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Estrogen- and xenoestrogen-induced ERK signaling in pituitary tumor cells involves estrogen receptor-α interactions with G protein-αi and caveolin I

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Abstract

Multiple physiologic estrogens (estradiol, estriol, and estrone), as well as xenoestrogenic compounds (including alkylphenols and bisphenol A), can act via nongenomic signaling initiated by liganding of the plasma membrane estrogen receptor-α (mERα). We examined heterotrimeric G protein involvement leading to extracellular-regulated kinase (ERK) activation in GH3/B6/F10 rat anterior pituitary tumor cells that express abundant mERα, and smaller amounts of mERβ and GPR30. A combination of microarrays, immunoblots, and quantitative immunoassays demonstrated the expression of members of all α, β, and γ G protein classes in these cells. Use of selective inhibitors showed that the $G_{\alpha i}$ subtype was the primary initiator of downstream ERK signaling. Using antibodies against the GTP-bound form of G_{α} protein subtypes i and s, we showed that xenoestrogens (bisphenol A, nonylphenol) activated $G_{\alpha i}$ at 15-30 sec; all alkylphenols examined subsequently suppressed activation by 5 min. GTP-activation of $G_{\alpha i}$ for all estrogens was enhanced by irreversible cumulative binding to GTPγS. In contrast, $G_{\alpha s}$ was neither activated nor deactivated by these treatments with estrogens. ERα and $G_{\alpha i}$ co-localized outside nuclei and could be immuno-captured together. Interactions of ERα with $G_{\alpha i}$ and caveolin I were demonstrated by epitope proximity ligation assays. An ERα/β antagonist (ICI182780) and a selective disruptor of caveolar structures (nystatin) blocked estrogen-induced ERK activation.

Conclusions—Xenoestrogens, like physiologic estrogens, can evoke downstream kinase signaling involving selective interactions of ERα with $G_{\alpha i}$ and caveolin I, but with some different characteristics, which could explain their disruptive actions.

Keywords

membrane estrogen receptor; GPR30; nongenomic; GH3 cells; bisphenol A; alkylphenols

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Introduction

The actions of the physiologic estrogen estradiol (E_2) in high pM to nM concentration ranges are most often associated with reproductive function, and with the development of cancers in reproductive tissues. Other endogenous estrogenic compounds can be more prevalent during other life phases where they can have significant effects on tissue development, function, and disease states. Estrone (E_1) is a significant estrogenic hormone contributor in both reproductive (~0.5-1 nM) and postmenopausal (150-200 pM) women and in men; estriol (E_3) levels are much higher in pregnant women (~10-100 nM) than in nonpregnant women (<7 nM) [1], and changes in free E_3 levels in pregnancy have been associated with complications of eclampsia [2] and the incidence of Down's syndrome in offspring [3]. All three of these physiologic estrogens are also produced by aromatases in a number of nonreproductive tissues where their effects may extend beyond reproductive functions [4,5]. Therefore, loss or enhancement of these physiological estrogenic effects due to interference by xenoestrogenic compounds could affect human and animal health in a large number of tissues and life stages. Nongenomic signaling actions of E_1, E_2, and E_3 at physiologic concentrations have been demonstrated [6-8], and we have shown that xenoestrogens can interfere with their activity via this mode of action [9,10].

Alkylphenol (AP) xenoestrogens are structurally related variants (in aliphatic side-chain lengths) and are commercially useful in a variety of processes requiring surfactants. The related bisphenol A (BPA) has a substituted phenol group instead of a long side chain, and is a widely used and environmentally distributed plastics monomer [11]. We have previously shown that these compounds also signal via nongenomic estrogen receptor pathways, but with distinct timing and nonmonotonic dose-response characteristics compared to that observed for physiological estrogens [12-14], suggesting a mechanism for the deleterious effects of xenoestrogens.

Rapid nongenomic actions can be triggered by estrogens and other steroids to generate a variety of second messengers and diverse pathway activities in many cell types [15]. One of the signaling paradigms known to be utilized by estrogens are those involving G proteins, as demonstrated in vascular tissues [16-18] and neurons [19]. The particular G_α protein involved in most of those responses is of the "i" subclass. Membrane versions of ERs (mERs) are located in caveolar membrane specializations in some tissues [20-22], making them available to partner with many other signaling proteins including G proteins involved in propagating nongenomic signals, leading to further downstream activation of kinases and phosphatases, including the mitogen-activated protein kinases (MAPKs) [23,24].

Traditionally, receptors that associate with heterotrimeric G proteins are seven-transmembrane receptors, such as the G protein-coupled receptor 30 (GPR30 or GPER) which binds estrogens and mineralocorticoids [25]. In oocytes and in ovarian and breast cancer cells GPR30 has been shown to activate $G_{\alpha s}$ or $G_{\beta\gamma}$ [26,27]. However, the classical "nuclear" ERα, has also been shown to interact with $G_{\alpha i}$ [16-19] in endothelial and brain tissues. In one case ERβ was specifically studied for this role, but was not found to be involved [18]. In our GH3/B6/F10 rat pituitary cells nongenomic estrogenic signaling operates via a membrane version of estrogen receptor-α (mERα) [28,29], with some mostly inhibitory participation by the other membrane ERs (mERβ and GPR30) shown by use of selective ligands in selective (nM) concentration ranges [10].

The functional endpoints for nongenomic signaling are varied, dependent upon cell type, and often involve rapidly generated second messengers (reviewed in [30]). All three mER types (mERα, mERβ, GPR30) are known to have some nuclear effects downstream of their initial perimembrane signaling activations (examples in [31-33]). Nongenomic and genomic

actions of estrogens are thus a continuum beginning at the plasma membrane and sometimes culminating in nuclear actions when sustained signaling warrants a commitment of the cell to new synthesis of macromolecules [34], though some nongenomic actions such as peptide secretion can terminate in actions devoid of nuclear participation [35]. We selected the GH3/B6/F10 rat pituitary tumor cell line for study based on its robust expression of mERα and previous demonstrations of a variety of rapid signaling responses to estrogens that culminate in functional changes (reviewed in [24]) such as prolactin release, cell proliferation, apoptosis, and changes in cell shape and attachment to substrate. We have previously shown that all MAPKs can be rapidly phospho-activated by a variety of estrogens in these cells and that resulting functional responses can be blocked when extracellular-regulated kinases (ERKs) are inhibited ([9,24], and references therein). Changes in the activation of MAPKs are generally associated with functions such as changes in cell number and the mechanisms leading to that outcome (cell division, cell differentiation, cellular apoptosis) (reviewed in [36,37]). Though prolactin release in these cells is initiated by Ca^{++} signaling [14], other aspects of secretion (eg. vesicle docking and refilling) probably involve other signaling cascades including those of kinases [38].

Our current studies examine if the actions of these three physiologic estrogens (E_1, E_2, E_3), and the APs/BPA operate proximally via mERα and G protein-coupled signaling mechanisms. We relate downstream activation of one class of MAPKs, the ERKs, to interactions with a specific G protein subclass – αi.

Experimental

Reagents and cell culture

We purchased phenol red-free Dulbecco modified Eagle medium (DMEM, high glucose) from Mediatech (Herndon, VA); horse serum from Gibco BRL (Grand Island, NY); defined supplemented calf sera and fetal bovine sera from Hyclone (Logan, UT); penicillin-streptomycin and trypsin EDTA from Mediatech (Manassas, VA); charcoal and Triton X-100 from Sigma (St. Louis, MO). NF023 and NF449 inhibitors were purchased from CalBiochem (San Diego, CA). All other materials were purchased from Fisher Scientific (Pittsburgh, PA) or Sigma-Aldrich (St. Louis, MO).

GH3/B6/F10 cells were routinely cultured in phenol red-free DMEM containing 12.5% horse serum, 2.5% defined-supplemented calf serum, and 1.5% fetal bovine serum with penicillin-streptomycin (50 U/ml). Cells were used between passages 13 and 20 to stably maintain the robust mERα expression levels [35,39] needed for our assessment of these nongenomic responses. Because serum levels of steroids can mask the responses we monitor, we removed small hydrophobic molecules, including steroids, from serum by stripping 4 times with dextran-coated charcoal; cells were grown in welled plates pre-coated with poly-D-lysine in these media for 48 hrs before treatments. For some treatments (BPA) we used multiple concentrations to avoid discrepancies that exist in the literature about activating vs. inhibiting effects of xenoestrogens (for example [40,41]) due to complex nonmonotonic concentration-responses that we have seen previously.

Antibodies (Abs) and Ab assay reagents

Abs to GTP-$G_{\alpha i}$ and GTP-G_{as} were from NewEast Biosciences (Malvern, MA); Abs to ERα (MC-20) and caveolin-1 (N-20) were from Santa Cruz (Santa Cruz, CA); ERα C542 Ab was from Assay Designs, Enzo Life Sciences International (Plymouth Meeting, PA). Our other G protein Abs were from Santa Cruz or CalBiochem: $G_{\alpha s}$ (k20); $G_{\alpha q}$ (E-17); G_{α}/1213 (A-20); several anti-$G_{\alpha i}$ subtypes [3 (C-10), o/t/z (D-15)]; $G_{\beta 3}$ (C-16). Vectastain kits with biotin-conjugated secondary Abs and ABC-AP color development reagents were from Vector

Laboratories (Burlingame, CA); fish gelatin from Bio-Rad (Hercules, CA); FITC and Cy3 conjugated secondary Abs from Jackson ImmunoResearch Laboratories (West Grove, PA); Duolink reagents were from Olink Bioscience (Uppsala, Sweden).

pERK plate immunoassay

GH3/B6/F10 cells were treated with nM concentrations of all estrogens (plus 10fM BPA) for 5 min, emphasizing this earlier time point as a known nongenomic response [12,42]. Briefly, 10,000 cells were plated in poly-D-lysine-coated wells of 96-well plates, deprived of serum steroids, and then treated with physiologic estrogens (E_1,E_2 or E_3), xenoestrogens (APs or BPA), 12-O-tetradecanoylphorbol 13-acetate (TPA, 20nM) as a positive control, or ethanol vehicle as a negative control. The cells were then fixed with 2% paraformaldehyde (PFA)/0.2% picric acid at 4°C for 48hr, permeabilized with 0.1% Triton X-100 for 1hr at RT, blocked with 0.2% fish gelatin, and exposed to Ab for phospho-ERKs 1 and 2 overnight at 4°C. Biotin-conjugated secondary Ab was then applied, followed by washing, development with Vectastain kit avidin-conjugated alkaline phosphatase, 0.1% Triton X-100 washes, and the addition of alkaline phosphatase substrate paranitrophenol phosphate (pNpp). The yellow product pNp was monitored at A_{405} nm in a model 1420 Wallac microplate reader (Victor, Perkin Elmer, Waltham, MA). The plates were then washed, dried, and stained with crystal violet solution as previously described [39,43] to estimate cell numbers for normalization. For inhibitor studies, selective G protein inhibitors used were: pertussis toxin (a $G_{\alpha i}$ + $G_{\alpha o}$ + $G_{\alpha z}$ inhibitor, 100ng/ml or 9.5 nM), NF449 ($G_{\alpha s}$ inhibitor, 50 M), and NF023 ($G_{\alpha i/o}$ inhibitor, 50 M). The ICI182780 selective inhibitor for ERsα/β was used at 1nM, and the caveolae-disrupting chemical nystatin [44] at 50μg/ml. Replicates of 7-8 wells were performed for pERK assays in each of 2-3 separate experiments.

$G_{\alpha i}$ and $G_{\alpha s}$ activation assays

The activation of $G_{\alpha i}$ and $G_{\alpha s}$ was determined by Ab recognition of the levels of these proteins that were GTP-bound in a quantitative plate immunoassay, adapted from our previously developed protocols [39]. This assay was optimized for cell permeabilization conditions, saturating amounts of Ab producing a signal above that of using secondary Ab alone, and for production of a differential signal for total vs. activated $G_{\alpha i}$, according to NewEast Biosciences company recommendations. Cells were plated at 10,000-13,000 cells/well in 96-well plates, and serum steroid-deprived (were incubated in DMEM without serum) for at least 2 hrs before treatment. Cells were then treated with physiologic estrogens or xenoestrogens for 15 sec to 8 min. The cells were then processed as described above for the pERK plate assays, except that cells were fixed with 4% PFA for 10 min, and 100μM GTPγS was additionally added, when required, with the permeabilization solution. The fixed cells were incubated with the primary Abs (anti-GTP-$G_{\alpha i}$ or anti-GTP-$G_{\alpha s}$) overnight at 4°C. Replicates of 7-8 wells were performed for GTP-G proteins over 2-3 separate experiments. When we originally developed these types of plate immunoassays for quantitating various receptor and activated signaling proteins, we used nonspecific IgGs instead of specific primary Abs as negative controls, to determine the specificity of this technique for identifying the protein of interest [39], and thus have not repeated those studies again here. We have now utilized this assay format to quantify a variety of proteins in GH3/B6/F10 cell membranes or cell interiors including: ERs and β, GPR30, clathrin, three activated MAPK subtypes, and the activated transcription factors Elk-1 and ATF-2 [32,39,42].

Co-immunocapture (Co-IC) and immunoblots

For the results shown in Fig. 4, after serum deprivation, cells were washed in fresh DMEM/1% stripped serum and treated with either 10nM E_2 or vehicle control (EtOH) for 10 min. To prepare whole cell lysates cells were washed in ice cold PBS and lysed by scraping from

plates into 1ml lysis buffer (0.25M sucrose, 20mM Tris, 1mM EDTA, 1mM PMSF, 1mM DTT, protease inhibitors, pH 7.8) followed by freezing and thawing three times. Lysates were microfuged at 10,000g for 10 min, the pellet discarded, and the supernatant saved on ice for immediate analysis.

Protein-G agarose beads were conjugated to ERα Ab by combining 30 ls of a 50% bead slurry and 1 g of the ERα Ab (MC-20) used for capture. The suspension was tumbled for at least 4 hrs at 4°C, then microfuged for 2 sec to retrieve the beads, which were then washed 3 times in lysis buffer. Then equal amounts of lysate protein [assessed by Pierce Bicinchoninic Acid (BCA) Protein Assays, Rockford, IL] were added to the Ab-conjugated protein-G beads and the mixture was tumbled overnight at 4°C. The suspension was microfuged for 5 sec, washed 3 times with cold lysis buffer, and proteins released by adding SDS-PAGE loading buffer (40 ls) and boiling for 10 min. Proteins were then electrophoresed and immunoblotted with different selective $G_{\alpha i}$ protein Abs (using a kit from CalBiochem) by standard procedures. As E_2 treatment did not appear to change the levels by visual inspection, the bands were not evaluated by densitometry. Cell compartment-specific antigen preparations (isolating cytosolic and membrane fractions) along with additional Abs recognizing ERα, and more sensitive detection schemes (Odyssey fluorescent Ab system; Li-Cor Biosciences, Lincoln NE) were tried, but we were unable to consistently detect these protein interactions under these more dissociating conditions. Nonspecific IgGs were included as a negative control in some experiments, instead of the primary Ab of interest, and no proteins in the size range of interest for G proteins or ERs were observed (data not shown).

Co-localization by immunofluorescence

GH3/B6/F10 cells were cultured on cover slips, washed twice with PBS, and then fixed with 4% PFA for 20 min. The cells were then blocked with Duolink II blocking buffer for 30 min at 37°C followed by incubating overnight at 4°C with primary Ab (MC-20) for the protein expected to be exposed outside of the plasma membrane of the cell (ERα). Then the cells were refixed with 2% PFA for 5 min, permeabilized with 0.1% Triton X-100 for 10 min, and then incubated with primary Ab for the second protein ($G_{\alpha i}$; C-10 Ab) for 2 hrs. Cells were then incubated with secondary Abs for each primary Ab conjugated to either FITC or Cy3 (Jackson ImmunoResearch Laboratories, West Grove, PA) for 2 hrs at 37°C, followed by washing and mounting in 4μl of Duolink II Mounting Medium with DAPI. The slides were kept at −20°C before being viewed with an Olympus BX51 fluorescence microscope with an Olympus DP71 camera (at 10x and 60x magnifications for Fig. 5).

Co-localization by epitope Proximity Ligation Assay (PLA)

We used a relatively new technique to determine the distance between two epitopes of interest in our studies. The revised PLA protocol [45] determines the nearness of partnered protein epitopes within 35nm [46]. Basically the technique detects nearby epitopes tagged with primary Abs of choice made in two different species, recognized in turn by two different anti-species Abs having attached oligonucleotides. When the two epitopes are sufficiently close (35 nm) the attached oligonucleotides can hybridize, producing a template for a rolling circle DNA amplification, which can then be probed efficiently with fluorescent oligonucleotide probes that will appear as discreet dots by fluorescence imaging. To identify a single protein, or to use as a control for the analysis of epitope nearness. epitopes representing two parts of the same protein are chosen. In our assays GH3/B6/F10 cells were cultured on cover slips overnight and washed twice with PBS before fixation with 4% PFA for 20 min, which does not permeabilize cells [47]. The cells were then blocked with Duolink blocking buffer for 30 min at 37°C, followed by incubation with primary Ab (i.e. in unpermeabilized cells for proteins such as ERα expected to be on the outside of the plasma

membrane) overnight at 4°C and washed. Then the cells were re-fixed with 2% PFA for 5 min and permeabilized with 0.1% Triton X-100 for 10 min. Subsequent incubation with primary Ab for proteins inside the plasma membrane ($G_{\alpha i}$, caveolin-1) was for 2 hrs at RT, followed by washing. Appropriate anti-species secondary Abs to which oligonucleotides had been conjugated (anti-rabbit PLA probe PLUS and anti-mouse PLA probe MINUS) were then incubated with the preparation for 2 hrs at 37°C, followed by treatment with Duolink ligation-ligase solution for 30 min at 37°C. Finally, cells were incubated with the Duolink amplification-polymerase solution for 100 min at 37°C and then labeling oligonucleotides, followed by washing and mounting on slides with 4μl of Duolink 2 Mounting Medium containing DAPI fluorescent dye for staining nuclei. The slides were kept at −20°C before being viewed with a Zeiss 510 Meta confocal microscope (63× magnification with an additional 2.4 × zoom magnification).

Statistics

Data from plate assays for pERK and GTP-charged G proteins were analyzed by one-way analysis of variance (ANOVA) followed by multiple comparisons vs. control group (Holm-Sidak method) using Sigma Stat v.3 (Systat Software, Inc.) Significance was accepted at $p<0.05$.

Results

All G protein classes are present in GH3/B6/F10 cells

First we asked which G protein subtypes are present in our subcloned (F10) cell line, as a first step in determining which and how many interactions with ERα might be possible. At the RNA level, microarray analysis demonstrated that members of all G_{α} protein families plus several β and subtype RNAs were consistently present (found in repeat analyses). We then confirmed the presence of all major subtypes by either immunoblots, plate immunoassays, or both, using commercially available Abs. Table 1 (supplemental materials) summarizes these findings. Some subclass-specific Abs can recognize more than one of the class members (eg. $G_{\alpha i\text{-}3,o}$ recognizes both i subtypes 3 and o). Correctly sized G proteins were observed in all immunoblots. Multiple members of most classes were detected.

G protein role in signal generation by physiologic and nonphysiologic estrogens

We then determined whether the major subclasses of the G proteins present were involved in the signaling pathways leading to ERK activation (measured by quantitative 96-well plate immunoassay, Fig. 1). For this we used several G protein class-selective inhibitors. These data were all consistent with the involvement of the $G_{\alpha i}$ subclass, as responses for all estrogens that activated ERK at this concentration were inhibited by both PTX and NF023 (panels A and B respectively). Because the responses to all physiologic and nonphysiologic estrogens were similarly affected by these inhibitors, it suggests that $G_{\alpha i}$ is involved in all of their actions. As the G s inhibitor NF449 was inconsistently effective (panel C, 4/8 estrogens), this isoform does not appear to be as clearly involved in mediating ERK activation via ERs by all of these estrogens. Because there are many different signaling streams that culminate in ERK activation, it is possible that some estrogens could engage $G_{\alpha s}$ or other G proteins and then secondarily activate ERK pathways by 5 min, and this could be yet another distinction between the actions of different estrogens.

For further studies on G_{α} proteins subcategories “i” and “s” we employed a relatively recently developed experimental tool -- Abs that recognize G proteins that are in an active state due to the binding of GTP [48]. Abs for both unmodified and GTP-bound $G_{\alpha i}$ recognized the expected 48MW protein in immunoblots of cell lysates from cells treated with either vehicle or 1nM E_2 for 5 min (data not shown). We then adapted our quantitative

plate immunoassay for use with these Abs recognizing activated $G_{\alpha i}$ and $G_{\alpha s}$ proteins. We demonstrated that $G_{\alpha i}$ could be activated at 15-30 sec (Fig. 2) by low concentrations of estrogens (BPA, nonylphenol, and E_2 as a trend but not significantly). However, after 5 min (300 sec) of treatment (Figs. 2 and 3A) this GTP-activation of $G_{\alpha i}$ was significantly suppressed, even below vehicle-treated levels, for those samples treated with AP estrogens only. Because GTP-binding can apparently fluctuate very rapidly, and may differ for different estrogens, it could therefore be difficult to demonstrate the time course for each estrogen without many more experiments, or techniques more amenable to elucidating such very rapid responses. Therefore, we decided to add GTPγS to these samples, to potentially trap the GTP-bound form by irreversible cumulative binding to this modified GTP (Fig. 3A & B). As all values for GTP-$G_{\alpha i}$ were higher after adding GTPγS, it suggests that the Ab to activated $G_{\alpha i}$ recognizes both GTP-bound and GTPγS-bound G proteins. While the reductions due to treatment with estrogenic APs were eliminated by this GTPγS treatment, $G_{\alpha i}$ 5 min activation levels were enhanced for treatment with the physiologic estrogens (E_1, E_2, E_3) and BPA, presumably by trapping of the activated forms so they could not be rapidly down-regulated (obscuring the response) after early activation. However, $G_{\alpha s}$ (Fig. 3B) was neither activated nor deactivated by any estrogens tested, and trapping of activated forms with GTP-γS did not increase any of these responses.

Complex formation between ERα, $G_{\alpha i}$, and caveolin-1 in pituitary cells

We tested whether G i proteins from GH3/B6/F10 cells could be co-immuno-captured with ERα (Fig. 4). Ab-bound ERα and its companion proteins were pulled out of cell lysates on Protein G beads and we detected the presence of $G_{\alpha i}$ in the eluted, electrophoresed and immunoblotted proteins. E_2 treatment for 10 min did not influence the association of ERα with $G_{\alpha i}$. We also isolated subcompartment-resident ERα (cytosol or membrane fractions) to try to determine if a compartmentalized subset of ERs associate with G proteins (data not shown). While these results supported the presence of these proteins in our cells (see supplementary material Table I), we could not consistently demonstrate co-capture. Therefore, we conclude that the association between ERα and G proteins is fragile, and that further manipulations that are required to isolate subcellular fractions carried out in dissociating conditions (centrifugations, washes, etc.) can easily destroy this association.

We examined our cells with imaging techniques that might demonstrate these protein associations in situ, where they would be less susceptible to disruption by dissociating experimental conditions. ERα and $G_{\alpha i}$ proteins visualized by Ab binding partially co-localized at non-nuclear sites (Fig. 5). Abs to ERα were applied before permeabilization (Ab MC-20 and Cy3-tagged secondary Ab, red staining) to favor the labeling of the membrane (and not the more plentiful nuclear) version of this receptor. Because these images are produced by conventional fluorescence micrography, we cannot determine whether the signals are on or underneath the cell membrane. Membrane and cytoplasmic staining over the nuclear region of these cells is usually sparse as the large nuclei of these cells protrude and thin the cytoplasm in that area, and place the membrane in a different focal plane [49]. After permeabilization of the cells, $G_{\alpha i}$ was stained with Ab C-10 and an FITC-tagged secondary Ab (green staining). These antigens gave a largely similar staining pattern. The merged image shows that their locations can overlap (yellow) in the non-nuclear compartment (nuclei stained blue with DAPI), but with the more abundant $G_{\alpha i}$ protein being the predominant unpaired signal, as expected.

Finally, we examined an alternative method for visualizing protein partners in combination with confocal imaging -- Duolink epitope PLAs (Fig. 6). We examined ERα using Ab C542 (applied before cell permeabilization) interacting with $G_{\alpha i}$ (using Ab C-10) or caveolin-1 (using Ab N-20), both latter Abs applied after cell permeabilization. Signals with this technique are visualized by Ab proximity-based ligation. Ab-attached oligonucleotides

hybridize wherever the Abs are near enough to each other to allow this. The hybridized oligos are then amplified by rolling circle DNA synthesis. Finally, the amplified DNA is probed with complementary Cy3-labeled oligos. We display individual mid-cell optical sections (left panels), and 3D reconstructions combining all optical slices for each sample (right panels). For orientation to the subcellular compartments, the nuclei were stained by DAPI (blue). The differences between positive and negative controls are remarkably clear. In addition, the positive control of ERα alone is an interesting visualization of the total population of mERα and its position on the cell, regardless of whether it is partnered with other proteins. The signals indicating ERα partnering with $G_{\alpha i}$ and caveolin-1 are both nonnuclear (C & D, respectively), and mostly coincident with the cell periphery. These cells usually have a small and irregular cytoplasmic rim around a large nucleus, as we have observed previously with other types of staining and 3D confocal reconstruction of images [47,49]. Inspection of the rotated orthogonal view of these images showed that signal dots that appear to be over nuclei in the 3D panels are really adjacent (and not inside) nuclei when viewed from the side (not shown). Note that the unpaired ERα signals in A are more plentiful and have a smaller diameter compared to the partnered proteins in C and D.

Caveolae and ERs are both involved in xenoestrogen and physiologic estrogen signaling endpoints (ERK)

Finally, we addressed the significance of ERs in caveolar locations, by perturbing various factors with selective inhibitors and monitoring ERK activation (Fig. 7). Interrupting caveolar structure with nystatin [50] destroyed the ability of all competent estrogens, both physiologic and nonphysiologic, to mediate ERK activation. Likewise, inhibiting the actions of ERα/β with the selective (at this concentration) inhibitor ICI182780 also destroyed the ability of most of these estrogens to elicit an ERK response. Where significant responses were not elicited by OP and NP, those concentrations have been previously shown to not cause ERK activation, due to the nonmonotonic nature of the concentration dependence of these signaling responses [14]. In the case of E_1 eliciting a response that the ICI compound was unable to inhibit, it is possible that there is involvement of GPR30 estrogen receptors, though we have seen the GPR30 selective ligand G1 enhance ERK responses only at very low (10^{-13} to 10^{-11}M) concentrations while inhibiting responses at nM or higher concentrations [10]. Though the ICI compound has also been shown by others to elicit actions via GPR30 [51,52], this was only at μM concentrations, well above the 10nM concentration used here.

Discussion

Both physiological estrogens and xenoestrogens cause nongenomic signaling leading to functional responses (reviewed in [24]), and we have again demonstrated that ability in these studies. However, while several downstream signaling components have been clearly linked to the actions of xenoestrogens, the question remained as to the proteins involved in organizing these actions at a site more proximal to membrane receptor stimulation. Here we have demonstrated the involvement of specific G proteins in the initiation of signaling by a variety of physiological and environmental estrogens. Our evidence for the interaction of ERα with both G i and caveolin proteins by multiple approaches further supports the idea that these proximal protein partners participate in the actions of both estrogens and xenoestrogens initiated at the membrane.

While G proteins of every major class are expressed in many cell types (including in our pituitary tumor cell line used in these studies), it appears that the $G_{\alpha i}$ subclass is so far, the one most often linked with the actions of mERα [16-18] with some exceptions ($G_{\alpha q}$; [18]). Our data agree with these ERβ$G_{\alpha i}$ signaling partnerships demonstrated in other cell types. Even though $G_{\alpha s}$ does not appear to be consistently involved in downstream ERK signaling

in the present studies, we have preliminary evidence (using inhibitors) that both $G_{\alpha i}$ and $G_{\alpha s}$ are involved in Ca^{++} signaling evoked by E_2 in this cell type (Bulayeva & Watson, unpublished observation). Therefore, it may be that different G_α proteins can partner with ERs selectively to mediate different downstream effects, and these other associations and activations may be on different time scales and affected differently by distinct estrogens.

We also examined the nature of the physical association of ERα with $G_{\alpha i}$ proteins, as well as the influence of different estrogens on this G protein's activation. The 15-30 sec GTP-activation of $G_{\alpha i}$ was subsequently (at 5 min) suppressed by some estrogens (the APs), even below vehicle-treated levels. This could indicate a compensatory response more robust for xenoestrogens than for physiologic estrogens. Because GTPγS increased measurable levels of GTP-activated protein in all samples, this Ab directed at "loaded" $G_{\alpha i}$ apparently recognizes GTPγS-bound $G_{\alpha i}$ as an activated form. Because activation of $G_{\alpha i}$ was not statistically significant in our time course beginning at 15 sec by E_2, actions by this physiological estrogen could be more rapid, and only observable when all activated G proteins were trapped at 5 min with irreversible and thus cumulative binding of GTPγS to $G_{\alpha i}$. This trapping of the active form essentially erased the compensatory down-regulation of the GTP-bound form seen with the AP treatments. Because G protein activation appears to be a very dynamic response over such a short time frame, it is difficult to determine optimal activation and deactivation profiles for each estrogen separately given the expense of these reagents. However, it is interesting to see that the APs appear to regulate GTP activation/deactivation cycles differently (slower/more robustly) than the physiologic estrogens and BPA. We recently saw a similar separation between these two subsets of estrogens and their ability to temporally shift ERK response phases [10].

Co-immuno-capture experiments were done under a variety of conditions, some providing evidence that ERα and $G_{\alpha i}$ can partner in these cells. However, we were only reliably successful when using whole cell lysates, perhaps because such weak protein-protein interactions are vulnerable to the extra experimental manipulations required when preparing membrane and cytosolic fractions. However, the co-localization immunocytochemistry and the PLAs were done in fixed cells that did not undergo the same disruptions, which perhaps allowed these weaker protein complexes to survive [53]. Our experiments do not distinguish between direct vs. indirect protein interactions, as both immunocapture and a distance of <35 nM in PLAs could allow for the participation of other proteins as "adaptors" for formation of this complex. It is interesting that the PLA signals were qualitatively different when detecting ERα alone (a large number of dispersed small signals), vs. ERα partnered with other proteins (fewer, larger signals). Partnered signals should be a subset of the total number and thus be fewer. Because receptors and other signaling proteins are known to cluster in caveolae, it is possible that we observed pools of these proteins (and thus larger diameter signals) [54,55]. The clustering of mERα signals is an immuno-staining pattern that we have also previously seen after treatment of cells with estrogens [56].

Conclusions

We showed that xenoestrogens of the AP class are potent activators of nongenomic signaling via G proteins. Because such responses are normally mediated by a spectrum of physiologic estrogens (E_1, E_2, and E_3) each having different life stage-specific significance, the actions of these xenoestrogens could disrupt many different normal estrogenic functions including fertility/cycling, pregnancy, post-menopausal signaling, and non-reproductive tissue signaling (e.g. bone, cardiovascular, and behavioral functions). Actions on these cells representing the anterior pituitary suggest that xenoestrogen disruptions could affect a wide variety of endocrine axis tissues that are regulated from the pituitary. Altogether, our results contribute new strategies and evidence for both physiologic and nonphysiologic estrogens

acting via mERα to selectively interact with and activate $G_{\alpha i}$. This $G_{\alpha i}$ response precedes and is necessary for the downstream activation of ERKs in these cells. Though nonphysiologic xenoestrogens also cause these responses, they cause them with distinct differences in timing and magnitude. Therefore, xenoestrogens are potent nongenomic signal activators, but they induce defective (altered) estrogenic responses.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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List of abbreviations

AP	alkylphenol
Ab	antibody
BPA	bisphenol A
GPR30 or GPER	G protein-coupled estrogen receptor
E_1	estrone
E_2	estradiol
E_3	estriol
ERK	extracellular-regulated kinase
mERα	membrane estrogen receptor-α
mERβ	(membrane estrogen receptor-β
MAPK	mitogen-activated protein kinase
PFA	paraformaldehyde
PLA	proximity ligation assay

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- $G_{\alpha i}$ initiates ERK activation by physiologic and xenoestrogens in pituitary cells.
- Estrogens activate $G_{\alpha i}$ (cause binding of GTP) by 15 seconds.
- Xenoestrogen-induced compensatory deactivation of $G_{\alpha i}$ is blocked by GTPγS.
- ERα physically partners with $G_{\alpha i}$ and caveolin I.
- ERs and caveoli are necessary for ERK signaling via estrogens.

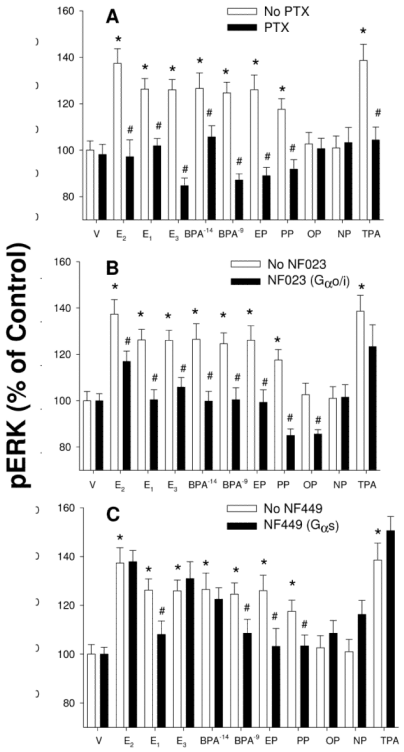

Fig. 1. G protein involvement in signaling leading to ERK activation triggered by three physiologic estrogens and alkylphenols

Cells were incubated with 1nM concentrations of estrogens except where an additional 10fM concentration is noted for BPA. Two concentrations of BPA were tested due to its consistent highly nonmonotonic concentration-response curve. ERK activation was measured using the pERK quantitative plate immunoassay. A. Cells were pretreated with 100ng/ml pertussis toxin (PT) overnight and then stimulated with estradiol (E_2), estrone (E_1), estriol (E_3), bisphenol A (BPA), ethylphenol (EP), propylphenol (PP), octylphenol (OP), nonylphenol (NP), or vehicle (V) for 5 min. Tetradecanoylphorbol acetate (TPA, or phorbol 12-myristate 13-acetate) was used as a positive control for post-receptor activation of pathways leading to ERK phosphorylation. B. Cells were pretreated with 50μM of the $G_{\alpha i}$-selective inhibitor NF023 for 30 min, and then stimulated by all estrogens named above for 5 min. C. As in B except cells were pretreated with 50μM of the $G_{\alpha s}$-selective inhibitor NF449. *=statistical significance ($P<0.05$) for estrogen treatments alone vs. vehicle control (100%); #=statistical significance ($P<0.05$) for inhibitor pretreatment before estrogen treatment vs. no inhibitor treatment. These results are mean ± SEM values of 24 samples assessed over 3 separate experiments.

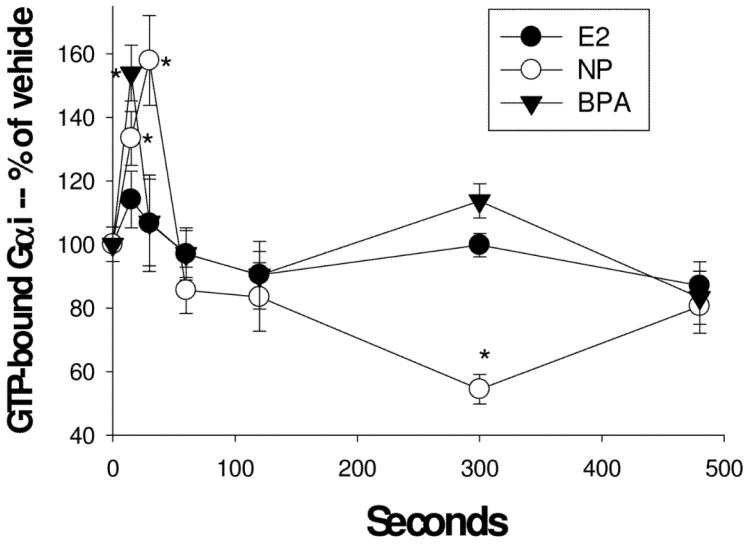

Fig. 2. Time-dependent activation of $G_{\alpha i}$ by a physiologic estrogen and two xenoestrogens

Cells were treated with 1nM estradiol (E_2), nonylphenol (NP), and bisphenol A (BPA). GTP-bound $G_{\alpha i}$ was measured with an Ab specific for the complex at various times shown in sec. n=24 samples (over 3 experiments) for each condition; *= statistical significance ($P<0.05$) for each estrogen/xenoestrogen treatment vs. the vehicle control (shown at time 0).

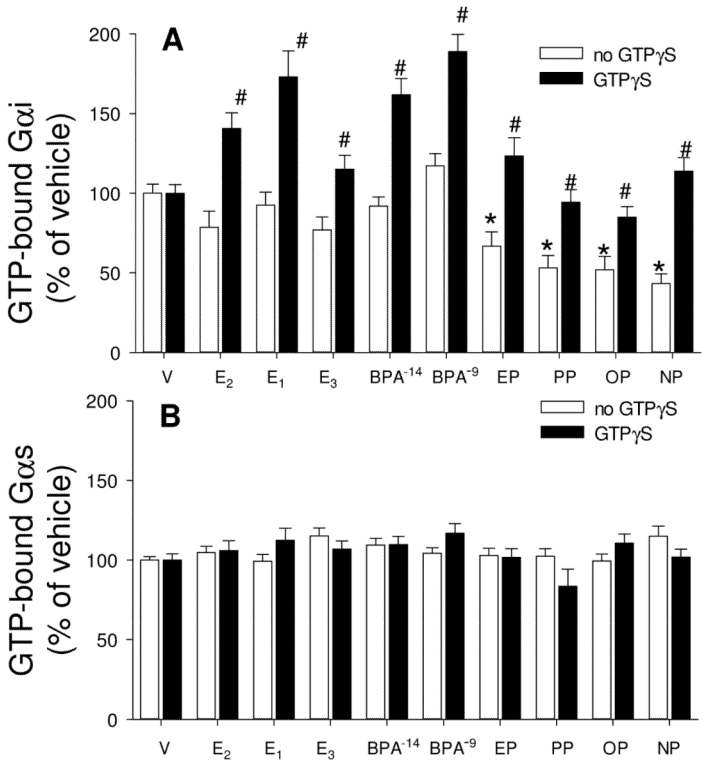

Fig. 3. Estrogens cause $G_{\alpha i}$ deactivation at 5 min and GTPγS binding shows cumulative activations; there are no such effects on $G_{\alpha s}$

Cells were treated with vehicle (V), estradiol (E_2), estrone (E_1), estriol (E_3), bisphenol A (BPA), ethylphenol (EP), propylphenol (PP), octylphenol (OP) and nonylphenol (NP) for 5 min, all estrogens at 1nM, except that BPA was additionally tested at 10 fM. GTP-bound $G_{\alpha i}$ (A) and $G_{\alpha s}$ (B) were measured by recognition with an Ab selective for these GTP-bound forms in the absence (white bar) or presence (dark bar) of 100μM GTPγS. Total $G_{\alpha i}$ and $G_{\alpha s}$ were also measured, but did not differ under any conditions of estrogen treatments (data not shown). For statistical annotations: For A *= statistical significance ($P<0.05$) from paired V control for the no GTPγS group. #= GTPγS-treated group statistical significance ($P<0.05$) from paired estrogen-treated levels; n=48 without, and n=32 with GTPγS treatment over 3 experiments. For B n=32 without and n=16 with GTPγS treatment over 2 experiments.

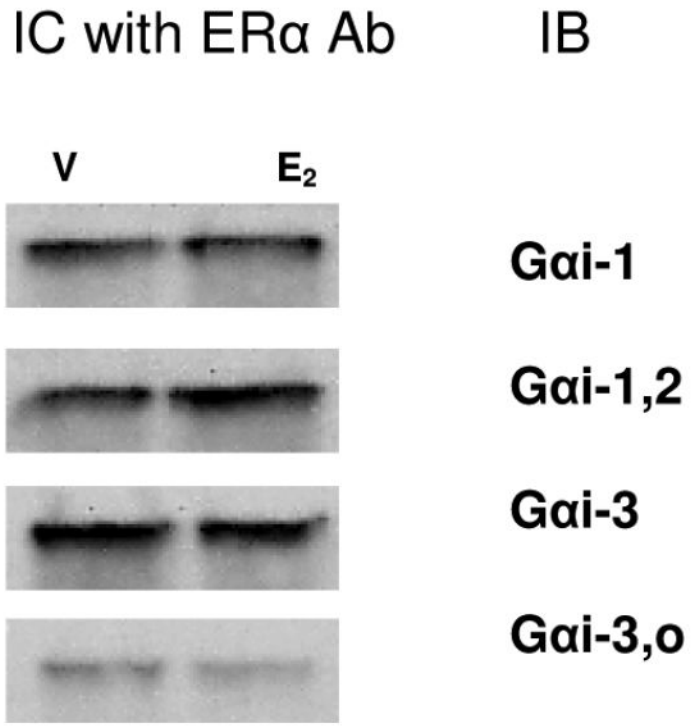

Fig. 4. ERα and $G_{\alpha i}$ proteins interact in whole-cell lysates

Lysates from cells treated with either vehicle (V) or 10nM E_2 (E_2) for 10 min were bound to specific Ab to ERα (MC-20 recognizing the C-terminus) and captured on Protein G agarose beads. Immuno-captured (IC) proteins were subsequently eluted, electrophoresed, blotted, and probed with four different subtype-selective $G_{\alpha i}$ Abs (IB), as indicated. All captured G proteins were ~48 MW, and no changes were noted due to E_2 treatment. A representative immunoblot from 3 experiments is shown.

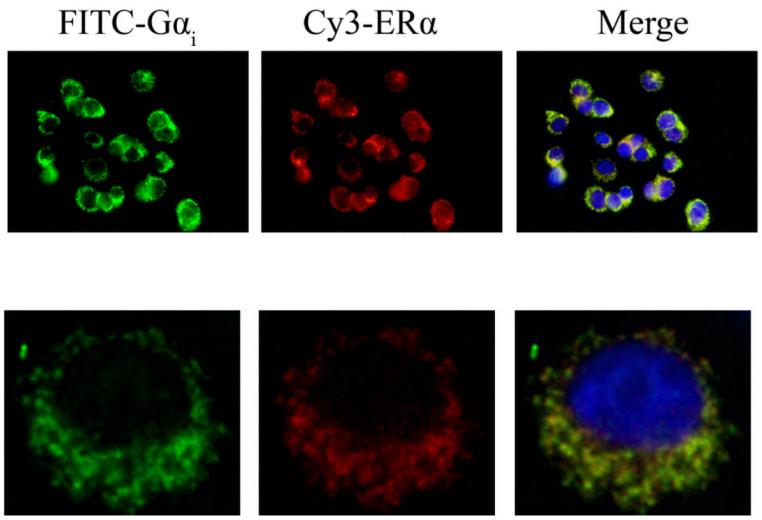

Fig. 5. ERα and $G_{\alpha i}$ proteins partially co-localize at non-nuclear sites

The subcellular location of ERα was assessed with Ab MC-20 followed by Cy3-tagged secondary Ab (red staining) in unpermeabilized cells. After permeabilization with 0.1% Triton X-100 for 10 min the subcellular location of $G_{\alpha i}$ was probed with Ab C-10 followed by an FITC-tagged secondary Ab (green staining). The top row shows a multi-cell field at 10X magnification and the bottom row shows a single cell at 60X magnification. The merged images display colocalization of ERα and G i as a yellow signal, and nuclei (blue) DAPI staining for comparison. Representative images from 2 experiments are shown.

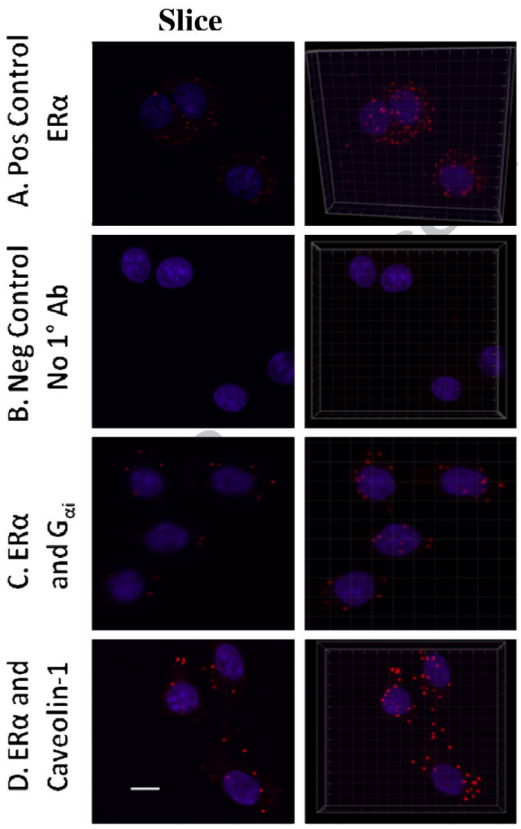

Fig. 6. ERα, and $G_{\alpha i}$ are in the same complex at or near the plasma membrane

Duolink epitope proximity assays for two different epitopes amplify a signal (red; cy3) due to the adjacency (<35nm) of epitopes. Each sample was also counterstained for nuclei (blue; DAPI). For each treatment, a representative image from 2 experiments is shown in the left panels as a single confocal slice through the middle of several cells. The representations in the right panels are 3D reconstructions of confocal slices into a composite image. The white bar in the lower left image measures 10 μM and the images were obtained using a 63X objective with a 2X optical zoom. A. Positive control: Two different ERα epitopes were assayed including one at the amino terminus (Ab ERα 21-32) and one at the carboxy terminus (Ab C542). B. Negative control: No primary Ab was included and there was no signal. C. Before permeabilizing the cells, the ERα Ab (C452) and a secondary Ab-linked Duolink probe was applied; after permeabilizing the cells the $G_{\alpha i}$ Ab (C-10) and its secondary Ab-linked Duolink probe was applied. The signal generated demonstrates close proximity of these two proteins in a non-nuclear pattern. D. ERα Ab (C542) and its probe were applied pre-permeabilization, and caveolin-1 Ab (N-20) and its probe were applied after cell permeabilization, again showing an interaction.

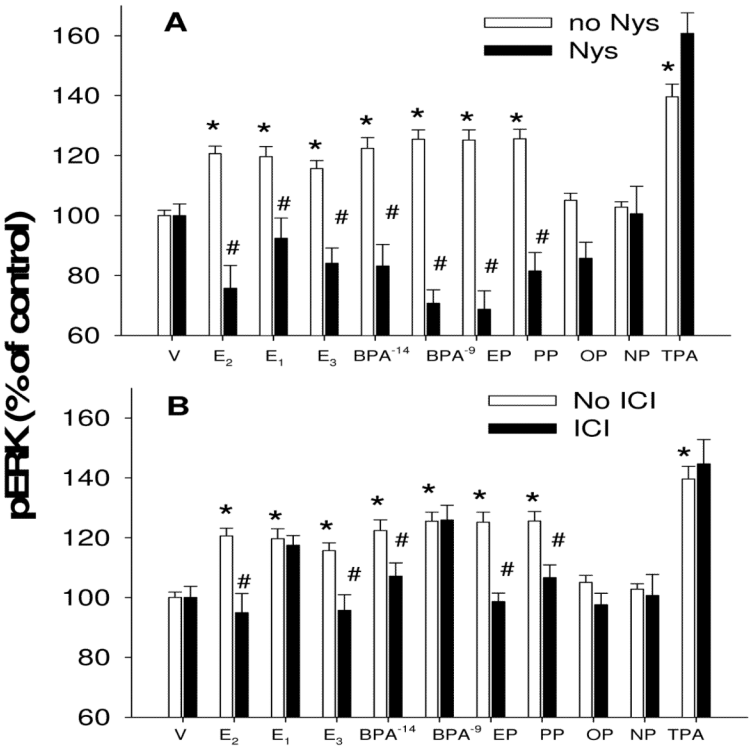

Fig. 7. Intact caveolae and ERs are involved in the ERK responses

Cells were pretreated (30 min) with 50μg/ml nystatin (A) which disrupts caveolar structure or (B) the selective ERα/β antagonist at this concentration, ICI 182780 (ICI, 10nM). Then cells were treated for 5 min with various estrogens before assessing ERK activation via the plate immunoassay. Physiologic estrogens at 1nM used to evoke an ERK response were estradiol (E_2), estrone (E_1), estriol (E_3). Xenoestrogens were bisphenol A (BPA), ethylphenol (EP), propylphenol (PP), octylphenol (OP), nonylphenol (NP). Tetradecanoylphorbol acetate (TPA) was used as a post-receptor positive control for ERK activation. Vehicle (V) treatment was the negative control. n=16 samples for each condition done in 2 separate experiments; *= statistical significance ($P<0.05$) for ERK activators vs. vehicle control; #= statistical significance ($P<0.05$) for inhibition of the stimulated level by inhibitors.