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Rapid Actions of Xenoestrogens Disrupt Normal Estrogenic Signaling

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Abstract

Some chemicals used in consumer products or manufacturing (eg. plastics, surfactants, pesticides, resins) have estrogenic activities; these xenoestrogens (XEs) chemically resemble physiological estrogens and are one of the major categories of synthesized compounds that disrupt endocrine actions. Potent rapid actions of XEs via nongenomic mechanisms contribute significantly to their disruptive effects on functional endpoints (eg. cell proliferation/death, transport, peptide release). Membrane-initiated hormonal signaling in our pituitary cell model is predominantly driven by mER α with mER β and GPR30 participation. We visualized ER α on plasma membranes using many techniques in the past (impeded ligands, antibodies to ER α) and now add observations of epitope proximity with other membrane signaling proteins. We have demonstrated a range of rapid signals/protein activations by XEs including: calcium channels, cAMP/PKA, MAPKs, G proteins, caspases, and transcription factors. XEs can cause disruptions of the oscillating temporal patterns of nongenomic signaling elicited by endogenous estrogens. Concentration effects of XEs are nonmonotonic (a trait shared with natural hormones), making it difficult to design efficient (single concentration) toxicology tests to monitor their harmful effects. A plastics monomer, Bisphenol A, modified by waste treatment (chlorination) and other processes causes dephosphorylation of extracellular-regulated kinases, in contrast to having no effects as it does in genomic signaling. Mixtures of XEs, commonly found in contaminated environments, disrupt the signaling actions of physiological estrogens even more severely than do single XEs. Understanding the features of XEs that drive these disruptive mechanisms will allow us to redesign useful chemicals that exclude estrogenic or anti-estrogenic activities.

Keywords

nongenomic; endocrine disruptors; environmental estrogens; G proteins; estrogen receptor- α ; mitogen-activated protein kinases

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Introduction

With too little estrogenic activity, a species cannot reproduce, and non-reproductive tissues also supported by estrogens (Es) can malfunction. However, too much estrogenic activity, or imperfect mimicry of estrogenic activity, as with xenoestrogens (XEs), can also cause some responsive organs to malfunction or develop cancers [1]. Therefore, Es must be very tightly regulated, and there are multiple hormonal regulatory mechanisms to ensure this control. Our studies examine the cellular control mechanisms by which XEs interfere with this regulation via the relatively novel rapid nongenomic signaling pathways. As the relatively insensitive genomic pathways often require very high doses (μM - mM) of XEs to be affected, and this is incongruous with animal studies showing actions at environmentally relevant concentrations, nongenomic signaling initiated at membrane receptors for Es can better explain the potent effects of XEs on functions.

Contamination of our environment with chemicals that can disrupt endocrine functions by mimicking Es is a growing problem, with many new compounds being adopted for various industrial and consumer uses [2-4]. It will become very difficult to keep up with the potential health threats posed by these chemicals if we do not decipher the mechanisms and decode the chemical structures that contribute to endocrine disruption. Unfortunately, mixtures of different XEs are common in the environment, so we must also begin to understand how XEs acting at the same receptors, signaling initiators, signaling integrators, and downstream functions can have potentially additive or even synergistic impacts [5] on stimulations or inhibitions of function. Though the hormesis effect [6] offers various explanations for why physiological hormone effects do not simply plateau, but are depressed at higher concentrations, these safety mechanisms may also prevent overstimulation and harmful consequences from mixtures of XEs. These disruptions occur in multiple functional systems influenced by endogenous Es including development, reproduction, metabolism, behavior, and immunity.

Hormonal influences are summed or “blended” together with actions caused by other important cellular regulators by funneling upstream signaling streams into downstream summative “nodes” such as the mitogen-activated protein kinases (MAPKs). The resultant activity determined by phosphorylation levels of a given signaling integrator in that class (like the extracellular regulated kinases [ERKs]) then goes on to deliver the message to downstream cellular machineries that coordinately control major cellular fates such as proliferation (together with malignant transformation), migration, differentiation, or death. This alteration of central kinase activation states by posttranslational modifications is a fundamental mechanism of cellular regulation. Such changes are differentially initiated by ligands (including Es and their analogs) binding at receptors at or near the cell surface [7]. MAPK responses oscillate with time and fluctuate up and down with concentration (are non-monotonic) [8-10]. Several types of mechanisms can be involved including different receptor populations and subtypes [11, 12], phosphatase activations, and the engagement of different signaling cascades [13-17].

Here we summarize our demonstrations of the rapid nongenomic signaling mechanisms by which XEs act very potently with nonmonotonic patterns in a pituitary lactotrope cell line. We also present examples of how XEs alone and in mixtures oppose the actions of endogenous Es, how chemical modifications of XEs alter but do not diminish their effects on signaling, and finally how XEs also affect a functional response.

Experimental

Reagents, cell culture, and treatments

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

Our use of non-transfected cell systems avoids artifacts due to receptor or other component overexpression and hetero-expression, where partners can be in short supply, and results can therefore be harder to interpret. 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 mER α 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 well plates pre-coated with poly-D-lysine in these media for 48 hrs before treatments. For XE treatments we used multiple concentrations to avoid discrepancies that exist in the literature about activating vs. inhibiting effects (for example [40,41]) due to complex nonmonotonic concentration-responses that we have seen previously. We challenged adult female levels of E₂ (1 nM) with various XEs singly and in combinations.

Antibodies (Abs) to GTP-G_{ai} and GTP-G_{as} were from NewEast Biosciences (Malvern, MA); Abs to unmodified G proteins were from Santa Cruz or CalBiochem. Abs to ER α (MC-20) and caveolin-1 (N-20) were from Santa Cruz (Santa Cruz, CA); ERK Abs were from Cell Signaling Technology, Beverly, MA. Vectastain kits with biotin-conjugated secondary Abs and ABC-AP color development reagents were from Vector Laboratories (Burlingame, CA). Duolink reagents were from Olink Bioscience (Uppsala, Sweden).

Co-localization by epitope Proximity Ligation Assay (PLA)

We used a relatively new technique to determine the in situ association of two proteins of interest in our studies. The revised PLA protocol [45] determines the nearness of partnered protein epitopes [46]. Potentially near epitopes are tagged with primary Abs made in two different species, recognized in turn by two different anti-species Abs having attached complementary oligonucleotides. When the two epitopes are sufficiently close (≤ 35 nm) the attached oligonucleotides hybridize, producing a template for a rolling circle DNA amplification, which is subsequently probed with oligonucleotides containing fluorescent nucleotides. Signals appear as discreet dots by fluorescence imaging. To visualize a single protein, epitopes from two parts of the same protein are chosen, and to show protein partnering, epitopes from each of the putative partners are probed.

GH3/B6/F10 cells were cultured on cover slips overnight and washed twice with PBS before fixation with 4% paraformaldehyde (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 overnight at 4°C and washed. In unpermeabilized cells proteins such as membrane ER are expected to be exposed on the outside of the plasma membrane. 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_{ai}, 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 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 confocal microscope.

Confocal images were acquired with a Zeiss LSM-510 Meta confocal microscope with a 63X water immersion objective (1.2 NA). Multi-track sequential acquisition was done with excitation lines at 364nm for DAPI and 543nm for the PLA red probe. Respective emissions were collected with 385-470 nm and 560-615 nm filters. Frame size was 512 × 512, and the final image was a collection of an 8-frame Kallman-averaging. The pinhole was properly adjusted to give the best confocal resolution according to the objective numerical aperture and wavelengths. The pixel size was 140nm. Optical slices were kept constant in both channels (364 and 543nm). Z-stack acquisition was done with 0.5 μm steps, and an additional optical zoom of 2.0 was applied over the region of interest. 3D renderings were done using Imaris 7.0 software.

pERK plate immunoassay

Briefly, 10,000 cells were plated in each well of a poly-D-lysine-coated 96-well plate, deprived of serum steroids, and then treated with physiological Es (E₁, E₂ or E₃), XEs (alkyl phenols [APs], bisphenol A [BPA], or bisphenol S [BPS]), 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% 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 A₄₀₅ nm in a model 1420 Wallac microplate reader (Victor, Perkin Elmer, Waltham, MA). The plates were then washed, dried, and stained with crystal violet (CV) solution as previously described [39,43] to estimate cell numbers for normalization. We assayed 7-8 replicate wells [9, 18, 19] in each of 2-3 separate experiments. In the development of these assays nonspecific IgGs instead of specific primary Abs were shown to give no signals [39]. We have now utilized this assay format to quantify a variety of proteins in GH3/B6/F10 cell membranes or cell interiors including: ERs α and β, GPR30, clathrin, three activated MAPK subtypes, and the activated transcription factors Elk-1 and ATF-2 [32,39,42]. These assays were recently automated using a Biomek FX liquid handling robotic device [20].

G_{αi} activation assays

These activations were determined by specific Ab recognition of GTP-bound G proteins in a quantitative plate immunoassay, adapted from our previously developed protocols [39] and see above. 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_{αi}, according to NewEast Biosciences company recommendations. Cells were plated at 10,000-13,000 cells/well as for ERK assays above and serum steroid-deprived (incubated in DMEM without serum) for at least 2 hrs before treatment. Cells were then treated with physiological Es or XEs for 10 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. The fixed cells were incubated with

the primary Ab (anti-GTP- $G_{\alpha i}$) overnight at 4°C. We assayed 7-8 replicate wells over 3 separate experiments.

PRL release assay

Based on our previous studies [32,38] ~600,000 cells were plated into poly-D-lysine-coated wells of 6-well plates overnight, and then hormone-deprived for 48 hrs in DMEM-1% 4X charcoal-stripped serum. Cells were then pre-incubated for 30 min in DMEM/0.1% BSA and exposed for 1 min to 1nM E_2 or different concentrations (10^{-15} – 10^{-7} M) of XEs, then centrifuged at 4°C, 350×g for 5 min. The supernatant was collected and stored at – 20°C until radioimmunoassay (RIA) for PRL. Cells were then fixed with 1ml of 2% paraformaldehyde/0.1% glutaraldehyde in PBS, and cell numbers determined via the CV assay. RIA PRL concentrations were determined with a Wizard 1470 Gamma Counter (Perkin Elmer) and normalized to CV values.

Statistics

Data from plate assays for pERK, GTP-charged G proteins, and PRL release 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 & Discussion

Complex Formation between ER α , $G_{\alpha i}$, and Caveolin-1

Central to the story of nongenomic mechanisms for XEs is a version of the estrogen receptor that is in the right place to initiate such signaling cascades from the membrane. We have in the past demonstrated this in a variety of ways using a spectrum of ER antibodies, selective antagonists and agonists, and antisense/siRNA strategies [12, 15, 21-26]. In Fig. 1 we show our newest visualization approach for these receptors – the proximity ligation assay [27]. When two epitopes within ER α (left panel) are probed we see the receptor's expression pattern in the membrane. When epitopes of other proteins are each probed for association with ER α then a signal for these subsets of partnering proteins are visible. We show this for both the $G_{\alpha i}$ protein (middle panel) and caveolin-1 (right panel), both shown by other approaches to be involved in joint actions with ERs [15, 28, 29].

Proximal Signaling

We study both proximal and downstream (integrative) signaling components in nongenomic pathways. Fig. 2 shows one of the most proximal and rapid responses we have observed to date, the activation (GTP-charging) of a $G_{\alpha i}$ protein. Responses occur and disappear within the 10-15 second time range which is faster than the appearance of calcium uptake responses that usually take at least 1 minute to develop [30, 31] and ERK activations that usually occur at about 3-5 min and thereafter (see below, and [9, 32]) in this pituitary lactotrope model. We do not yet know how many different downstream actions that this G protein activation directly or indirectly precedes, and we have not yet blocked this response and determined its ability to block individual downstream signaling events. It is more likely that a $G_{\alpha q}$ response would precede lagging L-type calcium channel openings [33]. However, we do know that $G_{\alpha s}$ responses do not change as a result of E_2 stimulation in this cell model [34].

Distal Signaling and Challenging Endogenous Estrogens with Xenoestrogens

Figure 3 shows the concentration dependence of ERK activation by ethyl phenol (EP) at varying concentrations, and a physiological concentration (1 nM) of estrone (E_1). It also shows one example of how XEs affect the actions of an already present endogenous

hormone. With increasing concentrations of added XEs a typical pattern is seen. The less effective (in this case lower) concentrations of XEs enhance the actions of an endogenous E, while more effective concentrations suppress the activity of the endogenous E. Some compounds show far more complex nonmonotonic concentration dependent patterns (such as for BPA), yet this rule is still true for 5 different structurally related XEs challenging 3 different endogenous Es [11].

XEs can also disrupt the temporal pattern of ERK activation by an endogenous E, shown in Figure 4. In this case the oscillating response to estriol (E_3) and EP is activation at 3-5 min, followed by deactivation around 10 min, followed by reactivation in the 30-60 min phase. But when EP and E_3 are added together, we see dephosphorylation at 2 min, followed by a larger and later activation. Thus the ERK signaling was initially damped and then rephased by the XE addition. Recent analyses of the kinetics of ERK responses reveals that the frequency of these oscillating episodes is actually important information that is passed on to the cell cycle regulatory machinery [8].

Mixtures of Xenoestrogens Disrupt More Severely

Our ability to measure these outcomes quantitatively gives us special insights into XE combinations' impacts on endogenous hormones. Figure 5 addresses the problem of how mixtures of XEs very common in most environmental exposure scenarios (BPA, BPS, and NP) affect ERK activation. Compared to the temporal profile of E_2 -induced ERK activation, we see disruption of this pattern when two contaminants are included, and even more interference when three contaminants are present. In reality, many contaminants are present in typical environmental samples, so it is plausible that this degree of severe disruption will occur.

Chlorinated BPA Inactivates ERK

BPA can be modified in several positions by chlorine, a common occurrence during waste water treatment and paper recycling. The resultant tri-chlorinated BPA species not only does not activate ERK, but instead causes a dephosphorylation of ERK (Figure 6). Lesser amounts of chlorination and some other modifications have less dramatic effects, though they also alter the response profile [20]. This is in sharp contrast to the inactivity of modified steroids or XEs (chlorinated, glucuronidated, or sulfated) on genomic responses [35-37].

XEs Have Rapid Functional Effects

Figure 7 demonstrates a downstream functional endpoint of estrogenic actions in these pituitary cells: PRL release at 2 min after E application. Several important features of nongenomic responses are displayed. One is the rapidity of even a downstream functional change brought on by acute signaling cascades, clearly not dependent upon later downstream activation of the gene expression component of this system (though a genomic response is necessary for eventually replenishing these peptides after secretion). The triggering response of calcium influx starts seconds to a minute earlier [30, 31]. In this concentration dependence study, it is obvious that both of these XEs act with a nonmonotonic concentration dependence, a feature that they share with physiological hormones [31, 38]. In addition, this response is extremely sensitive (triggered by sub-pM concentrations) as we have seen in many other nongenomic responses to XEs and physiological Es [9, 26, 30, 31].

Conclusions

In the end, one of the most important practical questions about the toxicity of these compounds is: At which concentration(s) are they active and are those concentrations

present in our environment? Because most researchers previously believed XEs to be weak Es (via the nuclear transcriptional pathway), the large majority of scientific studies about them have been done at very high concentrations (μM - mM). Because of non-monotonic response patterns, many of these studies may have missed the concentration ranges in which these compounds are the most active. Because nongenomic responses have only recently been examined, few studies have yet reported on these phenomena; more recently, our work and that of others suggest that XEs can be active down to the pM - fM level in some cellular assays of signaling or function [30, 39, 40]. This non-classical concentration dependence, perhaps related to hormesis for endogenous hormones [41], prevents accurate extrapolation from high doses to predict the actions of low doses or ineffective doses, and poses a great difficulty to those who must regulate environmental exposure levels. In addition, many previous studies have not paired XEs, either alone or in mixtures, with endogenous Es as we do here; this approach of directly challenging the actions of endogenous Es provides an important perspective for understanding their patterns of disruption. It is clear from our studies that mixtures of XEs disrupt endocrine signaling more than do individual XEs.

We use different endogenous hormones (E_1 , E_2 , and E_3) for our XE disruption experiments to show what broad spectrum effects these XEs can have. These different physiological E metabolites are more or less prominent at different life stages [42]. Therefore, XEs present a hazard not only for adult females, but also for developing embryos, fetuses, adolescents, pregnant females, males, and during both male and female aging. These examinations of several physiological Es help provide a better understanding of the endocrine basis of XE disruptions that lead to a spectrum of diseases or disease predispositions in both humans and other animals.

While a variety of tissues may be affected by XEs, effects on the pituitary are particularly important because disruptions of this central endocrine regulation can provoke malfunctions of the many other target tissues under pituitary control. The responses we observed were modified in small but significant increments, known to build by amplification as they progress down a signaling cascade. Multiple small changes add up to large ones, with the MAPKs acting as response summation nodes, as a “rheostat” for dialing the final functional response up or down. This allows MAPKs to integrate responses to multiple inputs from different contributory ligands before they orchestrate global responses such as cell proliferation, differentiation, or death.

The development of our sensitive, quantitative, and now automated [20] assays makes the detailed study of XE mixtures’ nongenomic effects newly approachable. By understanding how each specific XE imperfectly mimics and disrupts the actions of physiological Es in cellular signaling pathways, we can establish test criteria to inform the regulation or elimination of XEs in manufacturing and consumer products. In the future we could also use these assays to predict which potential substitute compounds will avoid health risks, thereby guiding safer product development [43]. This could prevent toxin-based health disasters, provide large savings to consumers and consumer industries that bear the costs of retooling with acceptable substitutes, and eliminate the need to judge and compensate for exposures to dangerous chemicals.

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Figure 1. ER α , G α_i , and Caveolin are complexed at the cell surface

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. Images show a Z-stack 3D rendering of 1 μ m confocal optical slices acquired with a 63X (1.2 NA) water immersion objective using a 2X optical zoom. Left panel: Two different ER epitopes were probed including one at the amino terminus (Ab ER α 21-32) and one at the carboxy terminus (Ab C542) before permeabilizing cells. Center panel: Before permeabilizing the cells, the ER α Ab (C452) and a secondary Ab-linked Duolink probe was applied; after permeabilizing the cells the G α_i Ab (C-10) and its secondary Ab-linked Duolink probe was applied. Right panel: ER α Ab (C542) and its probe were applied pre-permeabilization, and caveolin-1 Ab (N-20) and its probe were applied after cell permeabilization. A negative control omitting the primary Ab had little to no signal (not shown). Each red dot shows a proximity of two epitopes in a non-nuclear pattern.

Figure 2. Time-dependent activation of Gαi by a physiologic E and two XEs
 Cells were treated with 1nM estradiol (E2), nonylphenol (NP), and bisphenol A (BPA). GTP-bound Gαi was measured with an Ab specific for the modified protein at various times shown in sec using a plate immunoassay. n=24 samples (over 3 experiments) for each condition, normalized to cell number; *= statistical significance (P<0.05) for each E/XE treatment vs. the vehicle control (shown at time 0).

Figure 3. Concentration-dependent changes in pERK caused by a short-chain alkylphenol
Cells were treated for 5 min with a combination of 1 nM E₁ plus different concentrations of ethyl phenol (EP), and pERK was assayed. The lighter hashed horizontal bar indicates the pERK level and error range around 100% in vehicle-treated cells (V); the darker (upper) crosshatched horizontal bar indicates the pERK value and error in cells treated with 1 nM E₁ alone. **p* < 0.05 compared with vehicle-treated cells. #*p* < 0.05 compared with cells treated with E₁. Plate immunoassay values were normalized to cell number.

Figure 4. Time-dependent changes in pERK elicited by a combinations of a short-chain alkylphenol with E₃

Cells were co-treated with 1 nM EP and 1 nM E₃ for different times. The pERK levels were measured by plate immunoassay; the pNp signal generated for each well was normalized to cell number (by the CV assay).

Figure 5. ERK activation by E₂ is severely disrupted by mixtures of XEs

Rat pituitary cells were exposed to E₂ (10⁻⁹ M), E₂ in combination with BPS (10⁻¹⁴ M) and BPA (10⁻¹⁴ M), or in combination with BPA, BPS and NP (10⁻¹⁴ M) over a 60- min time course. Responses were measured by plate immunoassay; the pNp signal generated for each well was normalized to cell number (by the CV assay). Values are expressed as percentage of vehicle (V)-treated controls; error bars are S.E.M. n = 24 wells over 3 experiments.

*=p<0.05 compared to vehicle (V); #=p <0.05 compared to 10⁻⁹ M E₂.

Figure 6. Dose-response analysis of ERK phosphorylation (pERK) after exposure to BPA vs. trichlorinated BPA

The cells were exposed to a range of concentrations (in log increments) of BPA or trichlorinated BPA. pERK was measured by plate immunoassay at a 5 min exposure time and normalized to cell number (by the CV assay). The widths of the vehicle and E₂ (10⁻⁹ M) bars represent the means ± SE (n = 24 over 3 experiments). *p<0.05 when compared with vehicle (V). #p<0.05 when compared with 10⁻⁹ M E₂. E₂ (10⁻⁹ M) is significantly different from vehicle.

Figure 7. XEs cause PRL release

We measured PRL release into the culture medium by RIA after a 2-min exposure to individual XEs (10^{-15} M– 10^{-7} M). The amount of PRL secreted for each well was normalized to the CV value for cell number, and expressed as a percentage of vehicle (V)-treated controls. Error bars are means $\pm$ S.E.M.