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Capacitance increases of dissociated tilapia prolactin cells in response to hyposmotic and depolarizing stimuli

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ABSTRACT

Prolactin (PRL) is the major hormonal mediator of adaptation to hyposmotic conditions. In tilapia (*Oreochromis mossambicus*), PRL cells are segregated to the rostral pars distalis of the anterior pituitary facilitating the nearly pure culture of dissociated PRL cells. Membrane capacitance (C_m) was recorded at 1 Hz or higher for tens of minutes as a surrogate monitor of PRL secretion by exocytosis from cells under perforated patch clamp. The study compares secretory responses to trains of depolarizing clamps (100 at 2.5 Hz, from −70 to +10 mV for 100 ms) to the physiological stimulus, exposure to hyposmotic medium, here a switch from 350 to 300 mOsm saline ($[Ca^{2+}]$ 15 mM). Two-thirds of cells tested with each stimulus responded. In response to depolarizing clamps, C_m increased linearly at an average rate of 7.2 fF/s. The increase was also linear in response to hyposmotic perfusion, but the average rate was 0.68 fF/s. Response to depolarization was reversibly blocked in Ca^{2+}-omitted saline, or in saline with 30 μM Cd^{2+}. It was unaffected by 0.1 μM tetrodotoxin. By contrast, responses were reduced but not absent during perfusion of hyposmotic saline with Ca^{2+}-omitted; 30 μM Cd^{2+} appeared to enhance the hyposmotic response. BAPTA-AM eliminated responses to both stimuli, confirming that secretion was dependent on increases of intracellular $[Ca^{2+}]$. Together with previous observations from this laboratory of $[Ca^{2+}]_i$ with simultaneous collection and immunoassay of perfusate for PRL, we conclude that depolarization and hyposmotic stimuli initiate secretion by independent mechanisms.

1. Introduction

Physiological adaptation to changes in environmental osmolarity is fundamentally important both to whole animals and to their cells. Prolactin (PRL), secreted from anterior pituitary cells, is the major hormonal mediator of adaptation to hyposmotic conditions in fish [16]. This peptide hormone with the molecular structure of a cytokine is present with minor structural variation in all groups of vertebrates where it consistently has a role in osmotic and electrolyte regulation [20,41]. In fish, PRL stimulates ion retention and uptake and reduces ion and water permeability at the gills and other epithelial surfaces [26,33]. PRL receptors in tilapia are present in many tissues such as reproductive organs, brain, hematopoietic organs, and lymphocytes [41], as is also the case in mammals. In mammals it has major roles in reproduction, development of mammary glands and lactation (hence the name), and numerous additional actions are documented or proposed [20].

Tilapia tolerates transfer from sea water to fresh water provided there is a source of PRL: an intact pituitary gland, an explant of PRL cells or an injection of bovine PRL [11]. Plasma osmolarity of fresh water adapted fish is ~310 mOsm; upon transfer to sea water, it reaches levels of 355 mOsm. Over this range, levels of PRL are inversely proportional to medium osmolarity [35,54,46]. Secretion of PRL from isolated pituitaries or from dissociated PRL cells is similarly inversely proportional to the medium osmolarity [23,22,37]. Thus, PRL cells are their own osmoreceptors. It is noteworthy that release of PRL from bovine anterior pituitary tissue *in vitro* is also inversely related to the osmolarity of the medium [28].

In tilapia PRL cells are segregated in a separate lobe of the pituitary; the rostral pars distalis (RPD). This facilitates study of PRL cells without the presence of other cell types. As mentioned, dissociated PRL cells respond directly to reduced osmolarity by increasing their secretion of prolactin. As in essentially all secretory cells that have been examined, PRL is secreted by exocytosis from cytoplasmic storage granules triggered by a rise in cytoplasmic calcium ion concentration ($[Ca^{2+}]_i$). Previous work has shown that $[Ca^{2+}]_i$ of PRL cells increases when perfusate osmolarity is reduced, when the

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perfusate includes a depolarizing concentration of K^+ [44], when the culture is exposed to agents causing release of Ca^{2+} from cellular stores [43], and in response to glutamate [4]. All of these treatments produce increased PRL secretion. PRL cells are electrically excitable. They show spontaneous impulse activity arising from an endowment of voltage-gated ion channels producing neuron-like Na^+-, Ca^{2+}-, and K^+-currents [53].

This study compares secretion from isolated PRL cells stimulated by perfusion of hyposmotic medium and secretion elicited by trains of depolarizing pulses. Perforated patch clamping is used to track cell capacitance as a surrogate for secretion. Capacitance has been documented to increase as a result of the addition of granule membrane to the cell surface when the granule undergoes exocytosis in releasing hormone (e.g. [36]). Our observations lead to the conclusion that hyposmotic stimulation and electrical stimulation initiate secretion by independent mechanisms.

2. Materials and methods

2.1. Fish

Mature tilapia (*Oreochromis mossambicus*) of both sexes weighing 200–400 g were brought to the laboratory from a population maintained at the Hawaii Institute of Marine Biology outdoors in 5000 L tanks in fresh water, 22–26 °C. They were held for up to 1 month under similar conditions, except indoors in a ~250 L tank under artificial 12/12 lighting. Fish were fed Purina trout chow. All experiments were conducted in accordance with the principles and procedures approved by the Institutional Animal Care and Use Committee, University of Hawaii.

2.2. Cell dispersion and culture

Pituitaries from 2 to 6 fish were removed after decapitation with sterile instruments and a portion of the anterior pituitary including the rostral pars distalis consisting primarily of prolactin-secreting cells was removed in a sterile laminar flow hood under a dissecting microscope. The pituitary pieces were placed in sterile Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline adjusted with NaCl to an osmolality of 355 mOsm (PBS). For dissociation, the tissues were combined and placed in PBS containing 0.125% trypsin in a volume of 0.5 ml/RPD. After 45 min at 26 °C with gentle agitation, the cells were dispersed by trituration with a pipetter, and the cells spun down by centrifugation (~250 g/5 min). The cells were resuspended in trypsin-free PBS, pelleted, and resuspended for a total of three washes. The last time, the cells were resuspended in hyperosmotic (355 mOsm) culture medium. Aliquots of the suspended cells were then placed in a small volume on up to eight untreated 35 mm culture dishes (Primaria, Falcon 3801) and after leaving the cells to adhere for several minutes, the dishes were flooded with the hyperosmotic culture medium. The cultures were placed in humidified incubators (Billups-Rothenberg), gassed with a 95%O_2/5%CO_2 mixture, and held in the dark at room temperature (24–26 °C). Cultures were usually prepared in the afternoon and used for the patch-clamping experiments beginning the following morning and for up to 3 days. Similar experiments run on successive days provided responses that showed no systematic differences attributable to longer time in culture.

2.3. Culture media

Eagle's minimum essential medium (MEM), as 50× concentrate (Gibco) was diluted in Kreb's saline at 2 mL/L of final medium. The saline consisted of (mM) NaCl 155, KCl 2.35, $CaCl_2$ 2.1, $MgSO_4$ 1.4, KH_2PO_4 1.25, $NaHCO_2$ 25, glucose 2.8, and glutamine 2. The osmolarity was measured and adjusted to 355 mOsm by addition of 5 M NaCl if necessary. The medium was gassed with 95%O_2/5%CO_2 for 5 min and sterilized by filtration. Culturing and extracellular recording solutions were adjusted to 355 or 350 mOsm, values within the physiological range of observed blood osmolarity [54] at which basal secretion of prolactin is minimized [44]. This permits comparison of the electrophysiological observations with experiments in this laboratory on relations between changes of $[Ca^{2+}]_i$ and prolactin secretion [43–45].

2.4. Recording solutions

For recording, the culture medium was replaced by a balanced (hereafter "standard") saline of the following composition (mM): NaCl 140 or 130, KCl 2.34, $CaCl_2$ 15, $MgCl_2$ 2, glucose 5, Hepes 10, pH adjusted to 7.4 with NaOH, and osmolarity adjusted with mannitol to 350 mOsm. For recording of I_{Ca} Na^+ was largely replaced by 140 mM *N*-methyl-D-gluconate (NMG)-Cl. "Ca^{2+}-free" or "Ca^{2+}-omitted" saline consisted of the standard saline in which the 15 mM $CaCl_2$ was replaced with $MgCl_2$.

Saline for hyposmotic stimulation of the cells was made by reducing NaCl to 130 mM (for experiments in which the standard saline had 140 mM NaCl), or without change of the relative ionic composition by reduction of mannitol (for changes from standard saline having 130 mM NaCl).

The intracellular solution used for both whole-cell and perforated patch recording consisted of (mM): Cs-aspartate 110, NaCl 8, Hepes 20, $CaCl_2$ 3.5, EGTA 0.1, Mg–ATP 5, pH adjusted to 7.4 with CsOH.

For perforated patch clamping, Amphotericin B, 3 mg was dissolved in 50 μL DMSO with the aid of ultrasound [38,30]. This, in turn was diluted in 1.5 mL of distilled water, with sonication, and aliquoted at 5 μL/microcap tube for storage at −20 °C in a freezer without an automatic defrost cycle. For use in electrodes, 30 μL of the electrode solution was added to a microcap tube giving a concentration of amphotericin of 375 μg/mL. During handling the material was kept as much as possible in ice and wrapped in aluminum foil. Immediately before an electrode was to be used, the tip was dipped for a few seconds in electrode solution and then back-filled with the amphotericin solution. The tip was mounted and applied to the cell as rapidly as feasible. Waiting time after achieving a seal for access conductance to reach the criterion for beginning to acquire data, >20 nS, was usually 15–25 min.

2.5. Electrophysiology

Voltage-clamp recordings were obtained in the perforated patch-clamp configuration, using an EPC9B amplifier (Instrutech Corp., NY). Data acquisition, storage and analysis were performed using HEKA software (Instrutech Corp., NY) run on a Macintosh Centris 650. Signals were filtered with a corner frequency of 2.9 kHz. All experiments were recorded at room temperature (22–26 °C). Pipettes were pulled from Kimax thin-walled glass capillaries (1.5–1.8 mm OD) on a vertical puller (David Kopf Instruments, TW 150F-4). Pipettes were coated with dental wax to reduce capacitance and fire-polished with a microforge (Narishige, model MF-83). Pipettes filled with the intracellular solution and immersed in the bath had resistances ranging from 3 to 6 MΩ. Compensation for series resistance was <60%; capacitance transient cancellation was optimized; leak subtraction utilized a P/4 protocol using hyperpolarizing pulses. No corrections have been made for junction potentials.

Cell membrane capacitance (C_m) was recorded using the slow capacitance tracking facility of the EPC9. The measurement uses the time constant and resistance as assessed by a train of