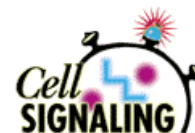


Inhibition of EGF-Induced ERK1 and ERK2 Enzyme Activation in NIH3T3 Cells by *in situ* Electroporation of a Nonpermeant Inhibitor



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A nonpermeant compound, [(dimethylamino)methyl]acrylo-para-[(hydroxy-benzoylsulfonyl)-oxy]phenone, inferred by *in vitro* studies to specifically inhibit EGF receptor function, was introduced by *in situ* electroporation into mouse NIH3T3 fibroblasts growing on indium-tin oxide-coated glass. Cells were subsequently stimulated with growth factors and assessed for activation of a downstream target, the extracellular signal regulated kinase (ERK1/2), by probing with highly specific antibodies. The results showed that this compound can inhibit EGF- but not PDGF-mediated ERK1/2 activation *in vivo*, demonstrating the specificity of this compound and the utility of the *in situ* electroporation approach for the study of tyrosine kinase action in intact cells.

INTRODUCTION

One of the more promising targets for cancer therapy is the epidermal growth factor receptor (EGFR) family, which has been implicated in a number of human carcinomas. Several synthetic compounds that are highly effective inhibitors of EGF *in vitro* are unable to cross the plasma membrane, hence their effectiveness and specificity for inhibiting EGF signaling *in vivo* cannot be tested. We describe a technique for the rapid, efficient and nontraumatic introduction of such nonpermeant compounds into large numbers of cells in culture. In this procedure, cells are grown on a glass slide coated with transparent and conductive indium-tin oxide (ITO) (Figure 1). The compounds of interest are introduced by the application of a precisely controlled electrical pulse that transiently opens pores in the cell membrane; these pores rapidly reseal upon cessation of the pulse (1,2). This technique has been used for the efficient introduction of a variety of peptides and proteins, including antibodies, oligonucleotides, radioactive nucleotides or other compounds, into adherent cells (3-9). Electroporated cells can subsequently be lysed for large-scale experiments or examined for morphologic and biochemical alterations using immunocytochemistry techniques.

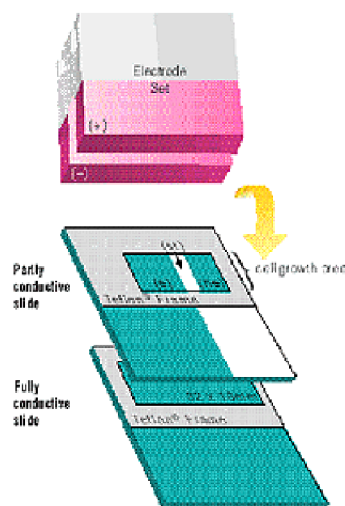


Figure 1. Diagram of the electroporation apparatus. Cells are grown on conductive, ITO-coated glass slides within a “window” cut into a Teflon® frame, as shown. The test compound solution is placed on and introduced into the cells using an electrical pulse delivered by the electrode set placed directly on the Teflon® frame (Ask Science Products, Kingston, Ontario). The upper slide represents a partly conductive slide assembly with electroporated (*e*) and nonelectroporated (*ne*) cells. Cells in areas (*st*) and (*ne*) are not electroporated because the ITO coating is stripped from area (*st*) so that no electrical pulse reaches cells in areas (*st*) and (*ne*). Cells in area (*e*) are electroporated (0.2 μ F, 40V, 4 pulses to an area of 4 x 4mm). Cells are subsequently fixed and stained as explained in the legend to Figure 3, Panels B-D. The lower slide represents a fully conductive slide. Cells are electroporated (30 μ F, 50V, 4 pulses to an area of 32 x 10mm), extracted and analyzed by Western blotting, as described in the legend to Figure 3, Panel A. Electroporation conditions are different for fully and partly conductive slides, due to the different sizes of the electroporated areas. Figure recreated from reference 9 with permission of authors and publisher.

To assess the effect of nonpermeant drugs, their introduction must be coupled with the measurement of inhibition of a specific downstream effector. For the rapid screening of a large number of compounds, an efficient readout is necessary. We took advantage of

recently developed antibodies that specifically recognize the dually phosphorylated, active forms of the extracellular signal regulated kinases (ERK; 10,11), also referred to as the Mitogen Activated Protein Kinases (MAPK), which are potently activated by many extracellular stimuli, especially growth factors and hormones. We examined the ability of a series of putative EGFR-selective drugs to inhibit EGF or PDGF activation of the ERK1 and ERK2 enzymes.

***IN SITU* ELECTROPORATION DOES NOT AFFECT ERK1/2 ACTIVITY OR THE STRESS PATHWAY**

The availability of the highly specific, affinity-purified antibodies to a series of ERK/MAPK superfamily members (i.e., MAPK, JNK and p38) provides the means for robust detection of these key signaling enzymes, thereby overcoming problems related to background levels often encountered with immunocytochemistry assays. To precisely assess small changes in morphology or gene expression levels, the presence of nonelectroporated cells growing on the same type of ITO-coated surface and adjacent to electroporated cells can provide valuable controls. This was achieved by plating the cells on a slide where the conductive coating was removed in a thin strip, exposing bare, nonconductive glass underneath. Application of the pulse results in electroporation of the cells growing on the left side of the strip, exclusively (Figure 1, area *e*). Cells growing on the strip (Figure 1, area *st*) or to the right of the strip (*ne*) do not receive a pulse because there is no contact between the positive contact bar and area (*ne*). In this configuration, electroporated cells (*e*) are being compared to nonelectroporated cells (Figure 1, area *ne*), while both are growing on ITO-coated glass.

Although ERK1 and ERK2 are potently activated by a variety of mitogens, growth factors or hormones, these enzymes are also activated by certain stress stimuli including ultraviolet light, endotoxin or osmotic imbalance (11). To examine whether electroporation *per se* has any effect on ERK1/2 activation in the absence of growth factor stimulation, NIH3T3 cells were plated on partly conductive slides (Figure 1), growth-arrested, electroporated in the presence of the PBS carrier alone and ERK1/2 levels assessed by immunocytochemistry. No ERK1/2 stimulation was observed in the range of 30-90V (Figure 2, Panel A), while the drugs could be introduced into essentially 100% of the cells using pulses as low as 40V (data not shown).

To examine other kinase cascades that are more potently activated by stressful conditions, we also examined the effect of electroporation upon the c-Jun amino-terminal kinase/stress-activated protein kinase (JNK/SAPK) and p38^{hog} enzymes. These are also members of the ERK/MAPK superfamily and are very responsive to a broad variety of stress-related stimuli including UV irradiation, heat or osmotic shock, protein synthesis inhibitors, endotoxins, ischemia or DNA damaging agents (11). Like the ERK1/2 enzymes, both JNK/SAPK and p38^{hog} are activated on the dual phosphorylation lip of these enzymes (9,10). To examine whether *in situ* electroporation can activate stress pathways, cells were serum-arrested, electroporated at different voltages and probed for activated forms of these kinases, using the respective phosphospecific antibodies. The results show no activation of JNK/SAPK or p38^{hog}, using pulses of up to 80V (Figure 2, Panels C and E). In addition, electroporation had no effect upon ERK1/2, JNK/SAPK or p38^{hog} activation by EGF or PDGF (data not shown).

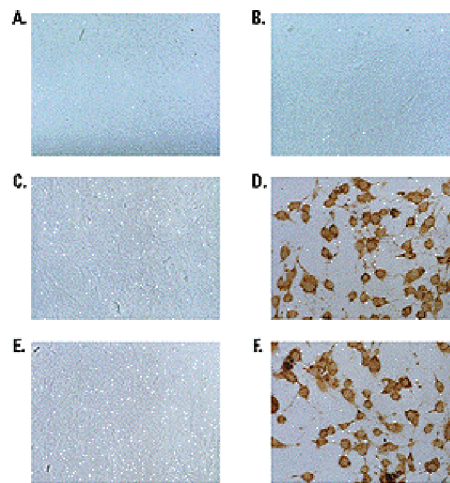


Figure 2. *In situ* electroporation does not affect ERK1/2 activity or the stress pathway. Panels A, C and E: NIH3T3 cells were plated on fully conductive slides, growth-arrested and electroporated (0.2 μ F, 70V, 4 pulses) in the presence of PBS containing 0.025% DMSO (diluent for compound 8). Ten minutes after the pulse, cells were fixed, permeabilized and incubated with the affinity-purified rabbit polyclonal antibodies, which recognize the dually phosphorylated, active forms of ERK1 and ERK2 (Panel A), JNK/SAPK (Panel C) and p38^{hog} enzyme (Panel E). Antibody signals were visualized using a biotinylated anti-rabbit secondary antibody, followed by avidin-biotin horseradish peroxidase complex and diaminobenzidine staining, according to manufacturer's instructions (VECTASTAIN[®] Elite ABC Kit, Vector Laboratories). Cells were photographed under brightfield illumination. Panels B, D and F: Control NIH3T3 cells were plated on conductive slides and the growth medium replaced with PBS. Cells were irradiated with UV light for 10 minutes using a hand-held lamp (model UVGL-58, UVP, Inc., San Gabriel, CA) on the long wavelength setting, at a distance of 1.5cm from the cells. Cells were then fixed and stained for activated ERK1/2 (Panel B), activated JNK/SAPK (Panel D) or activated p38^{hog} (Panel F), as described above. Images recreated from reference 9 with permission of authors and publisher.

COMPOUND 8 SELECTIVELY INHIBITS EGF-INDUCED ERK1/2 ACTIVATION

Incubation of [(dimethylamino)methyl]acrylo-para-[(hydroxy-benzoylsulfonyl)-oxy]phenone (compound 8; 12) with intact cells for extended periods of time had no effect upon cell growth or ERK1/2 activation, presumably due to its impermeant nature (12). To examine the ability of the drug to inhibit ERK1/2 activation by PDGF or EGF and to assess possible toxicity of the compound, as well as to examine the uniformity of introduction across the cell monolayer, as revealed by signal inhibition, NIH3T3 fibroblasts were grown on partly conductive slides. Following growth-arrest, the compound was introduced by electroporation. After incubation at 37°C for 5 minutes to allow the compound to home-in on its intracellular target(s), cells were stimulated with EGF or PDGF and at different times thereafter (1-10 minutes), were fixed and probed for activated ERK1/2 by immunocytochemistry (Figure 3, Panels B-D) or Western blotting (Figure 3, Panel A). This dramatic inhibition was uniform across the cell monolayer, in agreement with previous results showing that *in situ* electroporation can introduce the molecule of interest into essentially 100% of the treated cells (2,3). The use of lower concentrations of the compound resulted in progressively lower, although uniform, levels of inhibition. It is interesting to note that this inhibition extends into approximately three rows of cells in the nonconductive section (Panel C, *st*), where the cells were not electroporated. This is presumably due to the movement of the compound through gap junctions, as previously shown for Lucifer yellow (3). This provides additional evidence that the observed inhibition is due to the drug, rather than an artifact of electroporation. As shown by phase contrast microscopy (Figure 3, Panel D), there was no alteration in the morphology of the electroporated cells under these conditions.

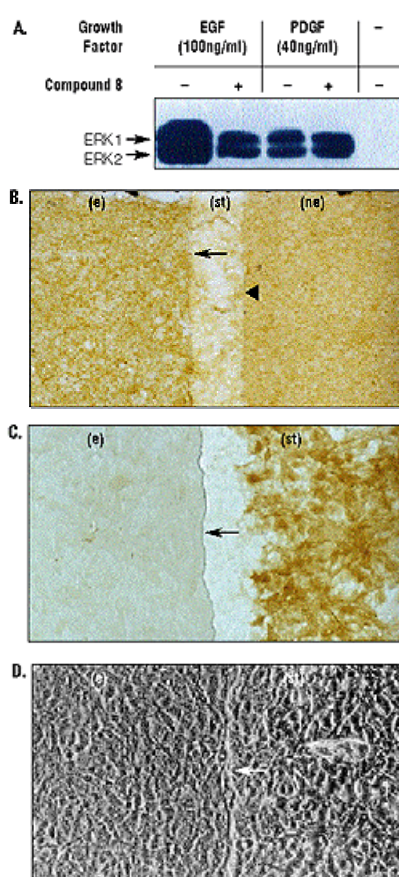


Figure 3. Compound 8 selectively inhibits EGF-induced ERK1/2 activation *in vivo*. Panel A: Quantitation of inhibition by Western blotting.

Compound 8 was electroporated (30 μ F, 50V, 4 pulses) at a concentration of 50 μ g/ml into serum-arrested NIH3T3 cells that were grown on fully conductive slides in an area of 10 x 32mm (see Figure 1). After a 5-minute incubation in DMEM, cells were stimulated for 5 minutes with 100ng/ml EGF (lanes 1 and 2) or 40ng/ml PDGF (lanes 3 and 4). Cells were extracted using 50mM HEPES (pH 7.4), 150mM NaCl, 10mM EDTA, 10mM Na₄P₂O₇, 100mM NaF, 2mM vanadate, 0.5mM PMSF, 10 μ g/ml aprotinin, 10 μ g/ml leupeptin and 1% Triton[®] X-100. Crude cell extract protein (300 μ g) was resolved by SDS-PAGE and analyzed by Western blotting, using Anti-ACTIVE[™] MAPK pAb. Signals were developed using an alkaline phosphatase-coupled donkey anti-rabbit antibody conjugate and chemiluminescence (Western-Star[™], Tropix, Bedford, MA) according to the manufacturer's instructions. The positions of the ERK isoforms are indicated. Panels B, C and D: Immunocytochemistry. Compound 8 was introduced by *in situ* electroporation (0.2 μ F, 40V, 4 pulses) into NIH3T3 cells growing on partly conductive slides (as in Figure 1) and growth-arrested in spent medium. Five minutes after electroporation, cells were stimulated with PDGF (Panel B) or EGF (Panel C) for 5 minutes, then fixed with 8% paraformaldehyde, incubated in 1% peroxide in PBS, permeabilized with 100% methanol at -20°C for 10 minutes and blocked with 1% BSA for 30 minutes. Cells were subsequently incubated with Anti-ACTIVE[™] MAPK pAb, which recognizes the dually phosphorylated, active forms of ERK1 and ERK2. Antibody signals were visualized using a biotinylated anti-rabbit secondary antibody followed by avidin-biotin-horseradish peroxidase complex and diaminobenzidine staining, according to the manufacturer's instructions (VECTASTAIN[®] Elite ABC Kit, Vector Laboratories). Cells were photographed under brightfield (Panels B and C) or phase contrast (Panel D) illumination. Magnification: Panel B, 40X, Panels C and D, 300X. Arrows (Panels B-D) point to the transition line between electroporated (e) and stripped (st) areas; arrowhead (Panel B)

points to the transition line between the stripped and control, ITO-coated (*ne*) areas. In [Panel B](#), cells growing on the left side of the slide (*e*) were electroporated, while cells on the stripped center zone (*st*) or right side (*ne*) did not receive a pulse. Note that the degree of ERK activation is the same on both sides of the slide for PDGF-stimulated cells ([Panel B](#)), while the compound dramatically reduced the EGF signal ([Panel C](#), area *e*). In [Panel C](#), the inhibition of the signal extends approximately three rows of cells into the stripped, nonelectroporated area (*st*), presumably because of movement of the compound through gap junctions (3). At the same time, the compound had no effect upon cell morphology, as shown by phase contrast microscopy in [Panel D](#). Images recreated from reference 9 with permission of authors and publisher.

SUMMARY

Unlike other techniques of cell permeabilization, such as streptolysin O (4) or scrape-loading (3), *in situ* electroporation does not affect cellular metabolism in any detectable way, presumably because the pores reseal rapidly so that the cell interior is restored to its original state. Previous results indicated that *in situ* electroporation does not increase the length of the G₁ phase of serum-stimulated cells and does not induce *fos* gene expression (2,3). The present work also demonstrates that this treatment does not affect the levels of activated ERK1/2 or the stress-activated kinases JNK/SAPK and p38^{hog}. These advantages illustrate the utility of this method for studying the consequences of efficient introduction of nonpermeant compounds upon cellular physiology. In addition, unlike microinjection, electroporation allows introduction of the material into large numbers of cells simultaneously, which facilitates large scale biochemical experiments. Moreover, the instant introduction into essentially 100% of the cells makes this technique especially suitable for kinetic studies of rapidly induced effector activation. In fact, this approach has been used to introduce a large variety of molecules, including peptides (2,5,7), proteins, oligonucleotides (6) or radioactive nucleotides (4), into adherent cells.

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