Estrogen Regulation of the Dopamine-activated GIRK Channel in Pituitary Lactotrophs: Implications for Regulation of Prolactin Release During the Estrous Cycle

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RUNNING HEAD: E₂ regulates GIRK channel expression in lactotrophs
ABSTRACT

Prolactin (PRL), synthesized and secreted from lactotrophs of the anterior pituitary gland, is tonically inhibited by hypothalamic dopamine (DA) throughout the female reproductive (estrous) cycle. Our lab has shown that DA hyperpolarizes these cells by activating GIRK channels; however, this response is only observed on proestrus. While the cellular mechanisms that allow for functional expression of this unique DA-signaling pathway are unclear, we hypothesized that activation of the DA-GIRK effector pathway is due to the rise in circulating estrogen (E_2) during the preceding day of diestrus. Thus, we examined the effects of E_2 on primary lactotrophs isolated from female rats. Treatment with a physiological concentration of E_2 (40-80 pg/mL, *in vivo* or *in vitro*) induced a proestrous phenotype in diestrous lactotrophs. These cells exhibited a DA-induced membrane hyperpolarization, as well as a secretory rebound of PRL following DA withdrawal (characteristic of proestrous cells). Internal dialysis of GTPγS demonstrated that E_2-exposure enabled functional expression of GIRK channels, and this regulation by E_2 did not involve the D_2R. The effect of E_2 was blocked by the receptor antagonist, ICI 182,780, and by the protein synthesis inhibitor, cycloheximide. Single-cell analysis revealed increased mRNA expression of GIRK channel subunits in E_2-treated lactotrophs. While E_2 is known to have multiple actions on the lactotroph, the present findings illuminate a novel action of E_2 in lactotrophs – regulation of the expression of a DA effector, the GIRK channel.
KEYWORDS: prolactin, dopamine, single-cell RT-PCR, estrous cycle
INTRODUCTION

Prolactin (PRL) is synthesized and secreted by lactotroph cells of the anterior pituitary (AP) gland. This peptide hormone, well known for its role in lactation, mammary gland development, and fertility (1, 14, 20, 31, 48, 57), exhibits a dynamic secretory pattern throughout the female reproductive cycle (5, 62). On any day of the reproductive cycle, PRL secretion (and synthesis) is inhibited by hypothalamic dopamine (DA) (2, 49). This principle inhibitory signal is transduced through the D2 type DA receptor (D2R) in the lactotroph membrane (6, 47), which is coupled to a pertussis toxin-sensitive G-protein pathway and multiple effector proteins (9, 21, 59).

In female rodents, a precipitous drop in DA on the day of proestrus allows for the periovulatory surge in serum PRL (19). Our lab has shown that on this day DA activates an inwardly rectifying K+ current that rapidly hyperpolarizes the lactotroph membrane (22, 26), due to activation of G-protein coupled inward rectifying K+ channels (GIRKs, of the Kir 3.0 gene subfamily). The function of this DA-activated K+ channel (KDA) is not observed on any other day of the estrous cycle (22), indicating that DA-signaling in the lactotroph switches on proestrus, and this change may contribute to the unique pattern of PRL secretion observed on proestrus. Indeed, a PRL secretory rebound elicited by the abrupt withdrawal of DA in vitro is characteristic only of proestrous lactotrophs and requires the membrane hyperpolarization activated during the presence of DA (22).

Since E2 is a critical factor in producing the proestrous surge of PRL in vivo, we hypothesized that the “switch” in DA signaling is dependent on the rise in estrogen (E2) during the preceding day of diestrus II and due to direct actions of E2 on the lactotroph. Therefore, we
tested whether manipulation of E₂, either *in vivo* or *in vitro*, could alter the lactotroph’s electrophysiological and secretory responsiveness to DA. In the present study, we show that the proestrous phenotype, in which DA activates K\textsubscript{DA} and withdrawal of DA produces a PRL secretory rebound, is dependent upon E₂ *in vivo*. In addition, E₂ treatment of diestrous lactotrophs *in vitro* directly induces the functional expression of K\textsubscript{DA} and the secretory rebound following DA washout. This induction by E₂ occurs at a target distal to the D₂R and may be due to, at least in part, a significant increase in mRNA expression of GIRK channel subunits 1, 2, and 4.

**MATERIALS AND METHODS**

*Animals and Reagents*

Female Sprague Dawley rats ages 2 to 5 months (Harlan Laboratories; Frederick, MD) were maintained on a 14-hour light / 10-hour dark cycle (lights on at 06:00am). Only females demonstrating at least two consecutive 4-day estrous cycles (as determined by daily vaginal lavage) were used in this study. Food and water were available *ad libitum*. For *in vivo* manipulation of E₂, bilateral ovariectomies were performed on the morning of diestrus II (prior to the rise in endogenous E₂) through dorsolateral incisions with animals under isoflurane anesthesia. Seven days following surgery, animals received two subcutaneous silastic implants (30 mm long; i.d. 1.57 mm; o.d. 3.18 mm), containing either estradiol-17β (150 μg/ml) or vehicle (oil), and AP glands were harvested 48 hours later. All animal handling and procedures were carried out in a facility accredited by the American Association for the Accreditation of
Laboratory Animal Care, and were approved by the University of Cincinnati’s Institutional Animal Care and Use Committee.

Horse serum was purchased from HyClone (Thermo Scientific; Logan, UT); tetrodotoxin and ICI 182,780 were purchased from Tocris Bioscience (Ellsville, MO). Ovine erythrocytes were obtained from Colorado Serum Company (Denver, CO). Anti-rat PRL (arPRL-86) for the RHPA was generated in our laboratory and characterized as previously described (30). Reagents for the rat PRL (rPRL) RIA were purchased from Dr. A. Parlow through the National Hormone and Peptide Program (UCLA). Primers were purchased from Integrated DNA Technologies (San Diego, CA). All other reagents and culture media, unless otherwise noted, were purchased from Sigma Chemicals (St. Louis, MO).

**Anterior pituitary cell dissociation**

Animals were sacrificed by rapid decapitation between 08:00 and 10:00AM on specific days of the estrous cycle or 48 hours after receiving silastic implants, and AP cells were dissociated as previously described (26). In all experiments, a single AP gland was used for each cell preparation; therefore, each AP cell preparation represents an individual rat. Briefly, the AP gland (posterior pituitary removed) was minced and enzymatically dissociated in Hank’s Balanced Salt Solution (with Ca\(^{2+}\) and Mg\(^{2+}\)) containing 0.20% trypsin for 15 minutes at 37°C. The digested tissue was washed in Hank’s Ca\(^{2+}\), Mg\(^{2+}\)-free medium (Hank’s CMF) and triturated in Hank’s CMF containing deoxyribonuclease-I (0.075 mg/mL) and trypsin inhibitor (3.75 µg/mL). The final cell suspension was filtered through a sterile, 20-µm pore nylon mesh. This method routinely yielded 3-5 x 10\(^6\) cells per AP gland, with viability in excess of 96%. Cells used for electrophysiology studies or single-cell reverse transcription PCR (RT-PCR) were
immediately subjected to the reverse hemolytic plaque assay. Cells for perifusion were transferred to 60 mm Petri dishes containing 7 ml of culture medium (DMEM with 10% heat-inactivated horse serum; DMES) and incubated on an orbital shaker (40 rpm) at 37°C in 5% CO₂ for 1-3 days. For \textit{in vitro} manipulation of E₂, AP cells dissociated from each diestrous female rat were divided into two groups and treated overnight (~18 h) or for 48 hours \textit{in vitro} with either vehicle (EtOH) or E₂ (40-80 pg/mL; to mimic the rise in endogenous E₂ that occurs during late diestrus II, \textit{see reference 5}).

\textit{Perifusion}

Secretory activity of primary lactotrophs was monitored using a two-column perifusion system as previously described (22). Briefly, dissociated AP cells were mixed with pre-swollen polyacrylamide gel (Bio-Gel P2, 200-400 mesh; 5x10⁶ cells/1 ml mesh) then loaded into the chamber of the system. The perifusion medium was the same SES used in the electrophysiological experiments described below. Flow rate was 0.5 ml/min and the effluents were collected in 2-minute fractions into tubes containing 100 µl 2% BSA in PBS. Each perifusion experiment included a control and an E₂-treated cell preparation perfused by the same solutions. Each preparation was derived from an individual rat. Protein levels of rPRL were determined on individual effluent samples by homologous double-antibody radioimmunoassay (RIA). All samples from an individual perifusion experiment were included in a single assay.

\textit{Reverse hemolytic plaque assay (RHPA)}

The RHPA is a well-established method of identifying viable endocrine cell types based on antigen secretion (25, 60). Briefly, ovine erythrocytes (oRBCs) were coated with
Staphylococcus protein A in the presence of chromium chloride. The coated oRBCs [6% in DMEM/BSA (0.1%)] were mixed 1:1 with freshly dissociated AP cells (300,000 cells/mL). The mixture was infused into poly-L-lysined glass coverslip chambers (as described in reference 16) and cells allowed to plate for thirty minutes at 37°C (5% CO₂). The AP cell / oRBC mixture was treated as follows: (1) washed with DMEM/BSA (to remove non-adherent cells); (2) incubated with primary anti-rPRL antibody (arPRL-86; final dilution 1:200 in DMEM/BSA, 37°C, 5% CO₂) for ninety minutes; (3) washed with DMEM/BSA (to remove unbound antibody); (4) incubated with guinea pig serum complement (1:100 in DMEM/BSA, 37°C, 5% CO₂) or autologous rat serum (1:120) until plaques were visible on the coverslip (approximately 15 minutes). Once plaques began to form, complement was rinsed out and the chambers dismantled. Coverslips, with cells attached, were incubated overnight in culture medium (DMES; 37°C; 5% CO₂) without additives (proestrous cells) or in medium containing 17β-estradiol (E₂; 40-80 pg/ml) or vehicle (EtOH) (diestrous cells). In some experiments, cells were also treated with the E₂ receptor antagonist, ICI 182,780 (ICI; 40 ng/ml), or the protein synthesis inhibitor, cyclohexamide (Chx; 35µg/ml).

Electrophysiology

Membrane potentials and whole-cell currents of plaque-identified lactotrophs were recorded using the giga-ohm seal patch-clamp technique (29). Recording pipettes were filled with standard intracellular solution (SIS; 130 mM potassium aspartate, 20 mM KCl, 1 mM glucose, 10 mM HEPES) and, in some experiments, included 250 µM GTPγS. Cells were bathed in standard extracellular solution (SES; 145 mM NaCl, 5 mM KCl, 2 mM CaCl₂, 2 mM MgCl₂, 10 mM glucose). In the voltage clamp experiments, K⁺ currents were isolated by
including tetrodotoxin (TTX, 1 μM) to block voltage-dependent sodium channels and omitting CaCl\(_2\) (substituting MgCl\(_2\)) to reduce calcium currents. All solutions were adjusted to pH 7.3-7.4 and 295-305 mOsmoles.

Patch-clamp experiments were performed using either an Axopatch 1B or 1D amplifier with pClamp 8.0 software (Axon Instruments, Foster City, CA) and low-resistance (4-6 MΩ) glass microelectrodes. The recordings were carried out at room temperature in a Lexan recording chamber mounted on the stage of a Nikon Diaphot inverted microscope. A coverslip, with plaque-identified lactotrophs attached, served as the floor of the recording chamber through which external solutions were continuously perfused during each experiment. DA solutions (made fresh immediately before use) and high [KCl] solutions (40 mM, substituted for NaCl) were applied via a U-tube apparatus that was positioned next to the cell during each recording. The positioning of the U-tube device allowed for rapid application and immediate withdrawal of the test solutions without mechanical disturbance of the cell being recorded. Whole cell voltage responses under current clamp were recorded wideband (~50 kHz). Whole-cell K\(^+\) currents were recorded from single lactotrophs during a constant rate (0.44 V/s) ramp depolarization from -160 mV to +60 mV (-60 mV holding potential).

**Analysis of mRNA expression by RT-PCR**

Whole tissue samples [rat AP, cerebellum (Cb) and cardiac atrium (C. Atr)] dissected from a female rat were flash frozen in liquid nitrogen and stored at –80°C until analyzed. Frozen tissues were homogenized in ice-cold TRIzol and RNA was extracted according to standard protocol guidelines (Invitrogen, Cat #15596-018). Reverse transcription (RT) of 0.5μg of total RNA was completed in a final volume of 10μL following manufacturer’s protocol (QIAgen, Cat
Amplification of cDNA (1μL, unquantitated) from each RT reaction was completed by PCR in a final reaction volume of 12.5μL (Roche FastStart PCR, Cat# 04 738 314 001). Following an initial hot start at 94°C for 2 minutes, reactions were sequentially cycled 30 times for 45-second durations at the following temperatures: 94°C (denaturing); Tm°C (annealing; see below); and 72°C (extending). All reactions were incubated at 72°C for 5 minutes after the last cycle for final extension, and then maintained at 4°C. All PCR reactions for GIRK subunits 1-4 were completed at Tm = 58°C (primers sequences from reference 36).

For analysis of gene expression on a single-cell level, individual plaque-identified lactotrophs were collected in a heat-sterilized patch-pipette tip filled with sterile 1X PBS (pH 7.4). In this study, 10 plaque-identified lactotrophs from each treatment group (vehicle or E2) were collected from each of 5 rats. All samples were dispensed into a sterile 0.5-mL thin-walled PCR tube and 6μL of sterile dH2O was added immediately. Samples were frozen at –80°C until analyzed. All 6μL of non-quantitated sample was reverse transcribed into cDNA following manufacturer’s protocol (10μL final reaction volume; QIAgen, Cat #205310). Amplification of each cDNA sample was completed using “nested primers” in two consecutive PCR reactions. In general, the initial PCR reaction (with “outside” primers) was completed using 1μL of cDNA as the template in a final reaction volume of 12.5μL. The nested reaction (with “inside” primers) was completed using 1μL of the initial PCR reaction as template in a final reaction volume of 12.5μL. In general, PCR conditions were: 94°C for 2 minutes, [94°C for 45 seconds, Tm°C for 45 seconds, 72°C for 45 seconds] x N cycles, 72°C for 5 minutes, 4°C hold. Initial (“outer”) reactions were cycled with N = 40, and nested (“inner”) reactions were cycled with N = 35. Each sample was analyzed for expression of rPRL (gene accession number NM_012629) using outer primers (forward 5’-GCAGGGACACTCCTCCTCTGCT-3’ and reverse 5’-
ATGGGAGTTGTGACCAAACC-3'; Tm = 57ºC; product = 517bp) and inner primers (forward 5’-GCCAGAAAAGTCCCTCCGG-3’ and reverse 5’-CAATCCCTTCAAGAAGCCGC-3’; Tm = 59ºC; product = 178bp). Primer sequences and conditions for rGIRK subunits 1, 2, and 4 are described in (36). As another verification of cell phenotype, samples were also screened for the rat glycoprotein hormone alpha subunit (rGPHα; gene accession number NM_053918) using the following outer primers (forward 5’-GCTGTCATTCTGGTGCTGCT-3’ and reverse 5’-GCACTCCGTATGATTCTCCA-3’; Tm = 55ºC; product = 306bp) and inner primers (forward 5’-GCCCCCATCTACCAGTGAT-3’ and reverse 5’-GCATTCCCCATTACTGTGGC-3’; Tm = 56ºC; product = 155bp).

Statistical analysis

Membrane potential before and during DA treatment was compared using a Student’s t-test. Inward K+ current data in the GTPγS experiments were subjected to one-way analysis of variance (ANOVA) followed by Tukey’s multiple comparisons post-hoc test. PRL release from AP cells without drug challenge remained stable throughout the period of perifusion (data not shown; see reference 27). Secretory responses of each treatment group to drug challenge were analyzed by comparison to extrapolated basal release using two-way ANOVA and Bonferroni post-hoc analysis. Differences in secretory responses between vehicle- and E2- exposed cells were similarly analyzed. Differences between GIRK subunit expression data were also subjected to two-way ANOVA followed by Bonferroni’s post-hoc test. The overall percent expression of GIRK subunits in vehicle- versus E2-treated lactotrophs was analyzed using the Mann-Whitney U test. In all analyses, a p-value of < 0.05 was considered significant. Values are reported as mean ± SEM.
RESULTS

The results of the present experiments demonstrate that manipulation of E2, either in vivo or in vitro, can alter the lactotroph’s electrophysiological and secretory responsiveness to DA. As shown in Figures 1 and 2, both in vivo treatment and in vitro treatment with E2 produce lactotrophs with a proestrous phenotype. None of the lactotrophs (0 of 9) isolated from ovariectomized rats with vehicle implants demonstrated a DA-activated membrane hyperpolarization, even when a maximally effective concentration (with regard to inhibition of PRL release; see references 6, 9, 17) was used. In contrast, the majority of lactotrophs (10 of 12) from E2-implanted rats responded to DA with a membrane hyperpolarization (Figure 1A). The resting membrane potential (RMP) of vehicle- and E2-treated lactotrophs was not different (-37.8 ± 2.9 mV versus -38.5 ± 3.3 mV, respectively).

E2 has actions at multiple levels in the hypothalamic-pituitary axis. Therefore, we tested its effects directly on the lactotrophs by in vitro treatment of primary AP cells derived from diestrous rats. In agreement with previous findings (22), DA did not activate membrane hyperpolarization in control (vehicle-treated) diestrous lactotrophs (0 of 10; Figures 1B and C). In contrast, diestrous lactotrophs treated overnight with E2 did hyperpolarize in response to application of DA (11 out of 13) by an average of 17.0 ± 2.4 mV (Figures 1B and D). Again, RMP values of vehicle- versus E2-treated lactotrophs were not significantly different (-35.9 ± 2.1 mV versus -34.2 ± 2.2 mV, respectively).

Since the DA-induced hyperpolarization in proestrous lactotrophs is a critical mechanism in the secretory rebound of PRL that occurs subsequent to the withdrawal of DA (24, 27), we
examined the secretory responses of lactotrophs whose exposure to E2 was manipulated \textit{in vivo} or \textit{in vitro}. In perifusion, lactotrophs from ovariectomized, E2-treated animals responded to application of a physiological concentration of DA (53) with decreased PRL release, followed by a significant elevation in secretory rate over basal PRL levels after DA withdrawal ($p < 0.001$; Figure 2A). The enhanced release after DA removal was indeed a true “rebound secretion” and is not merely the release of PRL that had accumulated during the inhibitory phase. For example, the quantity of PRL released during the 10-minute application of DA was a total of 22.4 ± 3.2 ng below baseline, whereas the amount of PRL release during the subsequent rebound was 91.9 ± 22.5 ng over baseline. In contrast, lactotrophs derived from ovariectomized, vehicle-treated rats, while still responsive to the inhibitory action of DA, did not produce a secretory rebound following washout of DA (Figure 2A), but instead recovered to the pre-DA rate of PRL release. Cells from the vehicle-treated rats were inherently able to produce a PRL secretory rebound if directly hyperpolarized by the K$^+$ ionophore, valinomycin (Val). The amount of PRL released over baseline in the period following Val withdrawal was significantly smaller in vehicle-treated cells as compared to the E2-treated group (18.2 ± 5.4 \textit{versus} 82.2 ± 15.9 ng PRL, respectively; $p < 0.001$). This was most likely due to the fact that these cells had been without the trophic effects of E2 for approximately 9 days and, therefore, had lower intracellular stores of PRL. This interpretation is supported by the lower basal release from OVX + vehicle cells, which is ~60% of that from the E2-treated cells (although not significantly different), and by the secretory response to the Ca$^{2+}$ ionophore, A23187 (Figure 2A). Directly increasing Ca$^{2+}$ influx with A23187 significantly stimulated PRL release over baseline in both groups ($p < 0.001$), but the release from OVX + vehicle cells was again significantly less than that from OVX + E2 cells (32.4 ± 7.9 \textit{versus} 106.7 ± 20.9 ng PRL, respectively; $p < 0.001$). Regardless of the \textit{in vivo}
treatment, lactotrophs responded to the application of DA with decreased PRL release. Val and the Ca\(^{2+}\) channel blocker, verapamil (Vp), also inhibited PRL release during application. Vp directly blocks the influx of Ca\(^{2+}\) into the lactotrophs but does not change the membrane potential and, therefore, did not cause rebound secretion in either the vehicle-treated or E\(_2\)-treated group (Figure 2A).

We also observed similar changes in rebound PRL secretion following direct exposure of lactotrophs to E\(_2\). PRL release from diestrous lactotrophs treated overnight \textit{in vitro} with either vehicle or E\(_2\) (40 pg/mL) was significantly inhibited below baseline in the presence of DA, Val or Vp, \((p < 0.01)\) and significantly increased by application of thyrotropin-releasing hormone (TRH; \(p < 0.001\); Figure 2B). However, only E\(_2\)-treated cells responded to DA withdrawal with the anticipated rebound secretion of PRL \((p < 0.001 \text{ versus baseline PRL secretion})\), while PRL release from vehicle-treated cells returned only to basal levels (Figure 2B). Again, direct hyperpolarization using Val produced a secretory rebound upon its removal that was not dependent upon E\(_2\) treatment and occurred in both groups of diestrous cells \((p < 0.01); \text{Figure 2B}\). A significant difference in PRL release (during perifusion) was observed between the two \textit{in vitro} treatment groups only during the period following DA washout \((p < 0.01; \text{minutes 50 - 68, Figure 2B})\).

\textit{E\(_2\) action is distal to the D\(_2\)R}\n
To test whether E\(_2\) may be acting on components of the signaling pathway \textit{other than} the D\(_2\)R, GTP\(\gamma\)S was dialyzed by passive diffusion into patch-clamped lactotrophs via the recording pipette (Figure 3A). Spontaneous GIRK channel openings, even in proestrous lactotrophs, are rare enough to be undetectable in the absence of DA (16). However, we had previously observed
that internal dialysis of proestrous lactotrophs with GTPγS produced a slowly developing membrane hyperpolarization as activated G proteins accumulated in the presence of this non-hydrolyzable GTP analog (Figure 3B). This hyperpolarization was mediated by GIRK channels as determined by its Ba²⁺ sensitivity. DA application prior to the attainment of maximal hyperpolarization (~E_K) produced a rapid additional hyperpolarization that was not reversible (Figure 3B). The GTPγS-induced hyperpolarization was found to reach a plateau by 8 to 9 minutes, and DA application after this time did not produce any additional change in membrane potential (data not shown). This indicated that the same GIRK channels activated by DA were activated by the intracellular GTPγS. Therefore, we used this approach to bypass the D₂R in our current investigation of E₂ action. Using voltage clamp to control for the driving force, K⁺ current was measured during a ramp depolarization. Figure 3C illustrates the changing currents in a proestrous lactotroph immediately after whole-cell access (0 min) and after internal dialysis of GTPγS (10 min). An inwardly rectifying K⁺ current is present only after internal diffusion of the GTPγS, and this could be blocked by Ba²⁺. Note that outward K⁺ currents at more positive potentials are unchanged with time or Ba²⁺. Figure 3D summarizes the responses of 10 proestrous lactotrophs, and shows a significant increase in DA-induced K⁺ current (p < 0.001 versus break in).

The activation of GIRK channels by GTPγS did not occur in control diestrous lactotrophs (Figure 4A). However, in diestrous lactotrophs treated overnight with E₂ (40 pg/mL), internal dialysis of GTPγS induced a Ba²⁺-sensitive inward rectifying K⁺ current similar to that observed in the proestrous cells (Figure 4B). Again, the outward K⁺ current activated at more positive potentials was not altered. Figure 4C presents summarized data from multiple diestrous lactotrophs, showing the robust activation of GIRK current by E₂ treatment (p < 0.001 versus
vehicle-treated cells). This current was blocked when cells were treated with E2 in combination with the E2 receptor antagonist ICI 182,780 (40 nM) or the protein synthesis inhibitor, cycloheximide (Chx, 35 μM).

_E2 induces GIRK channel subunit expression in primary rat lactotrophs_

The ability of ICI 182,780 and Chx to block the effect of E2 indicated that the steroid was acting through its classical receptor to affect gene transcription. Therefore, we investigated the possibility that E2 might up-regulate the expression of the GIRK channel subunits themselves. Whole tissues were subjected to RT-PCR analysis to verify detectability of all four mammalian GIRK subunits (Figure 5A). Consistent with the findings of other laboratories, cardiac atrium (C.Atr) expressed GIRK 1 and 4 (10, 39); cerebellum (Cb) expressed GIRK 2 and 3 (35). Transcripts for GIRK channel subunits 1, 2, and 4 (but not GIRK 3) were detected in whole rat AP. Therefore, we analyzed the expression of these three GIRK subunits in individual, plaque-identified diestrous lactotrophs treated overnight with vehicle or E2 (80 pg/mL). All single-cell samples (100 lactotrophs, 5 rats) were screened for rPRL, rGPHα (common to the glycoprotein-secreting anterior pituitary cell types: gonadotrophs and thyrotrophs), and GIRK subunits 1, 2, and 4. A representative gel of ten plaque-identified lactotrophs (Figure 5B) illustrates that all single-cell samples yielded a strong signal for rPRL with no signal for rGPHα, indicating that lactotrophs (and not thyrotrophs or gonadotrophs) were isolated. Of the 50 cells analyzed per treatment group, E2-treated lactotrophs exhibit significantly greater expression of one or more GIRK subunit transcripts (50 ± 3% cells expressing detectable transcript) when compared to vehicle-treated controls (16 ± 1% expressing detectable transcript; _p < 0.05_; Figure 6). Also, in independent preparations of pituitary cells derived from five rats, a significant increase in the
expression of all three GIRK subunits (1, 2, and 4) was observed in E$_2$ treated lactotrophs (10 lactotrophs per treatment groups for each preparation; $p < 0.001$ versus vehicle-treated cells; Figure 6).

**DISCUSSION**

We have previously identified and characterized a GIRK channel as a physiological effector of DA action in pituitary lactotrophs (22, 26). Importantly, the functional expression of this DA-activated channel could be observed only in lactotrophs isolated from female rats in proestrus, and not from animals on other days of the reproductive cycle (22). In the present paper we demonstrate that it is E$_2$ that controls the functional activation of the D$_2$R-GIRK pathway. Moreover, this action of E$_2$ involves components of this pathway that are distal to the D$_2$R and includes the up-regulation of GIRK channel subunits. This represents a novel action of E$_2$ on the pituitary lactotroph.

Pituitary PRL exhibits dynamic secretory patterns associated with reproduction in the mammal. These include bouts of high PRL secretion during the menstrual and estrous cycles, in pregnancy, in response to mating, and in response to suckling (20). The periovulatory surge of PRL on the afternoon of proestrus in the rodent has been extensively studied (5, 62, reviewed in reference 15), although the exact mechanisms contributing to this surge are far from settled. The primary regulatory input to pituitary lactotrophs is inhibitory, and this tonic inhibition is mediated by hypothalamic DA acting on D$_2$Rs in the lactotroph membrane. In all mammalian species, removal of hypothalamic DA or blockade of the D$_2$Rs results in an increase of PRL secretion. Throughout the estrous cycle, DA acts to inhibit PRL secretion from the AP.
However, a precipitous drop in DA delivered to the anterior pituitary occurs midday on proestrus, just prior to and during the PRL surge (12, 38). In addition, a rapid decrease in the density of D₂Rs in the AP occurs at the onset of the PRL surge (52). These two changes constitute a dramatic withdrawal of dopaminergic tone at the beginning of the proestrous PRL surge.

It is clear that the rise in circulating E₂ on the preceding day (diestrus II) is the signal that initiates the hypothalamic-pituitary activities producing the surge of PRL on proestrus. Immunoneutralization of E₂ on diestrus II, but not on proestrus, blocks the proestrus surge of PRL (51), and E₂ administration to ovariectomized rats initiates daily afternoon surges of PRL that exhibit the same timing as the normal proestrous surge (50). E₂ acts on both the DA neurons in the hypothalamus and the lactotrophs to promote PRL secretion. The synthesis and activity of tyrosine hydroxylase (the rate-limiting enzyme in DA synthesis) in TIDA neurons are inhibited by E₂ (3, 53). These changes result in an E₂-induced drop in DA in the hypophyseal portal vessels (8). At the lactotroph, E₂ stimulates PRL synthesis through its classical receptor, directly activating PRL gene transcription (42). In addition, E₂ can inhibit lactotroph responsiveness to DA (55), although the mechanisms underlying this reduced responsiveness have not been completely elucidated.

In the present study, we have demonstrated that one mechanism by which E₂ alters lactotroph responsiveness to DA is by activating an additional transduction pathway – the GIRK channel. The electrophysiological phenotype of lactotrophs (specifically, whether DA can activate a GIRK current or not) is dependent upon the estrous status of the animal from which they are derived (22). In other words, lactotrophs prepared from proestrous rats (the day we know that DA input to the anterior pituitary falls) hyperpolarize in response to DA, while
lactotrophs obtained on other days of the cycle do not. As we have previously reported, these phenotypes are observed immediately following dissociation (ex vivo) and remain stable in culture (in vitro) for as long as we have tested them (up to 4 days). Also coupled to this phenotype is the ability of the lactotrophs to produce a secretory rebound upon DA withdrawal (22). These data establish a temporal link between GIRK function and proestrus, when a reduction of dopaminergic tone underlies the PRL surge (see model in Figure 7). Manipulations of E2, both in vivo and in vitro, that mimic the transition from diestrus II to proestrus resulted in a switch to the proestrous phenotype in lactotrophs.

E2-induction of the D2R-GIRK channel pathway, enabling DA to hyperpolarize the lactotroph membrane, appears to be through its classical receptor and attendant signaling pathways. The E2 receptor (ER) antagonist, ICI 182,780, blocked E2 induction of GTPγS-activated GIRK current. ICI is considered a “pure” antagonist (64) that competes with E2 for binding at both ERα and ERβ. E2 is a known transcription factor in pituitary lactotrophs and we found that treatment with Chx also blocked the ability of E2 to induce GTPγS activation of GIRK current. Together, these data indicate a genomic action of E2 in the regulation of this signaling pathway (Figure 7).

To investigate potential E2-targets in the lactotroph that could allow for the observed switch in DA-signaling to a GIRK-dependent mechanism on proestrus, we considered the essential components of that pathway. The first element is the D2R, which couples to Gαi and thereby inhibits adenylate cyclase (11, 17). D2R density, without a change in affinity, has been shown to decrease in the afternoon of proestrus or with exposure to high concentrations of E2 (52). The present studies used E2 concentrations comparable to those of late diestrus II or early proestrus morning (5), which do not alter the density of D2Rs in the AP (54). Ligand binding
parameters may not reveal altered intracellular interactions with transduction proteins, however. To definitively rule out E₂-induced changes in D₂R, we exploited our discovery that the GIRK channel current could be measured with intracellular dialysis of GTPγS into proestrous lactotrophs. Thus, in the absence of D₂R activation, we found that E₂ exposure, either in vivo or in vitro, could switch the diestrous phenotype of lactotrophs to a proestrous phenotype, with GTPγS activating the Ba^{2+}-sensitive IRK current.

The second essential component of the D₂R-GIRK pathway is the pertussis toxin-sensitive heterotrimeric G protein complex. While the Gaᵢ subunit inhibits adenylate cyclase, the dissociated βγ complex directly binds to GIRK proteins to open the channel (33, 40, 44, 61, 65). E₂ treatment of ovariectomized rats has been reported to decrease the percentage of lactotrophs with immunoreactive Gaᵢ3 (43). Immunoneutralization of Ga subunits can increase the availability free βγ complexes and, therefore, the activity of βγ signaling. Thus, without a concomitant decrease in βγ proteins, a reduction in Gaᵢ could be one mechanism to increase the ability of DA to activate GIRK channels. The βγ subunit itself is another potential target of E₂. Bayliss and colleagues have shown that different βγ isoforms have varying abilities to activate GIRK channels and, in fact, β₅ acts as a dominant negative inhibitor when co-expressed with other β subunits (41). These different activities are associated only with the various isoforms of the β subunit and do not change with different isoforms of the γ subunit. It is currently unknown which isoforms of these subunits are in lactotrophs, but an E₂-induced change in their expression pattern could dramatically alter the ability of DA to activate GIRK.

The third essential component in this pathway is the GIRK channel itself. E₂ has been shown to increase the density of another channel, the T-type Ca^{2+} channel, in the PRL-secreting GH₃ cells (58), so we wanted to determine if it could have a similar effect on GIRK subunits.
Because GIRK transcripts and proteins are expressed at low levels (23), we chose to measure them using single-cell RT-PCR. The single-cell RT-PCR analysis technique has been utilized in several applications (4, 7, 34, 36) since it was first described by Eberwine et al. (13, 63). E2 significantly increased the percentage of lactotrophs with detectable levels of GIRK transcripts. This increase in mRNA expression was observed for all three GIRK channel subunits found in the rat AP gland – GIRKs 1, 2, and 4.

Using patch clamp analysis, approximately 85% of proestrous lactotrophs respond to DA with a robust hyperpolarization (22). The electrophysiological data from E2-treated lactotrophs in the current experiments closely agree with this percentage (hyperpolarization was observed in 10 out of 12 cells, Fig. 1A and in 11 out of 13 cells, Fig. 1B). This is larger than the population in which we could detect GIRK expression. Since we know the channel functions in 85% of these cells (with GIRK identity verified by Ba\(^{2+}\) sensitivity), we assume that the levels of GIRK transcript in 30-35% of the lactotrophs are below detectable limits of single-cell RT-PCR. This discrepancy between electrophysiology and single-cell RT-PCR has been reported before for another Kir channel (66). Although the single-cell RT-PCR may under-estimate the number of lactotrophs actually expressing GIRK subunits, our data demonstrate that estrogen treatment increases expression enough to significantly increase detection.

Our findings are consistent with several other reports that have demonstrated the ability of E2 to serve as a “first messenger,” modulating cell signaling pathways involving G-protein coupled receptors in other cell types (reviewed in (37)). These reports suggest several potential mechanisms by which E2 could modulate the coupling of GPCRs to GIRK channels, including the alteration of mRNA expression for transcripts that encode proteins involved in neurotransmission and neuropeptide secretion. To our knowledge, however, this is the first
demonstration of E2 activation of a GPCR-GIRK signaling pathway in which the K+ channel has been identified by its inward rectification and by Ba2+ sensitivity – the two hallmarks of the Kir channel family. Moreover, these data show a direct upregulation of the GIRK channel gene expression, indicating that in lactotrophs E2 action may be more direct. It is possible (and quite likely) that E2 alters several intracellular cascades that impinge upon the D2R-βγ-GIRK transduction pathway. GIRK1 subunits in heterologous expression systems can be phosphorylated, enabling βγ gating of the channel (46) and phosphatidylinositol 4,5-bisphosphate (PIP2) has been shown to directly activate inward rectifying K+ channels in cardiac myocytes (32). The convergence of multiple E2-regulated cellular processes may be required for the almost “all-or-nothing” switch in lactotroph GIRK function that we describe here. Nevertheless, our data clearly show that exposure to E2 directly alters the expression of this DA-signaling effector in lactotrophs and elicits a functional change in the secretory responses of these cells.

In the present studies, we found no E2-induced change in responsiveness to the inhibitory actions of DA. The majority (approximately two-thirds) of spontaneous PRL release is dependent upon the influx of Ca2+ through L-type VGCCs. This is demonstrated by the response to verapamil in the present (Figure 2) and previous studies (23, 30). In the absence of electrical or chemical stimulation, approximately 30% of lactotrophs exhibit spontaneous depolarizations (“spiking activity”) of the membrane potential (24). The Ca2+ influx during these depolarizations support elevated cytosolic [Ca2+] that, in turn, supports a higher rate of PRL release from these cells than from electrically quiescent cells (30, 45). Hyperpolarization of the lactotroph membrane closes VGCCs, mediating the inhibitory effect of valinomycin on PRL release. In proestrous lactotrophs, the DA-induced hyperpolarization has the same effect. We and others
have demonstrated that this is not a direct action of DA on the VGCCs, but an indirect, voltage-dependent closing of VGCCs (27, 56) (see Figure 7). On other days of the estrous cycle, DA does not alter the lactotroph membrane potential but is still an effective inhibitor of PRL release. Clearly there occurs a switch in the mechanism(s) by which DA causes this inhibition.

The qualitative, and perhaps most physiologically significant, change in PRL secretion induced by E₂ treatment was the generation of a rebound release of PRL following DA withdrawal. We have previously shown that this secretory rebound is dependent upon the influx of Ca²⁺ through L-type VGCCs (24) and requires the preceding membrane hyperpolarization activated in the presence of DA (27). In the majority (~67%) of quiescent lactotrophs, application and withdrawal of a hyperpolarizing agent (DA in proestrous cells, or Val) results in initiation of spiking activity upon recovery of the RMP (24). This electrical activation of previously quiescent cells produces a prolonged rise in cytosolic Ca²⁺ concentration that correlates temporally with the rebound release of PRL and supports the hypothesis that a population of inactivated L-type VGCCs is recruited in response to the application and withdrawal of DA (30). Previous work by our lab shows that these channels are fully “re-primed” when the membrane potential is held at -60mV (27). In vivo, the activation of the D₂R-GIRK pathway by the morning of proestrus would keep lactotroph membranes at the negative potentials needed for full recovery of VGCCs from inactivation. These channels would remain closed, but in a state capable of activation upon membrane depolarization (Figure 7). Such depolarization would occur in response to the effective withdrawal of dopaminergic inhibition in the afternoon. The availability of all or most VGCCs would also make the lactotrophs more responsive to hypothalamic stimulatory factors, contributing to the surge potential of the lactotroph population.
PERSPECTIVES AND SIGNIFICANCE

The ability of DA to activate a hyperpolarizing signal transduction pathway in lactotrophs only on proestrus fits with the secretory requirements of the female reproductive cycle. PRL synergizes with luteinizing hormone (LH) as a luteotrophic hormone in rodents and is released in a surge coincident with the ovulatory surge of LH. As described above, the ability of the DA-induced hyperpolarization to recruit quiescent lactotrophs into active, highly-secreting cells upon the loss of DA tone in the afternoon of proestrus supports this surge release. However, chronic elevations in circulating PRL can be anti-gonadotrophic, disrupting female fertility (19). The use of non-hyperpolarizing mechanisms of DA inhibition on the other days of the estrous cycle may be a safeguard to avoid large, surge-like releases of PRL during transient interruptions in DA (as seen during stress, for example) on those days. The present data demonstrate that E2 controls this “switch” in D2R signaling in rat lactotrophs to the proestrus phenotype. E2 induces the functional expression of the D2R-GIRK signaling pathway through a genomic mechanism that affects components distal to the D2R. Our data support the hypothesis that rising E2 levels during diestrus alter the mechanism of DA signaling in normal lactotrophs that contributes to the unique secretory profile of PRL secretion on proestrus, and here we also identify a novel target of E2 action.
TEXT FOOTNOTES = none
ACKNOWLEDGEMENTS

GRANTS

DISCLOSURES = none
REFERENCES


25. **Gregerson KA, Einhorn L and Oxford GS.** *Modulation of potassium channels by dopamine in rat pituitary lactotrophs: a role in the regulation of prolactin secretion?* In:


FIGURE LEGENDS

Figure 1. E₂ induces functional expression of the D₂R-GIRK pathway in lactotrophs.  

**A** and **B**: Mean membrane potentials of lactotrophs before (Ctrl) and during DA (1 μM) application.  **A**: Cells were derived from ovariectomized rats treated *in vivo* with vehicle [open circles; 9 cells from 2 independent preparations (2 rats)] or E₂ (solid circles; 12 cells, 2 rats).  **B**: Diestrous lactotrophs were treated *in vitro* with vehicle (open circles; 10 cells, 4 rats) or E₂ (solid circles; 13 cells, 4 rats).  In both **A** and **B**, the Vₘ of E₂-exposed cells (but not vehicle-treated cells) was significantly hyperpolarized in the presence of DA (**p < 0.001** versus Ctrl; Student’s *t*-test).  

**C** and **D**: Representative current-clamp recordings of diestrous lactotrophs treated overnight *in vitro* with vehicle (**C**) or E₂ (**D**; 80 pg/ml), illustrating DA-induced hyperpolarization in E₂-treated cells and absence of response in vehicle-treated cells. Control solution (SES) did not alter the Vₘ, demonstrating absence of mechanical disturbance by U-tube application, while 50 mM KCl caused membrane depolarization in all cells.

Figure 2. E₂-treated AP cells produce a secretory rebound of PRL following withdrawal of DA.  

**A**: AP cells derived from ovariectomized rats treated *in vivo* with vehicle implants (open circles) or E₂ implants (solid circles).  **B**: AP cells, derived from intact diestrous rats, treated *in vitro* with vehicle (open circles) or E₂ (40 pg/mL, solid circles).  In both **A** and **B**, only cells exposed to E₂ produced a PRL secretory rebound following withdrawal of DA.  Both
vehicle- and E₂-exposed cells responded with a significant increase in PRL secretion following
Val withdrawal or upon stimulation by A23187 (A) or TRH (B). Only significant PRL release
over baseline rate is indicated by asterisks (*p < 0.05 ; **p < 0.01 ; ***p < 0.001 ; 2-way
ANOVA with Bonferroni post-hoc analysis of drug-challenged release versus basal release).
PRL release was significantly reduced from baseline (p < 0.05) in all groups by DA, Vp, and Val
(not indicated with asterisks). Symbols represent means ± SEM from five (A) or three (B)
independent perifusions, each representing an individual animal.

Figure 3. Internal dialysis of GTPγS causes membrane hyperpolarization of proestrous
lactotrophs independent of D₂R activation. A: Plaque-identified, proestrous rat lactotrophs
were dialyzed with GTPγS (250 μM) from the patch clamp recording pipette. B: Dialysis of
GTPγS into the lactotroph after whole-cell access (“cell break-in”) resulted in a gradually
developing hyperpolarization measured in current clamp. DA (300 nM) application during this
time caused rapid hyperpolarization that was not reversible. C: K⁺ currents measured during
ramp depolarization in a proestrous lactotroph, immediately after cell break-in (0 min), 10
minutes later, and then in the presence of 250 μM BaCl₂ (Ba⁺⁺). D: Summary of inward
rectifying K⁺ currents in proestrous lactotrophs (10 cells, 2 rats) dialyzed with GTPγS. Currents
at different times following whole-cell access were compared using 1-way ANOVA with
Tukey’s multiple comparisons post-hoc test (***p < 0.001).

Figure 4. Barium-sensitive IRK is activated by GTPγS dialysis of diestrous lactotrophs
treated in vitro with E₂. A: Internal dialysis of GTPγS (250 μM) does not activate IRK current
in vehicle-treated, diestrous lactotrophs. B: Diestrous cells treated overnight with E₂ (40 pg/ml)
develop Ba$^{2+}$-sensitive IRK during internal dialysis with GTP$\gamma$S.  

C: Summary of IRK currents recorded from diestrous lactotrophs dialyzed with GTP$\gamma$S.  E$_2$-treated lactotrophs have an increased IRK over control diestrous lactotrophs.  The effect of E$_2$ is blocked by co-incubation with ICI-182,780 (ICI, 40 nM) or cycloheximide (Chx, 35 $\mu$M).  Numbers in bars indicate number of lactotroph cells tested in each treatment group.  Cells were derived from 4 independent preparations (4 rats).  Treatment groups were compared using 1-way ANOVA with Tukey’s multiple comparisons post-hoc test ($***p < 0.001$).

Figure 5. Transcripts for GIRK channel subunits 1, 2, and 4 (but not GIRK3) are expressed in the rat AP and are detectable in single identified lactotrophs.  RNA isolated from tissues or cells was reverse transcribed and the cDNA for each was amplified by PCR using nested (outer and inner) primers.  A: To test the nested PCR primers, whole rat AP was used.  Transcript for GIRK channel subunits 1, 2, and 4 (but not 3) are detected in whole rat AP.  Control tissues used were cardiac atrium (C. Atr, for GIRK1 and GIRK4) and cerebellum (Cb, for GIRK2 and GIRK3).  B: A representative gel of PCR products amplified from 10 single plaque-identified lactotrophs.  Positive (+) control is RT reaction from whole rat AP (lane 12).  Negative (-) control has RT reaction replaced with dH$_2$O (lane 13).

Figure 6. E$_2$ significantly increases GIRK channel subunit expression in primary rat lactotrophs.  RNA was collected from single, plaque-identified lactotrophs, reverse transcribed, and the cDNA amplified by PCR using nested primers against GIRK channel subunits 1, 2, and 4.  These data represent the number of lactotrophs expressing each GIRK channel subunit from five independent experiments (5 rats; 10 cells analyzed in each treatment group per rat).  A
significant, overall increase in GIRK channel subunit expression was observed after *in vitro* treatment with 80 pg/mL E2 compared with vehicle-treated cells (***p < 0.001; 2-way ANOVA with Bonferroni post-hoc test). *Inset:* A summary of all preparations (5 rats) shows that *in vitro* E2-treatment (80 pg/mL overnight) significantly increases the percent of lactotrophs expressing one or more GIRK channel subunits as compared to vehicle-treated cells (*p < 0.05; Mann-Whitney U test).

**Figure 7. Model of E2 action on D2 receptor (D2R) signaling in the lactotroph.** On diestrus II (left panel), the D2R is not coupled to the GIRK channel. Rising levels of circulating E2 (dashed line) during this time, however, result in a functional switch in DA signaling to include GIRK channel activation. This transduction pathway is complete by the morning of proestrus (right panel), when DA activates membrane hyperpolarization (ΔVₘ). The more negative membrane potential “primes” the lactotroph population by removing inactivation (illustrated as a ball-and-chain blocker) of voltage-gated Ca²⁺ channels (VGCC). When DA levels delivered to the AP via the portal vessels (dashed-dotted line) drop on the afternoon of proestrus, the primed lactotrophs depolarize, initiating increased Ca²⁺ influx through VGCC to support PRL release and contribute to the PRL surge (solid line). E2 does not induce this functional switch in signaling by acting on the D2R, but by significantly increasing the expression of GIRK channel subunits (solid arrow). E2 may also regulate expression of the G-protein βγ subunit isoforms (dotted arrow, ?), which are known to directly bind to and activate the GIRK channel.
Figure 1

A

○○○ Ovex + veh  ●●● Ovex + E₂

Vₘ (mV)

Ctrl  DA

B

○○○ Diestrus-veh  ●●● Diestrus-E₂

Vₘ (mV)

Ctrl  DA

C

Diestrus-veh

DA  KCl  KCl

20 sec

D

Diestrus-E₂  KCl  DA  SES

***

Ovex + veh  Ovex + E₂

Ctrl DA

Ovex + veh  Diestrus-E₂

Ctrl DA

Diestrus-veh

DA  KCl  KCl

20 sec

Diestrus-E₂  KCl  DA  SES

***
Figure 2

A

- Ovex + E₂
- Ovex + vehicle

PRL Released (ng/2min)

Time (minutes)

B

- E₂ in vitro
- control (vehicle)

PRL Released (ng/2min)

Time (minutes)
Figure 3

A

GTPyS

GTPyS

B

Cell break-in

DA

VM

-20

-80

-160

-120

-80

-40

0

120

Ba++

10 min

Membrane (ramp) Potential (mV)

0 min

-80

-160

<30 sec after break-in

K+ Current (pA/50mV)

***

Ba2+

wash

180

120

60

0

***

control

>10 min after break-in

K+ Current (pA)

***

<30 sec after break-in

control

Ba2+

wash
Figure 4

A. Vehicle-treated

B. E2-treated

C. Bar graph showing K⁺ current (pA/50mV) for different conditions:
- Control
- E₂
- E₂+ICI
- E₂+Chx

* * *
Figure 5

A

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B

(10) Individual plaque-identified lactotrophs

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Figure 6

The diagram illustrates the number of lactotrophs expressing GIRK subunit mRNA under different treatments. The x-axis represents the GIRK subunits (GIRK1, GIRK2, GIRK4), and the y-axis represents the number of lactotrophs expressing GIRK subunit mRNA.

- **Vehicle** treatment shows a lower expression of GIRK subunits compared to **E₂** treatment.

The inset graph shows a significant difference (*p < 0.05*, **p < 0.005**) between the Vehicle and E₂ groups, with E₂ treatment resulting in higher expression levels.