Ionotropic glutamate receptor activation increases intracellular calcium in prolactin-releasing cells of the adenohypophysis

Frederick P. Bellinger, Bradley K. Fox, Wing Yan Chan, Lori K. Davis, Marilou A. Andres, Tetsuya Hirano, E. Gordon Grau, and Ian M. Cooke. Ionotropic glutamate receptor activation increases intracellular calcium in prolactin-releasing cells of the adenohypophysis. Am J Physiol Endocrinol Metab 291: E1188–E1196, 2006. First published July 5, 2006; doi:10.1152/ajpendo.00207.2005—Endocrine cells of the anterior pituitary are controlled by the central nervous system through hormonal interactions and are not believed to receive direct synaptic connections from the brain. Studies suggest that some pituitary cells may be modulated by the neurotransmitter glutamate (5, 16). We investigated prolactin (PRL)-releasing cells of the anterior pituitary of a euryhaline fish, the tilapia (Oreochromis mossambicus), for the presence of possible glutamate receptors (GluRs). Fura-2 imaging addressed the ability of glutamate to increase intracellular calcium. We observed a dose-dependent increase in intracellular calcium with transient perfusion (1–2 min) of glutamate (10 nM to 1 mM) in two-thirds of imaged cells. This increase was attenuated by the ionotropic GluR antagonist kynurenic acid (0.5–1.0 mM). The increase was also blocked or attenuated by antagonists of L-type voltage-gated calcium channels. The GluR agonist α-amino-3-hydroxy-5-methylisoxazole propionic acid (AMPA; 100 μM) produced intracellular calcium increases that were reversibly blocked by the selective AMPA antagonist 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX). In contrast, the selective agonist N-methyl-D-aspartate (NMDA; 100 μM to 1 mM in magnesium-free solution with 10 μM glycine) had no effect on intracellular calcium. Radioimmunoassays demonstrated that glutamate stimulated PRL release. CNQX but not the NMDA receptor antagonist 2-amino-5-phosphonovaleric acid blocked this release. Antibodies for mammalian AMPA- and NMDA-type GluR produced a similar punctate immunoreactivity in the periphery of PRL cells. However, the NMDA antibody recognized a protein of a different molecular mass in PRL cells compared with brain cells. These results clearly indicate the presence of GluRs on tilapia PRL cells that can stimulate PRL release.

pituitary; endocrine; osmoregulation; calcium imaging; radioimmunoassay

The anterior pituitary, or adenohypophysis, is derived from nonneuronal epithelial tissue that fuses during embryonic development with the neural crest-derived neurohypophysis (posterior pituitary) (17). Cells of the adenohypophysis release a variety of peptide hormones involved in growth, stress, metabolism, and sexual differentiation and function. In mammals, a complex portal system of capillaries carries blood and signaling molecules between the neurohypophysis and adenohypophysis. In teleost fish, this system is limited to a thin wall of capillaries between the anterior and intermediate lobes. The portal system brings hormones and releasing factors to cells of the anterior pituitary from neurosecretory cells in the hypothalamus. In addition, it carries hormones released from pituitary cells to the circulation.

The major excitatory neurotransmitter in the vertebrate central nervous system is the amino acid glutamate. Recent research suggests that many nonneuronal cells also have glutamate receptors (16). Although glutamatergic innervation of endocrine cells of the anterior pituitary has not been described, growing evidence suggests that glutamate can influence hormone release from these cells. Studies in rats suggest that glutamate induces secretion of prolactin (PRL) by acting through N-methyl-D-aspartate (NMDA) receptors (20, 26). However, most evidence is indirect (see DISCUSSION), and few studies at the cellular level have been done. Glutamate signaling could represent a means for modulation of the anterior pituitary by neurons of the hypothalamus.

The euryhaline fish tilapia (Oreochromis mossambicus), which can adapt quickly between saltwater and freshwater conditions, provides an ideal model for the study of osmoreception (13). An internal decrease in salt concentrations, that is, a decrease in interstitial fluid osmolality, induces a rapid release of the peptide hormone PRL, which acts on gill and other epithelial cells to stimulate uptake of salts from the environment (21). PRL-secreting cells make up 95–99% of cells in a lobe of the anterior pituitary called the rostral pars distalis (RPD) (17). Thus the tilapia PRL cells can be easily dissected and cultured to permit study of their osmoreceptor and secretory mechanisms in vitro.

Isolated PRL cells in culture respond appropriately to changes in osmolality (13). Recent work using this model has demonstrated that changes in cell volume stimulate putative stretch-activated receptors, allowing calcium to enter the cell and trigger PRL release (28, 29). In the present study, we examined PRL-releasing cells of the tilapia for the activity of possible glutamate receptors. The results clearly indicate the presence of glutamate receptors that can activate voltage-gated calcium channels and stimulate the release of PRL.

MATERIALS AND METHODS

Fish. Tilapia (O. mossambicus) were raised and maintained at the Hawaii Institute of Marine Biology. They were raised in 5,000-liter tanks containing fresh water at 22–26°C under natural light and fed twice daily with Purina Trout Chow (~2% of body wt per day). All

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experimental procedures were preapproved by the Institutional Animal Care and Use Committee, University of Hawaii.

Cell culture. For calcium imaging and immunohistochemistry, the RPDs were dissected from tilapia pituitaries as described by Nishioka et al. (24) and placed in 0.1% trypsin (Sigma T4799, St. Louis, MO) in dissociation medium consisting of the following (in mM): sucrose 260, KCl 2.35, HEPES 10, glucose 5, adjusted to 330 mosmol/kgH2O with sucrose and pH 7.4 with KOH. Following a 45-min incubation, RPDs were rinsed five times with PBS and then incubated for 5 min in dissociation medium containing 0.1% trypsin inhibitor (Sigma T-9128). RPDs were again rinsed five times in PBS and dissociated mechanically by gentle trituration. Cells were recovered by a 5-min centrifugation using a desktop swinging bucket centrifuge at ~250 g. Recovered cells were resuspended in culture medium and plated on 22-mm poly-L-lysine-coated coverslips and kept in 3 ml of culture medium at 28°C. Culture medium contained the following (in mM): NaCl 133, KCl 3.6, MgSO4 1.4, NaHCO3 2.5, CaCl2 1.2, HEPES 25, supplemented with minimal essential medium (MEM) amino acid solution (Gibco/Invitrogen, Carlsbad, CA; diluted to 1× from 50× stock), adjusted to pH 7.4 with NaOH and 330 mosmol/kgH2O with NaCl. All media were adjusted to 330 mosmol/kgH2O, the measured average blood osmolality of tilapia in brackish water (36), since hyposmotic solution (~300) has a maximal basal release of PRL, preventing further increases, and hyperosmotic solution (~355) inhibits release (13). As previous studies indicated (17), the RPD contains up to 99% PRL cells; we define “PRL cells” in this study as all cells cultured from RPD.

Cultures used for radioimmunoassay were prepared from pituitaries collected aseptically in isosmotic medium (Kreb’s bicarbonate-Ringer solution, 330 mosmol/kgH2O, pH 7.4) as described by Wigham et al. (35), supplemented with penicillin (100 IU/ml), streptomycin (0.1 mg/ml), and nystatin (250 IU/ml, Sigma). RPDs were dissected, pooled, diced with a sterile razor, and trypsinized for 1 h at room temperature in 2.5 ml of trypsin-EDTA solution (0.25% trypsin + 0.002% EDTA in PBS, pH 7.4). Tissues were frequently aspirated through a pipette during trypsinization to promote dissociation of cells. The process was terminated by the addition of 0.5 ml (20%) fetal bovine serum (Sigma). Cells were counted on a hemocytometer under a light microscope, and their viability was determined by Trypan blue exclusion. Cells were then plated at a density of 1.0 × 105 cells/well into a 96-well plate (Falcon, Primaria 96; Becton Dickinson, Franklin Lakes, NJ) at a volume of 200 μl/well of isosmotic medium supplemented with 10% fetal bovine serum. The cells were preincubated overnight at 28°C under a humidified atmosphere of 95% O2 and 5% CO2.

Calcium imaging. Cells were loaded with fura-2 AM (0.5 μg/ml; Molecular Probes, Eugene, OR) for 2 h before imaging. Coverslips with adhering cell cultures were placed in an imaging chamber (~75 μl) and perfused with culture medium at a rate of 2–5 ml/min. Agonists and antagonists for glutamate receptors were added to culture medium in separate reservoirs and applied by manually changing the perfusion medium. Ratiometric calcium imaging was performed by taking images at 340- and 380-nm wavelengths at 10-s intervals with an intensified charge-coupled device camera interfaced with an inverted microscope. Cell image intensities were analyzed online using Universal Imaging software. Up to 30 cells within the field of view were imaged and provided data referred to as a single experiment. Averages were calculated for cells from the same coverslip. Each type of experiment was repeated at least three times to validate the results. A repeated-measures ANOVA design was used to indicate significant changes from baseline or control values using Prism software (GraphPad, San Diego, CA).

Approximate internal calcium concentrations were calculated as described by others (14), using the dissociation constant (Kd) value of 466 determined for goldfish endocrine cells (18). Minimum and maximum fluorescence (Fmin and Fmax, respectively) and ratio (Rmin and Rmax, respectively) values were determined by measuring the fura-2 signal in a series of known calcium concentrations varying from 0 to 1,305 nM (Molecular Probes). From these calibration solutions, we estimate for the conditions of these experiments that a 340- to 380-nm ratio of 0.5 would indicate an intracellular calcium concentration of ~175 nM, a ratio of 1 would indicate 350 nM, a ratio of 2 would indicate 600 nM, and a ratio of 4 would indicate 1 μM. We found PRL cells to have baseline internal calcium concentrations of 231 ± 7 nM (mean ± SE). Because these calculations do not take into account many factors affecting cell cytoplasm measurements (11, 15), the concentration values were used only to calculate percent changes in internal calcium, and only ratio values are reported here. Data are presented as ratios of single cells or averages of responding cells from a single experiment.

A solution with high KCl (55 mM) was added at the end of every experiment as a positive control. “Dead space” in the tubing between the solution switching valve and the recording chamber caused delays in responses of ~1 min. The latency between onset of KCl perfusion and the onset of the subsequent calcium response was measured for each experiment and used to correct the lag times for arrival of solution at the cells.

Radioimmunoassay. PRL released in the medium was estimated by homologous radioimmunoassay (1), as modified by Yada et al. (36). The tilapia pituitary secretes two distinct PRL molecules, PRL177 and PRL185, that are encoded by separate genes (37). Since we found no significant difference in response to various treatments between the two forms of PRL (28), only PRL185 release was examined.

Before each experiment, PRL cells were washed once with 200 μl of serum-free medium and allowed to acclimate for 1 h. A final 200-μl volume was added containing either L-glutamate (1 mM, Sigma), glutamate with the glutamate blocker 2-amino-5-phosphonovoleric acid (APV; 25 μM), glutamate (1 mM) with the glutamate blocker 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX; 10 μM), glutamate (1 mM) with both APV (25 μM) and CNQX (10 μM), medium containing a depolarizing concentration of KCl (55 mM KCl), prepared by adding 55 mM KCl and subtracting the osmotically equivalent amount of NaCl, or control medium without treatment. APV and CNQX were purchased from Tocris Biosciences (Eastville, MO). The incubated medium was removed after 15 min. Medium samples were stored at –20°C for further analyses of PRL release by homologous radioimmunoassay. All treatments were tested in sextuplicate, and the experiments were repeated four times. PRL release was expressed as percent control (n = 4 replicates). Replica experiments were pooled before statistical comparisons with one-way analysis of variance (ANOVA) followed by Dunnett’s multiple comparison post hoc test for differences from the untreated control, using Prism software.

Immunohistochemistry. Newly plated cells were fixed in 4% paraformaldehyde at room temperature for 4 h. After three washes in phosphate buffer (PB) containing 0.2% Triton X-100, cells were blocked using 0.1% BSA in PBS. We have found that greater amounts of mammalian albumin or use of mammalian serum only increased the background for teleost cell immunohistochemistry and thus were not used for blocking or washes. Primary antibody was added overnight [anti-glutamate receptor (GlurB)2/3, 1:1,000; anti-NR1 (NR1 is an NMDA receptor subunit), 1:1,000, purchased from Chemicon; and anti-PRL185, 1:10,000, described previously (1)]. All primary antibodies were from rabbit serum. The primary antibody was omitted in some cultures as a negative control, and cells cultured from fish cerebellum or olfactory bulb were used as positive controls (data not shown). After five washes in PB with 0.1% Triton X-100, cells were labeled with secondary anti-rabbit antibody conjugated to Alexa488 or Alexa594 for 4 h at room temperature. Following an additional four washes, coverslips with labeled cells were mounted on slides using Vectashield (Molecular Probes) and viewed under an epifluorescence or confocal microscope. Fluorescent images under low power (5–10×) epifluorescence with an Olympus BX51 microscope were compared with corresponding differential interference contrast (DIC) images to determine the percentage of immunolabeled cells. Confocal...
images between 1–2 µm taken serially through the z-axis with a Bio-Rad MRC 1024 confocal microscope were used to determine the cellular location of immunoreactivity.

Western blot analysis. Whole brains and RPDs were homogenized in lysis buffer (20 mM Tris·HCl, pH 7.4; 10% sucrose; 1% Triton-X; 1 mM phenylmethylsulphonyl fluoride; 0.1% aprotinin; all from Sigma) and centrifuged at 10,000 g for 10 min at 4°C. Supernatants were removed and stored at −20°C until use. Protein concentrations were quantified using a Bradford protein assay (Bio-Rad, Hercules, CA).

SDS-PAGE was performed in a precast Tris·HCl gradient gel with 4–15% gradient of total acrylamide (Bio-Rad). Protein extracts were heated to 95°C for 5 min in the presence of 2.5% 2-mercaptoethanol in 4× loading buffer (10% glycerol, 2% SDS, 0.75% Tris·OH, 0.005% bromophenol blue). Gels were run at 100 V constant for 2 h and then placed in transfer buffer (0.58% Tris·OH, 0.29% glycine, 0.38% SDS, 20% methanol). Proteins were transferred to nitrocellulose membranes (Bio-Rad) using an electrotransfer apparatus (Invitrogen, Carlsbad, CA). Post-transfer, the membranes were washed for 1 min in Tris-buffered saline solution (TBS; 20 mM Tris·OH, 500 mM NaCl, pH 7.5) with 0.05% Tween 20 (TBST) and then placed in blocking solution (5% milk in TBS) for 2 h. Membranes were cut and incubated overnight in blocking solution with primary antibodies at the following titers: anti-GluR2/3, 1:100, and anti-NR1, 1:50. Subsequently, the membranes were washed with TBST three times for 10 min. Membranes were incubated in peroxidase-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch, West Grove, PA) in TBST for 2 h. Membranes were washed two times with TBST and once with TBS alone. Antibody binding was detected with an enhanced chemiluminescence Western blotting analysis system (Amersham, Piscataway, NJ).

RESULTS

L-Glutamate increases intracellular calcium in PRL cells. Application of L-glutamate by perfusion produced a rapid and robust increase in intracellular calcium, immediate in onset, and peaking in 30–60 s (Fig. 1A). Onset of the decay immediately followed the peak response before commencement of glutamate washout (Figs. 1 and 2). Decay from the peak response was typically much slower than the initial response, taking up to 5 min for complete recovery to baseline calcium concentrations, although response kinetics varied between cells. About 58% of PRL cells responded to glutamate (determined from 5 separate experiments with 36 of 62 responsive cells). Responses were significantly reduced by 0.5–1 mM kynurenic acid, a selective antagonist for ionotropic GluRs, to 37 ± 11% (mean ± SE) of peak glutamate responses (19 cells, P < 0.05; Fig. 1A). Glutamate responses were also reduced by omitting calcium from the external medium (data not shown); however, small responses were still detectable under these conditions. Responses were dose dependent, with a calculated EC50 of 7.2 µM (Fig. 1B). Calcium increases were detectable in individual cells at glutamate concentrations as low as 1 nM, although mean calcium responses were not significantly above baseline values for concentrations <1 µM.

GluR activation in PRL cells activates voltage-gated calcium channels. Intracellular calcium increases in response to glutamate were blocked or greatly reduced with co-application of 50 µM cadmium chloride, a general calcium channel antagonist, to 9 ± 8% of peak glutamate responses in 31 cells from three separate experiments, (Fig. 2A). This concentration of cadmium attenuated most of the calcium increase induced by medium with 55 mM KCl (Fig. 2B) to 17 ± 11% of peak responses in 19 cells from three experiments. The L-type voltage-gated calcium channel (VGCC) antagonist nifedipine (10 µM) significantly reduced the glutamate-mediated intracellular calcium increases to 18 ± 6% of peak glutamate responses (18/18 cells from 4 separate experiments, P < 0.05; Fig. 2C). Another L-type channel antagonist, nimodipine (2 µM), also significantly attenuated the glutamate-mediated calcium increase (34 ± 9% of peak responses, 15/15 cells from 3
experiments, $P < 0.05$; Fig. 2D). These results indicate that activation of GluR leads to the opening of VGCCs by depolarizing the cell membrane.

$\alpha$-Amino-3-hydroxy-5-methylisoxazole propionic acid but not NMDA receptors increase intracellular calcium in PRL cells. The selective GluR agonist $\alpha$-amino-3-hydroxy-5-methylisoxazole propionic acid (AMPA; 100 $\mu$M) produced a large increase in intracellular calcium with an onset and time course similar to that of glutamate (Fig. 3). Onset was immediate, increasing rapidly to a peak signal within 1 min. Decay began before the end of AMPA perfusion and required several min. Approximately 63% of AMPA-treated PRL cells responded (33 of 52 cells from 4 experiments); both glutamate and AMPA application produced similar results in cells where both agonists were applied. Kynurenic acid blocked AMPA responses by 32–100% in 45% of responding cells (10 of 23) and reduced responses in others by 30–60%. All AMPA responses were completely blocked by the AMPA receptor antagonist CNQX ($P < 0.01$). CNQX also blocked responses to 1 mM glutamate in $\sim$70% of responding cells (10 of 14) and reduced remaining responses by at least 50%. AMPA responses were absent in calcium-free medium and reversibly blocked with 50 $\mu$M cadmium chloride (data not shown).

The selective GluR agonist NMDA did not produce any increase in intracellular calcium at concentrations as high as 1 mM (222 cells from 11 separate experiments) (Fig. 4A). Even when magnesium was omitted from the extracellular medium and 10 $\mu$M glycine included, NMDA failed to produce any measurable response. The NMDA receptor antagonist APV had a minimal, nonsignificant effect on glutamate-induced calcium increases (data not shown), with no effect on baseline calcium levels or when applied with NMDA. NMDA (1 mM) also failed to prolong AMPA-induced calcium increases when applied immediately following a brief (30–60 s) application of 100 $\mu$M AMPA (Fig. 4B).

L-Glutamate induces PRL release. Treatment of PRL cells with 1 mM glutamate for 15 min produced a 50% increase in release of PRL (Fig. 5). The effect of glutamate was blocked by the AMPA receptor antagonist CNQX (10 $\mu$M) but not by the NMDA receptor antagonist APV (25 $\mu$M). The glutamate-mediated PRL release was significantly less than the release induced by depolarizing cells with high potassium (55 mM).
These results on PRL release data closely parallel the intracellular calcium increases shown in Figs. 3 and 4, suggesting that glutamate causes release of PRL by increasing intracellular calcium concentrations through activation of AMPA receptors. PRL cells contain GluR protein. Cells were immunopositive for two different ionotropic GluR antibodies tested (Fig. 6). An antibody recognizing the mammalian AMPA receptor subunit GluR2 (with some cross-reactivity with GluR3) produced positive immunoreactivity in 97% of cultured RPD cells (34 of 35 cells counted from 2 cultures, comparing fluorescence with DIC images). Surprisingly, an NMDA receptor subunit (NR1) antibody produced similar reactivity in 67% of cultured RPD cells (200 of 298 cells from 2 cultures), despite the inability of NMDA to increase intracellular calcium levels. Immunostaining with both antibodies was punctate, resembling receptor labeling in neurons (12). Immunoreactivity to both receptor types was found mainly along the periphery of thin (1–2 μm) cross sections of cells imaged with confocal microscopy, indicating that the labeled antigens were associated with the cell membrane. Examples of anti-GluR2/3 and anti-NR1 labeling in confocal cross sections are shown in Fig. 6, C and D, respectively. This labeling pattern was also observed with these antibodies in cells cultured from tilapia olfactory bulb or cerebellum, and no labeling was observed in PRL cells when the primary antibody was omitted (data not shown). In contrast, PRL antibody labeling was internally concentrated in small areas that appear to be granules (Fig. 6E). We report that 92.3% of cultured cells were PRL immunopositive (372 of 403 cells from 4 cultures). This percentage is slightly less than previously reported for tilapia RPD and is smaller than the percentage for AMPA-immunopositive cells; however, this may simply reflect the titer of the antibody or indicate some release of PRL during the fixation process.

We used Western blot analysis to confirm the specificity of these antibodies. The AMPA receptor antibody labeled a thick
band of protein between 105 and 115 kDa from brain tissue (Fig. 6F, *top*). In protein extracted from pituitary RPD, this antibody labeled a doublet of bands, one at ~105 kDa and the other at ~115 kDa. This possibly indicates different isoforms or posttranslational modifications. The NR1 receptor antibody recognized a protein from brain tissue that was ~110 kDa (Fig. 6F, *bottom*). However, the protein recognized in RPD was larger by ~10 kDa than that found in brain extract. This may indicate a modified form of the NR1 protein, or a different protein with some homology to the NMDA receptor. A larger amount of RPD protein was required to produce bands of similar intensity compared with brain protein (23 \*g for RPD as opposed to 3 \*g for brain), signifying that these proteins are not as abundant in RPD as in neuronal tissue.

**DISCUSSION**

This study demonstrates the presence of glutamate receptors on PRL-secreting cells that are capable of raising intracellular calcium levels. Responses were largely attenuated in the presence of the general calcium channel antagonist cadmium as well as by the L-type VGCC antagonists nifedipine and nimodipine. This suggests that glutamate application leads to activation of VGCCs to increase intracellular calcium. Previously, nifedipine attenuated the calcium increase resulting from treatment with high KCl (29), indicating a direct role for L-type VGCCs in PRL secretion. Calcium imaging analysis demonstrated the activity of an AMPA receptor subtype of glutamate receptors. However, calcium increases were not seen in response to NMDA application, despite the higher calcium permeability of NMDA receptors compared with AMPA receptors (22). Glutamate-stimulated PRL release as measured by radioimmunoassay was inhibited by AMPA but not NMDA receptor antagonists. Immunocytochemistry and Western blot studies also demonstrated the presence of glutamate receptors in tilapia PRL cells. These results suggest that glutamate is capable of inducing PRL release through AMPA receptor-mediated membrane depolarization, leading to activation of VGCCs.

The activation of AMPA receptors, which are primarily permeable to sodium ions, rapidly depolarizes the membranes of excitatory cells (10). With continued glutamate application, the inward sodium current rapidly deactivates to a steady-state current as low as one-eighth of the peak current (33). The initial depolarization could activate the L-type calcium channels, which have a slow inactivation rate (4). A large initial influx of calcium could take several seconds to return to baseline levels. The incomplete block of glutamate-mediated calcium increases with antagonists for L-type channels indicates that L-type channels mediate some but not all of the increase in intracellular calcium. In previous experiments, L-type channels accounted for approximately one-third of VGCC current at positive membrane potentials (35a). These experiments also demonstrated that net calcium current of these cells has an activation threshold of approximately ~10 mV with a peak current at +10 mV. Additionally, calcium entering through L-type channels may induce calcium release from intracellular stores (6).

We also found NMDA receptor immunoreactivity in the periphery of PRL cells with a specific antibody. However, Western blot analysis showed that this antibody recognized a protein of a slightly larger molecular mass than that found in the brain. This may indicate a modification of the receptor subunit. It may also indicate that the antibody recognizes another membrane-associated protein with homology to the NMDA receptor. The NMDA receptor subunits were recently cloned in another teleost fish, the zebrafish (*Danio rerio*), and the NMDA receptor subunits showed 90% homology with human NR1 receptor subunits, suggesting a high level of evolutionary conservation (8). However, because teleost fish have an extra chromosomal replication relative to other vertebrates, there are 10 subunit genes in zebrafish paralogous to the NR1 gene and 4 NR2 genes identified in mammals. It is possible that a receptor formed with a particular combination of subunits is nonfunctional or not expressed on the cell surface, does not respond to NMDA, or is not permeable to calcium. Additionally, the presence of NR3 receptors, yet to be cloned in teleosts, inhibited NMDA responses by acting as a dominant negative in previous studies (9, 23). These subunits have also been reported to convert the function of NMDA receptors to an excitatory glycine receptor (7). However, this finding is controversial, and we did not observe excitatory responses with glycine alone (data not shown). NMDA receptor subunits may associate with other proteins, altering their functions. For example, the NR2B subunits are known to form complexes with ryanodine receptors in rat myocytes (30). In any case, tilapia PRL cells did not respond to NMDA with increased intracellular calcium. Additionally, the NMDA receptor antagonist APV did not block either glutamate-mediated calcium increases or glutamate-mediated PRL release. Thus NMDA

**Fig. 5.** Glutamate induces release of PRL via AMPA but not NMDA receptor activation. PRL cells were treated for 15 min with normal medium (untreated), 1 mM glutamate alone, glutamate with either 10 \*mM CNQX, 25 \*mM APV, or both, or high KCl (55 mM). Extracellular medium was collected and radioimmunoassayed for release of PRL. **P < 0.01 for each treatment vs. untreated (ANOVA with Dunnett’s post hoc test).
receptors as defined pharmacologically do not appear to be involved in PRL secretion in the tilapia.

Several recent studies have indicated that glutamate can act on endocrine cells of the adenohypophysis. Release studies using radioimmunoassays have shown that NMDA and other glutamate receptor agonists can also induce release of PRL and other hormones in mammalian anterior pituitary cells (19, 20, 26). However, because of the heterogeneous cell population of the mammalian pituitary, the possibility of PRL cells being activated secondarily by other cells with glutamate receptors cannot be ruled out (17). Glutamate, NMDA, and kainic acid increased intracellular calcium concentrations of cultured rat pituitary cells that are also responsive to thyrotropin-releasing hormone (TRH) (34). Subsequent immunohistochemical investigation indicated that 36% of cells containing PRL responded to glutamate, and 66% responded to TRH. Interestingly, TRH stimulates release of PRL from the tilapia pituitary only after pretreatment with estrogen (3). In preliminary experiments, we found that only PRL cells cultured from female tilapia respond to TRH, although PRL cells from both sexes responded equally to glutamate (Bellinger FP, Andres MA, and Cooke IM, unpublished data). Thus the apparent correlation between TRH

Fig. 6. Immunoreactivity of rostral pars distalis (RPD) cells for glutamate receptors. A: immunoreactivity to the AMPA receptor subunit glutamate receptor (GluR)2/3. The secondary antibody is labeled with Alexa488. B: immunoreactivity to the NMDA subunit NR1, with an Alexa594 secondary antibody. A and B are z-axis projections from stacks of 2-μm focal slices through cells. C: serial confocal microscope sections (2-μm thick) through the center of a PRL cell showing GluR2/3 immunoreactivity. Note the peripheral labeling, indicating the antigen is associated with the cell membrane. D: serial confocal microscope sections (1.8-μm thick) through the center of a PRL cell showing NR1 immunoreactivity. E: PRL cells labeled with anti-PRL antibody, imaged with confocal microscopy. Dark regions within cells are nuclei. Scale = 10 μm. F: Western blot using anti-GluR2/3 (top) and anti-NR1 (bottom) antibodies for protein samples from brain or RPD. Arrows at left indicate approximate molecular mass estimated from migration of standard protein markers.

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and glutamate receptors in mammalian PRL cells (34) is not found in tilapia. Additionally, we found no evidence of functional NMDA receptors in tilapia PRL cells. This stands in contrast to observations that NMDA stimulated PRL release in cultured rat pituitary cells (20, 26, 34).

In tilapia, axonal fibers from neurosecretory cells in the supraoptic nucleus of the hypothalamus terminate on the basement wall of the RPD, in close proximity to adenocorticotropin (ACTH) cells and stellate cells (25). Previous studies have indicated that these fibers do not enter the RPD (27). In goldfish, glutamate-immunopositive fibers have been detected in the anterior pituitary in proximity with endocrine cells; however, the source of these fibers is unknown (32). The stellate cells in tilapia send projections among the PRL cells (25). In rats, stellate cells have been shown to receive direct glutamatergic and GABAergic innervation from the hypothalamus (31). It is possible that these cells directly elicit hormonal responses by release of glutamate onto anterior pituitary cells, although to date no one has investigated this possibility. The role of the stellate cells and whether they release signaling molecules remains to be explored.

Glutamate may also have autocrine and/or paracrine actions in addition to its role as a neurotransmitter (16). Glutamate receptors and transporters are found in the pancreas, adrenal gland, osteoblasts in bone, and testis. The normal human blood glutamate concentration of 10 μM (2) is adequate for activation of glutamate receptors. This suggests that peripheral cells with glutamate receptors would be tonically activated by serum glutamate unless protected by extracellular structures.

The present study demonstrates the regulation of PRL secretion in tilapia by the AMPA-type glutamate receptors acting through VGCCs. Tilapia PRL cells do not appear to have active NMDA receptors as found in mammals. These findings increase our understanding of regulation of hormone release from the anterior pituitary. In euryhaline fish such as tilapia, the primary role of PRL is osmoregulation in fresh water (13). Determining the role of glutamate-mediated release of osmoregulating hormones could greatly improve our knowledge of this process and could assist in the treatment of osmoregulatory disorders.

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REFERENCES


