**Endocrine,** *vol. 20, no. 1, 67–74, February 2003* 0969–711X/03/20:67–74/$20.00 

# Functional Expression of the Dopamine-Activated $K^+$ Current in Lactotrophs During the Estrous Cycle in Female Rats

## *Correlation with Prolactin Secretory Responses*

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**It is well established that hypothalamic dopamine (DA) is the major physiologic regulator of prolactin (PRL) secretion, exerting a tonic inhibition throughout most of the estrous cycle. A dramatic drop in the amount of DA perfusing the anterior pituitary occurs in the afternoon of proestrus and is critical for the production of the surge of PRL that occurs at that time. In my laboratory, we have identified and characterized a DA-activated $K^+$ channel ($K_{DA}$) in lactotrophs derived from proestrous rats that underlies DA-induced membrane hyperpolarization of lactotrophs. We have also demonstrated that this hyperpolarization plays a critical role in both the inhibition of PRL release from proestrous cells and the PRL secretory rebound that occurs following DA withdrawal. We now report that the ability of DA to activate the $K_{DA}$ channel and elicit hyperpolarization in primary lactotrophs changes dramatically during the estrous cycle. Lactotrophs isolated from cycling female rats were studied using whole-cell voltage clamp. DA (1 μ*M*) elicited a robust membrane $K^+$ current in the majority of proestrous lactotrophs (86%; 24.0 ± 2.9 pA). By contrast, DA activated a considerably smaller membrane current (3.3 pA) in very few lactotrophs isolated from rats on either diestrus or estrus (8 and 0%, respectively). Using a perifusion system to examine temporal patterns of PRL release, we found that following application and withdrawal of DA, proestrous cells produced a robust secretory rebound, but diestrous and estrous cells did not. However, DA inhibited PRL release to the same extent regardless the stage of the cycle from which the cells were derived. These data are consistent with the presence of multiple DA effectors in lactotrophs and demonstrate that their relative importance shifts dramatically with changes in the endocrine status of the animal. We propose that the DA-activated $K^+$ channel ($K_{DA}$) is a critical effector governing the unique secretory profile of PRL observed in proestrous animals.**

Received December 20, 2002; Accepted December 20, 2002.

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**Key Words:** Dopamine; prolactin; estrous cycle; inwardly rectifying $K^+$ channels; GIRKs.

### Introduction

Dopamine (DA) is widely appreciated as the major physiologic regulator of prolactin (PRL) secretion from the anterior pituitary gland *(1)*. DA released from the hypothalamic tuberoinfundibular dopaminergic neurons exerts tonic inhibitory control of PRL release via activation of $D_2$ DA receptors on the lactotrophs *(2,3)*. A drop in dopaminergic tone and dissociation of DA from its receptor appears to be another physiologic signal leading to a stimulatory phase of PRL secretion *(4–6)*. In fact, dopaminergic input to the anterior pituitary gland during the estrous cycle of the rat is inversely correlated with circulating levels of PRL, being constant through most of the cycle but dropping significantly just prior to the initiation of the afternoon proestrous surge of PRL *(7)*.

Ovarian steroids modulate the regulation of PRL secretion via actions both in the hypothalamus and directly on the lactotroph. The slow rise in circulating estrogen that begins on diestrus 2 and continues through proestrus is required for both the luteinizing hormone (LH) and the PRL surges that occur in the afternoon of proestrus *(8)*. In addition to influencing the activity of the neuroendocrine dopaminergic neurons *(9)*, estrogen alters lactotroph responsiveness to DA *(10)*, but the mechanisms by which this occurs are not known.

In my laboratory, we have identified and characterized a voltage-independent, inwardly rectifying potassium channel in lactotrophs derived from proestrous rats that is activated by DA ($K_{DA}$). Activation of $K_{DA}$ leads to hyperpolarization of the lactotroph membrane and cessation of calcium-dependent action potentials *(11,12)*. In vitro studies from my laboratory have demonstrated a critical role for this $K_{DA}$ channel in both the inhibition of PRL release by physiologic (nanomolar) concentrations of DA and the stimulatory phase of PRL secretion elicited by DA withdrawal *(13,14)*.

We now report that the functional expression of this DA-activated $K^+$ current in lactotrophs varies dramatically over the reproductive cycle. Whereas a robust hyperpolarization is activated by DA in the majority of proestrous lactotrophs,

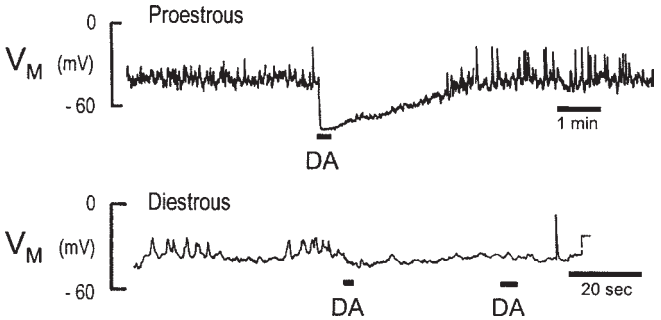


**Fig. 1.** Membrane potential ($V_M$) responses of primary lactotrophs to DA during whole-cell current clamp. Lactotrophs were derived from intact female rats on various days of the estrous cycle. Brief (~2–5 s) application of DA ($10^{-6}$ *M*) induced a robust hyperpolarization in proestrous lactotrophs (top trace) but little or negligible membrane potential change in diestrous lactotrophs (bottom trace).

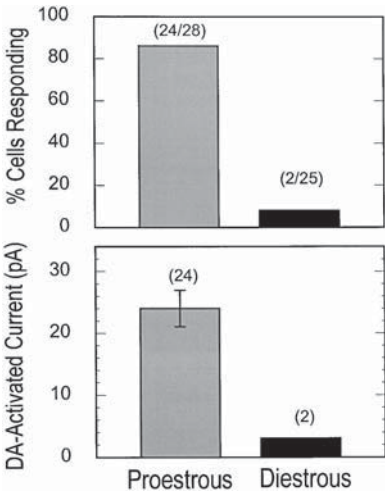


**Fig. 2.** Summary of membrane current responses of primary lactotrophs to DA. Lactotrophs were derived from intact female rats between 8:00 AM and 9:00 AM on various days of the estrous cycle and identified by reverse hemolytic plaque assay (RHPA). DA ($10^{-6}$ *M*) was applied for 5 s to voltage-clamped cells (holding potential of –40 mV) and peak current responses were measured. (**Top**) Percentage of lactotrophs that responded to DA with a membrane current change (number of responders/total number of cells tested is shown in parentheses above each bar). No lactotrophs derived from estrous rats responded (16 tested) and, therefore, data from estrous lactotrophs are not shown. (**Bottom**) Mean membrane current change in those cells responding (number of cells is indicated in parentheses above each bar).

little to no change in membrane potential occurs in lactotrophs derived from other stages of the cycle. The present study examined DA-regulated PRL release in vitro to determine how this variable activation of one effector may underlie altered secretory responsiveness of lactotrophs to DA.

## Results

In identified lactotrophs derived from female rats on the morning of proestrus, brief (~2 s) application of 1 μ*M* DA activated a $K^+$ current, resulting in rapid hyperpolarization of the membrane potential that lasted for several minutes (Fig. 1, top trace). We have previously shown that this hyperpolarization is activated in proestrous lactotrophs by concentrations of DA that are in the range of those measured in hypothalamic-pituitary portal blood (5–500 n*M*; *[15]*. We chose to use a maximally effective concentration of DA (1 μ*M*) in the present electrophysiologic studies for the purpose of comparing the number of responsive lactotrophs on different days of the estrous cycle. We found that when lactotrophs derived from either diestrous 2 or estrous animals were studied, very few were found to respond to DA with any change in membrane potential. The lower trace in Fig. 1 illustrates a diestrous cell in which a weak response appeared to be elicited by 1 μ*M* DA. An initial application of DA coincided with a small (~6.5 mV), slowly developing hyperpolarization. A second application of DA had no effect. To control for driving force on $K^+$, lactotrophs from proestrous, diestrous 2, and estrous rats were examined in voltage clamp. As summarized in Fig. 2, a robust current (24.0 ± 2.9 pA) was activated by DA in the majority of proestrous lactotrophs (24 of 28; 86%). In marked contrast, very few diestrous lactotrophs responded to DA with a change in membrane current (2 out of 25; 8%), and those responding showed a minimal response (3.3 pA). No lactotrophs tested from estrous rats (0 of 16) exhibited a DA-activated membrane current.

We have previously published evidence that the DA-induced hyperpolarization in proestrous lactotrophs is a critical mechanism in both the inhibition of PRL release during DA application and the secretory rebound of PRL that occurs subsequent to the withdrawal of DA *(13,14,16)*. Thus, we chose to examine the PRL secretory responses to DA in diestrous and estrous cells, in which the hyperpolarization was virtually absent. Initially, studies were conducted in which proestrous and diestrous pituitary cells were compared side-by-side, from dissociation through perifusion and radioimmunoassay (RIA); these data are presented in Fig. 3. As predicted, if the DA-induced hyperpolarization were necessary for the PRL secretory rebound after DA withdrawal, diestrous cells produced almost no secretory rebound following washout of DA (solid symbols). Note the robust secretory rebound from proestrous lactotrophs following washout of DA (open symbols). Diestrous lactotrophs are inherently capable of producing a secretory rebound if transiently hyperpolarized as demonstrated by the response to application and withdrawal of valinomycin. Valinomycin is a $K^+$-selective ionophore, which incorporates into the plasma membrane and directly mediates a hyperpolarizing $K^+$ current. Note the similarity in amplitude and temporal profile of the valinomycin-induced PRL rebound between proestrous and diestrous cells (Fig. 3). Surprisingly, DA (50 n*M*) was equally effective in inhibiting PRL release from diestrous and proestrous cells. Later, the secretory responses of estrous

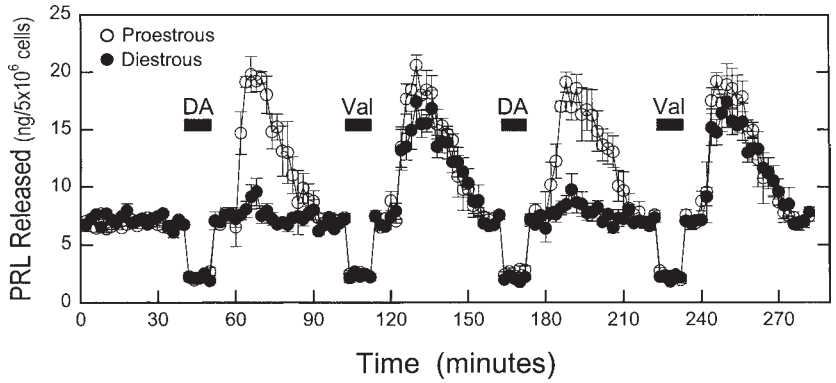


**Fig. 3.** PRL secretory responses of proestrous (○) and diestrous (●) pituitary cells to challenges of DA (50 n*M*) and valinomycin (Val, $10^{-9}$ *M*). Each test substance was introduced to the perifusion system for 10 min (time periods indicated by the solid bars), and each challenge was separated by 50 min of perifusion with control standard extracellular solution (SES). Two columns were run simultaneously in each perifusion experiment, proestrous cells in one and diestrous cells in the other, both perifused with control and test solutions from the same reservoirs. Symbols represent means ± SE of four independent perifusions. Valves regulating inflow to columns were actuated every 30 min to verify that mechanical switching of solutions did not in itself cause changes in secretion.

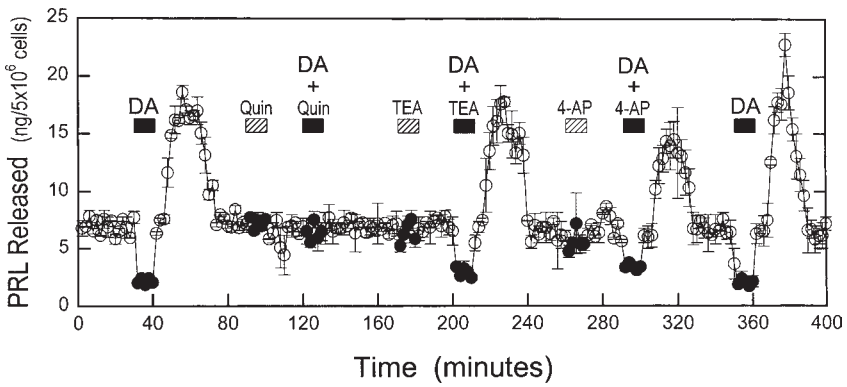


**Fig. 4.** Effects of known $K^+$ channel blockers on DA-induced secretory responses of proestrous pituitary cells. Cells were perifused with DA (50 n*M*) for 10-min periods (indicated by solid bars) in the absence or presence of quinine ($10^{-4}$ *M*), TEA (10 m*M*), or 4-AP (5 m*M*). The $K^+$ channel blockers were also applied alone (time periods indicated by hatched bars) to test for independent effects on PRL release. PRL release during exposure to the drugs is represented by the solid symbols (●) and release during perifusion with control SES is represented by the open symbols (○). Symbols represent means ± SE of three independent perifusions.

lactotrophs were examined in perifusion and found to behave similarly to diestrous cells. DA still inhibited PRL release from estrous cells but did not produce a secretory rebound on following withdrawal (Fig. 6). Transient hyperpolarization of the lactotroph membrane with valinomycin did, however, produce a PRL rebound following drug washout (Fig. 6).

To determine whether the DA-activated $K^+$ channel ($K_{DA}$) was necessary for the inhibitory action of DA in the proestrous cells, we sought a way to selectively block this channel. Unfortunately, $K_{DA}$ is resistant to pharmacologic blockers of $K^+$ channels with the exception of quinine at 100 μ*M*. Quinine is not very selective, even at much lower concentrations. However, this is the only drug that we have found to effectively block $K_{DA}$ activation *(17)* and, therefore, we chose to use it to dissect DA's actions on PRL release. Two other known $K^+$ channel blockers were also tested to evaluate selectivity of effects in this system: tetraethylammonium (TEA) and 4-aminopyridine (4-AP). In proestrous cells, 100 μ*M* quinine completely blocked both the inhibition of PRL during DA application and the PRL rebound following DA washout (Fig. 4). TEA (10 m*M*) had little effect on either response of PRL release to the application and withdrawal of DA. A small inhibition of both inhibitory and rebound responses was seen when 5 m*M* 4-AP was present during the application of DA. Single-channel analysis has revealed that the $K_{DA}$ channel is insensitive to TEA (10 m*M*), partially blocked by 4-AP (5 m*M*), and completely blocked by quinine (100 μ*M*) *(17)*. Thus, the effects of these three $K^+$ channel blockers on DA-induced secretory responses of proestrous lactotrophs are consistent with their effects on the DA-induced $K^+$ current and hyperpolarization.

When proestrous and diestrous lactotrophs were compared side-by-side, we found that 100 μ*M* quinine reduced DA inhibition of PRL release from diestrous cells by more than half (55% reduction) although the same solution completely blocked DA inhibition of PRL release from proestrous cells (Fig. 5). A similar response was observed in pituitary cells isolated from estrous rats. Quinine did reduce DA

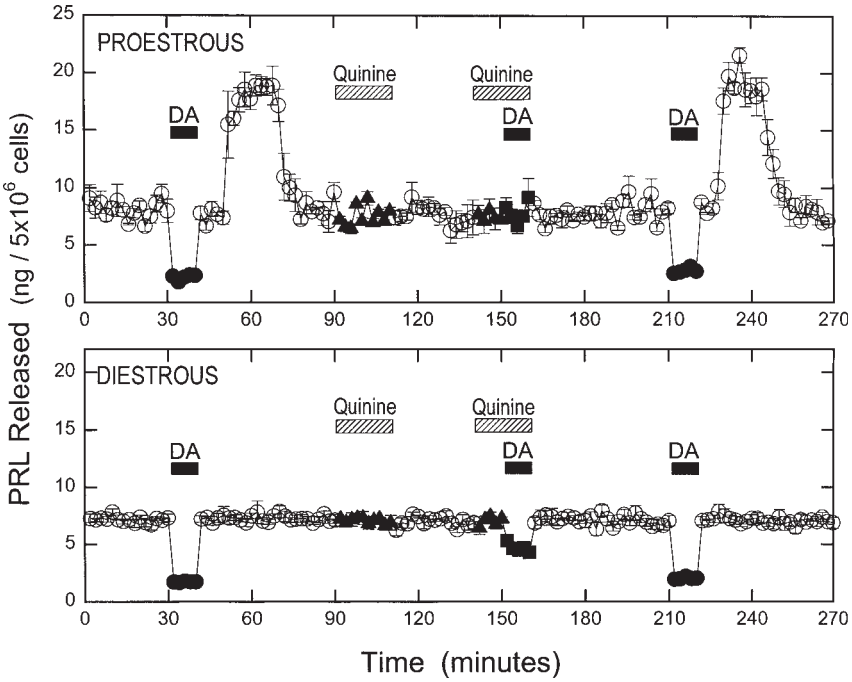


**Fig. 5.** Effect of quinine ($10^{-4}$ *M*) on DA-induced secretory responses of proestrous and diestrous pituitary cells. Cells were perifused with DA (50 n*M*) alone (●), quinine alone (▲), or DA in the presence of quinine (■). Drug applications were separated by perifusion with control SES (○). Two columns were run simultaneously in each perifusion experiment, proestrous cells in one and diestrous cells in the other, both perifused with control and test solutions from the same reservoirs. Symbols represent means ± SE of three independent perifusions.

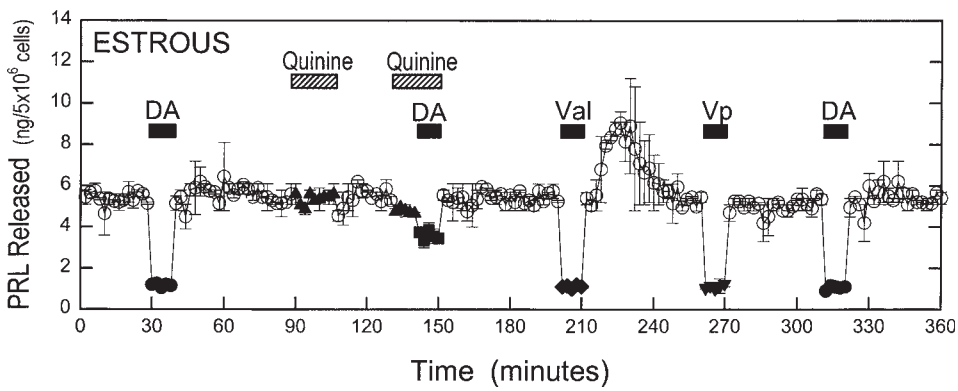


**Fig. 6.** PRL secretory responses of pituitary cells derived from rats on the morning of estrus. Cells were challenged with DA (50 n*M*) in the absence (●) or presence (■) of quinine ($10^{-4}$ *M*), with quinine alone (▲), valinomycin ($10^{-9}$ *M*, ◆) or verapamil (20 μ*M*, ▼). Time periods of drug application are indicated by the bars. Drug applications were separated by perifusion with control SES (○). Symbols represent means ± SE of three independent perifusions.

inhibition of PRL release (by 57%) but did not completely block it (Fig. 6).

## Discussion

The data presented herein demonstrate a dramatic change in the functional expression of one effector of DA's actions in the lactotroph. The DA-activated hyperpolarizing $K^+$ current is present in the majority (>85%) of lactotrophs derived from proestrous rats and is virtually absent in lactotrophs on other days of the estrous cycle. DA withdrawal elicits a robust rebound secretion of PRL only from proestrous cells. This supports our previous conclusion that the hyperpolarization induced by DA plays a key role in producing the marked rise in PRL release observed when DA is removed *(13)*. Indeed, lactotrophs derived on other days of the cycle exhibit negligible hyperpolarization in response to DA and do not produce a secretory rebound of PRL on withdrawal of DA. Moreover, a PRL secretory rebound can be elicited from the estrous and diestrous cells when a transient hyperpolarization is produced directly by the application and withdrawal of the ionophore valinomycin.

The present data also indicate that there occurs a shift in the functional dominance of DA's effectors in the lactotrophs over the estrous cycle. The hyperpolarizing current is not only necessary for inducing the subsequent secretory rebound, but also appears necessary for the inhibitory action

of DA in proestrous lactotrophs. Quinine, which blocks the $K_{DA}$ channel *(17)*, also completely blocks the DA inhibition of PRL release from proestrous cells. Yet, in the present controlled in vitro experiments, DA is equally effective in inhibiting PRL release from diestrous and estrous cells. Moreover, quinine cannot completely block DA's inhibitory effect on PRL release from these cells. These data indicate that a mechanism other than $K_{DA}$ and DA-induced hyperpolarization mediates DA's inhibitory effect in diestrous and estrous lactotrophs.

Quinine is not a selective blocker of specific $K^+$ channel subtypes and, in fact, acts on a wide variety of systems and effectors *(18)*. The partial block of DA inhibition of PRL release from estrous and diestrous cells must be due to actions other than blockade of the $K_{DA}$ channel. This makes it possible that half the block observed in proestrous cells is also due to actions of quinine other than $K_{DA}$ block. Unfortunately, selective pharmacologic block of the $K_{DA}$ channel has been very elusive. Inwardly rectifying $K^+$ channels can be blocked by barium, but this is not a feasible tool for studying the secretory events since barium has direct actions on PRL release *(19)*. However, the present studies clearly show that quinine can completely block DA's effects on proestrous cells while there is a substantial portion of DA's action on other days of the cycle that is independent of quinine. That the $K_{DA}$ channel is mediating the DA-induced secretory responses of proestrous lactotrophs is supported by the data shown in Fig. 4. Again, while the three $K^+$ channel blockers used in the present study are not perfectly selective, DA-regulated PRL release exhibits the same sensitivity to these compounds as does the $K_{DA}$ channel, determined in single channel analysis experiments *(17)*.

Ovarian steroids are likely candidates for triggering the changes in the functional expression of $K_{DA}$ activity. We initially chose to compare cells derived from rats on the mornings of diestrus 2 and proestrus because of the known differences in exposure to estrogen. Circulating estrogen begins to rise on diestrus 2, gradually increasing until mid-day on proestrus, when a more rapid rise occurs *(20)*. It is known that this gradual rise in estrogen is necessary for both the LH and PRL surges that occur in the afternoon of proestrus since immunoneutralization of estradiol on diestrus through early proestrus will block these surges *(8)*. DA input to the anterior pituitary and circulating PRL levels are the same on the mornings of diestrus 2 and proestrus. Yet, the hypothalamo-hypophysial axis has been committed to the surge responses even if estrogen is later withdrawn on proestrus *(8)*. High levels of estradiol can reduce the numbers of anterior pituitary $D_2$ receptors *(21)* and, indeed, the numbers are lower in the afternoon of proestrus *(22)*. However, both $D_2$ receptor density and affinity are the same on the mornings of diestrus and proestrus *(22,23)*. Thus, we proposed that changes in lactotroph responsiveness to DA, independent of the $D_2$ receptor, may be observed by the morning of proestrus, as indeed we show herein.

Lactotrophs contain classic nuclear estrogen receptors (ERs) of both the ERα and ERβ subtypes *(24)*, and estrogen is a known transcription factor in these cells. Estrogen stimulates transcription of PRL *(25,26)* and the thyrotropin-releasing hormone receptor in mammotropic cells *(27)*. However, recently, rapid (5 to 6 min) actions of estradiol on $GH_3$ cells have been reported *(28)* that may be mediated by a plasma membrane–bound ER. Whether estrogen is in fact inducing the functional expression of $K_{DA}$ will require further studies. If it is, mechanisms could be proposed for either the traditional nuclear receptor, acting on transcription, or a membrane-bound receptor. Based on the present data, we do not know if the $K_{DA}$ channel is actually expressed during those times when DA does not induce hyperpolarization. Channel openings are not observed in the absence of DA even in proestrous lactotrophs *(17)*, and the $K_{DA}$ channel is not activated by changes in membrane potential. Therefore, the lack of current activation by DA in estrous and diestrous cells does not discriminate between the possibilities that the channel is absent vs present but not coupled to the $D_2$ receptor vs present and directly blocked or inactivated. Again, the elusiveness of the $K_{DA}$ channel makes a pharmacologic investigation difficult. Labeled agonists or antagonists are simply not available for binding studies. $K_{DA}$ is a GIRK (G protein-activated, inwardly rectifying $K^+$) channel and we have recently identified GIRK1 and GIRK4 as excellent candidate subunits for the $K_{DA}$ channel *(29)*. However, pituitary cells other than lactotrophs also express transcripts for these proteins (unpublished findings), so any analysis of these GIRKs using specific antibodies will require purified lactotrophs. Thus, estrogen may act on the lactotrope in several possible ways. As a transcription factor it may be inducing expression of the $K_{DA}$ channel proteins themselves and/or other proteins necessary in the $D_2$ receptor–$K_{DA}$ transduction pathway. Through a membrane-bound receptor, estrogen may have direct actions on the $K_{DA}$ channel.

Whatever the mechanism(s) that induces functional expression of $K_{DA}$ in proestrous lactotrophs, there also must be a mechanism that turns off this process. The phenotype at the time of dissociation of the pituitary cells (d 0) is the phenotype that is retained throughout the culture time in vitro. In other words, lactotrophs derived from diestrous rats exhibit negligible DA-activated hyperpolarization throughout at least 4 d in culture. Likewise, the majority of lactotrophs derived from proestrous rats respond to DA with robust hyperpolarization through this time. These periods long outlast the shifts in phenotype observed if the cells remain in vivo. Progesterone seems a likely candidate for switching off the $K_{DA}$ response because circulating levels of this steroid rise to very high levels late in proestrus *(20)*. In addition, in ovariectomized, steroid-treated rats, replacement of estradiol alone will result in daily afternoon surges of both PRL and LH *(30–32)*. These daily surges will continue as long as the estradiol is maintained. If progesterone is administered, the surges are larger, with magnitudes similar to the endogenous

surges, but they occur only once despite continued presence of estradiol *(33)*. Perhaps elevated progesterone limits the functional expression of $K_{DA}$ to the day of proestrus and thereby contributes to the limitation of the PRL surge to that one day. If so, the mechanism by which progesterone alters $K_{DA}$ function may be indirect since rat lactotrophs do not express nuclear progesterone receptors *(34)*. Alternatively, progesterone may act on the lactotroph through a membrane-bound receptor.

If activation of the hyperpolarizing $K_{DA}$ channel is not mediating DA inhibition of PRL release in estrous and diestrous lactotrophs, then what is? It has been clearly established that $D_2$ receptor activation in lactotrophs leads to inhibition of adenylate cyclase and a drop in cyclic adenosine monophosphate (cAMP) production *(3,35)*. While elevations in cAMP levels have been shown to stimulate PRL release, several groups have shown that the inhibition of cAMP is not required for the inhibition of PRL by DA *(36–39)*. Changes in the ability of DA to inhibit cAMP also cannot account for the variation in $K_{DA}$ activation seen on the different days of the cycle since the internal cAMP concentration in all recorded cells was "clamped" at 2 m*M*.

DA might inhibit PRL release on estrus and diestrus by inhibiting voltage-gated calcium channels (VGCCs). PRL release on these days of the cycle is sensitive to block by calcium channel blockers (see Fig. 6), so calcium influx is still the major drive for PRL secretion throughout the estrous cycle. There is experimental evidence for DA inhibition of VGCCs in cultured lactotrophs derived from lactating rats *(40,41)* and in $GH_4C_1$ cells expressing human $D_2$ receptors ("$GH_4C_1/D_2$-DAR"; *[42]*. In those studies, DA treatment of the PRL-secreting cells produced a decrease in calcium current ($I_{Ca}$) measured under voltage-clamped conditions. A 27% decrease in $I_{Ca}$ was observed after one or more minutes of DA exposure *(42)* while prolonged treatment (≥24 h) with DA was required for maximal (57–61%) reductions in $I_{Ca}$ *(41,42)*. Voltage-independent inhibition of $I_{Ca}$ is not seen in response to acute applications of DA *(43)*, as would be expected if the $D_2$ receptor were directly coupled with VGCCs. Thus, this mechanism does not account for the immediate drop in cytosolic [$Ca^{2+}$] observed in proestrous lactotrophs in response to DA *(14)*, and we have demonstrated that this does not account for DA inhibition of PRL release in proestrous cells *(13)*. Rapid and maximal inhibition of PRL is seen within 2 min of the application of DA in estrous and diestrous cells (Figs. 5 and 6), so the slowly developing block of VGCCs appears not to mediate this effect. It is possible that DA inhibition of PRL release on estrous and diestrous involves a step downstream of $Ca^{2+}$ influx. Careful studies of DA action on cytosolic [$Ca^{2+}$] and PRL release during the different stages of the estrous cycle will be required for that determination.

To summarize, the present data show that the ability of DA to activate the inwardly rectifying $K^+$ channel in female lactotrophs varies dramatically over the estrous cycle. In fact, it is very close to an all-or-nothing response with >85% of proestrous lactotrophs exhibiting a robust hyperpolarization and 0–8% of lactotrophs on other days of the cycle producing negligible changes in membrane potential. The tight correlation between DA-induced hyperpolarization and the rebound secretion of PRL elicited by DA withdrawal demonstrates the critical role that the $K_{DA}$ channel plays in the production of the secretory rebound. Our data also indicate that the $K_{DA}$ channel is the primary effector in DA inhibition of PRL release from proestrous lactotrophs, but that another effector must mediate this inhibition on other days of the cycle. While it has been appreciated that $D_2$ signaling in lactotrophs, as in other cell types, most likely involves multiple effector pathways, this is the first example of a complete switch from one effector to another in mediating the same cellular response in a given cell type. From these data, we propose that the $K_{DA}$ channel plays a critical role in the unique secretory profile of PRL secretion observed on the day of proestrus.

## Materials and Methods

### *Reagents*

Sources of reagents were as follows: trypsin (~270 U/mg of protein; Worthington, Freehold, NJ); DNase I, dopamine-HCl, valinomycin, verapamil, quinine-HCl, 4-AP, TEA, MgATP, 2 adenosine 3',5'-cyclic (cAMP) monophosphate, creatine phosphate, and creatine phosphokinase (Sigma, St. Louis, MO); culture media (Irvine, Santa Ana, CA); horse serum (HyClone, Logan, UT); ovine erythrocytes (Waltz Farms, Smithburg, MD); and Bio-Gel P-2 beads (Bio-Rad, Richmond, CA). Reagents for the RIA of rat PRL (rPRL) were provided by Dr. Albert Parlow through the National Pituitary Agency (National Institute of Diabetes and Digestive and Kidney Diseases [NIDDK]). Rabbit anti-rPRL for use in the plaque assay was generated in our laboratory and characterized as previously described *(14,44)*.

### *Anterior Pituitary Tissue and Cells*

Anterior pituitary cells were harvested from Sprague-Dawley-derived female rats obtained from Charles River (Wilmington, MA) and maintained on a 14-h:10-h light/dark cycle (lights on at 4:00 AM) with food and water available ad libitum. All animals were housed and cared for strictly in accordance with US Department of Agriculture regulations and the NIH Guide for the Care and Use of Laboratory Animals in a facility accredited by the American Association for the Accreditation of Laboratory Animal Care. Estrous cyclicity was determined by daily vaginal smears. Animals demonstrating at least two consecutive 4-day cycles were killed between 8:00 AM and 9:00 AM, and the anterior pituitary glands (with neurointermediate lobes removed) were rapidly dissected free. Pituitary cells were prepared as previously described *(44)*. Briefly, the anterior pituitary gland was minced in 2 mL of Hank's balanced salt solution (HBSS) and then incubated in HBSS containing

0.15% trypsin for 15 min at 37ºC. Following two washes with Hank's $Ca^{2+}$-, $Mg^{2+}$-free medium (Hank's CMF), the tissue fragments were mechanically dispersed by trituration in Hank's CMF containing 1 mg% DNase I, and the cells were separated from any remaining fragments by passing the suspension through a sterile 20-µm-pore nylon mesh. Cell yield was quantified using a hemocytometer, and viability was determined to always be >95% based on trypan blue exclusion. Cells for perifusions were transferred to 60-mm Petri dishes with 7 mL of serum-containing Dulbecco's minimum essential medium (DMES: 6% horse serum) and incubated on an orbital shaker (40 rpm) at 37ºC in 5% $CO_2$/95% air for 1–3 d. Cells to be used in electrophysiologic studies were immediately subjected to the RHPA to identify PRL-secreting cells as described subsequently.

### *Perifusion*

Dynamic PRL release was monitored using a perifusion system. The system consisted of a chamber constructed of a siliconized glass column (1 cm id) and two Teflon plungers each with a central bore (1 mm id). The surface of the bottom plunger was covered with nylon mesh (10-µm pores) to support the cell/Bio-Gel mixture, which filled the 1-$cm^3$ volume chamber. Inflow and outflow tubings (0.5 mm id Teflon) were connected to the top and bottom plungers, respectively, with flangeless Teflon fittings. Control and test solutions (prewarmed in a water bath) were pumped simultaneously to a switch complex placed just before the column. At the complex, each solution passed through a filter (P/N LFAA-35; Lee, Westbrook, CT) and then through a miniature electrically actuated three-way valve (P/N LFAA-1201618H; Lee). A remote panel switch activated both valves together, permitting either solution to enter the column. The other solution flowed to waste. This enabled minimal mixing of solutions as they moved through the tubing and into the column. Thus, the actual change of solution over the cells took place very quickly, in an almost stepwise manner, resulting in very accurate concentration exposures. The dead spaces between the valves and the column and from the column to the fraction collector were ~39 and ~99 µL, respectively. These volumes were a small fraction of the 1-mL samples collected; therefore, no correction for delays between solution changes and sample collection was required. The closed system enabled the entire column, as well as the solution reservoirs, to be immersed in a water bath to maintain temperature (37ºC). Two such systems were run simultaneously, proestrous cells in one and diestrous cells in the other, both perifused with control and test solutions from the same reservoirs. Perifusions of cells derived from rats in estrus were run separately at a later date. Therefore, those data are presented separately. In all experiments, because of the instability of DA at physiologic pH, DA solutions were made up no more than 10 min prior to application to cells.

Cells from a single pituitary gland were used in each column. Thus, each experiment represents one proestrous animal and one diestrous animal. Each experiment was repeated at least three times. After 1–3 d in culture, the cells were mixed with an inert matrix of polyacrylamide gel (Bio-Gel P2, 200–400 mesh) that had been preswollen overnight (0.25 g in 5 mL of 0.9% NaCl) and loaded into the chamber of the perifusion system. The perifusion medium was the same SES used in the electrophysiologic experiments. Flow rate was 0.5 mL/min, and the effluents were collected in 2-min fractions into tubes containing 100 µL of 2% bovine serum albumin (BSA) in phosphate-buffered saline (PBS). The cells were allowed to adjust to the perifusion conditions for 20–30 min before collections began. This equilibration period is not indicated in the figures, where the time scale begins with the first sample collected. Samples were stored at –20ºC until assayed for PRL. rPRL was determined on individual perifusion samples by homologous double-antibody RIAs. Values are expressed in terms of NIDDK rPRL-RP-3. All samples from an individual perifusion experiment (one proestrous prep and one diestrous prep) were included in a single assay. Perifusions of cells derived from rats in estrus were performed separately at a later date and the samples assayed in a separate RIA.

### *Reverse Hemolytic Plaque Assay*

RHPA, modified as described previously *(14,44)*, was used to identify lactotrophs for electrophysiology. Briefly, aliquots of dissociated pituitary cells, from those preparations used for perifusion, were plated together with an excess of protein A–coated erythrocytes on poly-L-lysine-coated glass cover slips in modified Cunningham chambers *(45)*. The chambers were placed in a 95% air, 5% $CO_2$ atmosphere at 37ºC for 45 min to allow cells to attach to the cover slip. Unattached cells and excess erythrocytes were rinsed away with DMEM (without phenol red) containing 0.1% BSA (DMEM-BSA). PRL antiserum (arPRL-86; final dilution, 1:200; *[44]*) was then introduced into the chambers and allowed to incubate for 60 min. Chambers were again rinsed, and areas of hemolysis (plaques) surrounding the PRL-secreting cells were initiated with serum complement. The source of complement was the serum harvested from the donor of the pituitary cells *(14)*. This autologous serum was used at a final concentration of 1:140 or 1:160 for 15–20 min. At the end of this incubation, the chambers were dismantled while submerged in DMEM containing 10% deactivated horse serum and 0.04 mg/mL of gentamycin (DMES), and cover slips with cells attached were transferred immediately to six-well plates containing DMES. Cells were maintained in culture for 1–3 d until used for patch clamp studies.

### *Patch Clamp Studies*

Whole-cell membrane potential and current recordings from plaque-identified lactotrophs were made according to the giga-ohm seal patch clamp technique *(46)*. Access resistances of the recording electrodes ranged from 4 to 6 MΩ. The standard intracellular solution comprised 130 m*M*

K-Asp, 20 m*M* KCl, 10 m*M* glucose, 10 m*M* HEPES, 2 m*M* MgATP, 2 m*M*, and a nucleotide-regenerating system composed of 20 m*M* creatine phosphate and 50 U/mL of creatine phosphokinase. Cells were bathed in SES comprising 145 m*M* NaCl, 5 m*M* KCl, 2 m*M* $CaCl_2$, 1 m*M* $MgCl_2$, 10 m*M* glucose, and 10 m*M* HEPES. All solutions were adjusted to pH 7.3 to 7.4 and 295–305 mos*M*.

Patch clamp experiments were performed at room temperature in a Lexan recording chamber mounted on the stage of a Nikon Diaphot inverted microscope. Recordings were made using an Axopatch 1B patch clamp (Axon). A cover slip with the plaque-identified pituitary cells attached comprised the floor of the recording chamber through which external solutions were continuously perfused during the experiment. Application of DA was accomplished by a U-tube device that could be positioned next to a cell to apply and withdraw the test solution rapidly while minimizing mechanical disturbance. Again, DA solutions were made up no more than 10 min prior to use. Whole-cell voltage responses under current clamp were recorded wideband (~50 kHz) on videotape using a digital audio processor interface sampling at 44 kHz (Sony PCM701). Whole-cell current responses under voltage clamp were sampled at 12-bit resolution by an A/D converter, and the data were stored on computer and analyzed with pCLAMP software (Axon).

## Acknowledgments

I am grateful to Negar Golesorki and Dmitri Eroshenko for their excellent technical assistance. This work was supported by National Institutes of Health grants DK-40336 and DK54966.

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