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Mechanisms of membrane estrogen receptor-α-mediated rapid stimulation of Ca^{2+} levels and prolactin release in a pituitary cell line

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Bulayeva, Nataliya N., Ann L. Wozniak, L. Leanne Lash, and Cheryl S. Watson. Mechanisms of membrane estrogen receptor-α-mediated rapid stimulation of Ca^{2+} levels and prolactin release in a pituitary cell line. *Am J Physiol Endocrinol Metab* 288: E388–E397, 2005. First published October 19, 2004; doi:10.1152/ajpendo.00349.2004.—The role of membrane estrogen receptor-α (mERα) in rapid nongenomic responses to 17β-estradiol (E_2) was tested in sublines of GH3/B6 rat prolactinoma cells selected for high (GH3/B6/F10) and low (GH3/B6/D9) mERα expression. E_2 elicited rapid, concentration-dependent intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) increases in the F10 subline. Lack of inhibition by thapsigargin depletion of intracellular Ca^{2+} pools, together with abrogation of the response in Ca^{2+}-free medium, suggested an extracellular source of Ca^{2+} for this response. The participation of voltage-dependant channels in the E_2-induced $[Ca^{2+}]_i$ increase was confirmed by the specific L-type Ca^{2+} channel inhibitor nifedipine. For comparison, the D9 mERα-depleted subline was insensitive to steroid action via this signaling mechanism. $[Ca^{2+}]_i$ elevation was correlated with prolactin (PRL) release in the F10 cell line in as little as 3 min. E_2 caused a much higher PRL release than KCl treatment (which caused maximal Ca^{2+} elevation), suggesting that secretion was also controlled by additional mechanisms. Participation of mERα in these effects was confirmed by the ability of E_2-peroxidase (a cell-impermeable analog of E_2) to cause these responses, blockage of the responses with the ER antagonist ICI 182 780, and the inability of the E_2 stereoisomer 17α-E_2 to elicit a response. Thus rapid exocytosis of PRL is regulated in these cells by mERα signaling to specific Ca^{2+} channels utilizing extracellular Ca^{2+} sources and additional signaling mechanisms.

prolactinoma cell line; intracellular Ca^{2+}; L-type channel; exocytosis

GH3/B6 CELLS ARE A CLONAL LINE of rat lactotrophs that can release the polypeptide hormone prolactin (PRL) in response to different stimuli. Whereas some mechanisms cause slow, synthesis-based release of PRL, others allow for rapid release of PRL from storage vesicles. Estradiol (E_2) has been shown to regulate synthesis of PRL via genomic mechanisms (13). In addition, genomic estrogenic effects influence PRL release through protein upregulation of L-type voltage-dependent Ca^{2+} channel proteins required for exocytosis and through upregulation of PKC, which influences the generation of Ca^{2+} currents and exocytosis by phosphorylation (33). However, in addition to classic genomic (protein synthetic) effects, physiological concentrations of E_2 can rapidly stimulate a variety of second-messenger pathways in diverse cell types (30, 31). These include generation of cAMP and nitric oxide, activation of kinases (MAPK, phosphatidylinositol 3-kinase), and elevation of intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) levels (3, 8, 12, 18, 22, 27, 28). Rapid $[Ca^{2+}]_i$ level increases elicited by E_2 can come from intracellular stores and be initiated by capacitative Ca^{2+} entry through store-operated Ca^{2+} channels in mouse neurons (2), breast cancer cells (17), human endometrial cells, and rat distal colon (9). E_2 can also modulate voltage-dependent Ca^{2+} channel activity in macrophages (1) and vascular smooth muscle cells (19). In the GH3/B6 cell line, rapid effects of E_2 on membrane excitability have also been observed; E_2 can activate Ca^{2+} currents by increasing the action potential frequency or by the reversal of dopamine-mediated inhibition (10, 11). E_2 is also able to increase PRL secretion at 10 min in the GH3/B6 cell line (34), at 1–5 min in our sublines expressing increased amounts of membrane estrogen receptor-α (mERα; Refs. 24–26), and within 10 min in lactotroph primary cultures (6).

Dufy et al. (11) demonstrated the similarity between E_2- and thyroid hormone (TRH)-regulated effects on Ca^{2+} currents through voltage-dependent Ca^{2+} channels. TRH, as well as other neuropeptides (VIP family proteins, angiotensin), is able to produce a rapid PRL release from lactotrophs, and all of these peptides act via receptors coupled to different types of G proteins (G_q, G_i, G_s, and so forth) on the plasma membranes of lactotrophs. Activation of G proteins, in turn, leads to an increase in PLC activity, which produces inositol trisphosphate that binds to a receptor on the endoplasmic reticulum and releases Ca^{2+}. Voltage-dependent Ca^{2+} channel activity can also be modulated by phosphorylation of the channel's subunits via PKC or, in the case of cAMP production, PKA (13). Therefore, Ca^{2+} levels are raised in this cell type via a variety of specific pathways and mechanisms.

In the present study, using GH3/B6/F10 (F10) and GH3/B6/D9 (D9) sublines enriched and depleted for mERα, respectively (23), we examined the role of this membrane steroid receptor in these processes. Our goal was to link the presence of the mERα receptor directly to specific mechanistic pathways. Our more detailed examination of the mechanism of E_2-induced Ca^{2+} mobilization linked to this receptor also addresses the extent to which Ca^{2+} elevations are responsible for PRL secretion.

MATERIALS AND METHODS

Reagents. Phenol red-free DMEM was purchased from Mediatech (Herndon, VA). Horse serum was from GIBCO Invitrogen (Carlsbad, CA); defined-supplemented calf sera and fetal bovine sera were from Hyclone (Logan, UT). We purchased paraformaldehyde and glutaraldehyde from Fischer Scientific (Pittsburgh, PA). Nifedipine, thapsigargin, and 2,5-di-(*t*-butyl)-1,4-hydroquinone (tBHQ) were purchased from Calbiochem (San Diego, CA), and fura 2-AM was from Molecular Probes (Eugene, OR). From the National Institute of Diabetes and Digestive and Kidney Disease's National Hormone and Pituitary

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Program (NIDDK, Baltimore, MD), we purchased rat PRL-RP-3 standard and anti-rPRL-s-9. ^{125}I-labeled rat PRL was from Perkin-Elmer (Wellesley, MA). The ER antagonist ICI 182 780 was purchased from Tocris (Ellisville, MO) or Zeneca Pharmaceuticals (Cheshire, UK). The C542 antibody, which recognizes the COOH terminus of ERα, was from StressGen (Victoria, BC, Canada). All other reagents were purchased from Sigma-Aldrich (St. Louis, MO). For the E_2-peroxidase (E_2-P) conjugate purchased from Sigma, we administered 10 nM on the basis of the E_2 concentration in the complex. To eliminate free E_2 molecules from the E_2-P reagent, it was centrifuged through a Millipore filter (cutoff mol wt 10,000) just before use in these assays.

Cell culture. The GH3/B6/F10 and GH3/B6/D9 clonal rat prolactinoma cell lines were further selected for high and low expression of mERα, respectively, using C542 antibody according to immunopanning methods previously reported (26). Cells were routinely cultured in DMEM containing 12.5% horse serum, 2.5% defined-supplemented calf serum, and 1.5% fetal bovine serum. For individual experiments, cells were deprived of steroids for 48 h after plating by substitution of culture media with DMEM containing 5 μg/ml insulin and transferrin and 5 ng/ml sodium selenite plus 0.1% BSA, 20 mM sodium pyruvate, and 25 mM HEPES (DMEM-ITS). Cells were incubated in DMEM alone for 1 h just before all experiments. For treatments, E_2 was dissolved in ethanol (EtOH) at a 10^{-2} M concentration to create a stock solution, and was subsequently diluted into experimental media to yield final concentrations from 10^{-8} to 10^{-12} M. The EtOH concentration used as vehicle control was 0.0001%.

Ca^{2+} measurements. GH3 cells (10^5 cells) were plated on poly-D-lysine-coated coverslips (25 mm^2) inside wells of a six-well plate. Just before each experiment, the cells were washed in a normal Ringer solution (120 mM NaCl, 1.25 mM $CaCl_2$, 4.7 mM KCl, 1.2 mM $MgCl_2$, 20 mM HEPES, 10 mM glucose, and 0.1% BSA; pH 7.4) and loaded with 2 μM fura-2 AM (diluted in normal Ringer) for 1 h at room temperature (RT). The cells were then washed twice and incubated in Ringer solution for 20 min at RT before imaging. For some experiments, we used a Ca^{2+}-free Ringer solution (Ca^{2+} chelated with 2 mM EGTA in Ringer) or a KCl solution (in Ringer; the NaCl concentration was reduced to 105 mM, and 20 mM KCl was added). Ionomycin (1 μM) was

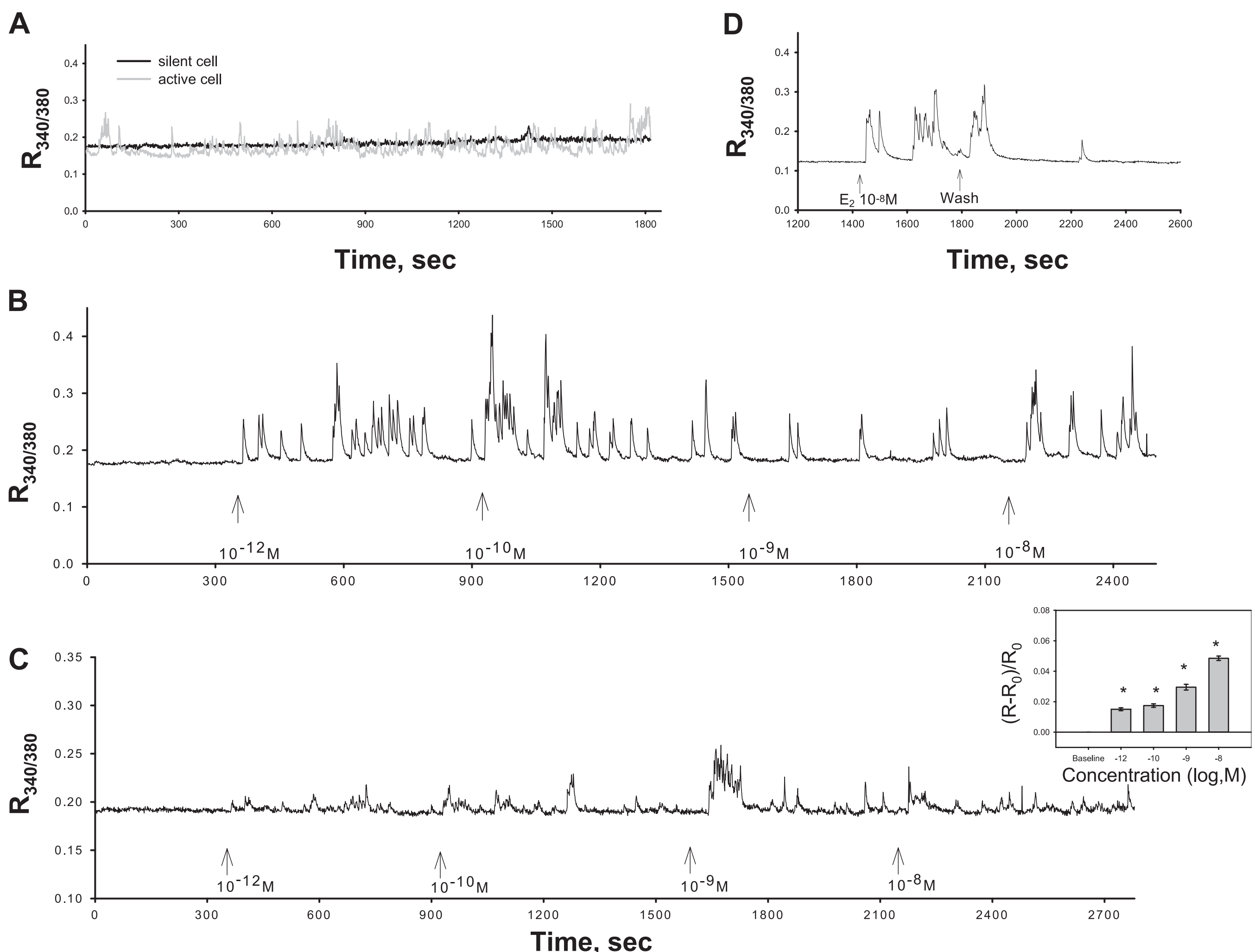

Fig. 1. 17β-Estradiol (E_2) can produce dose-dependent and rapid intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) increases in GH3/B6/F10 cells. *A*: control recordings from silent and spontaneously active cells over the time period used for the experiments that follow. E_2-induced time- and concentration-dependent $[Ca^{2+}]_i$ changes from a single cell (*B*) and from 27 cell traces averaged from 4 different experiments (*C*) are shown. The trace from a single cell (*D*) shows reversibility of the 10^{-8} M E_2 effect on $[Ca^{2+}]_i$ changes. The *x*-axes of all graphs show the time elapsed in s. The *y*-axes in *A–D* are labeled $R_{340/380}$, showing the ratio (R) of fluorescence signals collected at 510-nm emission using 340- or 380-nm excitation wavelengths. Arrows indicate the times of E_2 applications at shown concentrations. Bar graph (*inset* in *C*): means ± SE of 27 cells from 4 different experiments; the calculation presents the difference between stimulated and basal levels ($R - R_0$) during a 5-min treatment period, normalized to the basal level (R_0). *Statistical significance for E_2 treatment by ANOVA ($P < 0.05$) vs. ethanol (EtOH) vehicle (0.0001%)-labeled baseline.

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used in Ringer solution containing high $CaCl_2$ (10 mM). In the Ca^{2+}-free solution experiments, we added E_2 quickly after the solution change (within 5 min) to prevent response changes due to Ca^{2+} leakage from intracellular stores.

E_2 and other reagents were administered with a microperfusion pump system (Bioptechs, Butler, PA) at a rate of 2 ml/min. The dead time between the vials of treatment solution and the cell chamber (Molecular Probes) was 20 s; the solution was pumped as close to the cells as possible. All experiments were done at RT. Imaging was performed using a TE200-IUC Quantitative Fluorescence Live-Cell and Multidimensional Imaging System equipped with a Nikon EPI 200 fluorescence microscope and a digital monochrome-cooled charge-coupled device Roper Coolsnap HQ camera (Roper Scientific, Tucson, AZ). Signals were collected from regions of interest corresponding to a single cell with a ×40 objective (1.3 NA) using the MetaFluor program (Universal Imaging, Downingtown, PA). Background measurements were made from an area without cells. Signals were obtained in dual excitation mode (340/380 nm), and the $[Ca^{2+}]_i$ was calculated as a ratio ($R_{340/380}$) of emission data collected at 510 nm after background subtraction (15). To quantitate the degree of Ca^{2+} elevation, data were represented as a change in fluorescence ratio ($R - R_0$) during a 5-min treatment period, normalized to the basal $[Ca^{2+}]_i$ level (R_0).

PRL release. GH3/B6 sublines (5×10^5 cells/well) were plated in poly-D-lysine-coated six-well plates. Just before each experiment, the medium was removed and new DMEM-0.1% BSA containing E_2 or vehicle (control) was added. The cells were incubated for 3, 6, 10, or 15 min at 37°C and then centrifuged at 300 *g* for 5 min. The supernatant was collected and stored at −20°C. Each experiment was repeated four times.

PRL RIA. Concentrations of PRL in the media of GH3/B6 sublines were determined using components of the rat PRL RIA kit from the NIDDK. Briefly, RIA buffer (80% PBS, 20% DMEM, 2% normal rat serum), 100 μl of cold standard or unknown sample, ^{125}I-labeled rat PRL at 15,000 counts/tube (diluted in RIA buffer), and rPRL-s-9 antiserum (final dilution of 1:437,500 in RIA buffer) were combined and incubated overnight with shaking at 4°C. Anti-rabbit IgG (Sigma R-0881, 1:9 dilution) was added, and the samples were further incubated in a shaker for 2 h at RT. One milliliter of polyethylene glycol solution (Sigma P-6667; 1.2 M PEG, 50 mM Tris, pH 8.6) was then added, and the samples were incubated for an additional 15 min at RT. The samples were then centrifuged at 4,000 *g* for 10 min at 4°C, the supernatant was decanted, and the pellet was counted in a 1470 Wizard gamma counter. PRL concentrations were normalized to cell number [determined by the crystal violet (CV) assay].

CV assay. This procedure was used to normalize the PRL concentration to cell number (5). Briefly, after collection of the supernatant, cells were fixed in 2% paraformaldehyde-0.1% glutaraldehyde for 30 min at RT. They were then washed with water and allowed to dry completely. A 0.1% CV solution was then added to each well, and the plates were incubated for 30 min at RT. Washing and drying were repeated as before. The dye was extracted with 10% acetic acid solution and read at A_{590} nm on a 1420 Wallac microplate reader (Perkin-Elmer, Boston, MA).

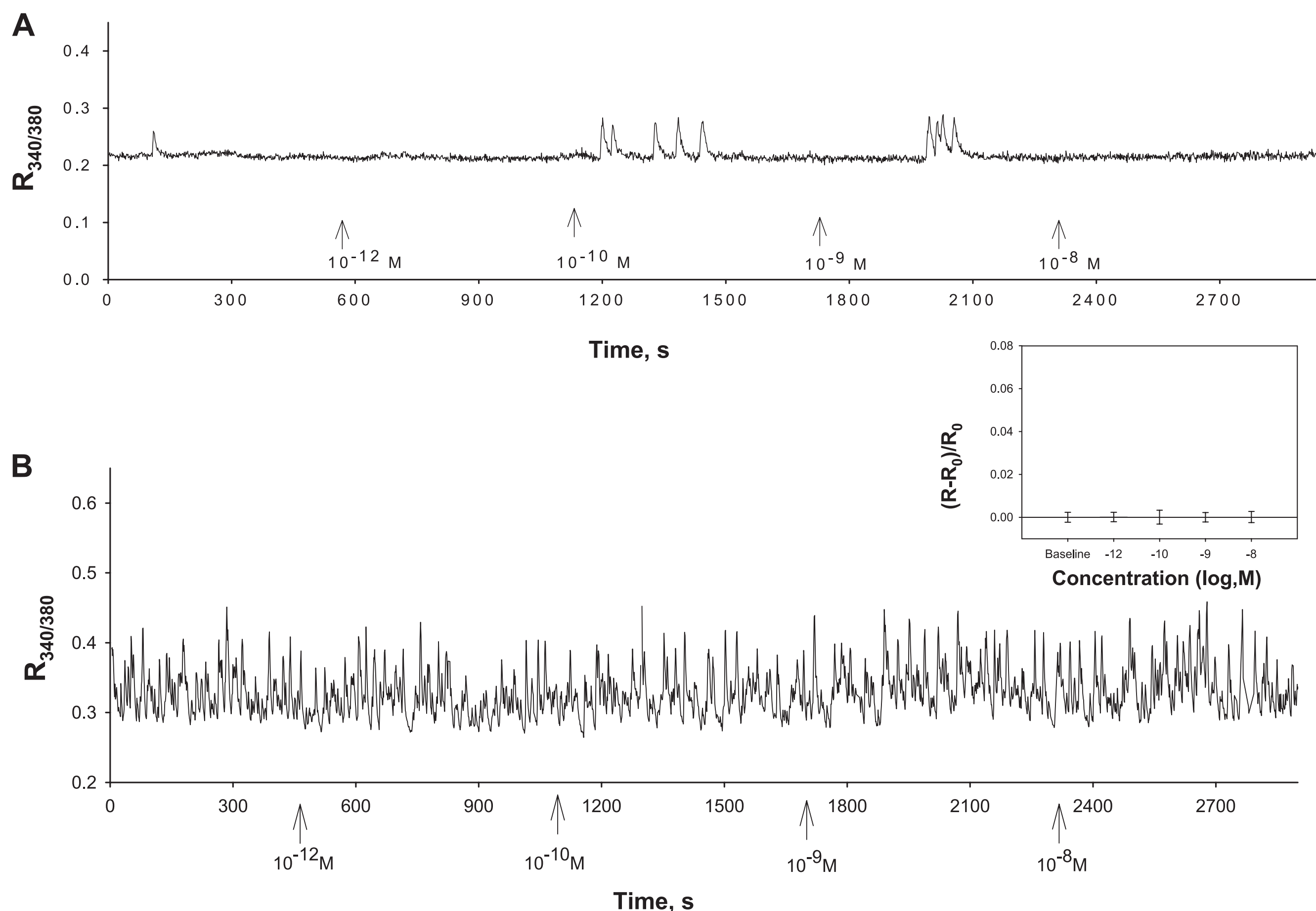

Fig. 2. Lack of $[Ca^{2+}]_i$ changes due to E_2 action on cells with very low membrane estrogen receptor-α (mERα) levels (GH3/B6/D9 cells). Traces show the absence of E_2-induced time- and concentration-dependent $[Ca^{2+}]_i$ changes from a single cell (*A*) and from 12 cells (averaged) from 2 different experiments (*B*). Bar graph (*inset*): normalized Ca^{2+} level changes as means ± SE for 28 cells from 3 different experiments.

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Statistics. Data were compared for significance of differences using a one-way ANOVA test, followed where appropriate by a Mann-Whitney test (accepting significance at $P \leq 0.05$). The Sigma Stat program was used for these calculations (version 3; Jandel Scientific, San Rafael, CA).

RESULTS

$[Ca^{2+}]_i$ changes due to E_2 action. The basal $[Ca^{2+}]_i$ level in the F10 cell line was measured at an average of 103.6 ± 20 nM; ~30% of cells displayed spontaneous $[Ca^{2+}]_i$ oscillations, and the rest of cells were silent (Fig. 1*A*). However, E_2 administration rapidly (within 1 min) increased the amplitude and frequency of Ca^{2+} oscillation and produced significant effects even at the low concentration of 10^{-12} M (Fig. 1*B*). About 70% of the cells responded to the hormone. Among those responders, ~40% were spontaneously active in the 5 min before hormone application, and ~60% were silent without oscillation during this time. Vehicle (EtOH) treatment at 0.0001% did not produce any changes in basal $[Ca^{2+}]_i$ levels (data not shown). The averaging of the traces from individual cells (Fig. 1*C*) produced an apparent lessening in the amplitude of the Ca^{2+} response to E_2 due to the misalignment of peaks, but increased frequency of responses were still clearly visible over the averaged cell population. Ca^{2+} spikes remained consistent for the entire time of E_2 action (5 min) and continued to be present for a period of time during washing with PBS. However, the E_2 effect on $[Ca^{2+}]_i$ was reversible, taking ~5 min to wash out and cease to affect Ca^{2+} activity (Fig. 1*D*). Increasing the E_2 concentration from 10^{-12} to 10^{-8} M sequentially did not produce a desensitization in the Ca^{2+} response (Fig. 1*B*, trace) but increased the response in a dose-dependent manner when the response values were calculated by looking at the increase in Ca^{2+} levels over background ($R - R_0/R_0$, calculated for each cell before averaging the values; Fig. 1*C*, *inset*). Although D9 (mERα depleted) cells showed a low spontaneous Ca^{2+} activity similar to that of F10 cells, E_2 administration did not change Ca^{2+} levels, even at the highest (10^{-8} M) hormone concentration (Fig. 2, *A*, *B*, and *inset*). Again, the different phasing of the composite cell traces gave the appearance of higher frequency activations in Fig. 2*B*, but this did not correspond to any dose effect of E_2 (*inset*). The cell-impermeant E_2 analog, E_2-P, was able to produce a more intensive $[Ca^{2+}]_i$ response compared with E_2. However, the

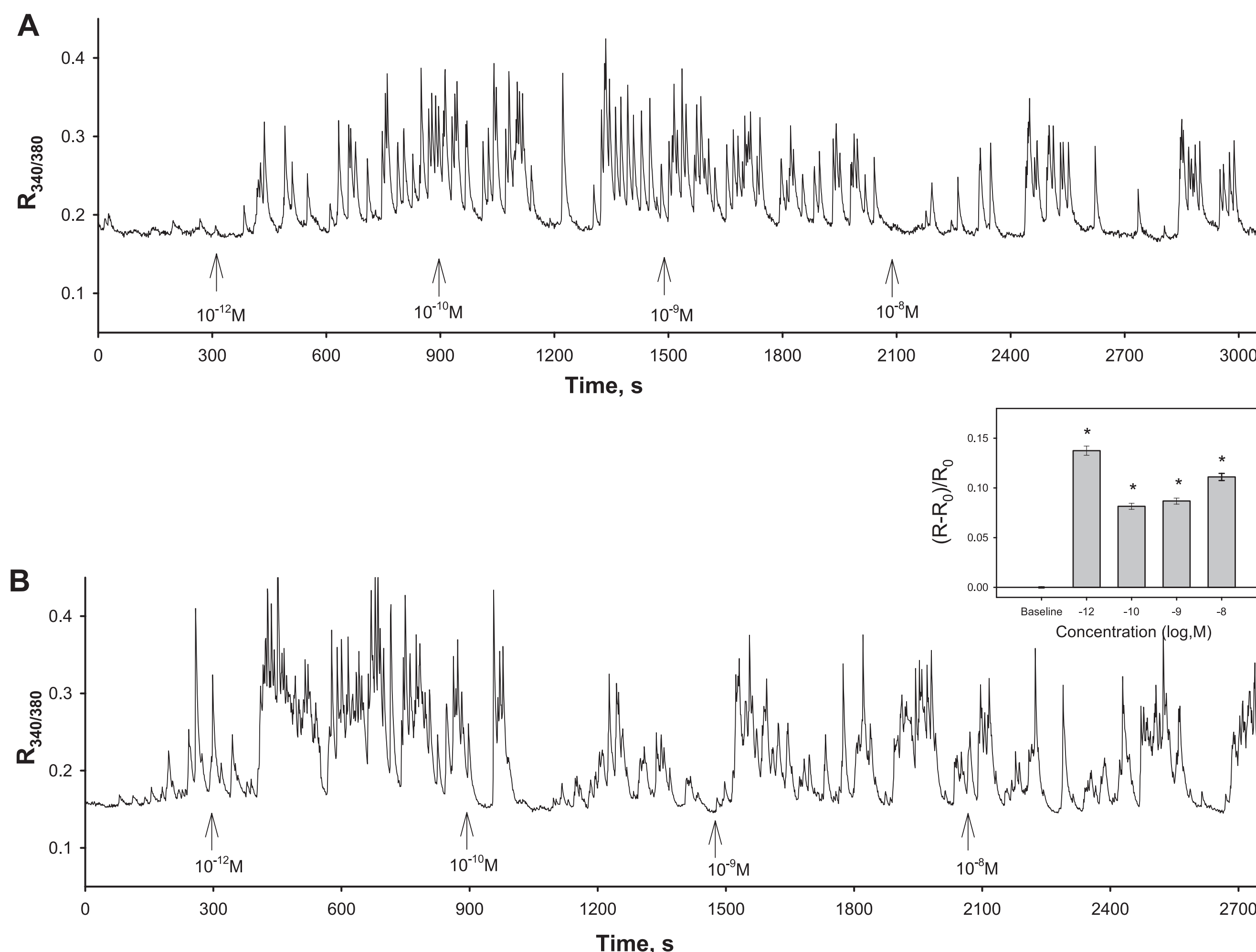

Fig. 3. $[Ca^{2+}]_i$ changes in F10 cells due to E_2-peroxidase (impeded ligand) stimulation. Traces show time- and concentration-dependent E_2-peroxidase-induced $[Ca^{2+}]_i$ changes from a single cell (*A*) and from the averaged traces of 4 cells (*B*). Concentrations represent the amount of E_2 present in the conjugate. Bar graph (*inset*): normalized Ca^{2+} level changes as means ± SE for 10 cells from 3 different experiments. *Statistical significance for E_2-peroxidase treatment by ANOVA ($P < 0.05$) vs. EtOH (0.0001%) vehicle control (baseline).

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amplitude of this Ca^{2+} response was slightly decreased on application of higher hormone concentrations (Fig. 3, *A*, *B*, and *inset*).

Mechanism of $[Ca^{2+}]_i$ elevation in GH3/B6/F10 cells. To estimate the contribution of Ca^{2+} from intracellular compartments in E_2-induced $[Ca^{2+}]_i$ changes, we applied the selective endoplasmic reticulum Ca^{2+}-ATP pump blockers thapsigargin (Tg) and tBHQ (which are irreversible and reversible, respectively). Because of emptying of Ca^{2+} from the intracellular stores, these reagents were able to significantly increase $[Ca^{2+}]_i$ levels (Fig. 4) but did not affect the cell's subsequent response to E_2. After the initial E_2 application, two subsequent treatments with Tg ensured complete depletion of the Tg-sensitive intracellular Ca^{2+} stores, demonstrated by no further Ca^{2+} elevation by the second Tg application (Fig. 4*B*). The amplitudes of the first and second (post-Tg) Ca^{2+} responses to E_2 were not significantly different from each other.

To test the involvement of extracellular Ca^{2+} in hormone-stimulated signaling, we removed Ca^{2+} from the extracellular solution by chelation (Fig. 5, *A* and *B*). Cells were first tested for their ability to respond to 10^{-8} M E_2 stimulation, followed by washing. A Ca^{2+}-free solution was then added, resulting in elimination of cell activity, producing a slight decrease in basal $[Ca^{2+}]_i$ level. When E_2 was subsequently added, it did not produce any changes in $[Ca^{2+}]_i$. The most common way for Ca^{2+} to enter neuroendocrine cells from the extracellular environment is through voltage-dependent Ca^{2+} channels. To further confirm the participation of voltage-dependent Ca^{2+} channels in this E_2 effect, we applied the specific L-type Ca^{2+} channel blocker nifedipine (Fig. 5, *C* and *D*). The cells were first tested for their ability to respond to E_2 (10^{-8} M). Then, nifedipine was perfused onto the cells alone, followed by nifedipine plus E_2. This blocking agent was able to significantly block spontaneous Ca^{2+} oscillations after hormone washout as well as prevent E_2-induced $[Ca^{2+}]_i$ elevation. Although 1 μM was effective, a more pronounced blocking effect was observed at 10 μM.

PRL release is due to E_2 action via membrane ERα. GH3 cell sublines with enriched and depleted membrane ERα levels (F10 and D9 cells, respectively) were tested for their ability to rapidly release PRL in response to E_2. To determine that both cell lines had equivalent levels of PRL stored and ready for release, 20 mM KCl was applied. This treatment causes massive cellular depolarization and consequent activation of voltage-dependent Ca^{2+} channels, which results in significant $[Ca^{2+}]_i$ increases. This will usually result in exocytosis in neuroendocrine cells. Both cell lines were able to release a Ca^{2+}-sensitive PRL pool after KCl depolarization (compare Fig. 6, *A* and *B*). E_2 induced a rapid (within 3 min after application) PRL secretion in F10 cells (Fig. 6*A*), as expected (26). Released PRL stayed constant at longer test times up to 15 min, indicating that all of the releasable pool was quickly dumped. However, D9 cells were not sensitive to the E_2 application (Fig. 6*B*), even though they had plentiful KCl-

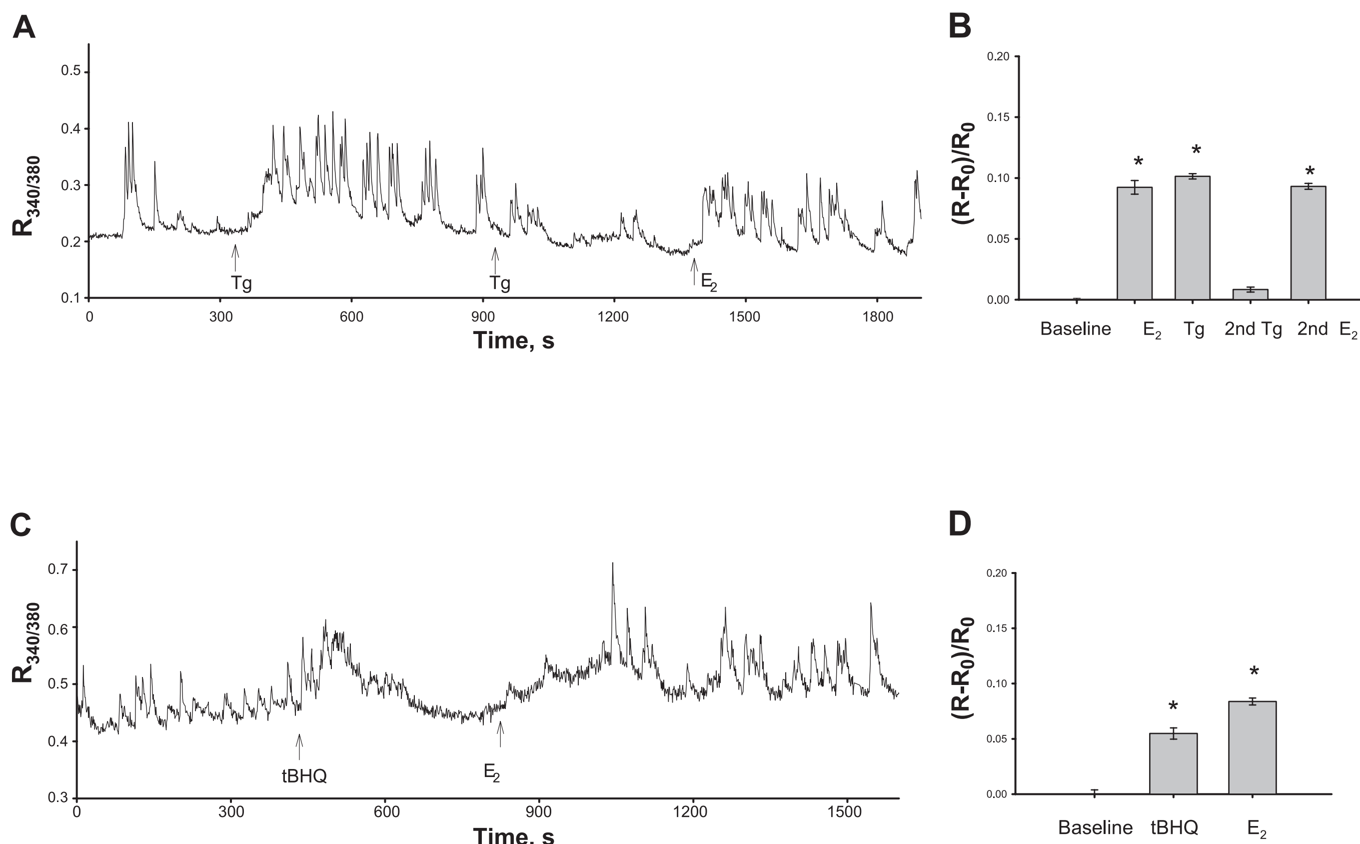

Fig. 4. Lack of participation of $[Ca^{2+}]_i$ stores in E_2-stimulated Ca^{2+} increases. Cells were treated twice with 1 μM thapsigargin (Tg) for 10 min (*A* and *B*) or with 30 μM 2,5-di-(*t*-butyl)-1,4-hydroquinone (tBHQ) for 7 min (*C* and *D*), followed by the addition of 10^{-8} M E_2. *A* and *C*: traces from single cells. *B* and *D*: bar graphs show the normalized Ca^{2+} level changes as means ± SE of 14 or 9 cells, respectively, from 3 different experiments. *Statistical significance by ANOVA ($P < 0.05$) compared with vehicle treatment (0.0001% EtOH; baseline).

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releasable PRL. All E_2 concentrations tested were effective in PRL release in F10 cells (Fig. 6*C*), but with the bimodal dose response pattern similar to that seen previously (32).

At 10^{-8} M, E_2 produced a significantly higher PRL release from F10 cells at 3 min than did KCl (Fig. 7), although the Ca^{2+} level elevation was much higher in the case of KCl. Therefore, Ca^{2+} elevation could not be solely responsible for PRL release. However, blocking the L-type voltage-dependent Ca^{2+} channel with nifedipine (10 μM) totally prevented E_2-induced PRL release measured at 3 min (Fig. 8). Therefore, the Ca^{2+} increase seems to be a necessary initiator of PRL release, but subsequent E_2-induced mechanisms that influence exocytosis events may be Ca^{2+} independent.

Hormone and receptor specificity of the estrogen-induced $[Ca^{2+}]_i$ *and PRL responses.* The 17β-E_2 stereoisomer of 17α-E_2 was unable to stimulate $[Ca^{2+}]_i$ changes or PRL release in F10 cells (Fig. 9). The synthetic antagonist of estrogen receptor ICI 182 780 prevented rapid E_2-induced $[Ca^{2+}]_i$ increases and PRL secretion and did not produce any changes in PRL secretion or basal Ca^{2+} levels when used alone.

DISCUSSION

The effects of E_2 on Ca^{2+} levels and PRL release in GH3/B6 cell sublines strongly depends on the presence of mERα on the cell's plasma membrane, as cells with very low mERα expression (the D9 subline) did not respond to E_2 compared with F10 cells (which have high levels of mERα). Additional evidence for the participation of mERα was the response to the cell-impermeant analog of E_2, E_2-P. A more pronounced $[Ca^{2+}]_i$ elevation caused by this impeded ligand may be explained by the reagent's continued presence on the plasma membrane (where it may continue to stimulate) compared with free E_2, which can readily diffuse inside the cells rapidly after application. Alternatively, because some conjugates may contain more than one E_2 molecule, this reagent may artificially cluster receptors by binding to more than one at a time, amplifying the signal. The slight desensitization seen after concentration-dependent E_2-P "overstimulation" can probably be explained by similar reasoning. Very low (lower than nanomolar) E_2 concentrations produced both Ca^{2+} elevation and PRL release, so these responses are physiologically relevant. Inhibition of these E_2-induced effects by the ER antagonist ICI 182 780 and the lack of stimulation by stereoisomer 17α-E_2 provide additional evidence for E_2 action through a known ER protein.

Using the fluorescent dye fura 2, we confirmed that extracellular Ca^{2+} was the main source of $[Ca^{2+}]_i$ changes due to E_2 action, since the $[Ca^{2+}]_i$ rise was completely blocked by the absence of Ca^{2+} in the medium. In addition, intracellular Ca^{2+} store depletion with Tg or tBHQ was unable to prevent E_2-induced $[Ca^{2+}]_i$ elevation. Others have shown that Ca^{2+} entry through the plasma membrane can produce Ca^{2+}-induced Ca^{2+} release from the endoplasmic reticulum (29) and that, in lactotrophs isolated from pituitaries of male rats (6), PRL secretion was not sensitive to extracellular Ca^{2+} removal.

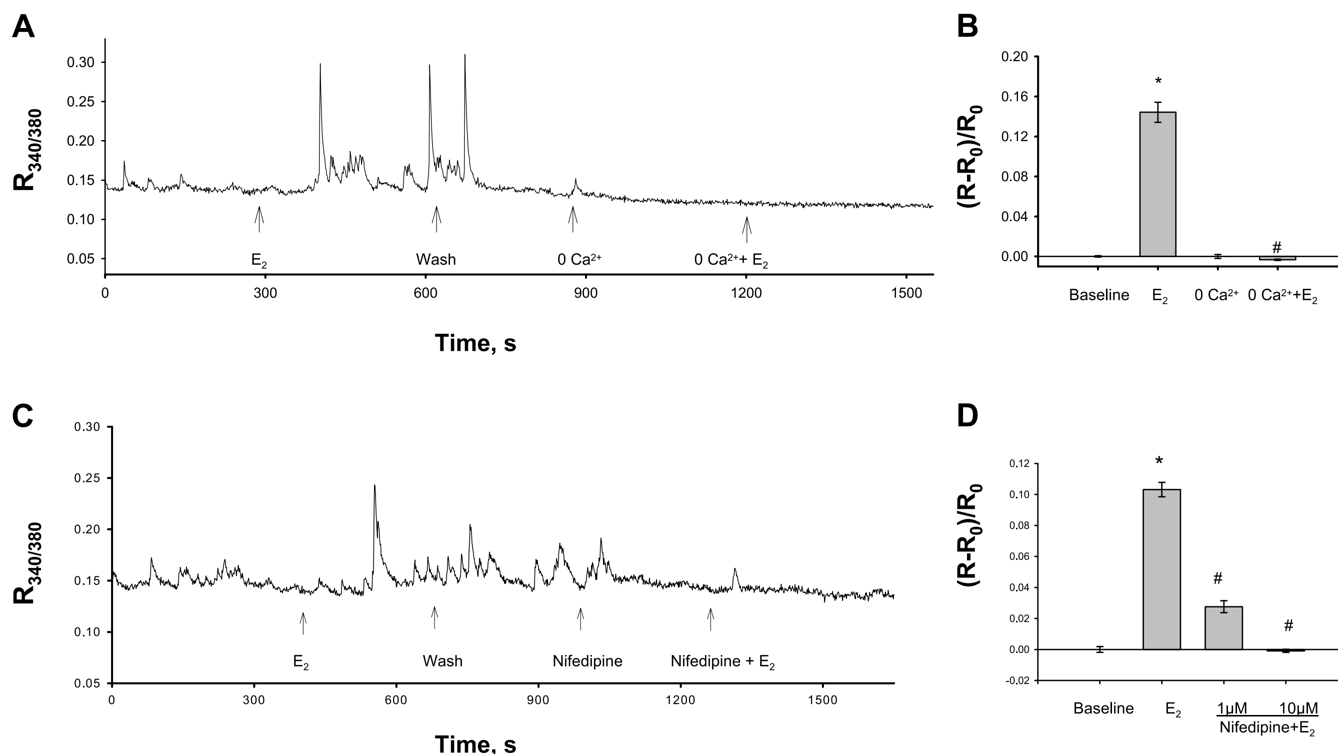

Fig. 5. Role of extracellular Ca^{2+} in E_2 action. *A*: elimination of Ca^{2+} by chelation (Ca^{2+} free) from the extracellular solution can prevent $[Ca^{2+}]_i$ stimulation by E_2 in F10 cells (single-cell trace). *B*: quantitation of the responses from 12 cells from 3 different experiments (means ± SE). *C*: trace from a single cell treated with 10 μM nifedipine shows that the voltage-gated Ca^{2+} channel blocker nifedipine can prevent the effects of E_2 on $[Ca^{2+}]_i$. *D*: bar graph of nifedipine effects as averaged values for normalized Ca^{2+} level changes; values are means of 23 cells from 4 experiments using 1 μM nifedipine, and of 8 cells from 3 experiments using 10 μM nifedipine. *Statistical significance of E_2-treated differences by ANOVA ($P < 0.05$) compared with baseline (vehicle treatment of EtOH at 0.0001%) in *B* and *D*. #Statistical significance of differences (at $P < 0.05$) compared with treatment with E_2 alone in *B* by the Mann-Whitney test and in *D* by ANOVA.

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However, in our study, the similar amplitude of E_2-induced Ca^{2+} increase before and after Tg application demonstrated an independence of Ca^{2+} response from Tg-sensitive intracellular stores. This discrepancy might be explained by the differences between primary cultures vs. established cancer cell lines.

Consistent with our studies is a previous investigation of E_2 effects on electrical membrane properties in the parent GH3/B6 cell line, done with intracellular microelectrode recording (11), showing that E_2 can elicit action potentials that were sensitive to the Ca^{2+} channel blocker D600, an earlier and less selective blocker. However, the possible participation of intracellular

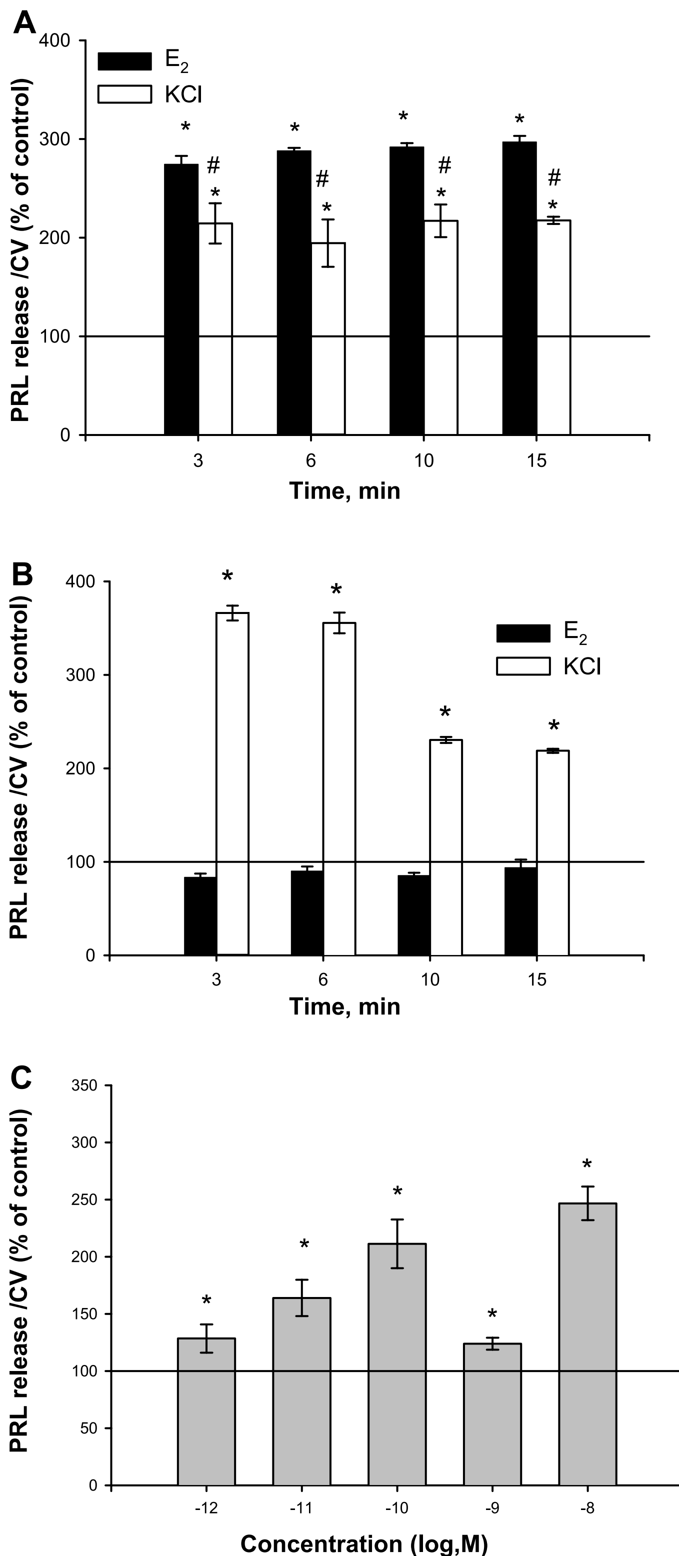

Fig. 6. Time- and concentration-dependent prolactin (PRL) release from cells with high mERα levels (F10 cell line) vs. cells with very low mERα levels (D9 cell line). *A*: time-dependent changes in PRL secretion from the F10 cell subline after E_2 or KCl treatment; $n = 20$ for each condition over 5 experiments. *B*: PRL release in the D9 cell subline after E_2 or KCl administration; $n = 24$ over 6 experiments, and $n = 20$ over 5 experiments, respectively. *C*: PRL secretion from F10 cells after treatment with different E_2 concentrations at the 3-min time point ($n = 24$ over 6 experiments). All data are means ± SE. CV, crystal violet. *Statistical significance by ANOVA ($P < 0.05$) compared with vehicle controls (0.0001% EtOH treatments, referred to as baseline = 100%). #Statistical significance by ANOVA ($P < 0.05$) for E_2 vs. KCl treatment in *A*.

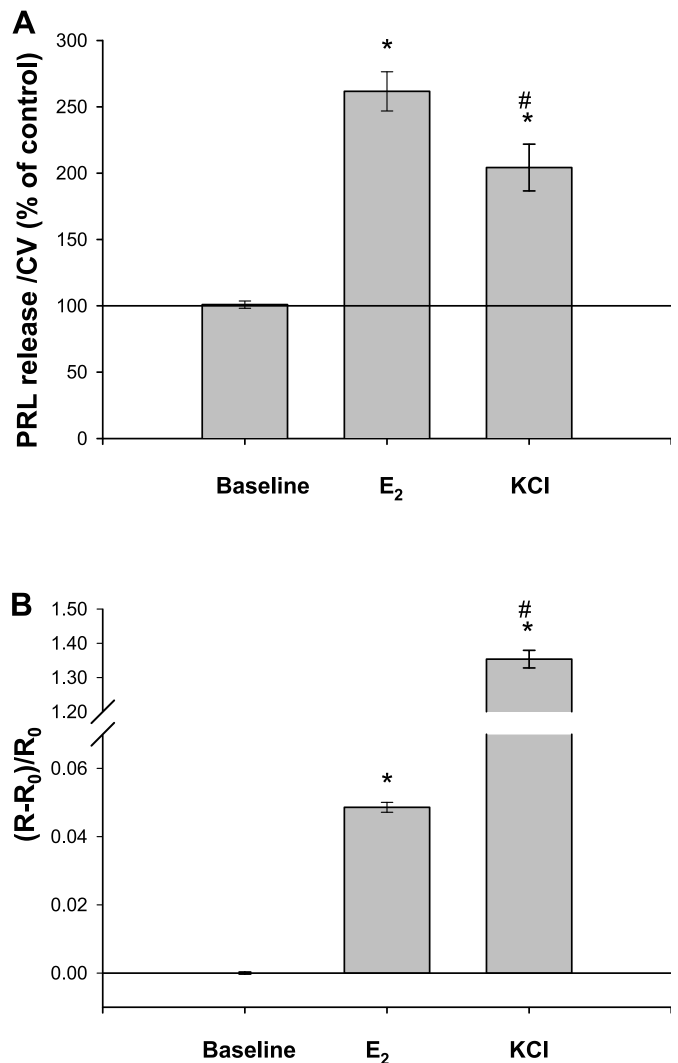

Fig. 7. PRL secretion levels do not directly correlate with the amplitude of $[Ca^{2+}]_i$ changes. *A*: PRL secretion after E_2 ($n = 16$ samples over 4 experiments) or KCl treatment ($n = 12$ samples over 3 experiments). *B*: $[Ca^{2+}]_i$ changes after E_2 or KCl treatment ($n = 27$ cells each from 4 experiments). Data are means ± SE. *Statistical significance by ANOVA ($P < 0.05$) compared with vehicle controls (0.0001% EtOH treatment, labeled baseline = 100%). #Statistical significance by Mann-Whitney test ($P < 0.05$) of KCl treatment compared with E_2 treatment.

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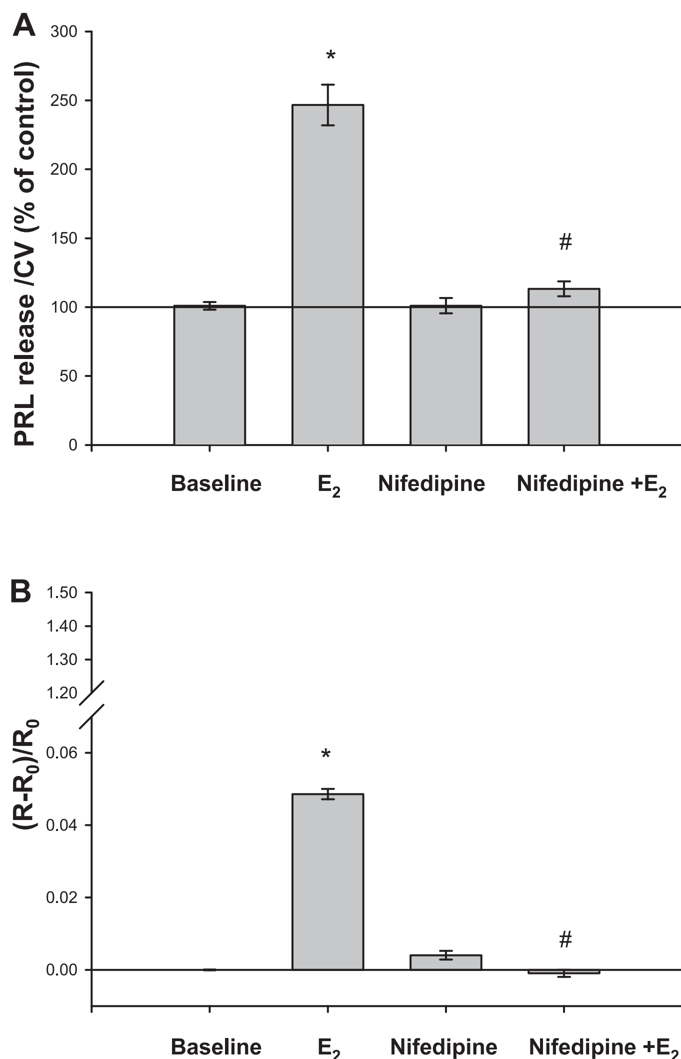

Fig. 8. E_2-stimulated PRL release is sensitive to nifedipine. *A*: PRL secretion after E_2, nifedipine, or nifedipine + E_2 treatment (n = 16 over 4 experiments for each). *B*: $[Ca^{2+}]_i$ changes after E_2, nifedipine, or nifedipine + E_2 treatment (8 cells from 3 experiments for all conditions). Data are means ± SE. *Statistical significance by ANOVA (P < 0.05) compared with vehicle controls (0.0001% EtOH treatment). #Statistical significance by Mann-Whitney test (P < 0.05) compared with E_2 treatment.

Ca^{2+} stores in the E_2-induced membrane signaling and PRL release response was not investigated in these early studies.

In our studies, the selective L-type Ca^{2+} channel blocker dihydropyridine nifedipine was able to inhibit the E_2-stimulated $[Ca^{2+}]_i$ increase in a concentration-dependent manner. These results are consistent with the recent findings that the GH3/B6 parent cell line expresses P-, Q-, and low amounts of T- but primarily L-type voltage-dependant Ca^{2+} channels (14). Higher concentrations of nifedipine (10 μM) were shown to inhibit ~90% of the L-type Ca^{2+} channel currents while being ineffective in blocking other Ca^{2+} channel subtypes in one study (14). However, others have shown that P- and Q-type Ca^{2+} channels in melanotrophs (which have the same pituitary origin as lactotrophs) are extremely sensitive to dihydropyridines (with half-maximal blockage at 200–500 nM; Ref. 21). Therefore, these Ca^{2+} channels, in addition to the L-type, could be involved in E_2 stimulations, but this question needs further clarification regarding the sensitivity of specific channel subtypes to nifedipine.

The precise protein interactions or signaling cascades involved in the regulation of voltage-dependent Ca^{2+} channels by E_2 via a membrane ER remain to be determined. The structure/conformation of mERs on the plasma membrane and their repertoire of interacting proteins are not yet known. Voltage-dependent Ca^{2+} channels may be opened by depolarization due to the inactivation of K^+ currents by E_2. It was previously shown that E_2 at nanomolar concentrations can rapidly increase a cell's excitability by inhibition of A-type K^+ currents in gonadotropin-releasing neurons (7) and closing of K^+ (ATP) channels in pancreatic ss cells (22). Another possible mechanism for voltage-dependent Ca^{2+} channel regulation is the phosphorylation of channel subunits by PKC or PKA (16, 33), which can be activated in other cell types via E_2 action.

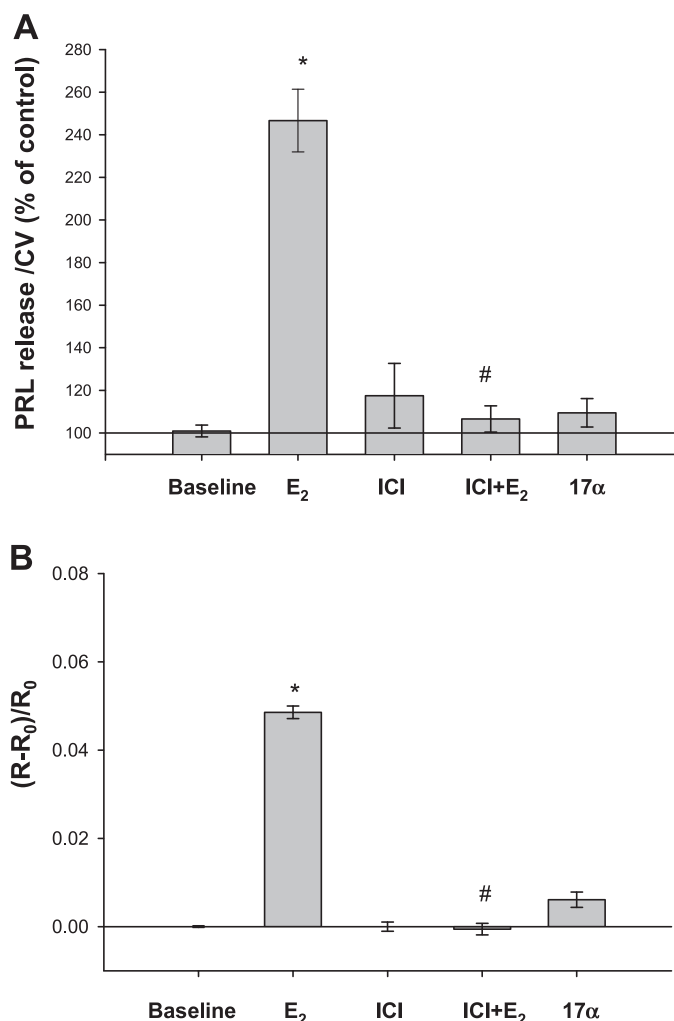

Fig. 9. Hormone and receptor specificity of the E_2-induced PRL and Ca^{2+} responses. *A*: PRL release after ICI 182 780 (1 μM, 40 min) pretreatment and 17α-E_2 treatment (n = 16 samples from 4 experiments). *B*: $[Ca^{2+}]_i$ levels after ICI 182 780 pretreatment (n = 21 cells from 4 experiments) and 17α-E_2 treatment (11 cells from 3 experiments). Data are means ± SE. *Statistical significance by ANOVA (P < 0.05) compared with vehicle controls (0.0001% EtOH treatment). #Statistical significance by Mann-Whitney test (P < 0.05) compared with E_2 treatment.

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Voltage-dependent Ca^{2+} channel opening is thought to be the main trigger for PRL release, corresponding to the classic model for exocytosis from the literature (4). However, for neuroendocrine cells, generally three different stages of secretion have been observed. The most rapid (within 100 ms) stage involves readily releasable pools contained in vesicles that are fused with the plasma membrane, causing release of PRL, and are regulated largely via Ca^{2+} channel activity. A second, slower stage involves a docked vesicle pool interacting with a membrane fusion-inducing protein complex associated with the membrane [*N*-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) complex]. This pool can be regulated by Ca^{2+} level changes but also by PKC, ATP, cAMP, and PKA. A third reserve pool contains vesicles that are not yet docked but are available for the subsequent steps. Recruitment of these vesicles to the docked pools can be triggered by the stimuli above, or by an increase in GTP levels (20). Therefore, it is possible that E_2, using other signaling pathways, can induce recruiting actions in these second and third vesicle populations in our model. This would account for maximal Ca^{2+} levels (achieved with KCl application) causing only moderate (submaximal) PRL release. The discrepancy between high Ca^{2+} and low PRL responses after 10^{-9} M E_2 stimulation (Fig. 1*C*, *inset*, vs. Fig. 6*C*, respectively) may be explained similarly. However, because blocking of Ca^{2+} channels with nifedipine completely abolishes the PRL release response, the Ca^{2+}-releasable pool may have to be expelled before these other E_2-stimulated mechanisms are permitted to act.

In summary, E_2 is a potent regulator of these membrane-initiated neuroendocrine secretory functions, and a membrane form of ERα is involved in rapid PRL secretion. However, Ca^{2+} is probably only part of the mechanism responsible for this response. Details about the membrane machineries and their mode of interaction with membrane receptors for estrogens are still lacking. The portions of the signaling cascades examined in these studies (Ca^{2+} level changes leading to PRL secretion) are only part of the web of signaling intermediates that define estrogenic responses in these cells.

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GRANTS

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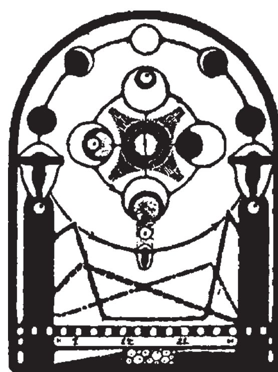

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