Cortisol rapidly suppresses intracellular calcium and voltage-gated calcium channel activity in prolactin cells of the tilapia (Oreochromis mossambicus)

Gregory N. Hyde,1 Andre P. Seale,2 E. Gordon Grau,2 and Russell J. Borski1
1Department of Zoology, North Carolina State University, Raleigh, North Carolina 27695; and 2Department of Zoology and Hawaii Institute of Marine Biology, University of Hawaii, Kaneohe, Hawaii 96744

Submitted 26 February 2003; accepted in final form 19 November 2003

Hyde, Gregory N., Andre P. Seale, E. Gordon Grau, and Russell J. Borski. Cortisol rapidly suppresses intracellular calcium and voltage-gated calcium channel activity in prolactin cells of the tilapia (Oreochromis mossambicus). Am J Physiol Endocrinol Metab 286: E626–E633, 2004. —Cortisol was previously shown to rapidly (10–20 min) reduce the release of prolactin (PRL) from pituitary glands of tilapia (Oreochromis mossambicus). This inhibition of PRL release by cortisol is accompanied by rapid reductions in 45Ca2+ and cAMP accumulation. Cortisol’s early actions occur through a protein synthesis-independent pathway and are mimicked by a membrane-impermeant analog. The signaling pathway that mediates rapid, non-genomic membrane effects of glucocorticoids is poorly understood. Using the advantageous characteristics of the teleost pituitary gland from which a nearly pure population of PRL cells can be isolated and incubated in defined medium, we examined whether cortisol rapidly reduces intracellular free calcium (Ca2+) and suppresses L-type voltage-gated ion channel activity in prolactin cells. Somatostatin, a peptide known to inhibit PRL release through a membrane receptor-coupled mechanism, similarly reduces Ca2+. Under depolarizing [K+]i, the L-type calcium channel agonist BAY K 8644, a factor known to delay the closing of L-type Ca2+ channels, stimulates PRL release in a concentration-dependent fashion (P < 0.01). Cortisol (and somatostatin) blocks BAY K 8644-induced PRL release (P < 0.01; 30 min), well within the time course over which its actions occur, independent of protein synthesis and at the level of the plasma membrane. Results indicate that cortisol inhibits tilapia PRL release through rapid reductions in Ca2+ that likely involve an attenuation of Ca2+ entry through L-type voltage-gated Ca2+ channels. These results provide further evidence that glucocorticoids rapidly modulate hormone secretion via a membrane-associated mechanism similar to that observed with the fast effects of peptides and neurotransmitters.

BAY K 8644; nongenomic actions; glucocorticoids; osmoregulation; nifedipine; L-type voltage-gated ion channels; teleost

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

function (26, 32), there are no reports to date that demonstrate nongenomic effects of glucocorticoids in lactotrophs other than the effect shown for tilapia (6, 9).

The present study was undertaken to determine whether cortisol reduces intracellular Ca\(^{2+}\) (Ca\(^{2+}\)) and voltage-gated Ca\(^{2+}\) channel activity in events that lead to reduced PRL release in tilapia. Microspectrofluorometry and the calcium-sensitive dye fura 2 were employed to measure real-time changes in Ca\(^{2+}\) in isolated PRL cells. This method, unlike \(^{45}\)Ca\(^{2+}\) influx studies (8), provides measures of Ca\(^{2+}\) that reflect mobilization from both extracellular and intracellular pools and the temporal resolution necessary for refining the rapid nature by which cortisol alters Ca\(^{2+}\) metabolism. Investigations were also designed to examine whether cortisol blocks PRL release induced by depolarizing [K\(^+\)] and the L-type Ca\(^{2+}\) channel agonist BAY K 8644 (BAYK), which, when used in conjunction, increase the probability that Ca\(^{2+}\) channels remain open (38). The actions of cortisol were compared with those of somatostatin (SRIF), a peptide known to work through membrane-associated mechanisms to rapidly alter PRL release (30, 41).

A principal impediment to the study of the function of a particular cell is the difficulty in isolating it within a morphologically complex tissue. To circumvent this problem, cell lines are often used to study stimulus-secretion coupling, but they may differ functionally from normal cells in their Ca\(^{2+}\) requirements, response to secretogogues, membrane receptor composition, and G-protein regulation (see Refs. 9 and 20). Our studies utilize the unique arrangement of the teleost pituitary, wherein a nearly pure population of PRL cells (95\%–99%) is segregated into a distinct region of the anterior pituitary, the rostral pars distalis (RPD) (20, 37). The RPD is easily isolated and can be studied in a completely defined, serum-free medium.

**MATERIALS AND METHODS**

**Static incubations.** Adult male tilapia (15–20 cm) were maintained in freshwater at a constant photoperiod (12:12-h light-dark) for ≥3 wk before all experiments. Fish were decapitated, and their pituitaries were removed and placed in hyperosmotic (360 mosmol/kgH\(_2\)O) medium. Medium consisted of a Krebs-Ringer bicarbonate solution (in mM: 2.35 KCl, 1.25 KH\(_2\)PO\(_4\), 25 NaHCO\(_3\), 1.4 MgSO\(_4\), 2.1 CaCl\(_2\), 140 NaCl) supplemented with glucose (0.5 mg/ml), L-glutamine (0.29 mg/ml), and a 2 ml/100 ml medium of 50 \(\mu\)M of hyperosmotic medium divided by medium + tissue in the incubation. Both PRLs are released at similar rates by those secretogogues examined to date, including osmotic pressure, cortisol, and SRIF (10). In research presented here, only the release of tPRL188 is shown, although both hormones responded similarly to experimental treatments.

For short-term static incubation studies, the RPDs of sexually immature male tilapia (10–12 cm long) were incubated under conditions identical to those described above, except that medium was changed every 30, 90, and 180 min. Tissues were sonicated in radioimmunounassay (RIA) buffer (0.01 M phosphate buffer, pH 7.3, containing 0.14 M NaCl, 1% BSA, 0.01% NaN\(_3\), and 0.1% Triton X-100) and kept frozen at –20°C. Medium and tissue PRL were quantified using a homologous RIA as previously described (3, 56), and hormone release is expressed as a percentage of the total amount of hormone in the incubations.

Ca\(^{2+}\) flux measurements. RPL cells were dissociated from RPD in phosphate-buffered saline (PBS: 0.5 M NaH\(_2\)PO\(_4\), 0.5 M Na\(_2\)HPO\(_4\), pH 7.25, adjusted to 360 mosmol/kgH\(_2\)O with NaCl) containing 0.125\% (wt/vol) trypsin for 45 min at room temperature. Cells were centrifuged (250 g, 5 min) and resuspended in 1 ml of PBS containing 0.125\% (wt/vol) trypsin inhibitor for 10 min. Cells were washed, resuspended in PBS alone, and then dispersed by 5–10 gentle passages through a plastic Pasteur pipette. After an additional rinse, cells were centrifuged and then resuspended in hyperosmotic medium. Prolactin cells were plated onto poly-L-lysine (0.1 mg/ml)-coated glass coverslips. Cells were incubated in hyperosmotic medium for ≥12 h before determination of Ca\(^{2+}\).

The experiments measuring Ca\(^{2+}\) were conducted at the Hawaii Institute of Marine Biology (Oahu, HI). PRL cells were loaded with 5 \(\mu\)M fura 2-AM, the membrane-permeable acetoxyethyl ester derivative of fura 2 (Molecular Probes, Eugene, OR), for 30 min at 28 ± 1°C. The fura 2-AM was solubilized in anhydrous dimethyl sulfoxide (Aldrich Chemical, Milwaukee, WI) to a concentration of 5 mM before its final dilution to 5 \(\mu\)M. The coverslip, plated with PRL cells, was then mounted onto a perfusion chamber. The perfusion chamber was placed on a microscope stage, and cells were continuously exposed to control medium before the start of the experiment.

Different experimental media were maintained in plastic 60-ml syringes connected to an eight-point manifold perfusate selector (Hamilton, Reno, NV) via one-way stopcocks and polyethylene tubing. The rate (0.5 ml/min) of perfusion through the chamber was maintained by keeping the height of the syringes and volume of all solutions in the syringes constant throughout the experiment. In experiments where a steroid hormone or BAYK was tested, all treatments contained the appropriate solvent (0.01% ethanol).

Simultaneous measurements of intensities of free fura 2 (380 nm) and fura 2 bound to Ca\(^{2+}\) (340 nm) were made with a dual-excitation spectrophotometer (ARCM-MIC-N; Spex Industries, Edison, NJ) coupled with a Nikon Eclipse TMD microscope (×40 oil immersion fluorescence objective, Nikon) and proprietary software (Spex Industries). Excitation light alternated between 340 and 380 nm (narrow bandpass filters, SPEX) by a computer-controlled

**AJP-Endocrinol Metab • VOL 286 • APRIL 2004 • www.ajpendo.org**
chopper mirror. Fluorescent emission (500 nm) intensity of fura 2 was detected from PRL cells every 2 s by a photomultiplier tube. All data are expressed as relative intensity, the ratio of fura 2 fluorescence excited at 340 nm to that excited by 380 nm from which background was subtracted. Shifts in this ratio (340/380) result directly from changes in Ca\(^{2+}\) (23).

Statistical analysis. Statistical differences in PRL release were analyzed using a one-way analysis of variance (ANOVA) test followed by the Fisher protected least significant difference test for predetermined comparisons of multiple treatments. All values for PRL release were expressed as means ± SE. Statistical differences in free Ca\(^{2+}\) were analyzed using repeated-measures one-way ANOVA followed by Tukey’s procedure for pairwise comparisons.

RESULTS

Before Ca\(^{2+}\) measurements, initial experiments were conducted to verify that dispersed PRL cells were viable and continued to respond to osmotic pressure, cortisol, and SRIF in a manner similar to the cells of intact tissues. The Trypan blue exclusion test showed that the viability of individual cells exceeded 90%. PRL release from dispersed PRL cells was enhanced by 95% during exposure to reductions in medium osmolality. A concentration of 200 nM cortisol and 300 nM SRIF reduced PRL release under hyposmotic stimulation by 32 and 35%, respectively (P < 0.05; data not shown). The similar response to these PRL regulators between dispersed cells and cells within the intact RPD (7, 10, 46) indicates that receptors and other membrane components central to hormone secretion remain functional and that the dispersion process does not interfere with the mechanisms of action of either cortisol or SRIF.

As observed with PRL release (7), Ca\(^{2+}\) remained low during exposure to hyposmotic medium. Cortisol, at a concentration (200 nM) measured in plasma of tilapia (8, 47), reduced Ca\(^{2+}\) within 60 s during exposure to hyposmotic medium (P < 0.05). The effect remained highly significant throughout the duration of cortisol exposure (P < 0.001; Fig. 1 and Table 1). Removal of cortisol from the treatment medium resulted in an equally rapid and full recovery of Ca\(^{2+}\), which returned to baseline hyperosmotic levels within 30 s (P < 0.01; Fig. 1 and Table 1). Reductions in medium osmotic pressure increase Ca\(^{2+}\) (21) and PRL release (19). In the present study, exposure to hyposmotic medium increases Ca\(^{2+}\) and, as seen under basal hyposmotic conditions, cortisol caused an immediate decline in the hyposmotic-induced rise in Ca\(^{2+}\), which became significant within 90 s (P < 0.001; Fig. 1 and Table 1). The recovery phase was also immediate and reached significance within 120 s (P < 0.05; Fig. 1 and Table 1).

SRIF, which inhibits \(^{45}\)Ca\(^{2+}\) and cAMP accumulation in tilapia RPD (20, 24), also rapidly reduces Ca\(^{2+}\) (Fig. 2 and Table 1). This reduction (P < 0.001) within 60 s is sustained over the course of the treatment, followed by an immediate recovery (P < 0.001) within 90 s) of Ca\(^{2+}\) levels upon removal of the hormone (Fig. 2 and Table 1).

To examine whether cortisol alters PRL release through regulation of L-type voltage-gated Ca\(^{2+}\) channel activity, we first determined the concentrations of BAYK that augment PRL release from the tilapia RPD. BAYK in the presence of depolarizing [K\(^{+}\)] increases PRL release in a dose-related manner (P < 0.001), reaching significance at 10 μM (P < 0.01; Fig. 3A). The level of PRL release achieved with BAYK and depolarizing [K\(^{+}\)] equaled that induced by hyposmotic medium. To verify that BAYK specifically targets L-type Ca\(^{2+}\) channels on tilapia PRL cells, we examined whether nifedipine, an L-type channel antagonist, blocked BAYK-induced PRL release. Nifedipine at concentrations of 10 and 50 μM completely overcame BAYK-evoked PRL release (P < 0.01; Fig. 3B).

Cortisol significantly lowered PRL release stimulated by the combined depolarizing [K\(^{+}\)] and 10 μM BAYK treatment (Fig. 4; P < 0.05, P < 0.01) during static incubations. The degree of PRL release inhibition increased with a greater concentration of cortisol (Fig. 4B; P < 0.05). The degree to which cortisol inhibits PRL release is similar to that observed when tissues were exposed to the peptide SRIF (P < 0.01; Fig. 3B) and to nifedipine (Fig. 3).

Cortisol reduces BAYK-evoked PRL release in a manner that is similar to that produced by SRIF over sustained incubations. It remained to be determined whether the steroid was effective within the time frame (≤4 h) over which it has been shown to act on PRL release in a nongenomic fashion at the level of the membrane (10). Figure 5 shows that cortisol reduces BAYK-evoked PRL release after 30 min (P < 0.05), 90 min (P = 0.0553), and 240 min (P < 0.01) of exposure.
Table 1. Period of onset and recovery of Ca\(^{2+}\) following exposure and withdrawal of cortisol (200 nM) and somatostatin (300 nM), respectively, during incubations in hyperosmotic (360 mOsmol) and hyposmotic (300 mOsmol) media

<table>
<thead>
<tr>
<th>Time to Onset and Recovery of Cortisol’s Effects</th>
<th>Time to Onset and Recovery of SRIF’s Effects</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatment</td>
<td>Time, s</td>
</tr>
<tr>
<td>Baseline</td>
<td>360</td>
</tr>
<tr>
<td>Onset</td>
<td>360 + F</td>
</tr>
<tr>
<td>Recovery</td>
<td>360</td>
</tr>
<tr>
<td>Baseline</td>
<td>290</td>
</tr>
<tr>
<td>Onset</td>
<td>290 + F</td>
</tr>
<tr>
<td>Recovery</td>
<td>290</td>
</tr>
</tbody>
</table>

Values are means ± SE. F, cortisol; SRIF, somatostatin. Intracellular Ca\(^{2+}\) (Ca\(^{2+}\)) is expressed as relative intensity of fura 2 fluorescence (340/380 nm) measured at 10-s increments for the 5 separate experiments displayed in Figs. 1B and 2. Ca\(^{2+}\) is presented as a percentage of control values represented by the original 300-s exposure to hyperosmotic medium (time 0 s). *P < 0.05, †P < 0.001 vs. baseline (time 0) at each respective 360 and 290 mosmol/kgH\(_2\)O exposure. 

**DISCUSSION**

These studies clearly show that cortisol, a steroid normally thought to act slowly on target cells through alterations in gene expression, reduces Ca\(^{2+}\) within seconds in isolated PRL cells. This fall in Ca\(^{2+}\) occurs well within the time resolution with which cortisol reduces PRL release during perfusion incubations (10–20 min) (8). This, along with prior evidence that the response of PRL release to cortisol is dependent on reductions in cytosolic Ca\(^{2+}\) (8), suggests that the ion is a central mediator of cortisol’s rapid inhibition of PRL release. Insofar as cholesterol (the precursor to all steroids) failed to alter Ca\(^{2+}\) (data not shown) or PRL release (8), the actions of cortisol on these parameters does not appear to result from a nonspecific perturbation or interaction of a steroid-like molecule with the plasma membrane. Rather, the PRL secretory response to cortisol appears specific to this seawater-adapting hormone. 17β-Estradiol and testosterone stimulate PRL release, whereas cortisone (cortisol’s conversion product; data not shown), aldosterone, 11-deoxycorticosterone, and the primary fish and mammalian progestins were without effect (8).

In the classical model of steroid action, steroids diffuse through the plasma membrane and bind cytosolic (glucocorticoid) or nuclear receptors. The activated steroid-receptor complex is then translocated to the DNA, where it binds palindromic sequences in conjunction with other transactivating proteins to alter gene transcription and subsequent protein synthesis (58). Through this pathway, steroids typically produce cell responses with latency periods ranging from hours to days (6, 34). Even after elimination of the steroid, a considerable lag period may exist before the cell response subsides. This lag period, which can last for hours, is needed to reduce the accumulation of newly synthesized proteins that was required for the initial cell response (49). In studies here, Ca\(^{2+}\) were reduced for as long as the cells were exposed to cortisol but promptly returned to the control level once the steroid was removed. The rapid onset and comparably rapid recovery of Ca\(^{2+}\) following the initial application and withdrawal of cortisol, respectively, support the idea that this steroid is not working through a genomically mediated process but may instead act via membrane-associated mechanisms. SRIF’s mode of action has been well characterized in mammalian pituitary cell systems (32, 41) but to a lesser degree in mammalian lactotrophs and tilapia PRL cells. Through the cAMP-independent pathway, SRIF binds one or more G protein-coupled membrane receptor(s) to increase K\(^+\) conductance, which secondarily reduces Ca\(^{2+}\) influx through voltage-sensitive channels. The subsequent decline in Ca\(^{2+}\) results in an inhibition of hormone release (11, 29). In agreement with studies on rat anterior pituitary cells, somatotrophs as well as PRL- and ACTH-secreting neoplastic cells (33, 36, 44, 48), we found that SRIF, like cortisol, reduces Ca\(^{2+}\) within seconds.

![Fig. 2. Effect of somatostatin (SRIF, 300 nM) on Ca\(^{2+}\) levels, expressed as the relative intensity of fura 2 fluorescence (340/380 nm) measured simultaneously from 25 prolactin cells during exposure to hyperosmotic (360 mosmol/kgH\(_2\)O) medium. Values represent the average Ca\(^{2+}\) trace from 5 separate experiments (mean ± SE). Before calculation of the average response of 5 different experiments, changes in Ca\(^{2+}\) from each experiment were first expressed as percentage of control values represented by the original 300-s exposure to hyperosmotic medium.](image-url)
and that this effect is immediately reversible upon removal of the peptide (Fig. 2 and Table 1).

Changes in Ca\textsuperscript{2+} may result from an increase or decrease in the release of Ca\textsuperscript{2+} from intracellular Ca\textsuperscript{2+} pools, or the influx of extracellular Ca\textsuperscript{2+} through various channel types, including L-type voltage-gated Ca\textsuperscript{2+} channels. Activation of these channels by depolarizing [K\textsuperscript{+}] rapidly stimulates PRL release and \textsuperscript{45}Ca\textsuperscript{2+} entry into tilapia RPD (22, 45). By stimulating the opening of L-type channels with depolarizing [K\textsuperscript{+}] and delaying their closing with BAYK, additional effects on PRL release were seen in this study: increased PRL release over that observed with depolarizing [K\textsuperscript{+}] alone. The ability of nifedipine, a specific antagonist of L-type Ca\textsuperscript{2+} channels, to block BAYK-induced PRL release from tilapia RPD provides further evidence that L-type voltage-gated Ca\textsuperscript{2+} channels were effectively targeted in these studies. Collectively, these studies and others (54) suggest that the PRL cells of teleost fish, like those of mammals (5, 32), are excitable with spontaneous action potentials playing an integral role to the secretory process, one which likely involves an influx of Ca\textsuperscript{2+} through voltage-sensitive channels.

Cortisol reduced BAYK-stimulated PRL release from tilapia RPD in as little as 30 min. In a previous study, cortisol was also shown to reduce \textsuperscript{45}Ca\textsuperscript{2+} accumulation and PRL release (8) within a similar time frame. These results suggest that cortisol inhibits PRL release by rapidly reducing Ca\textsuperscript{2+} via an inhibition of L-type Ca\textsuperscript{2+} channels and thereby an influx of extracellular Ca\textsuperscript{2+}. This is supported indirectly through studies that show
Several lines of evidence suggest that the early responses in Ca^{2+} and PRL release elicited by cortisol in tilapia are non-genomic and likely occur at the membrane. First, the time course over which cortisol alters BAYK-evoked PRL release (minutes) and Ca^{2+} (seconds) in particular is too short to encompass all steps in the intracellular signaling pathway leading to the synthesis of new proteins essential to a genomically mediated, classical steroid response (6, 34). Second, to a large degree, cortisol mimics the actions of SRIF and rapidly modulates the same signaling molecules (e.g., Ca^{2+} and cAMP) that transduce membrane receptor-coupled responses (9). Third, cycloheximide at concentrations that completely block de novo protein synthesis in tilapia RPDs is ineffective in overcoming cortisol’s inhibition of PRL release within at least the first 4 h of steroid application (10). Finally, membrane-impermeable cortisol (cortisol 21-hemisuccinate-BSA) inhibited PRL release in a dose-dependent fashion within this time period (10). Although evidence points to the presence of a putative membrane receptor on PRL cells, future studies are required to determine its identity and pharmacological signature. Evidence to date suggests that glucocorticoids may elicit rapid effects through various types of membrane-resident proteins. These include membrane corticosteroid-binding globulins (52), G protein-coupled receptor(s) distinct from the intracellular type (39), intracellular-like receptors (12), or perhaps other types (6, 31).

The concentration of cortisol used in this study falls within the physiological levels measured in tilapia and is below that typically found in stressed animals. During an initial 4-min nonstressed sampling period, plasma cortisol levels range from 20 to 50 nM in freshwater and 120 to 220 nM in seawater fish (8, 47). Over a more sustained sampling time course (5–9 min) that likely incorporates responses to stress (2), circulating cortisol levels rise two- to threefold and remain elevated in seawater (175–470 nM) compared with freshwater (100–150 nM) fish (47). It is well established that cortisol is an important seawater-osmoregulatory hormone and PRL is critical for freshwater adaptation in many euryhaline teleosts including the tilapia. Considering the opposing osmoregulatory actions of PRL and cortisol, we postulate that cortisol may serve the dual role of rapidly reducing the secretion of PRL, a seawater-antagonistic hormone, while itself activating osmoregulatory processes critical to seawater adaptation (53). It is also possible that cortisol may limit the rapid rise in circulating PRL that accompanies a stress response shown for a closely related tilapia species (O. niloticus (2)).

Plasma glucocorticoid levels can rise dramatically within minutes following a stress event or osmotic perturbation. Therefore, it is not surprising that this class of steroids may exert rapid as well as delayed effects on target tissues. We show that cortisol rapidly attenuates PRL release through a nongenomic mechanism that involves interactions with the plasma membrane and modulation of the Ca^{2+} messenger systems. Evidence is provided that suggests that the inhibition of PRL release by cortisol, like that of the fast-acting peptide hormones (SRIF), is mediated through a reduction of Ca^{2+} influx through L-type voltage-gated Ca^{2+} channels. Considering the strong logistic and heuristic advantages of the tilapia PRL cell model, it should provide a very useful system for discerning the cellular mechanisms underlying rapid actions of a “stress” hormone
known to influence and possibly impair several physiological processes, including behavior, reproduction, immune function, and osmoregulation.

ACKNOWLEDGMENTS

We thank Dr. Shira Fruchtman, Dr. Gregory Weber, and Wellington Tsai for valuable assistance and advice.

GRANTS

These studies were supported by National Science Foundation Grants IBN-9810326 and IBN-0215205 (R. J. Borski) and by US Department of Agriculture Grant 983506644, University of Hawaii Sea Grant NA68RG0041, and National Science Foundation Grant IBN-0133714 (E. G. Grau).

REFERENCES

Somatostatin blocks the potentiation of TRH-induced TSH secretion from perifused pituitary fragments and the change in intracellular calcium concentrations from dispersed pituitary cells elicited by prepro-TRH (PS4) or by tri-iodothyronine. J Mol Endocrinol 19: 87–97, 1997.

Russomarian F and Duval D. Dexamethasone-induced inhibition of prostaglandin production does not result from a direct action on phospholipase activities but is mediated through a steroid-inducible factor. Biochim Biophys Acta 712: 177–185, 1982.


