# Physiological Research Pre-Press Article

# Dependence of Spontaneous Electrical Activity and Basal Prolactin Release on Nonselective Cation Channels in Pituitary Lactotrophs

Abbreviated title: TRPC Channels and Excitability of Lactotrophs

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Authors have nothing to declare

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#### SUMMARY

All secretory anterior pituitary cells fire action potentials spontaneously and exhibit a high resting cation conductance, but the channels involved in the background permeability have not been identified. In cultured lactotrophs and immortalized GH<sub>3</sub> cells, replacement of extracellular Na<sup>+</sup> with large organic cations, but not blockade of voltage-gated Na<sup>+</sup> influx, led to an instantaneous hyperpolarization of cell membranes that was associated with a cessation of spontaneous firing. When cells were clamped at -50 mV, which was close to the resting membrane potential in these cells, replacement of bath Na<sup>+</sup> with organic cations resulted in an outward-like current, reflecting an inhibition of the inward holding membrane current and indicating loss of a background-depolarizing conductance. Quantitative RT-PCR analysis revealed the high expression of mRNA transcripts for TRPC1 and much lower expression of TRPC6 in both lactotrophs and GH<sub>3</sub> cells. Very low expression of TRPC3, TRPC4, and TRPC5 mRNA transcripts were also present in pituitary but not GH<sub>3</sub> cells. 2-APB and SKF-96365, relatively selective blockers of TRPC channels, inhibited electrical activity, Ca2+ influx and prolactin release in a concentration-dependent manner. Gd<sup>3+</sup>, a common Ca<sup>2+</sup> channel blocker, and flufenamic acid, an inhibitor of non-selective cation channels, also inhibited electrical activity, Ca<sup>2+</sup> influx and prolactin release. These results indicate that nonselective cation channels, presumably belonging to the TRPC family, contribute to the background depolarizing conductance and firing of action potentials with consequent contribution to  $Ca^{2+}$  influx and hormone release in lactotrophs and  $GH_3$  cells.

Keywords: TRPC channels, GH<sub>3</sub> cells, calcium influx, action potentials, resting membrane potential

#### INTRODUCTION

The endocrine pituitary functions are carried out by corticotrophs secreting adrenocorticotrophic hormone, thyrotrophs secreting thyroid-stimulating hormone, somatotrophs secreting growth hormone, lactotrophs secreting prolactin (PRL), and gonadotrophs secreting luteinizing and follicle-stimulating hormones (Kelberman *et al.* 2009). The common characteristic of these cells, as well as of immortalized pituitary cells, is their spontaneous excitability. Firing of action potentials (APs) reflects the expression of numerous voltage-gated channels on the plasma membrane of these cells, including voltage-gated and tetrodotoxin (TTX)-sensitive Na<sup>+</sup> (Na<sub>v</sub>) channels, voltage-gated Ca<sup>2+</sup> (Ca<sub>v</sub>) channels, voltage-gated K<sup>+</sup> channels, calcium-regulated K<sup>+</sup> channels, inwardly rectifying K<sup>+</sup> channels, purinergic P2X channels, and GABA<sub>A</sub> channels (Kwiecien and Hammond 1998, Stojilkovic *et al.* 2010). Spontaneous firing of APs in all pituitary cell types leads to the activation of Ca<sub>v</sub> channels and increased intracellular calcium ion concentration ([Ca<sup>2+</sup>]<sub>i</sub>), with the amplitude of transients determined by the pattern of firing (Stojilkovic *at al.* 2005). The T- and L-type Ca<sub>v</sub> channels play a role in spike depolarization, while voltage-gated K<sup>+</sup> channels and Ca<sup>2+</sup>-controlled K<sup>+</sup> channels are responsible for plateau bursting and repolarization of cells (Van Goor *et al.* 2001a, Tsaneva-Atanasova *et al.* 2007).

The physiological significance of electrical activity in lactotrophs is well established. These cells *in vitro* release PRL in the absence of external stimuli, and such secretion is termed basal or spontaneous release (Freeman *et al.* 2000). High PRL release is also observed in animals bearing ectopic pituitary grafts (Maric *et al.* 1982). In both cases, spontaneous APs and the associated  $Ca^{2+}$  influx account for high steady-state PRL release and any maneuver leading to silencing of electrical activity also abolishes  $Ca^{2+}$  influx and basal PRL release (Van Goor *et al.* 2001a). *In vivo*, spontaneous electrical activity and PRL release are controlled by hypothalamic dopamine, acting through dopamine D2 receptors. Activation of these receptors leads to silencing of electrical activity through activation of inwardly rectifying K<sup>+</sup> channels and inhibition of  $Ca_v$  channels (Missale *et al.* 1998). GH<sub>3</sub> cells, an established immortalized cell line derived from a rat anterior pituitary tumor, maintain pituitary-specific behavior in culture by secreting PRL and growth hormone (Cronin *et al.* 1980). These cells are spontaneously electrically active with high amplitude APs and often serve as a convenient cell model in studying electrophysiological properties of lactotrophs and somatotrophs (Lo *et al.* 2001).

Channels contributing to the spike depolarization and repolarization in spontaneously firing cells have been identified (Stojilkovic *et al.* 2010). In contrast, very little is known about channels controlling resting membrane potential and initiation of firing of APs. Resting membrane potential in these cells is between -50 and -60 mV, positive to the equilibrium potassium potential, indicating the presence of depolarizing conductance. TTX-sensitive Na<sub>v</sub> channels are expressed in pituitary cells, but do not play important role in control of resting membrane potential and spike depolarization in lactotrophs *in vitro* (Van Goor *et al.* 2001b). A TTX-insensitive background Na<sup>+</sup> (Na<sub>b</sub>) conductance was also identified in pituitary lactotrophs and GH<sub>3</sub> immortalized cells as necessary for spontaneous depolarization and PRL release (Simasko

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1994, Sankaranarayanan and Simasko 1996). However, the nature of these channels and the mechanism for their activation has not been identified.

Here we show that the abolition of Na<sub>b</sub> conductance by substituting extracellular Na<sup>+</sup> with large organic cations leads to a rapid and reversible hyperpolarization of the plasma membrane and inhibition of firing of APs, Ca<sup>2+</sup> influx and PRL secretion. Our results further indicate that this conductance takes place through non-selective cation channels, presumably the transient receptor potential - classic (TRPC) subfamily of these channels, which have been identified in pituitary cells using quantitative real-time PCR. TRPC channels conduct both Na<sup>+</sup> and Ca<sup>2+</sup>, are voltage independent and their role in spontaneous and receptor controlled electrical activity and calcium signaling has been indicated in other excitable cells (Clapham *et al.* 2005).

#### METHODS

#### Animals and Cell Cultures

Experiments were performed on anterior pituitary cells from normal postpubertal female Sprague Dawley rats obtained from Taconic Farm (Germantown, MD). Euthanasia was performed by asphyxiation with CO<sub>2</sub> and the anterior pituitary glands were removed after decapitation. Experiments were approved by the NICHD Animal Care and Use Committee. Anterior pituitary cells were mechanically dispersed after treatment with trypsin and cultured as mixed cells or enriched lactotrophs in medium 199 containing Earle's salts, sodium bicarbonate, 10% heat-inactivated horse serum, penicillin (100 units/ml), and streptomycin (100 µg/ml). A two-stage Percoll discontinuous density gradient procedure was used to obtain enriched lactotrophs (Lussier *et al.* 1991). Further identification of lactotrophs in single cell studies was achieved by the addition of dopamine (Tocris Bioscience, Ellisville, MO) and TRH (Bachem, Torrance, CA). Immortalized GH<sub>3</sub> pituitary cells were cultured in Ham's F12K medium supplemented with 15% heat-inactivated horse serum, and gentamicin (100 µg/ml).

#### Electrophysiological Measurements

Pituitary cells were plated on poly-L-lysine coated cover slips (15 mm diameter) in densities of 100,000 primary cells per cover slip and 10,000 immortalized cells per cover slip and cultured for 1-3 days prior to recording. All recordings were performed at room temperature using Axopatch 200B amplifier (Molecular Devices, Union City, CA). The amphotericine perforated patch-clamp technique was used to record membrane potentials and whole cell currents. Cells were continuously perfused with an extracellular solution containing (in mM): 145 NaCl, 5 KCl, 2 CaCl<sub>2</sub>, 1 MgCl<sub>2</sub>, 10 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES), and 10 glucose (pH 7.4). For Na<sup>+</sup>-free experiments, NaCl was replaced in 1:1 ratio by N-methyl-D-glucamine (NMDG), tetramethylammonium (TMA), or choline chloride (pH 7.4). Patch pipettes were pulled from borosilicate glass (World Precision Instruments, Sarasota, FL) and heat polished to a tip resistance of 5-7 M $\Omega$ . Pipette solution contained (in mM): 90 K-aspartate, 50 KCl, 3 MgCl<sub>2</sub> and 10 HEPES (pH 7.2). Prior to measurement, amphotericine B

was added to the pipette solution from a stock solution to obtain a final concentration of 200  $\mu$ g/ml. Recordings started when series resistance dropped below 100 M $\Omega$  for current-clamp or below 40 M $\Omega$  for voltage-clamp recordings. Series resistance was compensated to more than 60%. Drugs dissolved to a final concentration in extracellular solutions were delivered to the recording chamber by a gravity-driven microperfusion system RSC-200 (Bio-Logic USA, Knoxville, TN).

#### Single cell intracellular calcium measurements

For measurements of  $[Ca^{2+}]_i$ , cells were incubated in Krebs Ringer buffer with 2 µM Fura-2 AM (Invitrogen, Carlsbad, CA) at room temperature for 60 min. For Na<sup>+</sup>-free experiments, NaCl was replaced by NMDG and pH was brought to 7.4 by adding HCl. Coverslips with cells were then washed with Krebs-Ringer buffer and mounted on the stage of an Observer-D1 microscope (Carl Zeiss, Oberkochen, Germany) attached to an ORCA-ER camera (Hamamatsu Photonics, Hamamatsu City, Japan) and a Lambda 10-B filter wheel (Sutter, Novato, CA) with 340 and 380 nm excitation filters (Carl Zeiss, Oberkochen, Germany). Hardware control and image analysis was performed using Metafluor software (Molecular Devices, Downingtown, PA). Cells were examined under an oil immersion objective during exposure to alternating 340- and 380-nm light beams, and the intensity of light emission at 520 nm was measured. The ratio of light intensities,  $F_{340}/F_{380}$ , which reflects changes in  $[Ca^{2+}]_i$ , was followed in several single cells simultaneously at the rate of one point per second.

#### Prolactin secretion

Hormone secretion was monitored using cell column perifusion experiments. Briefly,  $1.5 \times 10^7$  cells were incubated with preswollen cytodex-1 beads in 60-mm petri dishes for 18 h. The beads were then transferred to 0.5-ml chambers and perifused with Hanks' M199 containing 25 mM HEPES, 0.1% BSA, and penicillin (100 U/ml)/streptomycin (100 µg/ml) at 37 °C for 2.5 h at a flow rate of 0.8 ml/min to establish stable basal secretion. Fractions were collected in 1-min intervals and their PRL content was later determined using radioimmunoassay. Primary antibody and standard for PRL assay were purchased from the National Pituitary Agency and Dr. A. F. Parlow (Harbor-UCLA Medical Center, Torrance, CA). [<sup>125</sup>I]PRL was purchased from PerkinElmer Life Sciences (Boston, MA).

#### **RT-PCR** Analysis

Total RNA from the primary pituitary cells was extracted using the RNeasy Mini Kit. Subsequently, 1 µg of total RNA was treated with DNAse I and reverse transcribed with SuperScript III First Strand Synthesis SuperMix for qRT-PCR (all from Invitrogen, Carlsbad, CA). Quantitative RT-PCR was performed using pre-designed Taq-Man Gene Expression Assays (Applied Biosystems) with LightCycler® TaqMan® Master mix and LightCycler 2.0 Real-time PCR system (Roche Applied Science). Gene expression levels of the target genes were determined by the comparative 2<sup>^</sup>(-delta delta C(T)) quantification method using GAPDH as a reference gene, where (delta delta C(T)) = (CT, target - CT,

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reference)sample - (CT, target - CT, reference)control. The Applied Biosystems pre-designed Taq-Man Gene Expression Assays were used: TRPC1-Rn00585625\_m1; TRPC2-Rn00575304\_m1; TRPC3-Rn00572928\_m1; TRPC4-Rn00584835\_m1; TRPC5-Rn00590142\_m1; TRPC6-Rn00677564\_m1; TRPC7-Rn01448763\_m1; GAPDH-Rn01462662\_g1.

## RESULTS

#### Dependence of electrical activity and PRL release on the background Na<sup>+</sup> conductance

To determine the role of Na<sub>b</sub> conductance in resting membrane potential and spontaneous electrical activity of pituitary cells, we first examined the effect of Na<sup>+</sup> removal from extracellular solution. We used lactotrophs and GH<sub>3</sub> cells that tend to fire APs spontaneously (Fig. 1A and C). However, the fraction of active cells is lower in lactotrophs than in GH<sub>3</sub> cells: about 60% and 90%, respectively. TTX, a Na<sub>v</sub> channel blocker, was ineffective in abolishing spontaneous electrical activity (Fig. 1C) in concentrations up to 10  $\mu$ M. In contrast, replacement of extracellular Na<sup>+</sup> with NMDG led to an instantaneous hyperpolarization of cell membranes both in lactotrophs (-15.1 ± 2.2 mV; n=6) and GH<sub>3</sub> cells (-28.3 ± 7.2 mV; n=10). The hyperpolarization was associated with a cessation of spontaneous firing of APs (Fig. 1A). Once the cells were returned to Na<sup>+</sup>-containing physiological buffer, the spontaneous electrical activity resumed. We also frequently observed an increase in the firing frequency during the initial period of the recovery phase (Fig. 1A), which could indicate that the level of  $[Ca^{2^*}]_i$  determines the frequency of spontaneous firing of APs. These experiments show that basal sodium conductance controls resting membrane potential and electrical activity in pituitary cells.

In further experiments we asked whether calcium influx and hormone secretion are regulated by Na<sub>b</sub> conductance. We clamped cells at -50 mV, a physiologically relevant voltage because the resting potentials in these cells were close to that value (Fig. 1A and 1C). In both cell types, TTX had no effect on holding current (data not shown), while complete replacement of extracellular Na<sup>+</sup> with NMDG caused a shift in the baseline, with the appearance of an outward current (Fig. 1B). A decrease in the noise of recording during NMDG application suggested that the shift in the baseline was due to a closure of cation channels and loss of a background depolarizing conductance. The amplitude of this outward-like current was 2.1±0.3 pA (n=7) in lactotrophs and 9.9±1.7 pA (n=13) in GH<sub>3</sub> cells. It was not abolished by the blockade of L-type Ca<sub>v</sub> channels with nimodipine or by the inhibition of hyperpolarization-activated HCN channels with 1 mM Cs<sup>+</sup> (data not shown). The same qualitative effects on membrane voltage and current were observed by substituting bath Na<sup>+</sup> with other large organic cations, including TMA and choline (data not shown).

Removal of bath Na<sup>+</sup> also led to cessation of calcium transients and rapid decrease in  $[Ca^{2+}]_i$  basal levels (Fig. 1D). Figures 1E and 1F show that basal PRL release from primary rat pituitary cells is high, indicating that spontaneous  $[Ca^{2+}]_i$  transients are sufficient to trigger hormone secretion. A transient replacement of Na<sup>+</sup> with large organic cations abolished such elevated basal PRL release by perifused

pituitary cells (Fig. 1E), while TTX had no effect on hormone secretion (Fig. 1F). Thus, spontaneous firing of APs and accompanied  $[Ca^{2+}]_i$  transients in lactotrophs and GH<sub>3</sub> cells depends on TTX-insensitive Na<sub>b</sub> conductance, and such electrical activity is critical for basal PRL release.

# Dependence of electrical activity and PRL release on Ca<sup>2+</sup> conductance

To resolve the identity of the channels contributing to Nab conductance-dependent Ca<sup>2+</sup> influx and PRL release, we first used Gd<sup>3+</sup>, an inhibitor of various Ca<sup>2+</sup>-conducting channels, including Ca<sub>v</sub> and TRPC channels (Clapham et al. 2005, Biagi and Enveart 1990, Lacampagne et al. 1994). Electrophysiological experiments in single GH<sub>3</sub> cells and lactotrophs showed that  $Gd^{3+}$  in 2  $\mu M$ concentration not only abolished firing of APs, but also hyperpolarized the cell membrane (Fig. 2A). However, this hyperpolarization was smaller than the one caused by removal of bath Na<sup>+</sup> (Fig. 1A). In single lactotrophs, addition of GdCl<sub>3</sub> abolished spontaneous calcium transients (Fig. 2B) similarly as replacement of Na<sup>+</sup> with NMDG (Fig. 1D). In perifused pituitary cells, Gd<sup>3+</sup> inhibited basal PRL release in a concentration-dependent manner, with an IC<sub>50</sub> value of about 2 µM (Fig. 2C). This raises the possibility that Na<sub>b</sub> channels conduct Ca<sup>2+</sup> in addition to Na<sup>+</sup>, i.e. that some of the non-selective cation channels account for or contribute to Nab conductance. Consistent with this hypothesis, flufenamic acid (FFA), a generic blocker of non-selective cation channels (Egorov et al. 2002, Ghamari-Langroudi and Bourgue 2002), also abolished spontaneous firing of APs in  $GH_3$  cells, and hyperpolarized the cell membrane by 5-10 mV (Fig. 3A). Furthermore, FFA inhibited spontaneous [Ca<sup>2+</sup>], transients in TRH-indentified lactotrophs (Fig. 3B) and basal PRL release in perifused pituitary cells (Fig. 3C). These physiological and pharmacological responses indicate that non-selective cation channels are involved in the background depolarization of pituitary lactotrophs and GH<sub>3</sub> cells.

#### Expression and contribution of TRPC channels to Nab conductance

In further work, we collected more evidence on expression and active involvement of TRPC subfamily of these channels in GH<sub>3</sub> cells. These cells express TRPC1 and TRPC6 mRNA transcripts (Fig. 3D, top), whereas pituitary cells also express mRNA transcripts for TRPC2, TRPC3, TRPC4, TRPC5 and TRPC7 (Fig. 3D, bottom). SKF96365, a widely used and relatively specific inhibitor of TRPC channels (Clapham *et al.* 2005), inhibited the electrical activity in GH<sub>3</sub> cells (data not shown). In lactotrophs, 10  $\mu$ M SKF96365 inhibited [Ca<sup>2+</sup>]<sub>i</sub> transients (Fig. 4A), in some cells after a transient increase in [Ca<sup>2+</sup>]<sub>i</sub> (data not shown). Consistent with these results, application of SKF96365 decreased PRL secretion from perifused pituitary cells in a concentration-dependent manner (Fig. 4B). 2-APB, another blocker of TRPC channels (Clapham *et al.* 2005), had similar effects on AP firing in GH<sub>3</sub> cells (not shown) and basal PRL secretion in perifused pituitary cells (Fig. 4C). PRL secretion was inhibited in a concentration dependent manner with an IC<sub>50</sub> value of 38  $\mu$ M. These results suggest that TRPC channels contribute to Na<sub>b</sub> conductance in lactotrophs and GH<sub>3</sub> cells, and that their contribution is physiologically relevant.

#### DISCUSSION

Lactotrophs and  $GH_3$  cells fire APs spontaneously and such electrical activity drives transients in  $[Ca^{2+}]_i$  (Van Goor *et al.* 2001a, Mollard *et al.* 1996, Schlegel *et al.* 1987) of sufficient amplitude to trigger PRL release (Zorec 1996). The role of bath  $Ca^{2+}$  and  $Ca_v$  channels in both spontaneous firing of APs and basal PRL release is well established. Basal PRL release is abolished in cells with blocked  $Ca_v$  channels, which are responsible for spike depolarization and the accompanied  $Ca^{2+}$  influx (Van Goor *et al.* 2001a). This is a unique characteristic of lactotrophs and somatotrophs; in other anterior pituitary cell types, spontaneous electrical activity does not cause significant hormone secretion (Stojilkovic *et al.* 1988).

In this study, we examined the role of bath Na<sup>+</sup> in electrical activity, Ca<sup>2+</sup> signaling and PRL release. We showed that replacement of bath Na<sup>+</sup> with large organic cations resulted in the membrane hyperpolarization and consequent abolition of spontaneous AP firing in pituitary lactotrophs and GH<sub>3</sub> cells. Experiments with voltage-clamped cells at resting membrane potential further revealed that replacement of bath Na<sup>+</sup> with organic cations caused a loss of basal depolarizing conductance. The Na<sup>+</sup> conductance is TTX-insensitive, it determines the resting membrane potential, and is necessary for the spontaneous firing of Ca<sup>2+</sup>-dependent APs as well as the associated Ca<sup>2+</sup> transients and PRL release. Others published similar observations in GH<sub>3</sub> cells (Simasko 1994) and lactotrophs (Sankaranarayanan and Simasko 1996), and termed this conductance Na<sub>b</sub> conductance. Its presence is not unique to PRL-secreting cells, but was also observed in other endocrine pituitary cells, including somatotrophs and gonadotrophs (Kucka *et al.* 2010). We have reported recently that Na<sub>b</sub> conductance plays an important role in multidrug-resistance protein-mediated cyclic nucleotide efflux in anterior pituitary cells (Kucka *et al.* 2010). However, the nature of the channels that enable this conductance and the mechanism for its activation has not been clarified.

Recently, we have also reported that Na<sub>b</sub> conductance could be mediated by TRPC channels, the largest family of cation-conducting channels (Tomic *et al.* 2011). In general, their activation leads to Na<sup>+</sup> and Ca<sup>2+</sup> influx and consequent depolarization of the plasma membrane (Clapham *et al.* 2005). Consistent with the role of Na<sub>b</sub> current in the control of resting membrane potential in normal and immortalized pituitary cells, some TRPC channels are constitutively active (Trebak *et al.* 2003, Nichols *et al.* 2007). Furthermore, TRPC4 was suggested as a molecular candidate for the nonselective cation channel responsible for the pacemaker activity in interstitial cells of Cajal (Kim *et al.* 2006). TRPC cation channels mediating persistent muscarinic currents also contribute significantly to the firing and mnemonic properties of projection neurons in the entorhinal cortex (Zhang *et al.* 2011). Leptin also depolarizes the plasma membrane via activation of TRPC channels in guinea pig kisspeptin neurons and proopiomelanocortin neurons (Qiu *et al.* 2010, Qui *et al.* 2011). These channels also contribute to agonist-induced depolarization of hypothalamic gonadotropin-releasing neurons (Zhang *et al.* 2008).

With such a tissue-wide distribution and involvement in electrical activity, here we give preliminary evidence for the further role of TRPC channels in  $Na_b$  conductance,  $Ca^{2+}$  signaling and hormone secretion of pituitary lactotrophs and  $GH_3$  lacto-somatotrophs. The mRNA transcripts for TRPC1 channels

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are highly expressed in normal and immortalized pituitary cells, whereas the expression of other subunits of this family of channels is below 5% of TRPC1-mRNAs. The blockers of TRPC channels, SKF96365 and 2-APB, and the nonselective blockers of cation channels including TRPC channels, FFA and  $Gd^{3+}$ , hyperpolarize the plasma membrane and inhibit spontaneous firing of APs, accompanied calcium influx and basal PRL release. Therefore, a TTX-resistant background-depolarizing Na<sup>+</sup> channels, presumably belonging to the TRPC family, are critical for firing of APs, accompanied Ca<sup>2+</sup> influx, and PRL release in lactotrophs and GH<sub>3</sub> cells.

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#### **FIGURE LEGENDS**

**Fig. 1.** Spontaneous electrical activity, calcium influx and basal prolactin (PRL) release of pituitary cells depend on a background Na<sup>+</sup> conductance. A and B, Effects of complete replacement of extracellular Na<sup>+</sup> with NMDG on electrical activity (A) and whole cell current (B) in lactotrophs (left panels) and GH<sub>3</sub> cells (right panels). Note the increased firing frequency after return to normal Na<sup>+</sup>-containing bath buffer. V<sub>h</sub>, holding potential. C, The lack of effects of 1  $\mu$ M TTX, a Na<sub>v</sub> channel blocker, on membrane potential (V<sub>m</sub>) and spontaneous firing of APs in lactotrophs (left panel) and GH<sub>3</sub> cells (right panel). D, Effect of complete replacement of bath Na<sup>+</sup> with NMDG on spontaneous calcium transients in lactotrophs. In this and following figures, data shown are representative of at least 5 recordings. Similar effects of replacement of bath Na<sup>+</sup> with NMDG were observed in other pituitary cell types. E, Effect of complete replacement of bath Na<sup>+</sup> with NMDG, TMA and choline chloride on basal PRL release in perifused pituitary cells. In parallel to electrical activity (Fig. 1A, left), notice the transient increase in PRL release after return of Na<sup>+</sup> containing buffer. F, The lack of effect of TTX on basal PRL release. Gray areas indicate duration of treatments.

**Fig. 2.** Effects of Gd<sup>3+</sup>, a non-specific blocker of calcium-conducting channels, on electrical activity in GH<sub>3</sub> cells and lactotrophs (A), calcium influx in lactotrophs (B) and PRL release in perifused pituitary cells (C) Notice the Gd<sup>3+</sup>-induced hyperpolarization of the plasma membrane in A.

**Fig. 3.** Effects of flufenamic acid (FFA), a blocker of various non-selective cation channels, on electrical activity in GH<sub>3</sub> cells (A), calcium influx in lactotrophs (B) and PRL release in perifused pituitary cells (from top to bottom 25, 50, 75 and 100  $\mu$ M) (C). D, Quantitative RT-PCR analysis of TRPC-mRNA expression in GH<sub>3</sub> cells (top). Data shown are mean ± SEM values of six experiments, using TRPC1-mRNA expression as 100%. Results for pituitary cells (bottom panel) are derived from Tomic *et al.* 2011. Notice the FFA-induced hyperpolarization of the plasma membrane in A.

**Fig. 4.** Effects of specific blockers of TRPC channels on calcium influx and PRL release. A, Inhibition of spontaneous calcium influx by SKF96365 in lactotrophs. B, Dose-dependent effect of SKF96365 on basal PRL release. From top to bottom: 10, 25, 50  $\mu$ M SKF96365. C, Dose-dependent effects of 2-APB on basal PRL release. Left, representative records in response to 0, 5, 10, 25, 50, 75 and 100  $\mu$ M 2-APB (from top to bottom). Right, concentration-dependence study revealed the IC<sub>50</sub> value of 38  $\mu$ M. Mean ± SEM values of four experiments are shown.



Fig. 1



Fig. 2



Fig. 3.

Fig. 4.

