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Estradiol Modulation of Calcium Dynamics in Pituitary MMQ Lactotroph Cells

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ESTRADIOL MODULATION OF CALCIUM DYNAMICS IN PITUITARY MMQ LACTOTROPH CELLS

A Thesis
Presented to
the Faculty of Natural Sciences and Mathematics
University of Denver

In Partial Fulfillment
of the Requirements for the Degree
Master of Science

by
Monika Pauckova
May 2012
Advisor: Dr. Nancy Lorenzon
Abstract

Pituitary lactotrophs are excitable cells that exhibit spontaneous, calcium influx triggering prolactin (PRL) secretion to stimulate lactation. Lactotrophs express estrogen receptors (ER) and are a well established estrogen-responsive cell system. 17β-estradiol (E$_2$) is known to directly affect lactotrophs by increasing PRL transcription and biosynthesis, intracellular Ca$^{2+}$ concentration ([Ca$^{2+}$]$_i$) and PRL secretion. This study demonstrates that the MMQ clonal cell line, isolated from the 7315a rat pituitary tumor, is a model lactotroph cell line that is E$_2$-responsive. Spontaneous and evoked Ca$^{2+}$ transients were especially sensitive to L-type channel block, but not affected by block of ω-conotoxin-GVIA-sensitive Ca$_V$ channels or TTX-sensitive voltage-gated Na$^+$ (Na$_v$) channels. A population of 17β-Estradiol-treated cells exhibited enhanced spontaneous Ca$^{2+}$ oscillation amplitude and frequency and depolarization-evoked Ca$^{2+}$ transients. E$_2$ treatment significantly enhanced the Ca$^{2+}$ response through dihydropyridine-sensitive voltage-gated Ca$^{2+}$ (Ca$_v$) channels, suggesting that 17β-Estradiol increased L-type Ca$^{2+}$ channel genomic expression.
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17β-Estradiol (E₂)
Dopamine Type 2 receptor (D₂R)
Endoplasmic reticulum (ER)
Endothelin receptor (ETₐ)
Estrogen Receptor α/β (ERα/β)
Estrogen response element (ERE)
Folliculo-stellate cells (FS cells)
High-voltage activated (HVA)
Intracellular calcium concentration ([Ca²⁺]ᵢ)
Low-voltage activated (LVA)
Membrane-associated estrogen receptor (mER)
Prolactin (PRL)
Prolactin Inhibiting Hormone (PIH)
Sarco/endoplasmic reticulum Ca²⁺ ATPase (SERCA)
Tetraethylammonium (TEA)
Thyrotropin-releasing hormone (TRH)
Voltage-gated calcium channel (Caᵥ channel)
Voltage-gated potassium channel (Kᵥ channel)
Voltage-gated sodium channel (Naᵥ channel)
Tetrodotoxin (TTX)
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INTRODUCTION

The Pituitary Gland

The pituitary gland plays a central role in body homeostasis during development and stress [1]. The gland is anatomically divided into the anterior pituitary (adenohypophysis), the intermediate lobe, and the posterior pituitary (neurohypophysis). Each receives blood through the hypophyseal portal system. The posterior pituitary stores antidiuretic hormones and oxytocin, which are produced in the paraventricular nuclei and supraoptic nuclei of the hypothalamus and are transported along the axons of the hypothalamo-hypophyseal tract to the posterior pituitary [1]. The anterior pituitary contains five major cell types that secrete peptide hormones necessary for the response to stress, metabolic homeostasis, growth, development, reproduction, and lactation: corticotrophs, thyrotrophs, gonadotrophs, somatotrophs, lactotrophs (Fig. 1). Because axons do not enter the anterior pituitary, hypothalamic control of the anterior pituitary is achieved by hormonal regulation. These hormones, known as inhibiting and releasing factors, are synthesized by neurons in the hypothalamus and are transported to the anterior pituitary by the hypophyseal portal system.
Figure 1: Five major hormone-secreting cell types of the anterior pituitary. Adapted from Ooi et al. [2].
The Physiology of Lactotrophs

The primary function of lactotrophs, which compromise about 15% of the total cell population of the anterior pituitary, is to secrete prolactin (PRL) [1, 2]. PRL, also known as luteotropic hormone or luteotropin, is a single-chain protein of 199 amino acids in humans with an approximate molecular size of 23 kDa [3]. While PRL is involved in several biological activities, the main physiological role of the hormone is to act on the mammary glands to support growth and development (mammogenesis), to stimulate milk production (lactogenesis) and to maintain milk secretion (galactopoiesis) [3, 4].

During pregnancy the mammary glands are prepared for lactation by the synergistic effect of the hormones: PRL, estrogen, progesterone, insulin, cortisol, and thyroxine [4]. PRL plays an important role in lactogenesis by signaling the secretory cells of the mammary glands to increase biosynthesis of the milk proteins, casein and lactalbumin. Throughout pregnancy, high circulating levels of estrogen promote PRL production. Although PRL levels gradually increase during pregnancy and peak during the third trimester, milk production is inhibited until parturition. Progesterone, which is initially secreted by the corpus luteum to sustain pregnancy and later secreted by the placenta, is responsible for inhibiting lactogenesis. Progesterone exerts its antilactogenic effects by directly acting on mammary tissue to antagonize the stimulatory action of PRL and inhibits the ability of PRL to induce synthesis of more PRL receptors [4]. At prepartum, progesterone levels suddenly drop and estrogen levels peak, stimulating PRL
levels to peak. As a result, the mammary epithelial cells are converted from a non-secretory state to a secretory state to allow for lactogenesis.

In nursing mothers, high levels of PRL secretion are maintained by the neuroendocrine reflex [4]. The suckling infant stimulates the nerve endings in the breast, which relays impulses to the hypothalamus to block the secretion of hypothalamic dopamine, a PRL-inhibiting hormone (PIH), resulting in a sharp rise in PRL serum levels. However, in order for the infant to receive milk, the hormone oxytocin must work in tandem with PRL. Oxytocin causes milk secretion by stimulating the contraction of the lactiferous ducts.

**Mechanism of Estrogen Action**

Estrogen is the dominant PRL-stimulating factor that regulates secretion at two levels: directly by binding estrogen receptors on lactotrophs and indirectly by acting within the hypothalamus to modify the activity of neuroendocrine neurons [3]. Estrogen exerts its direct effects on lactotrophs by upregulating PRL gene expression and altering lactotroph sensitivity to physiological stimulators and repressors of PRL secretion. For example, estrogen acts by decreasing the density of dopamine receptors, thus reducing the potency of dopamine as an inhibitor of PRL secretion [3]. In contrast, estrogen sensitizes lactotrophs to thyrotropin-releasing hormone (TRH), a PRL-releasing factor, by increasing TRH receptor density [3]. At the level of the hypothalamus, estrogen acts
on the inhibitory dopaminergic neurons to alleviate tonic inhibition imposed on lactotrophs.

Estrogen exerts its biological functions directly at the level of lactotrophs by binding to two estrogen receptors, ERα and ERβ, both belonging to the nuclear receptor family of transcription factors [5]. ERs are composed of three structurally and functionally distinct domains: the NH$_3$-terminal domain, DNA-binding domain (DBD) and the COOH-terminal ligand-binding domain (LBD) [6]. The NH$_3$-terminal domain is involved in protein-protein interactions and transcriptional activation of target-gene expression, the DBD is important in receptor dimerization and binding of receptors to specific DNA sequences, and the LBD is involved in ligand binding [6]. Lactotrophs primarily express ERα mostly localized in the nucleus, although other receptors are membrane associated (mERs) and anchored to the plasma membrane by scaffold proteins [10].

Estrogen signaling occurs through multiple pathways, classified either as classical, involving the estrogen response element (ERE), or non-classical, involving ERE-independent mechanisms (Fig. 2) [5]. The classical mechanism involves estrogen binding to the nuclear ER, dimerization of the receptor and subsequent direct binding to the ERE to modulate gene expression. Alternatively, gene regulation can be affected by indirect DNA binding of the activated ER through protein-protein interaction with transcription factors. In a ligand-independent pathway, growth factor signaling leads to the activation of kinases that phosphorylate and thereby activate ERs in the absence of
ligand [7]. In addition to the transcriptional effects of estrogen, a non-genomic mechanism may occur over seconds to minutes after estrogen exposure. Estrogen activation of mERs initiates second messenger pathways that modify the phosphorylation states of cytoplasmic proteins and ion channels, ultimately leading to a rapid physiological response (Fig. 2) [7]. Overall, estrogen directly effects lactotrophs by increasing PRL gene transcription, $[\text{Ca}^{2+}]_i$ and $\text{Ca}^{2+}$-regulated secretion.
Figure 2: Mechanisms of Estrogen Action. E₂ exerts its effects directly on lactotrophs through classical signaling involving the ERE and through an ERE-independent mechanism. As a result, E₂ increases PRL transcription, [Ca^{2+}]_i, and calcium-regulated secretion.
Lactotroph Remodeling

Lactotrophs are estrogen-responsive cells, and estrogen exposure *in vivo* and *in vitro* induces lactotroph proliferation [8]. Estrogen action on lactotroph cell growth acts through either proliferative or apoptotic mechanisms, mediated by estrogen-induced stimulatory and inhibitory acting growth factors such as TGF-α, TGF-β, and bFGF. TGF-β1 and TGF-β3 are produced and secreted by lactotrophs and have been characterized to have antagonistic effects on cell transformation [8]. While TGF-β1 is a potent inhibitor of estrogen-induced lactotrophic proliferation and PRL secretion, TGF-β3 stimulates proliferation. The production of TGF-β1 and TGF-β3 differs under estrogenic conditions. Upon E2 exposure, TGF-β1 expression is reduced, while TGF-β3 expression is increased to stimulate cell proliferation. In addition, neighboring folliculo-stellate (FS) cells in the pituitary mediate estrogen’s mitogenic action on lactotrophs. E2 induces TGF-β3 release from the lactotrophs, which acts on FS cells to release bFGF, which acts on lactotrophs to stimulate cell proliferation. Thus, lactotroph proliferation and apoptosis is dependent on the balance of estrogen-induced growth factors.

Lactotroph Excitability and Secretion

Lactotrophs are known to secrete high basal levels of hormone in the absence of stimuli, due to spontaneous firing of action potentials (APs) that drive sufficient Ca2+ entry to stimulate Ca2+-dependent exocytosis [9, 10]. The differences in expression of
ionic channels among pituitary cells is thought to underlie the cell type-specific patterns of APs, intracellular \( \text{Ca}^{2+} \) dynamics, and hormone secretion [11]. Primary lactotrophs, somatotrophs, and gonadotrophs each express varying levels of transient (i.e. T-type) and sustained (e.g., N- and L-type) \( \text{Ca}^{2+} \) channels, TTX-sensitive \( \text{Na}^+ \) channels, transient and delayed rectifying \( \text{K}^+ \) channels and multiple \( \text{Ca}^{2+} \)-dependent \( \text{K}^+ \) channel subtypes [12]. Lactotrophs exhibit low expression levels of TTX-sensitive \( \text{Na}^+ \) channels and high expression levels of \( \text{Ca}^{2+} \)-activated \( \text{K}^+ \) channels relative to gonadotrophs [12]. In addition, lactotrophs show higher functional expression of transient \( \text{K}^+ \) channels and lower expression of \( \text{Ca}_v \) channels relative to somatotrophs [12]. Furthermore, each channel type has a specific role that contributes to cellular excitability.

The main function of \( \text{Na}_v \) channels in excitable cells is to depolarize cells and generate the upstroke of the action potential (AP). Depending on the cell type, \( \text{Na}_v \) channels are either solely responsible for the rapid and regenerative upstroke of an AP or work in conjunction with low-voltage activated \( \text{Ca}_v \) channels to depolarize cells. In bovine lactotrophs, \( \text{Na}_v \) channels are critical for AP generation [13], and in lactotrophs expressing high levels of \( \text{Na}^+ \) channels, TTX application abolishes hormone secretion [14]. However, in the majority of rat anterior pituitary cells, inhibition of these channels does not affect the pattern of spontaneous electrical activity [14].

\( \text{Ca}_v \) channels are divided into two general types that differ in the voltage dependence of activation: high- and low-voltage activated channels (HVA and LVA). HVA channels require moderate to strong depolarization to open. HVA channels such as
L-, N-, P/Q-, and R-type Ca\(\text{v}\) channels, are distinguished by their single-channel conductance and pharmacology [13]. HVA channels inactivate incompletely and keep cells depolarized long enough to increase global intracellular \([\text{Ca}^{2+}]_i\) to trigger \(
abla \text{Ca}^{2+}\)-dependent processes. On the other hand, LVA Ca\(\text{v}\) channels require less depolarization for activation and subsequent inactivation. LVA channels are referred to as transient or T-type Ca\(\text{v}\) channels due to their gating kinetics. At resting conditions, T-type channels depolarize the cell to the threshold level for Na\(\text{v}\) and HVA Ca\(\text{v}\) channel activation. Thus, HVA and LVA Ca\(\text{v}\) channels serve two major functions in cells: electrogenic and regulatory [15]. In neurons and endocrine cells, Ca\(\text{v}\) channels give rise to APs similar to those generated by Na\(\text{v}\) channels, but with slower kinetics and smaller amplitude. In addition, intracellular \(
abla \text{Ca}^{2+}\) acts as a second messenger that regulates various cellular functions.

Potassium channels are largely responsible for setting the dominant negative resting membrane potential and repolarizing the membrane potential back to the resting potential. In general, opening of potassium channels reduces electrical excitability, while closure of the channels promotes excitability. In lactotrophs, dopamine induces hyperpolarization by acting on K\(\text{ir}\) channels, although it has been reported that dopamine also increases K\(\text{v}\) conductance and BK-K\(\text{Ca}\) conductance [13]. K\(\text{v}\) channels can be classified into four functional classes: fast activating delayed rectifier, slow delayed rectifier, A-type K\(^+\) channel, and EAG (ether-a-go-go) channels. Fast and slow delayed rectifier channels function as their name implies, according to their gating kinetics. A-
type channels show rapid activation and fast inactivation and recovery from inactivation. EAG channels have inward rectifying properties that contribute to resting membrane potential.

**MMQ Clonal Cell Line**

The MMQ clonal cell line used in this study is derived from the 7315a rat pituitary tumor and is one of three characterized lactotroph cell lines: the GH3 somatolactotroph cell lines, the 235-1 clonal lactotroph cell line, and the MMQ cell line [2]. MMQ cells are similar to primary lactotrophs since they only secrete PRL in a Ca\(^{2+}\)-regulated manner and express functional dopamine receptors (D\(_2\)R), type A endothelin receptors (ET\(_A\)) and oxytocin receptors [2]. Similar to primary pituitary cells, the secretory response in MMQ cells is regulated by calcium and cAMP [2]. Previously, MMQ cells have been used as a model system to study the biochemical effects of dopamine and other G-protein coupled receptors in regulating PRL release. However, little is known about the ion channel contribution to cellular excitability and Ca\(^{2+}\) dynamics, and E\(_2\)-regulation of Ca\(^{2+}\) dynamics in MMQ cells.
Thesis Objective

The objective of this thesis is to (1) characterize the contributions of several voltage-gated ion channels to cellular excitability and Ca^{2+} dynamics in MMQ cells and (2) to determine if the MMQ clonal cell line is an appropriate estrogen-responsive lactotroph model cell. In primary lactotrophs, 17β-estradiol is a major prolactin-stimulating factor that exerts its effects directly at the level of lactotrophs to increase PRL transcription and biosynthesis, intracellular calcium concentration ([Ca^{2+}]_i), and PRL secretion. Estradiol has been shown to increase L-type Ca^{2+} channels genomic expression in melanotrophs [16]. Therefore, the regulation of ion channels in pituitary cells is a dynamic process that is modulated by inhibiting and releasing factors, in order to control pituitary responsiveness during various physiological states such as development, the estrous cycle, and pregnancy. The Ca^{2+} dynamics of MMQ cells will be quantified by measuring spontaneous oscillation frequency and evoked Ca^{2+} transients due to potassium-induced membrane depolarization. Electrophysiology will be conducted to measure the contributions of voltage-gated Na^+ and high-voltage activated (N-/L-type) Ca^{2+} currents to Ca^{2+} dynamics. Fura-2AM Ca^{2+} imaging will quantify E_2 modulation of spontaneous Ca^{2+} oscillation frequency, evoked Ca^{2+} responses due to potassium-induced membrane depolarization, and relative changes of voltage-gated channel contribution to evoked responses.
MATERIAL & METHODS

Culturing MMQ cells

MMQ cells were cultured in either standard media (typical cell culture that has trace amounts of hormones and steroids), charcoal-stripped media (all hormones and steroids removed from media), or E2-enriched media (charcoal-stripped and supplemented with 1nM 17β-estradiol). Standard media was composed of F12K media with 15% horse serum, 2.5% fetal bovine serum, 100 unit/ml penicillin, 100µg/ml streptomycin, and 100µg/ml normacin. Charcoal-stripped media is similar in composition to standard media, with the substitution of standard horse serum and FBS by charcoal-stripped derivatives. E2-enriched media consists of charcoal-stripped media with the addition of 1nM 17-β estradiol (diluted from a 5.68mM stock in water).

Plating MMQ Cells

For calcium imaging experiments, cells were plated on micro cover glass (VWR Scientific Inc, Cat 48366 067) bottom plastic cell culture dishes (35mm x10mm). For electrophysiology experiments, cells were plated on plastic culture dishes. Prior to plating, the dishes were coated with ECL media for 1 hr at 37°C and afterwards rinsed three times with regular rodent ringers solution. Cells were maintained in a humidifier
incubator at 37°C with 5% CO₂. MMQ cells were plated for 30 minutes at 37°C prior to loading with Fura-2 AM.

**Fura-2 AM Loading**

After plating MMQ cells for 30 minutes, cells were rinsed twice with 5K⁺ external solution (see ‘Imaging Solutions’) prior to loading with the ratiometric dye Fura-2 AM (5μM diluted from 1mM stock in DMSO) in 5K⁺ external solution for 30 minutes at room temperature in dark conditions. Cells were rinsed three times with 5K⁺ external solution and were ready for imaging.

**Calcium Imaging Experiments**

Cells were visualized with an Olympus 1X71, with a 40x air objective lens, and images were captured using a Hamatsu Orca camera. Images were acquired and analyzed using Slidebook software (Intelligent Imaging Innovations Inc.), exposure times for 340 nm and 380 nm of light were 100 ms, and images were binned 8x8 at 2 second intervals. 100mM K⁺ puffs were applied to clusters of cells using PicoSpritzer® II (Parker Hannifin Corporation) at fixed four minute intervals with several puff durations: 200msec, 500msec, 1000msec, 5000 msec. Cells were perfused with either 10μM nifedipine (diluted from 2.28mM stock in ethanol), 1μM ω-conotoxin-GVIA (diluted from 228mM stock in water), or 1μM TTX (diluted from 1mM stock in water). Following the experiment, a brightfield image of the cells was taken.
**Imaging Solutions and Drug Application**

MMQ cells were imaged while bathed in a 5K+ external solution containing: 137mM NaCl, 5mM KCl, 1mM MgCl₂, 5mM CaCl₂, 10mM glucose, 10mM Hepes, pH 7.2 with NaOH. The 100mM K⁺ Solution was nearly identical to the 5K⁺ external solution, with the substitution of 42mM NaCl and 100mM KCl.

**Electrophysiological Recording**

Voltage-clamp recordings were performed at room temperature using an Axopatch 200B patch-clamp amplifier and were low-pass filtered at 2 kHz. Patch electrodes were pulled from borosilicate glass tubes (1.5 mm outer diameter, A-M Systems, Inc) using a Flaming/Brown Micropipette Puller (P-97; Sutter Instruments). Electrodes were heat polished to a final tip resistance of 3-6 MΩ. Data acquisition and analysis were done with a Digidata 1200 A/D interface in conjunction with Clampex8 and Clampfit software (Molecular Devices). For measuring I_{Ca} and I_{Na}, the extracellular medium contained (in mM): 120 NaCl, 2 KCl, 5 CaCl₂, 1 MgCl₂, 10 HEPES, 10 glucose, 20 TEA-Cl, 1μM TTX (added before use to block I_{Na}), pH 7.2 (adjusted with TEA-OH), Osm 310. The pipette solution contained (in mM): 135 CsCl, 1 MgCl₂, 1 CaCl₂, 10 CsEGTA, 10 HEPES, 3 MgATP (added fresh before use), pH 7.2 adjusted with CsOH, Osm 299. For measuring all voltage-dependent currents, the extracellular medium contained (in mM): 137 NaCl, 5 KCl, 1 MgCl₂, 5 CaCl₂, 10 glucose, 10 HEPES, pH 7.2 with NaOH, Osm 306. The internal pipette solution for measuring all currents contained...
(in mM): 70 KCl, 70 KMeSO\textsubscript{4}, 1 MgCl\textsubscript{2}, 10 HEPES, pH 7.2 with KOH. The perforated patch internal contained (in mM): 76 K\textsubscript{2}SO\textsubscript{4}, 10 KCl, 10 NaCl, 1 MgCl\textsubscript{2}, 5 HEPES, pH 7.35 with KOH. The pipette tip was filled with amphotericin-free solution and then backfilled with amphotericin B solution (60mg/mL amphotericin B stock in DMSO was diluted to a final concentration of 0.24 mg/mL). An average membrane capacitance (Cm) of 7.1 pF (n=14) was recorded in the MMQ cells.

**Statistical Analysis**

The evoked Ca\textsuperscript{2+} transients were quantified by calculating the response peak amplitude and area using IgorPro software. The amplitude of evoked transients was measured by subtracting the 340/380nm baseline ratio from the maximal peak ratio to obtain the Δ340/380 ratio. The area of each evoked response was obtained by summing the Δ340/380 ratio data points of the complete transient. Data were represented as ±SEM. Statistical analysis was performed using the paired t-test for two sample means and t-test two-sample assuming equal variances (tested by the f-test). Significance was considered at the 0.05 level. The 5s estrogen responders were analyzed after 48hr E\textsubscript{2} treatment.
RESULTS

We (1) pharmacologically described the types of voltage-gated currents expressed in MMQ cells, (2) characterized the Ca\(^{2+}\) excitability of cells cultured in normal media, and (3) investigated whether MMQ cells are E\(_2\)-responsive, and if so, whether E\(_2\) modulates the contribution of voltage-gated channels in enhancing [Ca\(^{2+}\)]\(_i\).

I. Characterization of Voltage-gated Ion Channel Currents in Normal MMQ Cells

The expression levels of ion channels determine cellular excitability and hormone secretion. Perforated patch recording of all voltage-gated channels present in MMQ cells reveal inward Na\(^+\) and Ca\(^{2+}\) currents and outward K\(^+\) currents (Fig. 3A). These currents were pharmacologically isolated to examine the contribution of various ion channel types to these currents. Na\(^+\) channels were isolated using conventional whole-cell patch clamp technique with TEA in the bath solution to eliminate outward K\(^+\) currents (Fig. 3B). A rapid activating and inactivating I\(_{Na}\) was observed at membrane potentials more depolarized than -40mV and reached a mean peak amplitude of 36.3 pA/pF (n=4) at +10mV (Fig. 3F). The application of 1\(\mu\)M TTX eliminated TTX-sensitive I\(_{Na}\), and the remaining isolated Ca\(^{2+}\) currents were examined using conventional whole cell recording (Fig. 3C). In response to depolarizing voltage steps, a Ca\(^{2+}\) current was activated at -60mV and reached a mean peak amplitude of 5.2 pA/pF (n=10) at 0mV (Fig. 3F).
Figure 3: Voltage-gated currents in MMQ Cells. (A) Perforated patch recording of voltage-gated currents (average 7.1pF membrane capacitance (n=14). (B) Whole-cell recording of inward current (I_{Na} and I_{Ca}). (C) Whole-cell recording of I_{Ca} from the same cell in (B) after the addition of TTX to block TTX-sensitive I_{Na}. (D) Step pulse protocol consisting of 100msec voltage steps from -70mV to 50mV from a holding potential of -70mV. (E) Current-Voltage (IV) plot of outward currents. (F) IV plots of I_{Ca} and I_{Na}. 
II. Characterization of MMQ Ca\textsuperscript{2+} Dynamics in Normal Culture Conditions

*Ca\textsuperscript{2+} Imaging Paradigm*

The following series of experiments characterize the Ca\textsuperscript{2+} excitability of MMQ cells cultured in normal media by measuring [Ca\textsuperscript{2+}]\textsubscript{i} using the calcium indicator dye, Fura-2AM (Fig. 4, A and B). A preliminary Ca\textsuperscript{2+} imaging study was conducted to determine appropriate Ca\textsuperscript{2+} imaging conditions. In resting 5K\textsuperscript{+} bath solution, MMQ cells generated spontaneous Ca\textsuperscript{2+} oscillations (Fig 4C, D). Imaging the cells at 100ms exposure time repeated every 2 seconds for 30 minutes did not result in cellular toxicity or artificial excitability, as illustrated by the steady-state 340/380 baseline (Fig. 4C). The large increase of intracellular Ca\textsuperscript{2+} in response to application of the Ca\textsuperscript{2+} ionophore, ionomycin, demonstrated that our evoked Ca\textsuperscript{2+} measurements were well below saturation of the indicator dye (Fig. 4D).

In addition to monitoring spontaneous Ca\textsuperscript{2+} oscillations, we characterized intracellular Ca\textsuperscript{2+} evoked by membrane depolarization. We initially quantified the effect of different durations of high K\textsuperscript{+} (100mM) application on spontaneous and evoked Ca\textsuperscript{2+} transients (Fig. 5). High K\textsuperscript{+} puffs were applied to clusters of cells at fixed four minute intervals with puff application durations set at 0.20 sec (threshold stimulus that evoked a detectable response above baseline), 0.5 sec, 1 sec, and 5 sec (evoked a maximal response below saturation) (Fig. 5, A-D). Although K\textsuperscript{+} puffs were observed to stimulate spontaneous oscillation frequency in quiescent cells, increasing the K\textsuperscript{+} duration did not increase spontaneous oscillation frequency (Fig. 5E). The evoked Ca\textsuperscript{2+} transients were
quantified by calculating the peak amplitude response (measured by subtracting the 340/380 baseline ratio from the maximal peak ratio to obtain the Δ340/380 ratio) and area (calculated by summing the Δ340/380 ratio data points) using IgorPro software. The evoked peak amplitude and area responses increased with increasing K⁺ puff durations (Fig. 5, F and G).
Figure 4: Calcium imaging of MMQ cells under normal resting (5K⁺) conditions. (A) Fura-2AM loaded MMQ cells in bright field illumination or (B) with fluorescence excitation 340nm. (C) Imaging paradigm with 100ms exposure every 2 sec for 30 min does not induce toxicity or artificial excitability. (D) Response to ionomycin application demonstrates that the maximal spontaneous Ca²⁺ oscillations (of E₂-treated cell) are well below saturation of the Ca²⁺ indicator.
**Figure 5:** Effect of different stimulation durations on spontaneous and evoked Ca\(^{2+}\) transients. (A-D) Representative cell traces for each high K\(^+\) puff duration. (E) Average spontaneous oscillation frequency. (F) Evoked peak amplitude response and (G) evoked area response from high K\(^+\). (0.2s n=85, 0.5s n=115, 1s n=120, 5s n=123). Data reported as mean ±SE.
Effects of Na\textsuperscript{+} Channel Block on Ca\textsuperscript{2+} Excitability

Depending on the cell type, Na\textsuperscript{+} channels are essential for eliciting APs, specific patterns of Ca\textsuperscript{2+} spiking and hormone secretion. All pituitary cells express TTX-sensitive Na\textsubscript{v} channels, and whole cell current recordings demonstrated that MMQ cells exhibit a substantial TTX-sensitive Na\textsuperscript{+} current (Fig. 3, B and F). We investigated the contribution of TTX-sensitive Na\textsuperscript{+} channels on spontaneous and evoked Ca\textsuperscript{2+} transients in MMQ cells. MMQ cells were imaged in resting 5K\textsuperscript{+} bath solution before bath perfusing 1µM TTX, a Na\textsubscript{v} channel blocker. Throughout the imaging study, cells were stimulated every 4 minutes by high K\textsuperscript{+} to evoke a Ca\textsuperscript{+} response (Fig. 7A). Spontaneous Ca\textsuperscript{+} oscillations were counted at each interval before and after TTX application. Na\textsubscript{v} channel block did not alter the average spontaneous oscillation frequency (Fig. 6A). Closer examination of the 5-s K\textsuperscript{+} puff data revealed no statistically significant difference between the pre-TTX and post-TTX oscillation frequency (spikes/sec= 0.07±0.01 and 0.08±0.01, respectively) (Fig. 7B). The evoked transient responses were calculated on an individual cell basis by averaging the first two evoked transients to obtain the pre-TTX response, and averaging the four transients recorded in TTX solution to obtain the post-TTX response (Fig. 7A). Na\textsubscript{v} channel block did not alter the amplitude or area of the evoked Ca\textsuperscript{+} transients at any K\textsuperscript{+} puff duration (Fig. 6, B and C). The 5-s K\textsuperscript{+} puff data revealed no statistical difference between pre-TTX and post-TTX evoked responses (peak ∆340/380 amplitude = 2.58±0.16 and 2.56±0.16, respectively, and area ∆340/380*sec= 28.14±1.35 and 27.13±1.56) (Fig. 7, C and D).
Figure 6: Effect of Na\(^+\) channel block on spontaneous and evoked Ca\(^{2+}\) transients at different K\(^+\) puff durations. (A) Spontaneous Ca\(^{2+}\) oscillation frequency before and after 1µM TTX. (B) Evoked peak amplitude and (C) evoked area of responses before and after TTX. (0.25s n=32, 0.5s n=52, 1s n=56, 5s n=64, P>0.05). Data reported as mean ±SE.
**Figure 7**: Effect of Na\(^+\) channel block on spontaneous and evoked Ca\(^{2+}\) transients. (A) Cells were bath perfused with 1µM TTX and Ca\(^{2+}\) transients were evoked by 5-s 100mM K\(^{+}\) puffs. (B-D) Re-plotted from figure 6. (B) Spontaneous Ca\(^{2+}\) oscillation frequency (n=40). (C-D) Evoked peak amplitude and response area of Ca\(^{2+}\) transients (n=64, p>0.05). Data reported as mean ±SE.
Effect of L-Type and N-Type Ca\(^{2+}\) Channel Block on Ca\(^{2+}\) Excitability

Lactotrophs exhibit spontaneous, extracellular Ca\(^{2+}\)-dependent plateau bursting activity, which is coupled to PRL secretion [17]. In rat lactotrophs, application of the L-type calcium channel blocker, nifedipine, and the non-specific Ca\(^{2+}\) channel blocker, Cd\(^{2+}\), inhibited PRL secretion and abolished AP firing [10]. Since voltage-gated Ca\(^{2+}\) entry is essential for lactotroph excitability, the following experiments investigated the contribution of L-type and N-type Ca\(^{2+}\) channels in generation of spontaneous Ca\(^{2+}\) oscillations and depolarization-induced (evoked) Ca\(^{2+}\) increases. Throughout imaging, cells were stimulated with high K\(^+\) every 4 minutes before and after bath application of 10μM nifedipine (Fig. 9A). A consistent decrease in spontaneous oscillation frequency was observed after L-type channel block (Fig. 8A). Nifedipine reduced the average spontaneous oscillation frequency by 71% (from 5s K\(^+\) data set, 0.07 ±0.01 spikes/sec to 0.02±0.01 spikes/sec, n=28, p<0.001) (Fig. 9B). The evoked peak amplitude and area of the Ca\(^{2+}\) response significantly decreased after nifedipine application for all stimulus durations (Fig. 8, B and C). The 5s K\(^+\) data set show that nifedipine application reduced the peak response amplitude by 71% (2.79±0.14 to 0.85±0.07) and the evoked response area by 68% (30.55±1.36 to 10.12±0.72, n=59, p<0.001) (Fig. 9, C and D). Thus, the main fraction of extracellular Ca\(^{2+}\) influx occurs through the L-type Ca\(^{2+}\) channel.

To determine the contribution of N-type Ca\(^{2+}\) channels on Ca\(^{2+}\) dynamics in MMQ cells, the specific N-type blocker, ω-conotoxin-GVIA was bath applied. Cells were stimulated at 4 minute intervals by 0.5s or 5s durations of high K\(^+\) (Fig. 11A). The
inhibition of N-type channels did not alter oscillation frequency (Fig. 10A). The oscillation frequency remained constant at 0.07 spikes/sec before and after conotoxin application for the 5-s K⁺ data set (Fig. 11B). However, conotoxin application did reduce the evoked peak amplitude and area responses at a statistically significant level (Fig. 10, B and C). The inhibition of N-type channels decreased the evoked peak amplitude by 14% (3.02±0.16 to 2.62±0.13) and evoked area by 9% (33.04±1.15 to 29.1±1.22, n=89, p<0.001) at statistically significant levels (Fig. 11, C and D).
Figure 8: Effect of L-type Ca\textsuperscript{2+} Channel Block on Spontaneous and Evoked Ca\textsuperscript{2+} transients at different K\textsuperscript{+} puff durations. (A) Spontaneous Ca\textsuperscript{2+} oscillation frequency before and after 10μM Nif. (B) Evoked peak amplitude and (C) evoked area of responses before and after Nif. (0.2s n=53, 0.5s n=63, 1s n=64, 5s n=59). Data reported as mean ±SE.
Figure 9: Effect of L-type Ca\textsuperscript{2+} Channel Block on Spontaneous and Evoked Ca\textsuperscript{2+} transients. (A) The first two evoked spikes were averaged to obtain the pre-nif response, the last two spikes recorded in TTX solution for post-nif response. Cells were perfused with 10µM nifedipine and stimulated with 5s K\textsuperscript{+} puffs. (B) Oscillation frequency decreased by 71\% after Nif (n=28, p<0.001). (C) Evoked peak amplitude decreased by 71\% and (D) evoked area decreased by 68\% (n=59, p<0.001). Data reported as mean ±SE.
Figure 10: Effect of N-type Ca\textsuperscript{2+} Channel Block on Spontaneous and Evoked Ca\textsuperscript{2+} transients at different K\textsuperscript{+} puff durations. (A) Oscillation freq before and after conotoxin (0.5s: n=48, 5s n=61, p>0.05). (B) Evoked peak amplitude (0.5s: n=51 p<0.01, 5s: n=89 p<0.001) and (C) area response before and after conotoxin (0.5s: n=51 p>0.05, 5s: n=89 p<0.001). Data reported as mean ±SE.
Figure 11: Effect of N-Type Ca\(^{2+}\) Channel Block on Spontaneous and Evoked Ca\(^{2+}\) transients. (A) Cells were stimulated with 5s K\(^{+}\) puffs and perfused with 1µM Conotoxins. (B) Conotoxin did not alter oscillation frequency. (C) Conotoxin decrease evoked peak amplitude by 14% (n=89, p<0.001) and (D) evoked area by 9% (n=89, p<0.001) (D). Data reported as mean ±SE.
III. Characterization of Charcoal-Stripped and Estrogen-Enriched MMQ Cells

Defining E\textsubscript{2} Responsive Cells

For the following series of experiments, MMQ cells were cultured in either charcoal-stripped media or estrogen-enriched media for three consecutive days. Charcoal-stripped media contains activated carbon that removes non-polar material such as certain growth factors, hormones and steroids, but has little effect on salts, glucose, amino acids, etc. Estrogen media contains charcoal-stripped media with the addition of 1nM 17β-estradiol. Pretreatment with E\textsubscript{2} caused a distinctive increase in spontaneous Ca\textsuperscript{2+} spiking behavior, which was not observed in the charcoal-stripped controls. Ca\textsuperscript{2+} imaging performed in 5K\textsuperscript{+} bath solution revealed that subpopulation of E\textsubscript{2}-responsive cells exhibited pronounced spontaneous Ca\textsuperscript{2+} oscillations with sustained peaks, compared to charcoal-stripped controls (Fig. 12, A and B). The removal of extracellular Ca\textsuperscript{2+} abolished spontaneous spiking of previously active MMQ cells (Fig. 12C).

The effectiveness of the 3 day treatment plan was analyzed, by comparing the evoked responses of charcoal-stripped cells to estrogen-treated cells. Each day, both charcoal- and estrogen-treated cells were imaged, and K\textsuperscript{+} puffs of different durations (0.2s, 0.5s, 1s, 5s) were applied at fixed 4 minute intervals. The evoked Ca\textsuperscript{2+} response peak amplitude and area in charcoal-stripped cells was similar across all 3 days, and the Ca\textsuperscript{2+} responses increased with stimulus duration (Fig. 13). In contrast, the average evoked peak amplitude and area responses of all estrogen-treated cells showed variable
sensitivity to K+ stimuli (Fig. 13). Some E2-treated cells exhibited distinct Ca2+ activity compared to charcoal-stripped, where as other treated cells demonstrated Ca2+ transients more similar to charcoal-stripped treated cells. Therefore, we established criteria to define E2-responders and additionally analyzed these cells separate from the total pooled E2-treated population. Histogram analysis examining evoked peak amplitude and area responses of charcoal-stripped versus estrogen treated cells defined an “estrogen-responsive cell” to exhibit a peak amplitude response greater than 5 and area greater than 50 (Fig. 14). From the established criteria, estrogen responders were pooled from the 5s K+ puff data sets and exhibited a statistically significant increase in spontaneous spiking amplitude and frequency, and evoked peak area and amplitude (Fig. 12 and 15). The average spontaneous oscillation amplitude was 0.26±0.02 and 2.67±0.24 Δ340/380, and frequency was 0.08±0.01 and 0.11±0.01 spikes/sec for charcoal-stripped vs E2 responsive cells, respectively (Fig. 12, D and E). The comparative evoked peak amplitude for charcoal vs E2 cells was 2.49±0.15 and 6.19±0.61, and evoked area was 28.29±1.39 and 76.07±6.33 (Fig. 15, C and D).
Figure 12: Spontaneous Ca$^{2+}$ oscillations of charcoal-stripped and E$_2$-responsive MMQ cells is dependent on extracellular Ca$^{2+}$. (A) Calcium imaging of charcoal-stripped cells and (B) E$_2$-treated cells under normal resting conditions (5K$^+$$)$. (C) The removal of 5mM Ca$^{2+}$ from bath abolished spontaneous Ca$^{2+}$ spiking. (D) Oscillation peak amplitude of charcoal-stripped cells (0.26±0.02, n=65), E$_2$-responders (2.67±0.24, n=42) and E$_2$-unresponsive cells (1.00±0.15, n=66). (E) Average oscillation frequency for charcoal-stripped cells (0.08±0.01 n=13), E$_2$ –responders (0.11±0.01 n=20), and E$_2$-unresponsive cells (0.09±0.01, n=76).
Figure 13: Evoked Ca$^{2+}$ responses in charcoal-stripped and pooled E$_2$-treated cells over 3 day treatment period. Cells were stimulated with different 100mM K$^+$ puff durations (0.2s, 0.5s, 1s, 5s) and the evoked peak amplitude and area was calculated. (For given day, n±20 (C) ±20 (E$_2$) for each puff duration)
Figure 14: Histogram analysis for defining estrogen-responsive cells. $E_2$-responders were defined as evoked peak amplitudes greater than 5 and evoked areas greater than 50. Moreover, $E_2$-responders lie more than three standard deviations above the mean of the charcoal-stripped responders. (purple= charcoal-stripped cells n=28, gray= $E_2$-treated cells n=67).
Figure 15: Spontaneous and evoked Ca\textsuperscript{2+} transients in charcoal-stripped and E\textsubscript{2}-responsive cells. Average evoked Ca\textsuperscript{2+} responses due to 5s K\textsuperscript{+} in (A) charcoal-stripped cells and (B) E\textsubscript{2}-responsive cells. Data pooled from preTTX and preNif experiments. (C) The evoked peak amplitude for charcoal-stripped cells (2.49±0.15), E\textsubscript{2} –responsive ( 6.19±0.61), and E\textsubscript{2}-unresponsive cells (2.36±0.20). (E) Evoked area for charcoal-stripped cells (28.29±1.39), E\textsubscript{2} –responsive (76.07±6.33), and E\textsubscript{2}-unresponsive cells (26.82±2.10). (Charcoal-Stripped n=101, E\textsubscript{2} –responsive n=19, E\textsubscript{2}-unresponsive n=95. p<0.001).
Effects of Na\textsuperscript{+} Channel Block on Ca\textsuperscript{2+} Excitability in Charcoal vs Estrogen Treated Cells

Na\textsubscript{v} channels are responsible for the generation and propagation of APs in neurons and excitable cells. In dorsal root ganglion neurons (DRG), E\textsubscript{2}-treatment has been shown to increase the mRNA expression of Na\textsubscript{v} channels [18]. Given that estrogen increases [Ca\textsuperscript{2+}]\textsubscript{i} responses of MMQ cells, we next investigated whether the increases in [Ca\textsuperscript{2+}]\textsubscript{i} was Na\textsuperscript{+} channel-dependent.

Charcoal-stripped and E\textsubscript{2}-treated cells were stimulated with different K\textsuperscript{+} durations (0.2s, 0.5s, 1s, and 5s K\textsuperscript{+}), and the effects of 1\textmu M TTX application on evoked responses were recorded over a 3 day period (Fig. 16). The overall spontaneous Ca\textsuperscript{2+} oscillation frequency, evoked peak amplitude and evoked peak area were calculated before and after TTX application. The charcoal-stripped cells showed relatively consistent responses over the three day period (Fig. 16). On the other hand, the E\textsubscript{2}-treated cells exhibited variable responses, largely depending on the number of estrogen responsive cells present in a particular imaging experiment (Fig. 16). E\textsubscript{2}-responsive cells were analyzed separately. TTX application did not change the spontaneous oscillation frequency, evoked peak amplitude and evoked peak area at a statistically significant level (Fig. 17, C-E).
**Figure 16:** Effect of Na\(^+\) channel block on evoked responses in charcoal-stripped and pooled E\(_2\)-treated cells over 3 day treatment period. (For given day, n±20 (C) ±20 (E\(_2\)) for each puff duration)
Figure 17: Effect of Na\(^+\) channel block on spontaneous and evoked Ca\(^{2+}\) transients in charcoal-stripped cells and E\(_2\)-responders. Average evoked Ca\(^{2+}\) responses due to 5s K\(^+\) in (A) charcoal-stripped cells and (B) E\(_2\)-responsive cells. TTX application did not change (C) the average oscillation frequency, (D) the evoked peak amplitude and (E) evoked area in charcoal-stripped, E\(_2\)-responders, and E\(_2\)-unresponsive cells at statistically significant levels (Charcoal-stripped n=44, E\(_2\)-responsive n=9, E\(_2\)-unresponsive n=40. Data are reported as mean ±SE, p>0.05).
Effects of Ca\(^+\) Channel Block on Ca\(^{2+}\) Excitability in Charcoal- vs E\(_2\)-Treated Cells

The main fraction of basal PRL secretion is due to regulated, Ca\(^{2+}\)-dependent exocytosis in response to Ca\(^{2+}\) influx through L-type Ca\(^{2+}\) channels [10]. Since L-type Ca\(^{2+}\) channels are largely responsible for increasing [Ca\(^{2+}\)]\(i\) in MMQ cells, we investigated whether estradiol modulates the contribution of the L-type Ca\(^{2+}\) channel to depolarization evoked Ca\(^{2+}\) responses. Charcoal-stripped and E\(_2\)-treated cells were imaged over a 3 day duration, stimulated with different durations of K\(^+\) and bath perfused with 10\(\mu\)M nifedipine (Fig. 19, A and B). The spontaneous Ca\(^{2+}\) oscillation frequency, evoked peak amplitude responses and peak area responses were calculated before and after nifedipine application for each day (Fig 18). Across the 3 day treatment period, charcoal-stripped cells showed consistent evoked Ca\(^{2+}\) transients (Fig. 18). In contrast, the E\(_2\)-treated population showed variable responses across days and stimulus durations (Fig. 18). E\(_2\)-responders were analyzed separately from the 5s K\(^+\) puff data set (Fig. 19). L-type channel block nearly abolished spontaneous oscillation frequency, reducing it by 75\% in charcoal-stripped cells (from 0.10±0.01 to 0.03±0.01, n=12) and 94\% in E\(_2\)-responders (from 0.11±0.01 to 0.01, n=11) (Fig. 19C). The evoked peak amplitude response was reduced by 81\% in charcoal-stripped cells (from 1.73±0.15 to 0.34±0.04, n=52) and by 80\% in E\(_2\)-resonders (from 7.40±0.95 to 1.43±0.33, n=10) (Fig. 19D). The evoked area was reduced by 82\% for charcoal-stripped cells (from 23.14±1.66 to 4.48±0.53, n=52) and by 80\% in E\(_2\)-responders (from 79.63±8.78 to 15.92±3.51, n=10) (Fig. 19E).
Figure 18: Effect of L-Type Ca^{2+} channel block on evoked responses in charcoal-stripped and pooled E_{2}-treated cells over 3 day treatment period. (For given day, n±20 (C) ±20 (E_{2}) for each puff duration)
**Fig 19:** Effect of L-Type Ca\(^{2+}\) channel block on spontaneous and evoked Ca\(^{2+}\) transients in charcoal-stripped cells and E\(_2\)-responders. Average evoked Ca\(^{2+}\) responses due to 5s K\(^+\) in (A) CS cells and (B) E\(_2\) cells. (C) Nif application reduced oscillation frequency by 75% in CS cells (from 0.10±0.01 to 0.03±0.01), by 94% in E\(_2\)-responders (from 0.11±0.01 to 0.01), and 88% in E\(_2\)-unresponsive cells (from 0.09±0.01 to 0.01). The evoked peak amplitude response was reduced by 81% in CS cells (from 1.73±0.15 to 0.34±0.04), by 80% in E\(_2\)-responders (from 7.40±0.95 to 1.43±0.33), and by 83% in E\(_2\)-unresponsive cells (from 2.50±0.24 to 0.42±0.04). (E) The evoked area was reduced by 82% in CS cells (from 23.14±1.66 to 4.48±0.53), by 80% in E\(_2\)-responders (from 79.63±8.78 to 15.92±3.51), and by 81% in E\(_2\)-unresponsive cells (from 25.52±2.19 to 4.93±0.56). (Charcoal n=52, E\(_2\)-responsive n=10, E\(_2\)-unresponsive n=55. p<0.001, data reported as mean ±SE ).
DISCUSSION

The MMQ clonal cell line, derived from the 7315a rat pituitary tumor, secretes PRL in a Ca\(^{2+}\)-regulated manner, and unlike the other existing lactotroph cell lines (GH3 somato-lactotroph cell line and 235-1 clonal cell line), the MMQ cell line expresses functional D\(_2\) receptors [13, 19]. Similar to primary pituitary cells, the secretory response of MMQ cells is regulated by calcium and cAMP, and MMQ cells express functional endothelin-type A (ET\(_A\)) and oxytocin receptors [13]. This thesis confirms that the MMQ cell line has retained the cell-specific lactotroph feature of estrogen-responsiveness, thus the MMQ clonal cell line is both a model lactotroph cell and estrogen-responsive cell model system.

This thesis examined Ca\(^{2+}\) excitability of MMQ cells cultured in normal media by quantifying spontaneous Ca\(^{2+}\) oscillation frequency and evoked Ca\(^{2+}\) responses from K\(^+\)-induced membrane depolarization. The spontaneous Ca\(^{2+}\) oscillation frequency of unstimulated cells was observed to be dependent on the presence of extracellular Ca\(^{2+}\) and sensitive to L-type Ca\(_v\) channel block. In contrast, spontaneous Ca\(^{2+}\) oscillation frequency was unaffected by the voltage-gated Na\(^+\) channel blocker, TTX, and the N-type Ca\(_v\) channel blocker, ω-conotoxin-GVIA. These results indicate that Ca\(^{2+}\) influx through
L-type VGCCs is largely responsible for increasing \([\text{Ca}^{2+}]_i\) to drive spontaneous cellular excitability.

Changes in membrane potential influence the gating of ion channels as well as \(\text{Ca}^{2+}\)-dependent functions through changes in free \([\text{Ca}^{2+}]_i\). This thesis pharmacologically characterized voltage-dependent channels in MMQ cells that are involved in the elevation of \([\text{Ca}^{2+}]_i\). Membrane depolarization stimulated a transient increase in \([\text{Ca}^{2+}]_i\), evident by the Fura-2AM fluorescent signal immediately reaching a peak and quickly decaying back to baseline. The addition of nifedipine (10\(\mu\)M) significantly reduced the evoked peak amplitude and area response by approximately 70%. Addition of \(\omega\)-conotoxin-GVIA (1\(\mu\)M) reduced evoked responses by approximately 10%. TTX (1\(\mu\)M) did not inhibit the \(K^+\)-induced increase in \([\text{Ca}^{2+}]_i\). The data demonstrate that potassium-induced membrane depolarization increases \([\text{Ca}^{2+}]_i\) due to influx of \(\text{Ca}^{2+}\) primarily through L-type and minimally through N-type \(\text{Ca}^{2+}\) channels. Consistent with the current results, studies performed on primary lactotrophs have shown that application of TTX did not alter PRL secretion, in contrast to application of nifedipine which inhibited PRL secretion [10]. One may infer that MMQ cells behave like the primary lactotrophs, and \(\text{Ca}^{2+}\) influx through the L-type and N-type channels is important for regulated, \(\text{Ca}^{2+}\)-dependent basal exocytosis.

Primary lactotrophs express intracellular and membrane associated receptors for estrogens and are a well established estrogen-responsive cell model system [2]. Estrogen directly acts on lactotrophs by increasing PRL transcription and biosynthesis, \(\text{Ca}^{2+}\) influx,
and PRL secretion by means of Ca\(^{2+}\)-regulated secretion. The estrogen-responsiveness of MMQ cells was assessed by examining the intracellular Ca\(^{2+}\) dynamics of cells grown in estrogen-enriched medium in comparison to charcoal-stripped cells which were cultured in the absence of steroids and hormones. Due to an estrogen-signaling mechanism that increased \([\text{Ca}^{2+}]_i\), E\(_2\)-responders exhibited robust, spontaneous Ca\(^{2+}\) oscillations (both in amplitude and frequency). In comparison to the potassium-induced Ca\(^{2+}\) transients observed in charcoal-stripped cells, E\(_2\)-responsive cells displayed evoked Ca\(^{2+}\) transients greater in peak amplitude and area.

We next examined if E\(_2\) modulates the contribution of TTX-sensitive Na\(_v\) and L-type Ca\(^{2+}\) channels to promoting cellular excitability. TTX application did not alter spontaneous Ca\(^{2+}\) oscillation frequency or evoked Ca\(^{2+}\) responses in charcoal-stripped and E\(_2\)-responsive cells, suggesting that Na\(_v\) activity is not altered by E\(_2\) treatment. The application of L-type channel blocker did significantly reduce spontaneous and evoked excitability in both charcoal-stripped and E\(_2\)-treated cells. Spontaneous Ca\(^{2+}\) oscillation frequency in charcoal-treated cells was reduced by approximately 75% and nearly abolished in E\(_2\)-treated cells by 94%. Prior to nifedipine application, spontaneous Ca\(^{2+}\) oscillation frequency was observed to be greater in E\(_2\)-treated cells. These results suggest that (a) Ca\(^{2+}\) influx through the L-type channel increases \([\text{Ca}^{2+}]_i\), to promote spontaneous Ca\(^{2+}\) oscillations and (b) increased Ca\(^{2+}\) influx through the L-type channel may be due to an E\(_2\)-induced increase in L-type channel expression. Although nifedipine application reduced the evoked Ca\(^{2+}\) response of estrogen responders by approximately
80%, a post-nifedipine transient, or residual Ca\(^{2+}\) influx, was still present. Perhaps E\(_2\) treatment may have increased the expression levels of nifedipine-insensitive Ca\(^{2+}\) channels, which are responsible for the residual Ca\(^{2+}\) influx observed in E\(_2\)-treated cells. Alternatively, an E\(_2\) signaling mechanism increased the mean open time of such nifedipine-insensitive Ca\(_v\) channels.

The mechanism by which E\(_2\) directly stimulates an increase in \([\text{Ca}^{2+}]_i\) remains to be determined. However, two calcium entry pathways are proposed to operate in lactotrophs: voltage-gated calcium influx (VGCI) and store-operated calcium entry (SOCE), the process in which depletion of Ca\(^{2+}\) stores in the endoplasmic reticulum (ER) induces Ca\(^{2+}\) influx from the extracellular space [17]. These pathways may be modulated during E\(_2\)-signaling. Possible mechanisms of E\(_2\) action on the lactotroph include classical signaling at the genomic level and nongenomic signaling, both of which may contribute to increasing \([\text{Ca}^{2+}]_i\). By means of classical signaling, E\(_2\) may act on the nuclear ER\(\alpha\) to increase the mRNA expression of HVA or LVA calcium channels (Ca\(_v\)1.1-1.3, 2.1-2.3, 3.1, 3.3). These Ca\(_v\) subunits have been detected in all endocrine pituitary cells [13]. In previous studies, estradiol has been reported to increase L-type current genomic expression in mouse melanotrophs [16] and increase the density of LVA T-type Ca\(^{2+}\) channels in rat pituitary GH\(_3\) cells by 4- to 5- fold that of the controls [10]. By means of non-genomic, short term signaling, E\(_2\) may promote mode 2 gating of the L-type Ca\(^{2+}\) channels, by increasing the mean open time via a phosphorylation event. Although these mechanisms are postulated and currently unknown in MMQ cells, it is known that E\(_2\) acts
on lactotrophs to increase PRL transcription and biosynthesis, $[\text{Ca}^{2+}]_i$ and $\text{Ca}^{2+}$-dependent exocytosis.

**Summary**

The MMQ lactotroph clonal cell line from the 7315a rat pituitary tumor is a model lactotroph cell that is estrogen responsive. Estrogen was found to increase global $[\text{Ca}^{2+}]_i$, spontaneous $\text{Ca}^{2+}$ oscillation amplitude and frequency, and evoked $\text{Ca}^{2+}$ transients. Estrogen did not modulate the relative contribution of TTX-sensitive $\text{Na}_v$ to $\text{Ca}^{2+}$ excitability, but did enhance the $\text{Ca}^{2+}$ response via dihydropyridine-sensitive $\text{Ca}_v$ channels.

**Future Direction**

It would be of interest to investigate whether $\text{E}_2$ modulates the protein expression levels of L-type and other $\text{Ca}_v$ channels types, such as T-type and R-type $\text{Ca}^{2+}$ channels. In addition to $\text{Ca}^{2+}$ imaging, western blot analysis would prove useful for quantifying changes in $\text{Ca}^{2+}$ channel expression.

A similar $\text{Ca}^{2+}$ imaging experiment to the one performed in this present study could be conducted to determine if $\text{E}_2$-signaling increases $[\text{Ca}^{2+}]_i$ by triggering $\text{Ca}^{2+}$ release from $\text{Ca}^{2+}$ stores through the IP$_3$ pathway. Cellular excitability would be monitored after the application of xestosponggin, a membrane-permeable blocker of IP$_3$R
channels, and thapsigargin, an inhibitor of the endoplasmic reticulum Ca^{2+} ATPase (SERCA) pump.

To characterize the non-genomic actions of E_2 and inhibit the involvement of nuclear estrogen receptors, one could repeat the experiments in this study using E_2 conjugated to BSA. The BSA-E2 conjugate is membrane-impermeable and therefore would only activate membrane-bound estrogen receptors to induce a number of relatively rapid effects.
BIBLIOGRAPHY


