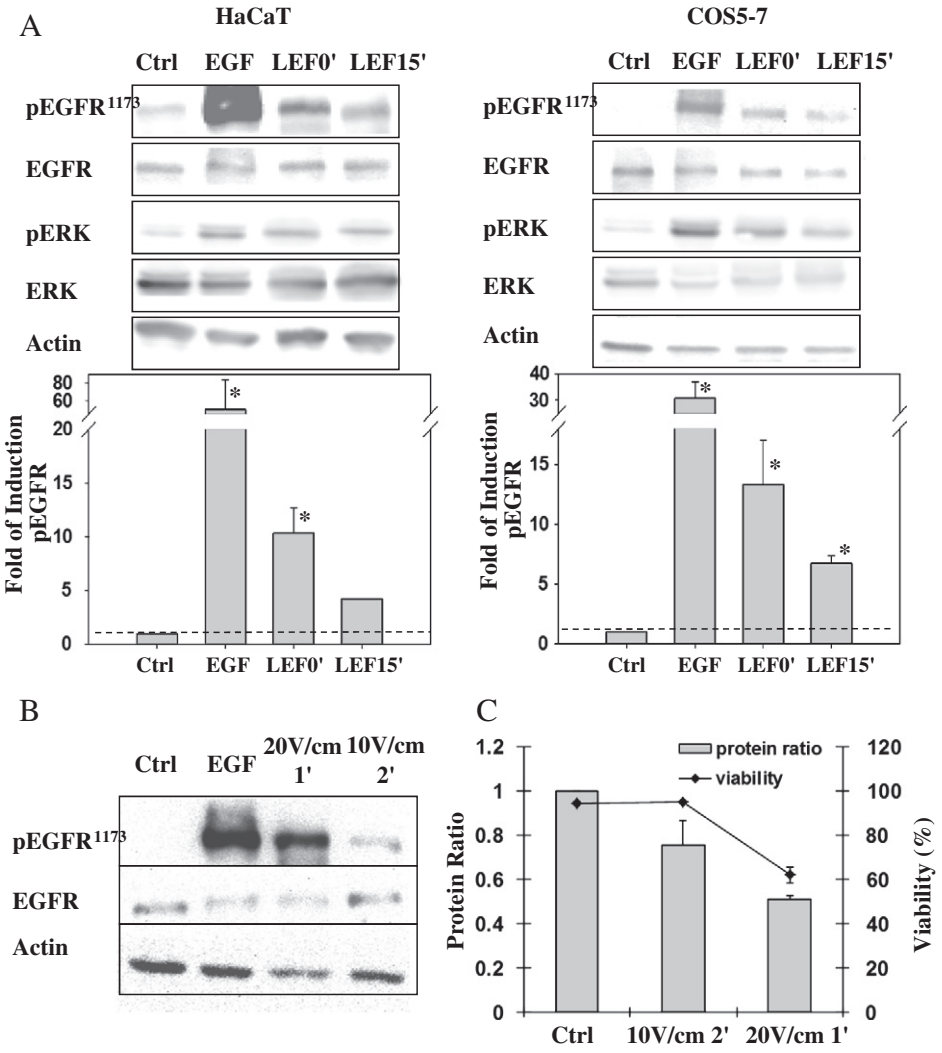


Fig. 1. LEF induced EGFR and ERK phosphorylation in both COS5-7 and HaCaT cells. Serum starved HaCaT and COS5-7 (*A*) cells were exposed to 10 V/cm for 2 min, in suspension, washed twice with cold PBS and lysed using RIPA buffer, immediately (LEF0′) or 15 min (LEF15′) after the exposure, or after an addition of 15 ng/ml EGF (EGF) for 5 min. The cell lysates were subjected to SDS-PAGE followed by Western blotting with pEGFR^{1173}, EGFR, pERK1/2, ERK2 and actin antibodies. The bar-charts below the blots represent computer-assisted densitometric evaluation of the ratio between phosphorylated EGFR and total amount of EGFR for each group, normalized to the ratio obtained from control unexposed cells. Data was extracted from 3 independent experiments (except for LEF15′ in HaCaT, where 2 experiments were conducted) and is expressed as mean ± SD (*) $p < 0.05$ relative to control by one-sample t-test. *B*. Serum-starved COS5-7 cells were exposed to 10 V/cm for 2 min, 20 V/cm for 1 min and 15 ng/ml EGF for 5 min as a positive control. Cells were washed with cold PBS and lysed using RIPA buffer. The lysates were subjected to SDS-PAGE followed by Western blotting with pEGFR^{1173}, EGFR and actin antibodies. *C*. Serum starved COS5-7 cells were exposed to LEF 10 V/cm for 2 min and 20 V/cm for 1 min at room temperature. Bar charts represent the protein left after the exposure relatively to the serum-starved unexposed (Ctrl) cells (protein ratio) and the line represents percent of viability as measured by FACS using PI exclusion.

Fig. 2A, addition of neutralizing antibodies to EGF completely blocked phosphorylation of EGFR induced by EGF (EGF-induced phosphorylation of ~20-fold for pEGFR^{1173} relative to serum starved cells), but had no effect on EGFR phosphorylation induced by LEF (~4-fold relative to serum-starved, unexposed cells), indicating that LEF-induced EGFR phosphorylation does not involve its ligands. Phosphorylation with or without the effect of neutralizing antibodies was also verified using the general anti-pTyr antibody, PY20.

3.2.2. LEF-induced EGFR and ERK phosphorylation depends on EGFR kinase activity

It is well established that binding of EGF to EGFR leads to increased EGFR kinase activity and ERK phosphorylation [16]. Therefore, we examined whether exposure to LEF induces EGFR and ERK phosphorylation through EGFR kinase activity. For that purpose, serum-starved cells were pre-incubated in the presence and in the absence of the selective inhibitor of EGFR tyrosine kinase activity, AG1478, prior to and during exposure to LEF (Fig. 2B). The levels of EGFR and ERK phosphorylation were then analyzed. Pre-incubation with AG1478 almost completely abolishes LEF-induced EGFR phosphorylation, implying that this phenomenon proceeds through EGFR tyrosine kinase activity. In addition, preincubation with AG1478 significantly decreases, though not completely, ERK phosphorylation. These experiments demonstrated that EGFR phosphorylation induced by LEF partially mediates LEF-induced ERK activation.

3.3. LEF-induced ligand-independent activation of EGFR occurs mostly near the anode

Exposure of cells to LEF in a physiological medium, where the electrodes are in direct contact with the conducting solution, is expected to lead to the formation of EF in the inter-electrode gap as well as to the production of electrolytic products at the electrode–solution interface. In order to distinguish between effects caused solely

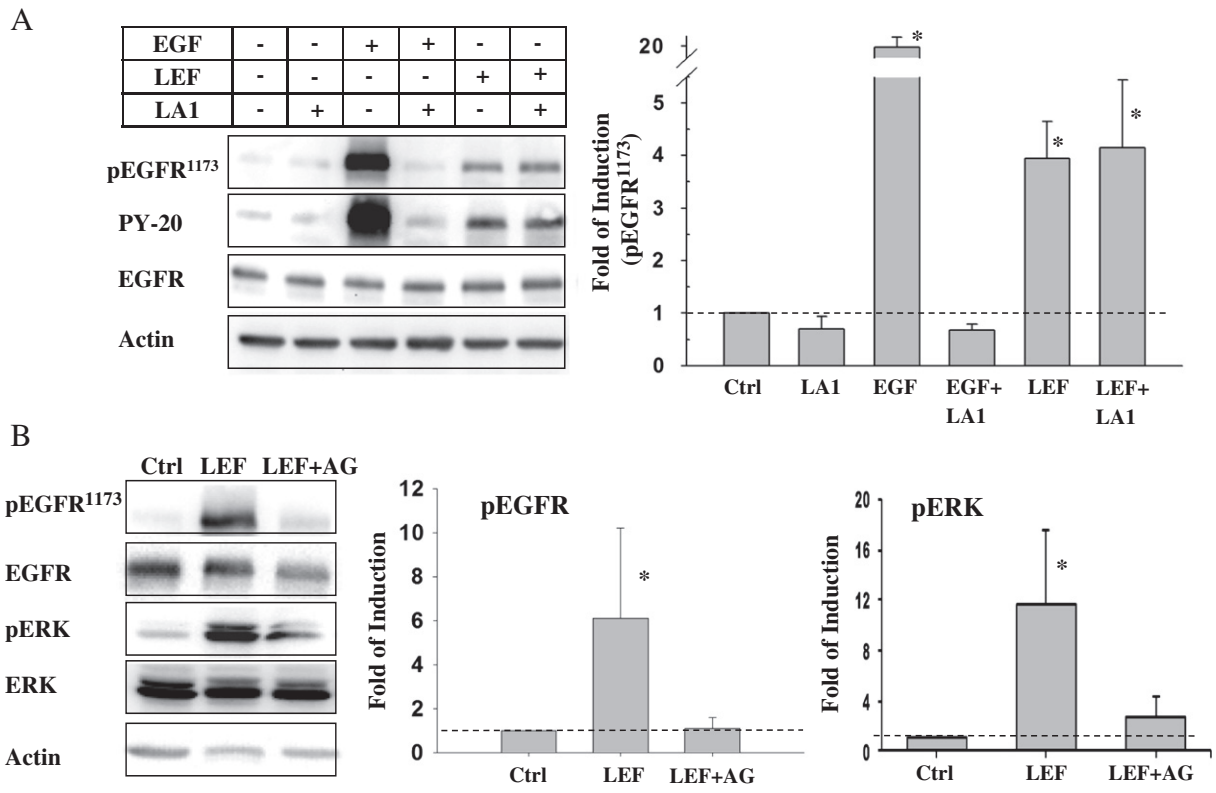


Fig. 2. EGFR phosphorylation induced by LEF is ligand-independent and sensitive to AG1478. *A.* Serum-starved COS5-7 cells were pre-treated with neutralizing antibodies (10 μg/ml, clone LA1) against EGF for 1 h and then exposed to LEF 10 V/cm for 2 min or to EGF (15 ng/ml) for 5 min as positive control. Cells were then lysed, subjected to SDS-PAGE followed by Western Blotting using the indicated antibodies (pEGFR^{1173}, anti-phosphotyrosine PY20, EGFR and actin). Bar charts on the right show the ratio between phosphorylated EGFR (pEGFR^{1173}) and total EGFR, normalized to the control ratio, for the six different groups. Data extracted from three independent experiments is expressed as mean ± SD (*)$p < 0.05$ relative to control by one-sample t-test. *B.* Cells were pre-incubated with 2 μM AG1478 (AG) for 1.5 h prior to and during exposure to LEF 10 V/cm for 2 min, lysed and prepared for Western Blot analysis using the indicated antibodies (pEGFR^{1173}, EGFR, pERK1/2, ERK2 and actin). The bar-charts in the middle represent densitometric evaluation of the ratio between phosphorylated EGFR and total amount of EGFR, normalized to control unexposed cells. The bar-charts on the right represent densitometric evaluation of the ratio between phosphorylated ERK and total amount of ERK, normalized to the ratio in the control, unexposed cells. Data was collected from four independent experiments and expressed as mean ± SD (*)$p < 0.05$ relative to control by one-sample t-test. The dotted line is set on a value of 1 (on the Y axis).

by direct effect of EF on cells and those related to electrochemical reactions at the electrode–medium interface (e.g., production of ROS, change of pH), we used an exposure chamber subdivided into three compartments (anode compartment, central compartment and cathode compartment) by semi-permeable conductive membranes, allowing passage of charges and solutes but preventing cells from electrophoretic transfer from one compartment to another (see Materials and methods). As expected, EGF caused a significant EGFR phosphorylation (~46-fold relative to serum-starved, unexposed cells, $p < 0.05$) (Fig. 3A and B). However, in the three-compartment exposure device LEF-induced EGFR phosphorylation occurred mostly near the anode (10-fold increase, $p < 0.001$) while the level of EGFR phosphorylation observed in the central compartment was close to that of control unexposed cells (1.4-fold rise, $p = 0.31$). The EGFR phosphorylation in the cathode compartment was much lower than in the anode compartment and not statistically different when compared to unexposed control cells (~2.2-fold increase, $p = 0.22$). The downstream ERK phosphorylation was also studied. It rose as anticipated following EGF stimulation (5.1-fold, $p < 0.05$). After LEF treatment ERK phosphorylation is higher by 4.3-fold in the anode compartment ($p < 0.001$), but also observed near the cathode (4.2-fold rise, $p < 0.05$) despite the fact that in the cathode compartment the EGFR phosphorylation is not significantly augmented. In the mid compartment the level of ERK phosphorylation was not different from the control unexposed cells (Fig. 3B). All p-values were calculated using two-tailed one-sample t-test.

3.4. The involvement of ROS in LEF-induced EGFR activation

To spot ROS produced by the electrochemical reactions at the electrode–solution interface, we used a cell-permeant indicator H_2DCF-DA suitable for the intracellular detection of a wide variety of ROS [17]. The indicator was preloaded into the cells for 1.5 h before exposing them to LEF. We could demonstrate that cells exposed to 20 V/cm for 1 min show increased level of probe fluorescence implying a 1.9-fold higher ROS level following the exposure. However, exposing the cells to 10 V/cm almost do not change level of probe fluorescence compared to the control unexposed cells (Fig. 4). When using TBPH as a positive control for oxidation, the relative ROS level rises by about two folds (Fig. 4). In order to reduce the intracellular oxidation, the cells were also preloaded with DHA which is an anti-oxidant agent [18]. As shown in Fig. 4, DHA concentration of 1 mM was enough to neutralize TBHP- and LEF-induced ROS production. Fig. 5A and B demonstrate the role of electrochemically produced ROS in LEF-induced EGFR activation. Decreased EGFR phosphorylation (by 26%) is observed in the presence of DHA following exposure to LEF 10 V/cm, despite the fact that level of ROS observed after 10 V/cm treatment is not significantly higher than in control. The level of EGFR phosphorylation also decreased (by 46%) in cells stimulated by EGF and pre-loaded with DHA, indicating involvement of ROS in EGF signal transduction. EGFR phosphorylation is abolished in cells pre-incubated with DHA and subsequently exposed to TBHP when blotted with non-site-specific general anti-phosphotyrosine antibody PY-20. Pre-incubating cells with AG1478 completely abolished EGFR phosphorylation following

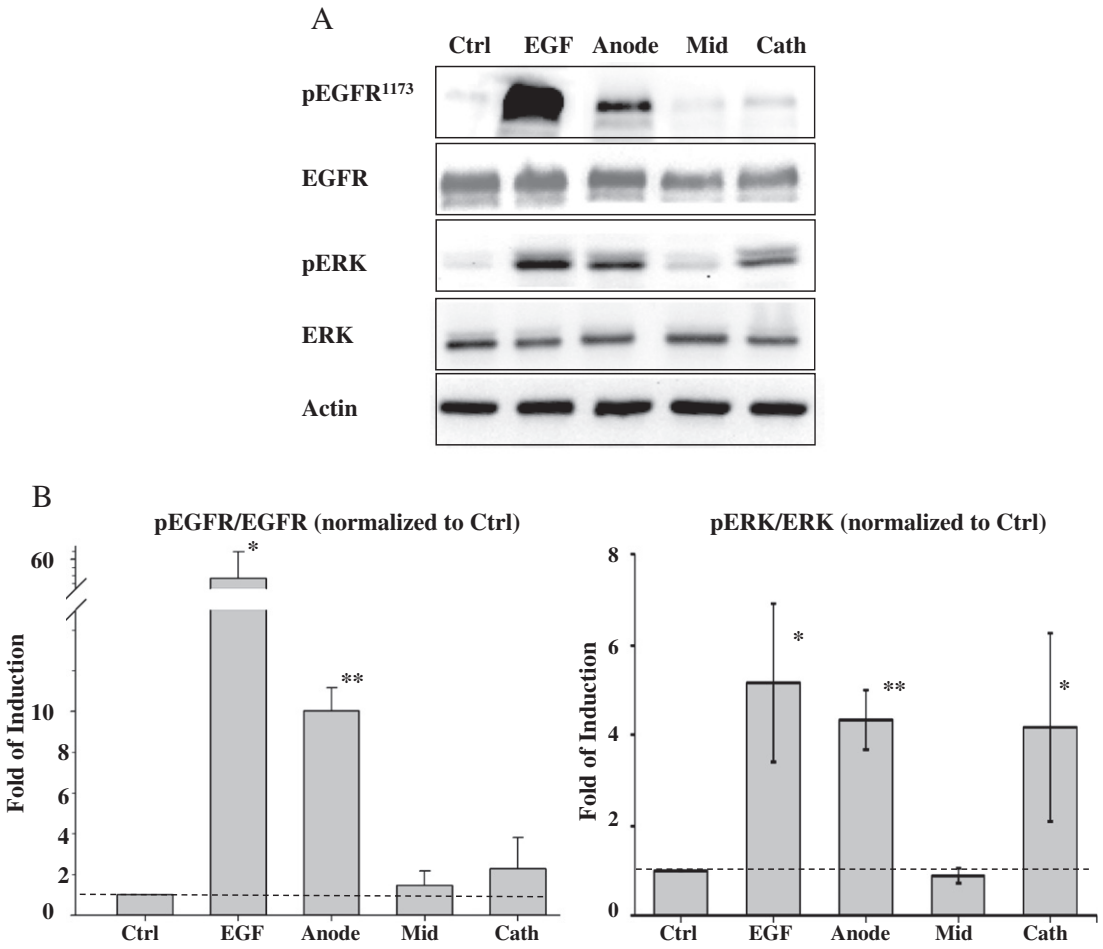


Fig. 3. Analysis of LEF-induced EGFR and ERK phosphorylation following exposure in the three-compartment device. *A*. Serum-starved COS5-7 cells were exposed to LEF 10 V/cm for 2 min in a chamber divided into three compartments, or to EGF (15 ng/ml) for 5 min as positive control. After the exposure cells from each compartment and cells from control groups were collected, washed and lysed for Western Blot analysis using pEGFR^{1173}, EGFR, pERK1/2, ERK2 and actin antibodies. *B*. The bar-charts below represent densitometric evaluation of the ratio between phosphorylated EGFR and total amount of EGFR, normalized to control unexposed cells (left graph), and the ratio between phosphorylated ERK and total amount of ERK, normalized to the control ratio (right graph). Data were collected from three independent experiments and expressed as mean ± SD. (*)$p < 0.05$, (**) $p < 0.001$, relative to control, by one-sample t-test.

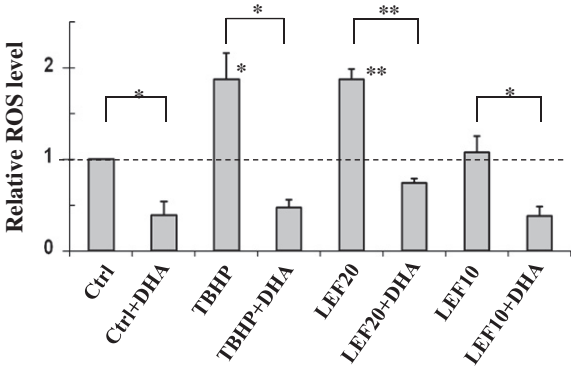


Fig. 4. Electrochemically produced ROS following exposure to LEF. FACS fluorescence analysis of COS5-7 cells exposed to 20 V/cm for 1 min (EF20) or 10 V/cm for 2 min (EF10), compared with control unexposed cells, and cells exposed to TBHP as positive control for oxidation. Cells in all groups were preloaded with H_2DCF 10 μM with or without DHA 1 mM, as indicated under the bar-charts, for 1.5 h, and then exposed to LEF as mentioned above, or to TBHP 1 mM for 5 min before FACS analysis. Data was collected from three independent experiments and expressed as mean ± SD. (*) $p < 0.05$, (**)$p < 0.001$. The comparisons were performed for each group relative to control using one-sample two-tailed t-test, and for each group with and without DHA, as indicated, using regular two-tailed t-test.

exposure either to EGF or LEF. These findings indicate that ROS production is partially involved in LEF-induced EGFR activation and that activation completely depends on EGFR kinase activity.

In addition, while downstream ERK activation can be clearly detected in cases where EGFR is activated by its ligand or upon LEF treatment (Figs. 2 and 5), ERK activation was not affected by pre-incubation with DHA (Fig. 2B). Though abolishment of EGFR phosphorylation by the specific receptor tyrosine kinase inhibitor AG1478 prevented ERK phosphorylation under stimulation by EGF, following exposure to LEF, AG1478 decreased ERK phosphorylation but not completely. This may imply that while LEF-induced EGFR phosphorylation induces ERK activation, ERK phosphorylation after LEF treatment may originate also from EGFR-independent pathways.

3.5. The involvement of pH changes in LEF-induced EGFR activation

It has been noted that transient acidification occurs in the anode compartment during the application of LEF (data not shown). In order to increase the extent of pH drop during exposure to LEF we lowered the buffering capacity of the medium in which the cells are treated. Thus, the cells were suspended in HBSS and different concentrations of Hepes buffer were added prior to exposure to LEF (Fig. 6). The results show

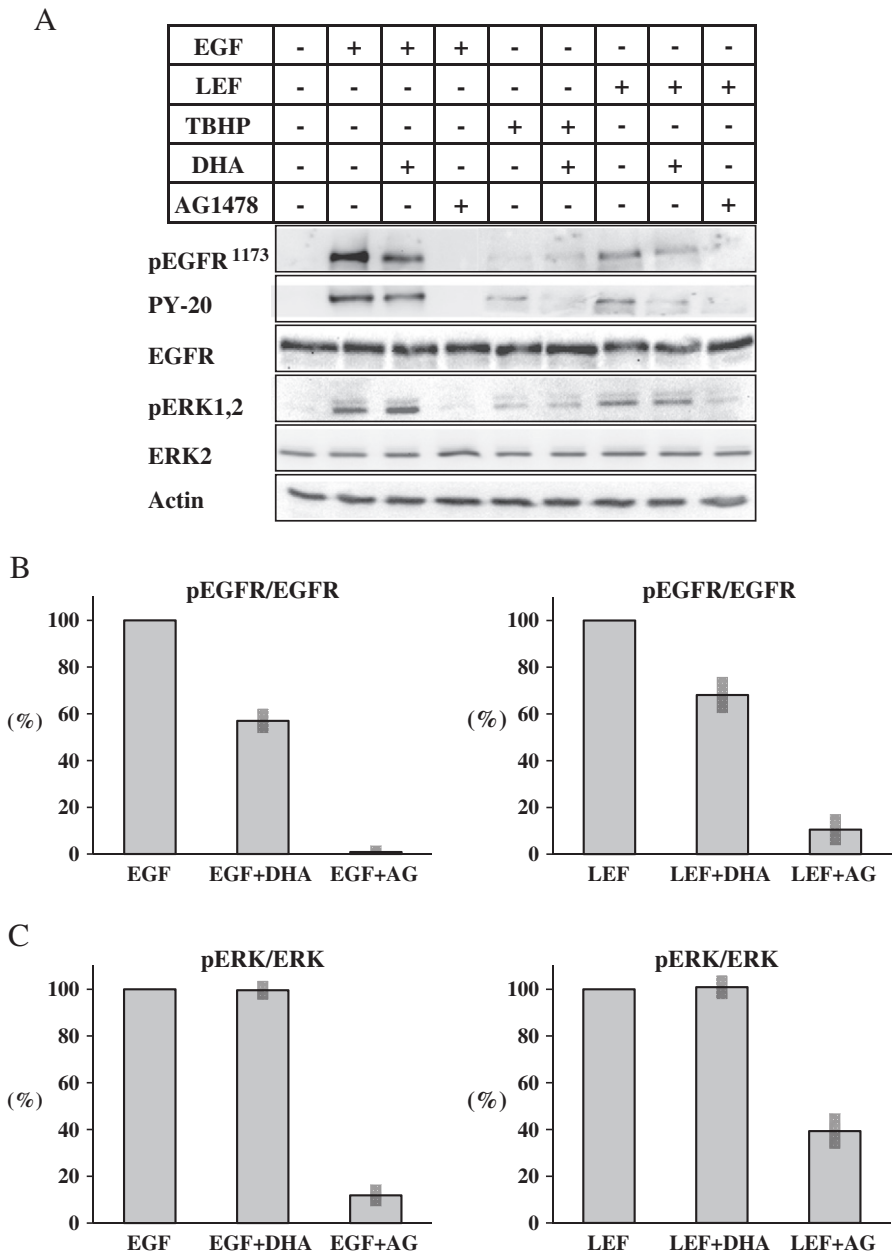


Fig. 5. The role of electrochemically produced ROS in LEF-induced EGFR activation. *A.* COS5-7 Cells were preloaded with AG1478 2 μM (AG) and DHA for 1.5 h where indicated, and exposed to 10 V/cm for 2 min. For positive control, 15 ng/ml EGF was added to cells for 5 min. Cells were then washed, lysed and prepared for Western Blot analysis and immuno-stained with antibodies as indicated on the left. *B* and *C* present pEGFR and pERK activity, respectively, where the phosphorylation activity was calculated as follows: Left graph — the ratio between phosphorylated protein (pEGFR or pERK) and total protein (EGFR or ERK) following EGF stimulation was defined as 100% and the 2 other EGF groups (EGF + DHA and EGF + AG) were normalized relative to it. Right graph — the ratio between phosphorylated protein (pEGFR or pERK) and total protein (EGFR or ERK) following exposure to LEF was defined as 100% and the 2 other LEF groups (EF + DHA and EF + AG) were normalized relative to it. The data are average of two independent experiments where bars represent the range.

that decreasing Hepes concentration causes increasing levels of EGFR phosphorylation. After exposure to LEF of 10 V/cm, EGFR phosphorylation levels, increased by 1.5-, 5.2-, 8.7-, 15.6- and 17-fold when compared to unexposed cells for Hepes concentrations of 100, 80, 60, 40 and 25 mM, respectively. Complementary experiments demonstrated that direct acidification of extracellular medium performed by adding HCl directly to HBSS for 1 min (to pH 1.5, 3.0) with subsequent neutralization by NaOH also causes EGFR activation. The level of EGFR phosphorylation increased by 13-fold and 7.8-fold for pH 1.5 and pH 3.0, respectively. It should be noted that levels of EGFR phosphorylation upon LEF stimulation or direct acidification are lower than following stimulation by 15 ng/ml EGF (72-fold rise in EGFR phosphorylation after incubation with its ligand).

In addition, Fig. 6 demonstrates ERK phosphorylation induced by pH drop, EGF and LEF treatment which was performed using different buffer capacities. One can observe that there is no direct proportion between the extents of EGFR- and ERK-phosphorylation. Though EGFR phosphorylation is much higher following EGF stimulation than after pH drop, ERK phosphorylation is almost similar. Furthermore, no dependence of ERK phosphorylation on medium buffer capacity during LEF treatment (except for 100 mM Hepes concentration) is observed. These facts imply that: (a) ERK phosphorylation may be saturated when EGFR phosphorylation level is high; (b) ERK phosphorylation may proceed also through EGFR-independent pathways thereby adding to the level of phosphorylated ERK. The statistical analysis using repeated measures

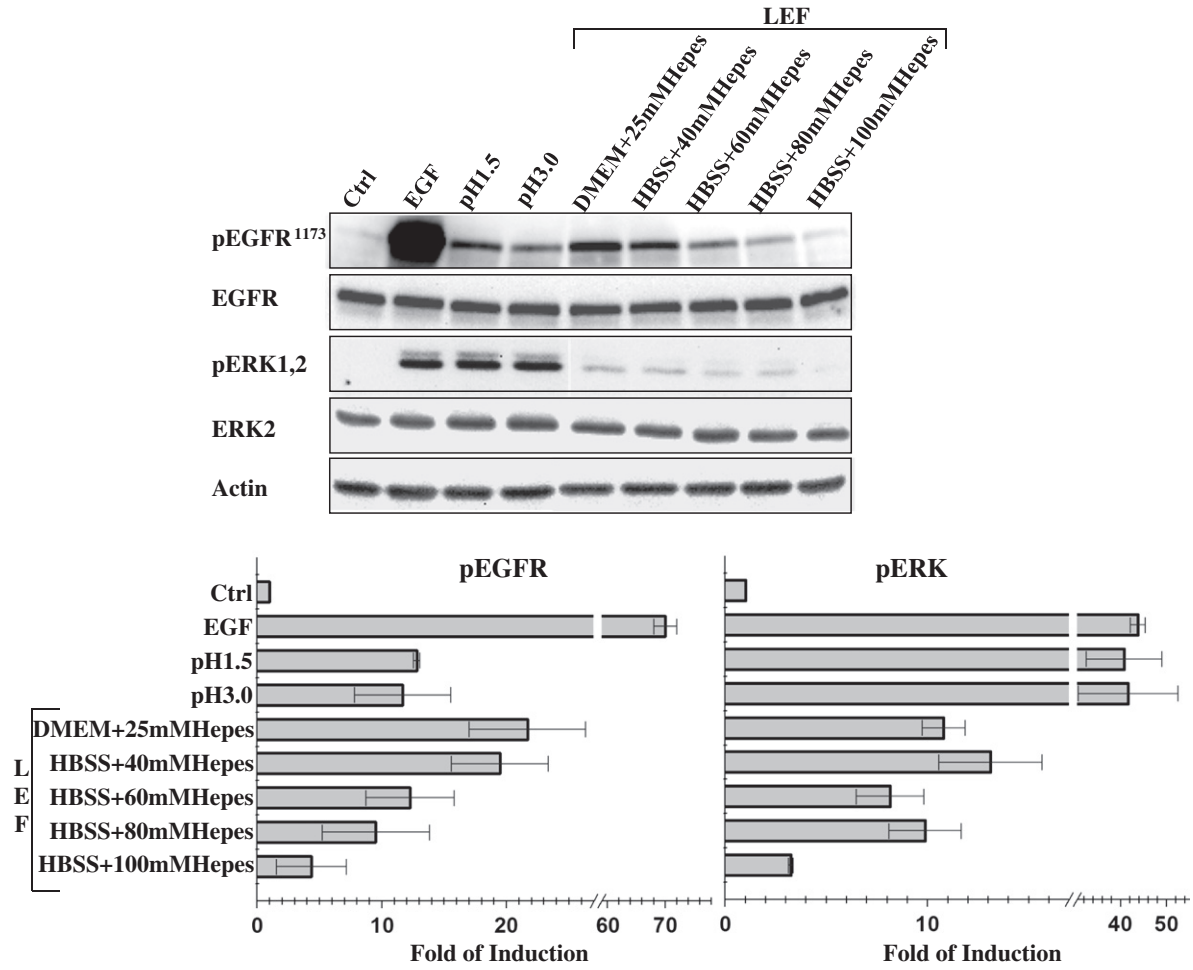


Fig. 6. Involvement of pH changes in EGFR phosphorylation. Serum-starved COS5-7 cells were exposed to LEF 10 V/cm for 2 min in HBSS with the specified concentrations of Hepes buffer, or to HCl added directly to cells suspension in HBSS (in order to produce pH 1.5 & pH 3) for 1 min. In addition, exogenously added EGF (15 ng/ml, 5 min) was used as a positive control. Cells were then washed, lysed, prepared for Western Blot and immuno-stained with antibodies as indicated on the left. Each bar-chart represents: left — the ratio between phosphorylated EGFR and total EGFR, normalized to the ratio in the control group; right — the ratio between phosphorylated ERK and total ERK, normalized to the ratio in the control group. The data are average of two independent experiments where bars represent the range.

analysis of variance for EGFR and ERK phosphorylation reveals a statistical significance ($p < 0.01$) regarding the effect of buffer capacity on the phosphorylation and a significant negative linear trend in these groups, i.e., decreasing buffer capacity is accompanied by increasing level of phosphorylation. These results indicate the role of pH changes in ligand-less EGFR and ERK phosphorylation.

3.6. LEF does not induce EGFR dimerization

It is known that upon EGF binding, EGFR undergoes dimerization and is then internalized [19]. As we observed ligand-independent activation of EGFR after exposure to LEF, and ligand-independent dimerization of EGFR was demonstrated, for example, following lipid raft disruption [20,21], we wondered whether dimerization of EGFR would also occur after exposure to LEF. Serum-starved COS5-7 cells were treated with either EGF, LEF or extracellular acidification and then incubated with the membrane-impermeable cross-linker BS^3 before cell lysis and western blot analysis using 5% SDS-PAGE. As shown in Fig. 7, EGF treatment clearly induced EGFR dimerization. However, induction of dimers could not be detected following exposure to LEF or extracellular acidification, though tyrosine phosphorylation was observed. These findings are similar to EGFR phosphorylation without dimer formation observed after H_2O_2 treatment [21,22]. It should be noted that the EGFR tyrosine kinase inhibitor AG1478 by itself can provoke receptor dimerization as was previously observed [23,24].

3.7. Low electric fields induce phosphorylation of different members of the receptor tyrosine kinase family in HaCaT cells

RTKs undergo tyrosine phosphorylation upon ligand binding. Since human immortalized keratinocytes, HaCaT cells, are known to express variety of tyrosine kinase receptors, e.g. ErbB family members [25], it was of interest to examine whether the exposure to LEF induces activation of different RTKs. HaCaT cells were serum starved for 24 h in serum-free DMEM before the experiment in order to avoid receptor activation by the growth factors present in fetal calf serum. Using human phospho-RTK array we were able to detect multiple tyrosine phosphorylated receptors in lysates of LEF-exposed compared to unexposed HaCaT cells (Fig. 8A). Exposure of cells to 10 V/cm for 2 min induced phosphorylation of the whole ErbB receptors family — ErbB1 (the EGFR), ErbB2, ErbB3, and ErbB4. The level of tyrosine phosphorylation of EGFR and ErbB4 increased by 6.5 and 5.4 fold respectively (Fig. 8B). The basal phosphorylation level of ErbB2 and ErbB3 almost could not be detected in the control cells but, as shown, underwent significant EF-induced phosphorylation. Other receptors which underwent

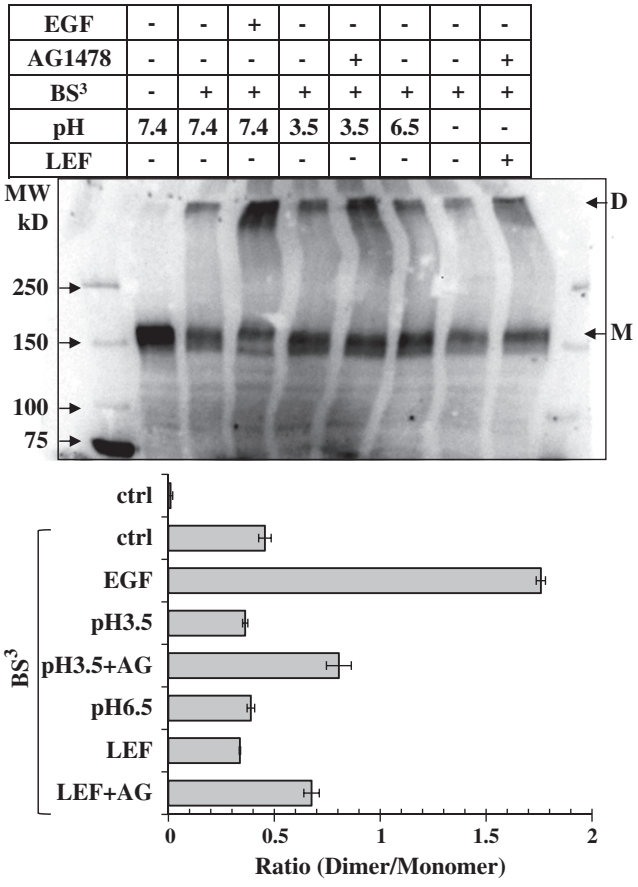


Fig. 7. Exposure to LEF or extracellular acidification does not induce EGFR dimerization. COS5-7 cells were serum-starved and exposed to either EGF (15 ng/ml, 5 min), LEF 10 V/cm for 2 min, pH 3.5 for 1 min or pH 6.5 for 2 min, in the presence and absence of AG1478. Cell-surface receptors were cross-linked in the presence of 2 mM BS^3, a membrane-impermeable cross-linker. Cell lysates were subjected to SDS-PAGE gels (5% polyacrylamide) followed by Western-blotting with anti-EGFR antibody. The positions of EGFR dimers (D) and monomers (M) are indicated. The bar-charts below represent the ratio of dimer/monomer for each group. The data are average of two independent experiments where bars represent the range.

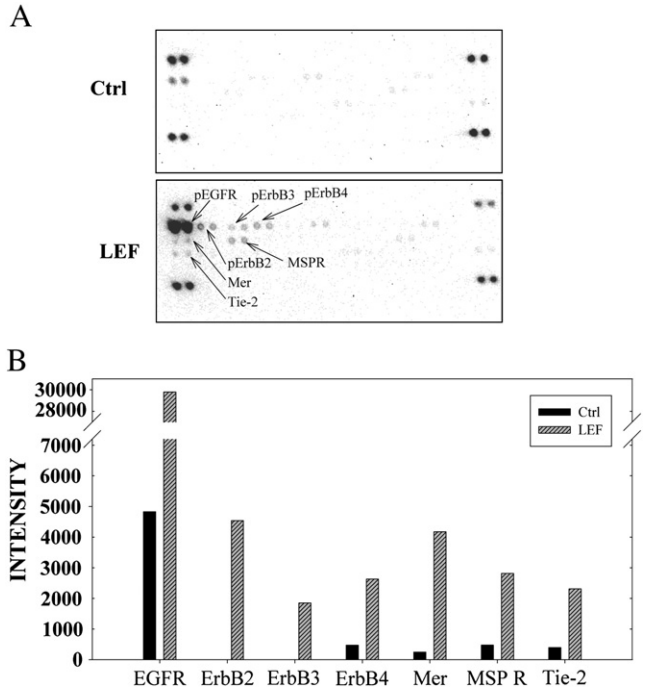


Fig. 8. The human phospho-RTK Array detects tyrosine phosphorylated receptors in HaCaT cells exposed to low EFs. *A.* Serum-starved HaCaT cells were exposed to 10 V/cm for 2 min at room temperature. Cells were washed, lysed and analyzed according to the manufacturer's instructions. Equal protein quantities (~200 μg) were incubated with the kit's membranes following determination of samples protein concentrations using the BCA total protein assay. The results were obtained on the kit's nitrocellulose membranes. *B.* The bar charts represent quantitative analysis of the array dot blot signals performed using TINA software (densitometric evaluation). Normalization of phosphorylation relative to the control sample could not be performed for all the receptors since the basal phosphorylation level of some of the receptors in the control group could not be detected.

significant phosphorylation detected by the array were Mer, MSP and Tie-2. It should be noted that while MSP and Tie-2 receptors were previously detected in human keratinocytes [26,27] though not in HaCaT cells, the Mer receptor, to the best of our knowledge, was not detected so far neither in keratinocytes nor in HaCaT cells. The results of RTK array emphasize the capability of LEFs to cause ligand-independent activation of not only of EGFR family but also RTKs of other families.

4. Discussion

4.1. LEF-induced activation of EGFR is ligand-independent but kinase-dependent and is attributed to electrochemical products

Our study demonstrates direct EGFR activation and signaling induced by LEF in serum free medium, and in the absence of exogenous EGF ligand, suggesting a ligandless activation pathway for EGFR. We show that LEF induced activation of endogenous EGFR, as evidenced by increased tyrosine phosphorylation, in both COS5-7 and HaCaT cells (Fig. 1), implying that this phenomenon is cell type-independent.

As shown in Fig. 2A, EGFR phosphorylation in COS5-7 cells was demonstrated to occur even following pre-treatment with a neutralizing antibody (LA1) that sterically blocks the ligand-binding site of EGFR and prevents the ligand-induced activation of it. As expected, pre-treatment with this antibody prevented EGFR phosphorylation induced by stimulation with EGF. Taken together, these data strongly support the notion that LEF triggers a ligand-independent EGFR activation. Moreover, using specific antibodies we were able to demonstrate that tyrosine phosphorylation of EGFR occurs at Tyr1173, a specific autophosphorylation site of EGF receptor tyrosine kinase. In addition, we showed that this ligandless phosphorylation depends completely on intrinsic EGFR kinase activity since the specific EGFR kinase inhibitor, AG1478, totally abolished LEF-induced EGFR phosphorylation, in the same way as AG1478 inhibited it upon treatment with EGF (Figs. 2B & 5A).

The ligand-independent activation of EGFR shown here may be attributed to the direct effect of EF on the cells and EGF receptors. The other possibility is that electrochemical products formed at the electrode–solution interface during electric stimulation cause EGFR activation.

The direct effect of the applied electric fields on EGFR might, in principle, be assumed on the basis of previous studies (for a review see [1]). Generally, following prolonged exposure to DC electric fields of 1 V/cm or less there is electrophoretical/electroosmotical relocation of EGFRs along the plane of cell membrane and their accumulation towards the cathode side. It was reported [9] that such asymmetrical distribution of receptors will cause directional migration of keratinocytes in the presence of EGF. Both receptor redistribution and cell migration were abolished following inhibition of EGFR kinase activity, suggesting a role for EGFR phosphorylation in the mechanism of cellular sensing the EF. Similarly, EGF was required together with β-integrin for the regulation of directional cell migration via Rac1 in response to galvanotactic stimulus [28]. In corneal epithelial cells, DC EFs were also demonstrated to induce upregulated expression of EGFR and F-actin reorganization. Cathodal accumulation of EGFR, actin and cathodal-directed migration did not occur in the absence of serum but were restored by adding

ligands to the medium [7,10]. It should be stressed out that all aforementioned effects of EF on EGFR activity and resulting biological consequences were observed only in the presence of extracellular ligands or serum. The answer to the question whether EF itself can activate the receptor [9] still remained unclear. In the case of pulsed electric fields of higher amplitude (10–20 V/cm) but with shorter duration (1–2 min) as applied in the present work the answer would be probably no. This emerges from our experiments with the three-compartment chamber. In such exposure setup the EF strength is identical in all three cathodal, central and anodal compartments though noticeable EGFR activation occurs only in cells located in the anodal compartment. Furthermore, there is no detectable EGFR phosphorylation in the central compartment, where cells are subjected to the EF but are not in contact with either of the two electrodes. Thus, the cells in the central compartment are not exposed to any significant electrochemical products (Fig. 3). If the direct contribution of EF to the ligandless activation of EGFR can be disregarded, one has to conclude that electrochemical products formed at the anode–solution interface are responsible for the ligandless activation of EGFR.

It is well established that ROS formation, as well as acidification, do occur at the anode–solution interface when EF of intensity similar to that used in the present study is applied to a physiological solution [29–31]. This suggests possible roles for ROS and high hydrogen ion concentration in eliciting the ligand-independent activation of EGFR. ROS are known to be produced by and regulate diverse cellular physiological responses such as signal transduction cascades and many critical events leading to cell proliferation, differentiation and migration [32–34]. They are also involved in pathological conditions and tumorigenesis [35,36]. In the present study we have shown the intracellular elevation of ROS intermediates induced by LEF by employing the H_2DCF-DA fluorescent probe which enables detection of a broad range of oxidizing intermediates during intracellular oxidative stress [17,37]. In addition, we could demonstrate that preloading of cells with the anti-oxidant, DHA, could neutralize the LEF-induced production of ROS [18]. Significant elevation of ROS level was observed only following exposure to 20 V/cm but not to 10 V/cm (Fig. 4).

This may be attributed to a scenario where at 20 V/cm ROS production overloads the scavenging capacity of the diverse cellular antioxidative defense systems and ROS are easily detected by the intracellular probe. However, at 10 V/cm ROS production is smaller and cell defense systems scavenge almost all ROS entering the cell, making it difficult to detect the overall elevation of ROS in the cell by the cytosolic fluorescent probe. Nevertheless, on their way into the cell ROS concentration may rise locally (e.g. near the cell membrane) where they can elicit their biological action. This notion is further supported by the fact that pre-incubation of cells with the anti-oxidant DHA prior to exposure to 10 V/cm caused almost a 30% decrease in EGFR phosphorylation compared with cells exposed to 10 V/cm without prior pre-incubation with the anti-oxidant (Fig. 5). These observations imply that ROS are involved in EGFR phosphorylation induced by LEF but their involvement is only partial and there are other mechanisms affecting this phosphorylation. Such conclusion is further supported by the experiments with ROS donor TBHP, that failed by itself to produce remarkable phosphorylation of EGFR at autophosphorylation site (Fig. 5A). The involvement of ROS in EGFR phosphorylation is consistent with previous findings according to which oxidative stress through ROS, and among them the H_2O_2 owing to its stability, increased tyrosine phosphorylation of EGFR and downstream signaling [12,22,35,38–43]. ROS can activate EGFR through different mechanisms such as oxidative inactivation of protein tyrosine phosphatases, activation of EGFR-associated protein tyrosine kinases (e.g. Src tyrosine kinase), direct activation by covalently cross linking the receptor or by stimulating the production of EGFR ligands, such as the cleavage of HB-EGF through metalloprotease activation [12,15,32,43–47]. In addition, our results show that pre-incubating cells with DHA before stimulation with EGF ligand significantly decreased EGF-induced EGFR phosphorylation. This observation is in accordance with previous findings suggesting that plasma-membrane localized EGFR can locally inactivate PTPs by inducing the production of ROS upon EGF binding [48,49], thereby enhancing the phosphorylation of the receptor.

LEF-induced EGFR tyrosine phosphorylation was completely abolished by its kinase inhibitor, AG1478 (Figs. 2 & 5), as demonstrated both by specific anti-pTyr1173 (an autophosphorylation site) and general anti-phosphotyrosine PY20 antibodies. The fact that EGFR phosphorylation detected by PY20 antibody completely disappeared in the presence of receptor kinase inhibitor AG1478 (Fig. 5A), supports the notion that LEF-induced EGFR phosphorylation originates mainly from its intrinsic kinase activity rather than via activation of receptor-associated kinases, such as Src, as reported to happen under oxidative stress [44,46,50]. However, some previous reports demonstrated that H_2O_2 induced EGFR phosphorylation in a manner dependent both on c-Src and on EGFR kinase activity [22,38].

The other major consequence of electrochemical reactions taking place at the anode–medium interface is elevation of proton concentration. The involvement of medium acidification in the ligandless activation of EGFR is evident from: (a) the existence of significant level of EGFR phosphorylation only in the anode compartment of the 3-compartment chamber, where pH during LEF exposure is low ($pH \leq 5.5$) and the absence of significant EGFR phosphorylation in the median and cathode compartments where pH is or neutral or alkaline ($pH \geq 10$), respectively (Fig. 3); (b) The dependence of LEF-induced phosphorylation of EGFR on the buffer capacity of the medium; upon increasing buffer capacity a decreasing level of EGFR phosphorylation is observed. Significant decrease in LEF-induced EGFR phosphorylation level from 15.6-fold down to 8.7-fold was observed upon increasing Hepes concentration in HBSS from 40 mM to 60 mM, corresponding to LEF-induced extracellular pH of 3.5 and 4.5 measured immediately following exposure to LEF (Fig. 6); (c) EGFR activation following brief direct acidification of the medium to pH 1.5–3 (Fig. 6). It should be noted that EGFR phosphorylation caused by direct acidification was not detectable at pH values higher than 4.5 (data not shown) while LEF treatment leading to pH even higher than 4.5 still led to EGFR phosphorylation. This may be partially attributed to the stimulatory effect of ROS, produced during exposure to LEF, on EGFR activation initiated by pH drop.

4.2. ERK activation following exposure to LEF

As it is well established that binding of EGF to EGFR causes downstream ERK activation, we have verified whether ERK cascade is activated also in cells exposed to LEF. Indeed we demonstrated significant ERK phosphorylation after LEF treatment (Figs. 1A, 2 and 3). But the LEF-induced ERK activation was found to be dependent on EGFR activation only partially. EGFR kinase inhibitor AG1478 decreased ERK activation considerably but not completely, although EGFR phosphorylation was completely abolished (Fig. 2B), in contrast with EGF-induced ERK activation which was completely eliminated in cells exposed to EGF in the presence of AG1478 (Fig. 5A). Thus we can assume that ERK activation in our experiments proceeds not only by conditional EGFR-dependent mechanism but also through EGFR-independent pathway(s). Actually, low extracellular pH was demonstrated previously to activate ERK independently of EGFR [51–53]. It is proposed [54] that such EGFR-independent ERK activation is linked to proton-activated G protein-coupled receptors OGR1 and G2A that activate phospholipase C and IP_3 formation. Subsequent Ca^{2+} release from intracellular stores recruits the MEK/ERK pathway. Since one of the results of LEF treatment is pH decrease near anode it is reasonable that ERK would be still activated following exposure to LEF by the aforementioned mechanism even if EGFR activation has been abolished. In addition, other mechanisms of ERK activation may exist, for example ERK was demonstrated to be activated following exposure to extracellular alkalosis [55]. Actually we show significant ERK phosphorylation also in the cathode compartment, where we observe substantial alkalosis during LEF treatment, albeit no noticeable EGFR activation is observed (Fig. 3). It is of interest to point

out that exposure of cells to DC EFs of physiological amplitude (e.g. 2 V/cm), in the absence of electrochemical byproducts, was shown to activate ERK and other signaling pathways in primary mouse keratinocytes and mouse peritoneal neutrophils in serum-free medium [56] though ERK activation induced by those EFs in corneal epithelial cells increased only when serum was present [10].

4.3. Possible mechanism for LEF-induced EGFR activation

It was previously elucidated that when EGF mediates EGFR dimerization, the kinase domain of one EGFR in the dimer pair binds to and activates the other, changing the conformation of its activation loop by an allosteric mechanism [57,58], thus removing the initial autoinhibition for receptor dimerization. As aforesaid, one of the mechanisms to induce EGFR phosphorylation by ROS, which is one of the LEF treatment products, is covalently cross-linking the receptor. However, different ROS have been shown to differently affect EGFR. For example, $ONOO^-$, the reaction product of NO and O_2^- was found, similarly to EGF, to generate covalently cross-linked receptor [43]. Unlike EGF and $ONOO^-$, H_2O_2 failed to induce detectable EGFR dimerization [21,22] although H_2O_2 induced stronger tyrosine phosphorylation of the receptor than following cholesterol depletion, which was also demonstrated to induce ligand-independent EGFR activation and dimerization [21]. We show that LEF and extracellular acidification did not induce EGFR dimerization compared with dimerization obtained following EGF stimulus (Fig. 7), raising the question of the requirement of dimerization during the process of EGFR activation.

Recent studies have shown the prominent role of the juxtamembrane region of several RTKs in kinase activation [59]. One of the mechanisms proposed by McLaughlin et al. [60] suggests an electrostatic based model according to which the positively charged residues 645–660 of the juxtamembrane EGFR domain together with the positive face of the kinase domain bind electrostatically to the negatively charged inner leaflet of the cell membrane, contributing to autoinhibition of EGFR. This mechanism was proposed to be the explanation for the role of Ca^{+2}/calmodulin complex. This complex binds rapidly to residues 645–660 of the juxtamembrane domain, reversing its net charge from +8 to −8 and repelling it from the negatively charged inner leaflet of the membrane. This leads to disengagement of the kinase domain from the membrane, allowing it to become fully active and phosphorylate an adjacent ErbB molecule or other substrate. Sengupta et al. [61] also used the electrostatic mechanism to explain the ability of the membrane-permeable calmodulin inhibitor, W-13, to stimulate autophosphorylation of EGFR in the absence of EGF, by binding to the cell membrane and decreasing the net negative charge on it, thereby facilitating the desorption of the juxtamembrane and the kinase region from the membrane. We would like to suggest that in our experimental system extracellular pH drop results in a rapid decrease in intracellular pH and binding of H^+ ions to the negatively charged lipids on the inner leaflet of the plasma membrane (mostly PS and PIP_2 with pK_a 4–5 and ~6.5 respectively [62,63]) thus leading to partial neutralization of inner leaflet charge. This, in turn, facilitates repelling the juxtamembrane and kinase domains from the membrane and increases the accessibility of part of the EGFR for the kinase. Since ligand-independent association in a preformed dimeric structure was observed for the ErbB family in vivo, it has been proposed [66] that receptor transmembrane domains have two dimerization motifs, corresponding to active and inactive receptor dimers. Namely, C-terminal GG4-like dimerization motifs would presumably correspond to inactive receptor state [66]. The removal of receptor autoinhibition in such homo- and hetero-dimers by electrostatic mechanism will cause the phosphorylation of the adjacent subunit. Since portion of preexisting receptor dimers is obviously small, this may explain the relatively low ligandless EGFR phosphorylation level in LEF- or acid-treated cells as compared to EGF-treated cells. Moreover, such electrostatic mechanism of the receptor tyrosine kinase activation does not oblige conformational changes in the extracellular domain and thus is not accompanied by the activation of dimerization loop. Indeed, no significant EGFR dimerization was detected in our study as discussed before. Though ROS, that are also generated during LEF treatment, failed in our experiments to produce any pronounced EGFR activation by themselves, they may nevertheless enhance EGFR response to other stimuli by inhibition of receptor-associated protein phosphatases and activation of receptor-associated protein kinases. In summary, we propose that exposure to LEF exerts its effects via intracellular acidification which leads to EGFR autophosphorylation and downstream signaling and that oxidative stress further modulates the EGFR response.

4.4. LEF-induced tyrosine phosphorylation of other RTKs

As discussed above the mechanism of action of other RTK families is similar to that of EGFR where the juxtamembrane region of the receptor provides another autoinhibition layer in addition to the requirement for juxtaposition of the kinase domain [59], and repositioning of the activation loop by an allosteric mechanism [58]. Therefore, it is not surprising that tyrosine phosphorylation of other RTKs occurred following exposure to LEF, as shown in Fig. 8. Using phospho-RTK array kit we identified RTKs in HaCaT cells which were activated by exposure to LEF (Fig. 8). We found that receptors of the EGFR family (ErbB receptors) were significantly more phosphorylated in the exposed cells, with ErbB2, ErbB3 and ErbB1 (EGFR) being most affected. Marques et al. [64] reported that HaCaT cells express the ErbB1, ErbB2, and ErbB3 family members though ErbB4 was not unambiguously identified. According to our results ErbB4 was 5.4-fold more phosphorylated in the exposed cells than in the untreated cells, meaning that ErbB4 not only is present but undergoes ligand-independent phosphorylation in HaCaT cells. Marques et al. [64] also reported that in the absence of ligand, only low phosphorylation was observed for ErbB2, and no phosphorylation was seen for ErbB1 or ErbB3, while our results demonstrated low phosphorylation for EGFR and ErbB4 and no phosphorylation for ErbB2 and ErbB3 in the starved unexposed cells. We attribute these differences to the experimental conditions and possible differences between the detection antibodies used by other groups compared to those imprinted on the phospho-RTK array membranes used by us. Since ErbB3, kinase-defective receptor, was also phosphorylated as a result of LEF treatment, it presumes the existence of heterodimers of ErbB3 with other family members with functioning kinase. Other RTK that were also found to be activated by LEF — Mer, Tie-2 and MSPR (RON) belong to Axl, Tie and Met RTK subfamilies respectively — are studied to a lesser extent than EGFR. Nevertheless they are thought to play important role in tumorigenesis and at least two of them — Tie-2 and RON were reported in human keratinocytes. Taking into account the ligand-independent activation, shown for all three RTKs, and the existence of juxtamembrane autoinhibition region shown at least for one of them — Tie-2 [59], it is possible to suggest that RTKs of quoted subfamilies share common activation mechanism with the EGFR subfamily in the absence of ligands (as discussed above). Interestingly, all mentioned RTK can activate downstream MAPK/ERK pathway and thus contributing to the portion of LEF-stimulated ERK phosphorylation that was found to be EGFR-independent.

4.5. Concluding remarks

We report here about ligandless EGF receptor activation taking place under low electric field stimulation or direct acidification of extracellular medium. RTKs of several other subfamilies may be activated as well. The proposed mechanism for RTK stimulation involves intracellular pH decrease, neutralization of negative charge on the inner leaflet of the cell membrane leading to repelling the juxtamembrane autoinhibition and kinase domains from the membrane and to kinase activation. Understanding RTK activation in the absence of extracellular ligands may shed the light on general mechanisms of RTK regulation in health and disease. The study of biological consequences of such ligand-independent

EGFR activation is now under way. The question whether exposure of cells to smaller pH decrease (e.g. to pH 6.0–6.5 as happens in solid tumors or inflammation) but for prolonged time will led to chronic EGFR activation, still remains to be answered.

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