

STEROID MODULATION OF BK CHANNEL GATING AND CELLULAR
EXCITABILITY IN NEUROENDOCRINE STRESS RESPONSES

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During a stressful event, the adrenal cortex increases the secretion of stress steroid hormones, known as glucocorticoids. Glucocorticoids have a variety of physiological effects aimed to rectify the disruption in homeostasis caused by a stressor. These effects are regulated through genomic mechanisms, but increasing evidence has supported the role of rapid (non-genomic) actions of steroid hormones in the regulation of physiology and behavior. BK channels are prominent regulators of cellular excitability, and are expressed in many different types of tissues including muscle and the brain. In the HPA stress response axis, BK channels could be vital in the control of the feedback mechanisms of the stress response. Stress steroids modulate BK channel splicing and other steroids including estrogens, xenoestrogens, and the androgen DHEA rapidly alter BK channel gating. By rapidly modulating adrenal chromaffin cells and anterior corticotropes, steroid hormones can regulate and fine-tune the stress response.

In the present study, I investigated the role of stress hormones in the rapid modulation of BK channels in different components of the HPA axis. Glucocorticoids released from the adrenal cortex during the stress response rapidly modulate BK channel gating and can facilitate or limit chromaffin cell excitability. By acutely modulating BK channels in chromaffin cells, glucocorticoids can affect adrenaline secretion. I used the heterologous expression system of HEK293 cells to investigate the mechanism responsible for the rapid modulation of BK channels. BK β 2 and β 4 subunits are differentially sensitive to the steroids corticosterone and DHEA.

Targeting auxiliary subunits of an K^+ ion channel is a novel mechanism for rapid modulation by steroid hormones and could give rise to differential modulation in tissues. Finally, I characterized BK channels that are present in anterior pituitary corticotropes and explored their role as important regulators of excitability. The anterior pituitary in addition to adrenal chromaffin cells could be a target for the acute modulation of stress steroids. Steroids can have both acute and genomic effects to regulate physiology and behavior. Understanding the mechanisms that modulate neuroendocrine interactions will provide valuable insight for stress related disorders.

BIOGRAPHICAL SKETCH

Jonathan T. King was born on October 4, 1976 in Kansas City, Missouri.

Jonathan shared his birth year with many memorable achievements and milestones including our nation's bicentennial, the formation of Apple computer, the release of the Ramones first self-titled album, the birth of Peyton Manning, and other political and social accomplishments. Jonathan grew up in the sleepy town of Olathe, Kansas, and enjoyed the midwest lifestyle as a child, but bigger better things were about to transpire in Jonathan's life. At the age of 9, Jonathan's family translocated to sunny southern California, and he was immersed in a natural paradise where 30 minutes in the west would take you to the peaceful Pacific Ocean and 30 minutes to the east would bring you to the snow-covered mountains. As a child, Jonathan was always exploring the outdoors, enjoying the year round sunshine and became fascinated by the ocean and life teeming from the abyss. His exposure to the natural world at this age molded Jonathan's interest in the natural sciences. Another hobby of Jonathan's as a young adult was music. During his formative years as a high school student he was a big fan of classic rock and "alternative" music and took up playing the guitar. Sometime during his freshman year in college Jonathan became infatuated with the culture of Great Britain and would frequent many local area public houses once he reached legal drinking age. In addition to science, music and the UK, Jonathan was also interested in electronics and technology. As a high school sophomore he took an industrial drafting class that inspired him to pursue a career in engineering.

When the time arrived for college, he decided to pursue a degree in mechanical engineering at California Polytechnic University at Pomona with aspirations of designing cars and mechanical tools. However, after just one year, Jonathan was disillusioned by the restrictive nature of mechanical engineering. He decided to completely switch majors and universities and started his sophomore year at California

State University, San Bernadino. He changed his major to biology, a subject he was passionate about since childhood, and while he was a biology major, he took a neurobiology course and instantly became enamored with the physiology and mechanics of the brain. During his junior year in college, Jonathan joined the lab of Dr. Jeff Thompson and began to research putative neurotrophic factors from retinal ganglion cells. He became enthralled with neuroscience and applied to several neuroscience graduate programs in California, and some programs outside the state. After an informative and successful recruitment visit, Jonathan decided to join the department of Neurobiology and Behavior at Cornell University in the summer of 2000. After one year of laboratory rotations he joined the lab of Dr. David McCobb studying the acute modulation of BK channels by steroid hormones and components of the neuroendocrine stress response. In addition to his research responsibilities in graduate school, Jonathan was also a teaching assistant for four semesters for Introduction to Neurobiology (BioNB 222) and for one semester in the Principles of Neurophysiology (BioNB 491), and through this experience he developed a passion for teaching.

To adjust to a new life in Ithaca, Jonathan and his core group of friends immersed themselves in the wonderment of the National Football League (NFL) during the cold winter months, other sporting events, and various recreational events throughout the year. These pastimes provided much needed rest and relaxation from the daily rigor of scientific discovery and progress.

As Jonathan departs Ithaca in late August 2007, he is reminiscent of the achievements and milestone from all the past years that have spanned his graduate career: especially the crowning of the Indianapolis Colts as Superbowl XLI champs. And he looks forward to a career in the field of Neuroscience, teaching and conducting research that will someday contribute to unlocking the secrets of the brain.

Dedicated to my family, for their support.

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With every major achievement in one's life, there are numerous pillars of reinforcement. Many people have helped me achieve my goals as a graduate student and I am eternally grateful to everyone who has supported my career thus far. First, I want to thank my family for their undying encouragement. My mom and dad, Eileen and Carl, are always a calming influence and my biggest advocates throughout my life. My sisters, Theresa and Grace are always available to provide countless hours of advice and counseling when I need it the most.

During my tenure at NB&B, I have met a countless number of friends, colleagues and professors that have all facilitated my success. I am forever indebted to Pete Lovell, who was a graduate student in the McCobb lab when I first joined, and taught me everything I know about electrophysiology. Several professors have been instrumental in my success at NB&B. I would like to thank all the members of my special committee, Andy Bass, David Deitcher and Manfred Lindau. They have helped to shape my scientific career and offered input during every stage of my graduate career. Ron Booker and Joe Fetcho have both been tremendous guides to me and offered precious advice about my future career. I would like to give my most sincere appreciation to my advisor and mentor David McCobb. I want thank David for accepting me into his lab with open arms. His guidance and extreme patience with me for all of these years have been an immense encouragement. David has such a passion for science that it has transferred over and invigorated my own fervor for science. Additionally, I would like to thank the NIH for grants NS40790 and GM07469 and the TA-ships received from NB&B for funding.

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Without my friends, my time at Cornell would have been extremely dull and monotonous. While here at NB&B I have solidified lifelong friendships that I will cherish throughout my life. When I first arrived at Cornell, I was immediately befriended by Jason MacLean and Damian Elias, who educated my naïve mind about the ins and outs of the Ithaca community and Cornell graduate life. Damian has since become a great friend and a close confidant. I must also extend gratitude to my other friends from NB&B: Joe Sisneros, Paul Forlano, Sujata Rao, Juliana-Rangel-Posada, and my generous friend Dustin Rubinstein. All of who have made my time at Cornell enjoyable. Most recently, my great friends Matt Arnegard, Dave McLean, and Jordan Moore have been a constant source of laughter and a solid core of friends. I thank Matt for all of his wisdom and advice, Dave for his camaraderie and wit and Jordan for his major toughness and being the voice of reason.

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CHAPTER 1

INTRODUCTION

An organism's response to stress involves two seemingly independent physiological systems acting on different time scales. The most immediate response, occurring within seconds, involves the activation of the sympathetic nervous system and the release of adrenaline from adrenal medullary chromaffin cells. Adrenaline immediately affects heart rate, vasoconstriction and metabolism. The other component of the stress response involves the HPA axis (hypothalamic- pituitary- adrenal) and the secretion of glucocorticoids from the adrenal cortex. Glucocorticoids directly affect metabolism, cardiovascular function, immune function, and reproduction on a slow time scale (minutes to hours) involving the transcription of a variety of genes.

In addition to the classic genomic mechanism of action by steroid hormones, evidence from the last 30 years show that steroids can modulate sexual behavior, hormone release, and intracellular Ca^{2+} , through rapid (nongenomic) mechanisms (Ramirez and Dluzen, 1987; Orchinik et al., 1991; Morley et al., 1992). Each component of the HPA axis (the hypothalamus, pituitary and adrenal gland) could be potential targets for rapid modulation by stress steroids. One of the potential targets includes adrenal medullary cells. Due to the unique structure of the adrenal gland, medullary chromaffin cells are episodically bathed by high concentrations of steroid hormones from the adrenal cortex. Glucocorticoids have well-established genomic effects on chromaffin cells (described in detail below), but their rapid effects have not been fully investigated. Anterior pituitary corticotropes are another potential target for rapid steroidal modulation. They are modulated genomically by glucocorticoids, but their electrophysiological characterization and rapid modulation by steroids remains largely unknown. Taken together, steroid hormones are promiscuous molecules that

target cytoplasmic steroid receptors in addition to membrane bound receptors to regulate the stress response.

In this dissertation, I provide evidence that steroid hormones produced from the adrenal cortex rapidly modulate BK channels through a non-genomic mechanism. This modulation is achieved through the differential sensitivity of BK β -subunits to a variety of stress and sex steroids. BK channels are important regulators of excitability in adrenal medullary chromaffin cells, and in anterior pituitary corticotropes. Steroids target BK channels in these cells to rapidly modulate the excitability and hormone release of these cells. The acute actions of steroids on components of the HPA axis contribute to complex feedback and feed-forward mechanisms of the stress response that tailor an individual's behavior.

Two Systems of the Stress Response:

Stressors in everyday life come in many forms. They range from physical stressors like hunger and cold to psychosocial stressors such as aggressive encounters, financial pressures, finding a potential mate, and public speaking. All of these stressors share one common theme, they disrupt the body's homeostasis (a state of optimal physiological processes). To rectify this disruption, the body has developed two physiological processes known together as the stress response. The most immediate response to stress is the activation of the sympathetic nervous system, also known as the "fight or flight response" (Cannon, 1929). During this response, there is activation of the sympathetic nervous system which innervates the adrenal medulla via the splanchnic nerve, triggering the release of adrenaline (or epinephrine) into the general circulation. Within seconds to minutes the physiological effects of adrenaline begin to take effect. These effects include an increase in heart rate, constriction of blood vessels, and mobilization of energy to muscles. The other branch of the stress

response operates on a relatively slower temporal scale, involves the hypothalamus, anterior pituitary, and adrenal gland and commonly referred to as the HPA axis. Once a stressor is perceived by the brain, there is activation of the hypothalamus, specifically, cells in the paraventricular nucleus (PVN) release corticotropin releasing hormone (CRH) into the portal blood system (Nelson, 2000). CRH then activates release of adrenocorticotrophic hormone (ACTH) from anterior pituitary corticotropes into the general circulation. Once ACTH reaches the adrenal glands it stimulates synthesis and release of the glucocorticoids from the adrenal cortex (in most rodents, birds and reptiles, the primary glucocorticoid is corticosterone; in most primates and carnivores it is cortisol, both will be abbreviated CORT) (Stratakis and Chrousos, 1995). Glucocorticoids regulate a variety of physiological actions including increasing carbohydrate, protein and lipid metabolism, increasing blood glucose levels, suppression of reproductive systems, and complex interactions with immune function (Munck and Naray-Fejes-Toth, 1994; Sapolsky et al., 2000).

Actions of Steroids

To mediate these physiological changes, glucocorticoids act through the classical mechanism of binding to cytosolic receptors, which act as transcription factors. There are two receptors that glucocorticoids bind to, the mineralocorticoid receptor (MR) and the glucocorticoid receptor (GR). MRs have a ten fold higher affinity for GCs than GRs (Reul and de Kloet, 1985). MR and GR expression occur throughout the brain, including the hippocampus and the cortex. The highest expression of GRs occurs in the CRH secreting cells of the hypothalamus and anterior pituitary corticotropes. MRs are hypothesized to be activate during basal secretion of GCs, and GRs become activated with stress induced rises in GC concentration. Activation of GRs with increased levels of GCs are thought to account for

physiological responses aimed to restore an organism's homeostasis (De Kloet and Reul, 1987). Once steroids are bound to their cytosolic receptors, the steroid-receptor complex translocates to the nucleus and binds to a specific nucleotide sequence of a gene, and facilitates or inhibits transcription.

The view that steroid hormones act primarily through classic genomic mechanisms is under constant revision as evidence emerges from rapid non-genomic effects on physiology and behavior. Recent evidence has shown that putative membrane receptors for steroids can be activated within seconds of exposure to steroids, are resistant to transcriptional inhibitors, and change cellular physiology and behavior (Orchinik et al., 1991; Wehling, 1994; Falkenstein et al., 2000) (Zinder and Dar, 1999).

The Adrenal Gland

At the intersection of these two systems of the stress response is the adrenal gland. The outer cortical layer of the gland, develops from the mesodermal region of the developing embryo, and is responsible for secreting various steroid hormones including stress steroids. The cortex is divided into 3 zones, and each produces its own type of steroid hormone. The zona glomerulosa produces mineralocorticoids such as aldosterone, the zona fasciculata produces and secretes glucocorticoids, and the zona reticularis produces sex steroids (Moon, 1961). Chromaffin cells, which make up the medullary region of the adrenal gland, are modified postganglionic sympathetic neurons that have lost their axons and dendrites (Hodel, 2001). Their name is related to the fact that these cells stain brown with chromium salts (Moon, 1961).

Chromaffin cells are prime targets for modulation by glucocorticoids. Their close proximity to the adrenal cortex exposes them to episodic pulses of steroids at higher concentrations than in systemic blood. Glucocorticoids mediate genomic

effects on chromaffin cells by increasing and maintaining enzymes that regulate the production of adrenaline (Ross et al., 1990; Hodel, 2001), and by increasing current through voltage gated Ca^{2+} channels (Fuller et al., 1997). More rapid effects have briefly been described for glucocorticoids and chromaffin cells. Dexamethasone (a synthetic glucocorticoid) was found to directly bind to the acetylcholine receptor, decreasing the cation flux into cultured chromaffin cells (Inoue and Kuriyama, 1989). Other rapid effects on chromaffin cells have not been explored.

The Structure and Function of the BK channel:

Ion channels are key regulators of cellular excitability. Complex interactions between Na^+ , Ca^{2+} and K^+ channels help shape the intrinsic electrical properties of chromaffin cells. Large conductance Ca^{2+} and voltage activated potassium (BK) channels are the most prominent voltage-gated K^+ channel in chromaffin cells. They enhance repetitive firing by facilitating Na^+ and Ca^{2+} channel de-inactivation, and show species specific differences in gating kinetics (Lovell et al., 2000).

The potassium ion channel family represents the largest, most diverse group of ion channels (Coetzee et al., 1999). They play important roles in setting the resting membrane potential, determining the shape and duration of the action potential, and regulating the firing rates and excitability of cells. For a subset of the K^+ ion channel family, an increase in K^+ permeability is enhanced by an increase in the cytosolic free Ca^{2+} (Latorre et al., 1989). These Ca^{2+} sensitive channels can subsequently be categorized by their unitary conductances. There are “small” conductance (<20 pS) Ca^{2+} activated K^+ channels (Blatz and Magleby, 1986; Lang and Ritchie, 1987), that contribute to the generation of long lasting hyperpolarizations (Vergara et al., 1998). A second category includes intermediate conductance Ca^{2+} sensitive K^+ (IK) channels, first identified in red blood cells (Gardos, 1958), with unitary conductances between

20-80 pS (Ishii et al., 1997). Finally, there are large conductance Ca^{2+} - and voltage-activated K^+ (BK for “Big” K) channels (Marty, 1981) that are characterized by single channel conductances of 200-300 pS in symmetrical 100-200 mM K^+ (Latorre et al., 1989). BK channels are expressed in a variety of tissues, including chromaffin cells, and play important roles in regulating cellular excitability (Vergara et al., 1998). Their large single channel conductance allows for the rapid repolarization of the membrane potential. The pore forming α -subunit of the BK channel was first cloned from *Drosophila Slowpoke (Slo)* (Atkinson et al., 1991). The *Slo* gene appears to be highly conserved across mammals (Butler et al., 1993; Pallanck and Ganetzky, 1994). Functional BK channels are formed by a tetramer of α -subunits encoded by the *Slo* gene. BK channels share a structural similarity to the voltage-gated K^+ channel extended family in the S1-S6 transmembrane regions, yet have an additional transmembrane region (S0) which places the NH_2 terminus on the extracellular side, and an extended carboxyl terminus, where the Ca^{2+} sensitive domain is located (Schreiber and Salkoff, 1997; Salkoff et al., 2006).

The physiological roles of BK channels have been explored through several genetic studies. In the nematode *C. elegans*, a null mutation of the *slo* gene increases the duration of neurotransmitter release (Wang et al., 2001). Also in *C. elegans*, *Slo*-mutants are highly resistant to ethanol, implicating their role in alcohol tolerance (Davies et al., 2003). Studies with *Slo* knockout mice have revealed anomalies at several levels, including locomotor deficiencies due to cerebellar ataxia, erectile dysfunction, incontinence, progressive deafness, and disruption of circadian rhythms (Meredith et al., 2004; Ruttiger et al., 2004; Sausbier et al., 2004; Werner et al., 2005; Meredith et al., 2006). BK channels have also been implicated in epilepsy. Mutations in the pore forming α -subunit lead to increased excitability, with generalized epilepsy and paroxysmal movement disorder (Du et al., 2005). It is not surprising that BK

channels have a wide range of physiological effects, since they are widely expressed throughout the body, including the brain.

Alternative splicing of BK channels, as well as the association with auxiliary β - subunits and modulation by intracellular messengers alters gating kinetics, Ca^{2+} sensitivity, voltage dependence and sensitivity to toxins (Xie and McCobb, 1998; Schubert and Nelson, 2001; Shipston, 2001; Tian et al., 2001).

Regulatory β -subunits of the BK channel

Accessory β -subunits are encoded by four different genes (β 1- β 4). All are comprised of two membrane spanning segments (Knaus et al., 1994; Brenner et al., 2000a; Meera et al., 2000; Uebele et al., 2000). Each β -subunit has distinct effects on BK channel properties, and is expressed in different tissues. The BK β 1-subunit was the first to be discovered and is expressed in smooth muscle, hair cells, and neurons (Knaus et al., 1994; Wallner et al., 1999; Orio et al., 2002). BK channels in cells that co-express β 1 along with α exhibit a relative negative shift in the voltage dependence of activation compared with cells expressing only the α -subunit. The presence of the β 1-subunit also slows the kinetics of activation and deactivation. BK channels in smooth muscles play an important role in vasoregulation. The targeted deletion of the BK β 1-subunits in smooth muscles leads to an increase in arterial tone and blood pressure (Brenner et al., 2000b). BK channels that include β 2 subunits exhibit an inactivating phenotype and are found in the brain as well as the adrenal medulla (Wallner et al., 1999; Xia et al., 1999). In chromaffin cells, BK channels that express the β 2-subunit and exhibit an inactivating phenotype facilitate the recovery of Na^+ channels from inactivation and allow for repetitive firing (Solaro et al., 1995). Cloning and expression of the BK β 3 subunit revealed that it also confers inactivation, but that inactivation properties differ between different splice variants of the subunit. BK β 3

subunits are expressed in the testis, pancreas and spleen (Xia et al., 1999; Behrens et al., 2000; Zeng et al., 2001). The $\beta 4$ subunit of the BK channel has high expression in the brain and decreases the apparent Ca^{2+} sensitivity of the channel (Brenner et al., 2000a; Meera et al., 2000). Studies using chimeric β -subunits also reveal that the extracellular loop of the $\beta 4$ -subunit alters the sensitivity of the channel to toxins such as charybdotoxin and iberiotoxin by decreasing toxin binding (Meera et al., 2000). $\beta 4$ -subunits have also been proposed to have a regulatory role in excitability and help protect the hippocampus from seizures (Brenner et al., 2005). BK channel functional diversity is greatly enhanced by association with β -subunits. With their effects on Ca^{2+} sensitivity, gating kinetics and differential tissue expression, they are poised to be prime targets for modulation by a variety of molecules.

Previous research has shown that BK channels are acutely modulated by steroids such as DHEA (dehydroepiandrosterone, an androgen produced by the adrenal cortex), estrogen, xeno-estrogens, and bile acids (steroidal anions) (Farrukh et al., 1998; Valverde et al., 1999; Behrens et al., 2000; Dick and Sanders, 2001; Fukao et al., 2001; Dopico et al., 2002; Sade et al., 2005). Other steroids could potentially target BK channels and have the potential to alter cellular excitability and the physiology of a wide variety of cells.

Rapid modulatory actions of the stress response by glucocorticoids remain largely unknown. By examining the immediate effects of stress steroids we can begin to understand how different stressors can exert both acute as well as chronic effects on the physiological response to stress. Since chromaffin cells reside in such close proximity to the cortex, we examined the effects that the stress steroids CORT and DHEA (both produced by the adrenal cortex) have on chromaffin cells. In chapter 2 of this dissertation, I present evidence that glucocorticoids released during the stress response have rapid effects on the electrical excitability of adrenal chromaffin cells,

and can facilitate or limit the firing of action potentials. By targeting BK channels in chromaffin cells, glucocorticoids can modify catecholamine secretion on a rapid time scale.

With evidence emerging that steroids such as estradiol target BK β 1-subunits (Valverde et al., 1999; Behrens et al., 2000), along with evidence showing that β -subunits are expressed in the adrenal medulla (King et al., 2006), we decided to test whether different β -subunits were differentially sensitive to steroids. In chapter 3, I describe how I used the heterologous expression system of HEK 293 cells to test the hypothesis that different BK β -subunits are sensitive to different steroid hormones. BK channels that express only the pore forming α -subunit are not sensitive to steroid hormones. The differential tissue expression of β -subunits could give rise to diverse rapid steroid actions.

The Pituitary Gland

A potential target of the HPA axis for rapid modulation of steroid hormones are corticotropes of the anterior pituitary gland. The pituitary gland is an endocrine gland that sits at the base of the brain and is connected to the hypothalamus through a vascular connection in the pituitary stalk. The gland is composed of the posterior pituitary (neurohypophysis- developmentally derived from the neural ectoderm) and the anterior pituitary gland (adenohypophysis- derived from Rathke's pouch). The anterior pituitary is comprised of multiple hormone secreting cells, including gonadotropes, thyrotropes, lactotropes, somatotropes and corticotropes. The excitable properties of some anterior pituitary cell types have been examined previously (for review see (Stojilkovic et al., 2005)). However, the excitable properties and characterization of K^+ channels in corticotropes have not been fully examined due to the difficulty in isolating corticotropes from other cell types in the anterior pituitary. In

chapter 4 of this dissertation, I present the first characterization of BK channels in “real”, non-tumor derived pituitary corticotropes. As in chromaffin cells, BK channels are the prominent voltage-gated K^+ channel present and contribute to the electrical activity of corticotropes. Steroid hormones could exploit the presence of BK channels in corticotropes to rapidly modulate excitability and hormone secretion.

The stress response encompasses an array of physiological processes that have been studied for decades (Selye, 1976). Over-activation of the HPA axis by stressors can lead to negative health consequences. Both genomic and non-genomic effects of stress steroid hormones are known to regulate physiology and behavior. Investigating the feedback mechanisms that tailor the stress response will provide valuable insight into organismal health.

REFERENCES

- Atkinson NS, Robertson GA, Ganetzky B (1991) A component of calcium-activated potassium channels encoded by the *Drosophila slo* locus. *Science* 253:551-555.
- Behrens R, Nolting A, Reimann F, Schwarz M, Waldschutz R, Pongs O (2000) hKCNMB3 and hKCNMB4, cloning and characterization of two members of the large-conductance calcium-activated potassium channel beta subunit family. *FEBS Lett* 474:99-106.
- Blatz AL, Magleby KL (1986) Single apamin-blocked Ca-activated K⁺ channels of small conductance in cultured rat skeletal muscle. *Nature* 323:718-720.
- Brenner R, Jegla TJ, Wickenden A, Liu Y, Aldrich RW (2000a) Cloning and functional characterization of novel large conductance calcium-activated potassium channel beta subunits, hKCNMB3 and hKCNMB4. *J Biol Chem* 275:6453-6461.
- Brenner R, Chen QH, Vilaythong A, Toney GM, Noebels JL, Aldrich RW (2005) BK channel beta4 subunit reduces dentate gyrus excitability and protects against temporal lobe seizures. *Nat Neurosci* 8:1752-1759.
- Brenner R, Perez GJ, Bonev AD, Eckman DM, Kosek JC, Wiler SW, Patterson AJ, Nelson MT, Aldrich RW (2000b) Vasoregulation by the beta1 subunit of the calcium-activated potassium channel. *Nature* 407:870-876.
- Butler A, Tsunoda S, McCobb DP, Wei A, Salkoff L (1993) mSlo, a complex mouse gene encoding "maxi" calcium-activated potassium channels. *Science* 261:221-224.
- Cannon WB (1929) Bodily changes in pain, hunger, fear and rage; an account of recent researches into the function of emotional excitement., 2nd Edition. New York, London: D. Appleton and Company.
- Coetzee WA, Amarillo Y, Chiu J, Chow A, Lau D, McCormack T, Moreno H, Nadal MS, Ozaita A, Pountney D, Saganich M, Vega-Saenz de Miera E, Rudy B (1999) Molecular diversity of K⁺ channels. *Ann N Y Acad Sci* 868:233-285.
- Davies AG, Pierce-Shimomura JT, Kim H, VanHoven MK, Thiele TR, Bonci A, Bargmann CI, McIntire SL (2003) A central role of the BK potassium channel in behavioral responses to ethanol in *C. elegans*. *Cell* 115:655-666.
- De Kloet ER, Reul JM (1987) Feedback action and tonic influence of corticosteroids on brain function: a concept arising from the heterogeneity of brain receptor systems. *Psychoneuroendocrinology* 12:83-105.

- Dick GM, Sanders KM (2001) (Xeno)estrogen sensitivity of smooth muscle BK channels conferred by the regulatory beta1 subunit: a study of beta1 knockout mice. *J Biol Chem* 276:44835-44840.
- Dopico AM, Walsh JV, Jr., Singer JJ (2002) Natural bile acids and synthetic analogues modulate large conductance Ca²⁺-activated K⁺ (BKCa) channel activity in smooth muscle cells. *J Gen Physiol* 119:251-273.
- Du W, Bautista JF, Yang H, Diez-Sampedro A, You SA, Wang L, Kotagal P, Luders HO, Shi J, Cui J, Richerson GB, Wang QK (2005) Calcium-sensitive potassium channelopathy in human epilepsy and paroxysmal movement disorder. *Nat Genet* 37:733-738.
- Falkenstein E, Tillmann HC, Christ M, Feuring M, Wehling M (2000) Multiple actions of steroid hormones--a focus on rapid, nongenomic effects. *Pharmacol Rev* 52:513-556.
- Farrukh IS, Peng W, Orlinska U, Hoidal JR (1998) Effect of dehydroepiandrosterone on hypoxic pulmonary vasoconstriction: a Ca(2+)-activated K(+)-channel opener. *Am J Physiol* 274:L186-195.
- Fuller L, Lu C, McMahan D, Alaudin E, Jorgensen M, Rau S, Sissen J, Jackson B (1997) Effect of dexamethasone on voltage-gated Ca²⁺ channels and cytosolic Ca²⁺ in rat chromaffin cells. *Neuroreport* 8:1169-1172.
- Gardos G (1958) The function of calcium in the potassium permeability of human erythrocytes. *Biochim Biophys Acta* 30:653-654.
- Hodel A (2001) Effects of glucocorticoids on adrenal chromaffin cells. *J Neuroendocrinol* 13:216-220.
- Inoue M, Kuriyama H (1989) Glucocorticoids inhibit acetylcholine-induced current in chromaffin cells. *Am J Physiol* 257:C906-912.
- Ishii TM, Silvia C, Hirschberg B, Bond CT, Adelman JP, Maylie J (1997) A human intermediate conductance calcium-activated potassium channel. *Proc Natl Acad Sci U S A* 94:11651-11656.
- King JT, Lovell PV, Rishniw M, Kotlikoff MI, Zeeman ML, McCobb DP (2006) Beta2 and beta4 subunits of BK channels confer differential sensitivity to acute modulation by steroid hormones. *J Neurophysiol* 95:2878-2888.
- Knaus HG, Folander K, Garcia-Calvo M, Garcia ML, Kaczorowski GJ, Smith M, Swanson R (1994) Primary sequence and immunological characterization of beta-subunit of high conductance Ca(2+)-activated K+ channel from smooth muscle. *J Biol Chem* 269:17274-17278.

- Lang DG, Ritchie AK (1987) Large and small conductance calcium-activated potassium channels in the GH3 anterior pituitary cell line. *Pflugers Arch* 410:614-622.
- Latorre R, Oberhauser A, Labarca P, Alvarez O (1989) Varieties of calcium-activated potassium channels. *Annu Rev Physiol* 51:385-399.
- Lovell PV, James DG, McCobb DP (2000) Bovine versus rat adrenal chromaffin cells: big differences in BK potassium channel properties. *J Neurophysiol* 83:3277-3286.
- Marty A (1981) Ca-dependent K channels with large unitary conductance in chromaffin cell membranes. *Nature* 291:497-500.
- Meera P, Wallner M, Toro L (2000) A neuronal beta subunit (KCNMB4) makes the large conductance, voltage- and Ca²⁺-activated K⁺ channel resistant to charybdotoxin and iberiotoxin. *Proc Natl Acad Sci U S A* 97:5562-5567.
- Meredith AL, Thorneloe KS, Werner ME, Nelson MT, Aldrich RW (2004) Overactive bladder and incontinence in the absence of the BK large conductance Ca²⁺-activated K⁺ channel. *J Biol Chem* 279:36746-36752.
- Meredith AL, Wiler SW, Miller BH, Takahashi JS, Fodor AA, Ruby NF, Aldrich RW (2006) BK calcium-activated potassium channels regulate circadian behavioral rhythms and pacemaker output. *Nat Neurosci* 9:1041-1049.
- Moon HD, ed (1961) *The Adrenal Cortex*. New York, NY: Harper & Brothers.
- Morley P, Whitfield JF, Vanderhyden BC, Tsang BK, Schwartz JL (1992) A new, nongenomic estrogen action: the rapid release of intracellular calcium. *Endocrinology* 131:1305-1312.
- Munck A, Naray-Fejes-Toth A (1994) Glucocorticoids and stress: permissive and suppressive actions. *Ann N Y Acad Sci* 746:115-130; discussion 131-113.
- Nelson RJ (2000) *An introduction to behavioral endocrinology*, 2nd Edition. Sunderland, MA: Sinauer Associates.
- Orchinik M, Murray TF, Moore FL (1991) A corticosteroid receptor in neuronal membranes. *Science* 252:1848-1851.
- Orio P, Rojas P, Ferreira G, Latorre R (2002) New Disguises for an Old Channel: MaxiK Channel beta-Subunits. *News Physiol Sci* 17:156-161.
- Pallanck L, Ganetzky B (1994) Cloning and characterization of human and mouse homologs of the *Drosophila* calcium-activated potassium channel gene, slowpoke. *Hum Mol Genet* 3:1239-1243.

- Ramirez VD, Dluzen D (1987) Is progesterone a pre-hormone in the CNS? *J Steroid Biochem* 27:589-598.
- Reul JM, de Kloet ER (1985) Two receptor systems for corticosterone in rat brain: microdistribution and differential occupation. *Endocrinology* 117:2505-2511.
- Ross ME, Evinger MJ, Hyman SE, Carroll JM, Mucke L, Comb M, Reis DJ, Joh TH, Goodman HM (1990) Identification of a functional glucocorticoid response element in the phenylethanolamine N-methyltransferase promoter using fusion genes introduced into chromaffin cells in primary culture. *J Neurosci* 10:520-530.
- Ruttiger L, Sausbier M, Zimmermann U, Winter H, Braig C, Engel J, Knirsch M, Arntz C, Langer P, Hirt B, Muller M, Kopschall I, Pfister M, Munkner S, Rohbock K, Pfaff I, Rusch A, Ruth P, Knipper M (2004) Deletion of the Ca²⁺-activated potassium (BK) α -subunit but not the BK β 1-subunit leads to progressive hearing loss. *Proc Natl Acad Sci U S A*.
- Sade H, Muraki K, Ohya S, Hatano N, Imaizumi Y (2005) Activation of large-conductance Ca²⁺ activated K⁺ channels by cannabinoids. *Am J Physiol Cell Physiol*.
- Salkoff L, Butler A, Ferreira G, Santi C, Wei A (2006) High-conductance potassium channels of the SLO family. *Nat Rev Neurosci* 7:921-931.
- Sapolsky RM, Romero LM, Munck AU (2000) How do glucocorticoids influence stress responses? Integrating permissive, suppressive, stimulatory, and preparative actions. *Endocr Rev* 21:55-89.
- Sausbier M, Hu H, Arntz C, Feil S, Kamm S, Adelsberger H, Sausbier U, Sailer CA, Feil R, Hofmann F, Korth M, Shipston MJ, Knaus HG, Wolfer DP, Pedroarena CM, Storm JF, Ruth P (2004) Cerebellar ataxia and Purkinje cell dysfunction caused by Ca²⁺-activated K⁺ channel deficiency. *Proc Natl Acad Sci U S A* 101:9474-9478.
- Schreiber M, Salkoff L (1997) A novel calcium-sensing domain in the BK channel. *Biophys J* 73:1355-1363.
- Schubert R, Nelson MT (2001) Protein kinases: tuners of the BKCa channel in smooth muscle. *Trends Pharmacol Sci* 22:505-512.
- Selye H (1976) *The Stress of Life*. New York: McGraw-Hill.
- Shipston MJ (2001) Alternative splicing of potassium channels: a dynamic switch of cellular excitability. *Trends Cell Biol* 11:353-358.

- Solaro CR, Prakriya M, Ding JP, Lingle CJ (1995) Inactivating and noninactivating Ca²⁺- and voltage-dependent K⁺ current in rat adrenal chromaffin cells. *J Neurosci* 15:6110-6123.
- Stojilkovic SS, Zemkova H, Van Goor F (2005) Biophysical basis of pituitary cell type-specific Ca²⁺ signaling-secretion coupling. *Trends Endocrinol Metab* 16:152-159.
- Stratakis CA, Chrousos GP (1995) Neuroendocrinology and pathophysiology of the stress system. *Ann N Y Acad Sci* 771:1-18.
- Tian L, Duncan RR, Hammond MS, Coghill LS, Wen H, Rusinova R, Clark AG, Levitan IB, Shipston MJ (2001) Alternative splicing switches potassium channel sensitivity to protein phosphorylation. *J Biol Chem* 276:7717-7720.
- Uebele VN, Lagrutta A, Wade T, Figueroa DJ, Liu Y, McKenna E, Austin CP, Bennett PB, Swanson R (2000) Cloning and functional expression of two families of beta-subunits of the large conductance calcium-activated K⁺ channel. *J Biol Chem* 275:23211-23218.
- Valverde MA, Rojas P, Amigo J, Cosmelli D, Orio P, Bahamonde MI, Mann GE, Vergara C, Latorre R (1999) Acute activation of Maxi-K channels (hSlo) by estradiol binding to the beta subunit. *Science* 285:1929-1931.
- Vergara C, Latorre R, Marrion NV, Adelman JP (1998) Calcium-activated potassium channels. *Curr Opin Neurobiol* 8:321-329.
- Wallner M, Meera P, Toro L (1999) Molecular basis of fast inactivation in voltage and Ca²⁺-activated K⁺ channels: a transmembrane beta-subunit homolog. *Proc Natl Acad Sci U S A* 96:4137-4142.
- Wang ZW, Saifee O, Nonet ML, Salkoff L (2001) SLO-1 potassium channels control quantal content of neurotransmitter release at the *C. elegans* neuromuscular junction. *Neuron* 32:867-881.
- Wehling M (1994) Nongenomic Actions of Steroid-Hormones. *Trends in Endocrinology and Metabolism* 5:347-353.
- Werner ME, Zvara P, Meredith AL, Aldrich RW, Nelson MT (2005) Erectile dysfunction in mice lacking the large-conductance calcium-activated potassium (BK) channel. *J Physiol* 567:545-556.
- Xia XM, Ding JP, Lingle CJ (1999) Molecular basis for the inactivation of Ca²⁺- and voltage-dependent BK channels in adrenal chromaffin cells and rat insulinoma tumor cells. *J Neurosci* 19:5255-5264.

Xie J, McCobb DP (1998) Control of alternative splicing of potassium channels by stress hormones. *Science* 280:443-446.

Zeng XH, Ding JP, Xia XM, Lingle CJ (2001) Gating properties conferred on BK channels by the beta3b auxiliary subunit in the absence of its NH(2)- and COOH termini. *J Gen Physiol* 117:607-628.

Zinder O, Dar DE (1999) Neuroactive steroids: their mechanism of action and their function in the stress response. *Acta Physiol Scand* 167:181-188.

CHAPTER 2

ACUTE MODULATION OF ADRENAL CHROMAFFIN CELL BK CHANNEL GATING AND CELL EXCITABILITY BY GLUCOCORTICOIDS*

Although adrenal glucocorticoids cortisol and corticosterone (CORT) have numerous ‘genomic’ effects on adrenomedullary chromaffin cells, acute modulatory actions remain largely unknown, despite rapid stress-related changes in secretion. We report that $1\mu\text{M}$ glucocorticoids rapidly modulate gating of chromaffin cell BK channels and action potential firing. In general, CORT, or the analog dexamethasone (DEX), increased channel activity in inside-out bovine patches, an effect not blocked by the glucocorticoid receptor (GR) antagonist RU38486. By contrast, these steroids profoundly inhibited BK activation in many rat patches, while facilitating activation in others. We show that BK inhibition arises from a negative shift in the voltage-dependence of BK inactivation paralleling that for activation. We report that rat cells characteristically exhibit greater repetitive firing ability than bovine cells in the absence of glucocorticoids. In both species, steroid application typically increased firing responses to smaller current injections, attributable to BK-enhanced repolarization and Na^+ channel deinactivation. However in rat cells, where BK inactivation is generally faster and more complete, glucocorticoids tended to dampen responses to stronger stimuli. Thus in the context of natural variation in BK gating, glucocorticoids can either promote or limit firing responses. We suggest that steroids exploit BK gating variety to tailor catecholamine output in species- and context-specific fashion.

*Used with permission. Originally published as Lovell PV, King JT and McCobb DP. Acute modulation of adrenal chromaffin cell BK channel gating and cell excitability by glucocorticoids. *J Neurophys.* 91: 561-570, 2004

INTRODUCTION

Regulation of protein expression by steroid hormones has long been recognized as an important mechanism underlying cellular plasticity (for review see McEwen, 1991). However, increasing evidence for much more rapid steroid-dependent modulation of voltage- and ligand-gated ion channels has begun to reshape our view of the physiological roles of steroids in the short-term (Falkenstein et al., 2000, Wehling, 1999; Moore, 1999; Makara and Haller, 2001). Among these are the glucocorticoids produced by the adrenal cortex, especially cortisol (in bovine and human) and corticosterone (in rat), together abbreviated CORT. CORT synthesis is under the regulatory control of pituitary adrenocorticotrophic hormone (ACTH), and represents the most important link between stress-related hypothalamic function and a myriad of body and brain responses. CORT secretion exhibits wide diurnal and stress-related fluctuations, and has an enormous variety of regulatory effects on, for example, metabolism, storage, and mobilization of nutrients, cardiovascular function, growth and reproductive function, and hippocampal structure and function (Sapolsky et al., 2000).

Historically, glucocorticoids have been credited with ‘genomic’ effects that initiate and maintain the phenotypic differences distinguishing chromaffin cells from sympathetic postganglionic neurons, for example, spherical shape (lack of axon outgrowth) and epinephrine versus norepinephrine synthesis (Hodel, 2001). Stress experience-related “tuning” of chromaffin cell protein expression by CORT, initially described for enzymes (Tank et al., 1986; Stochowiak et al., 1998; Betito et al., 1992, 1994) has begun to be extended to ion channels, including Ca²⁺ channels (Fuller et al., 1997a,b) and BK channel splice variants (Lai and McCobb, 2002). However, rapid surges in CORT release from adrenal cortex, to which underlying chromaffin cells are immediately exposed, may produce peak concentrations as high as 100 μM within the

rat adrenal medulla (Betito et al., 1992; Betito et al., 1994). Potential acute effects of these surges have received very little attention. Dexamethasone has been shown to decrease the amplitude of nicotinic acetylcholine-induced currents in porcine adrenal chromaffin cells (Inoue and Kuriyama, 1989), dramatically reducing nicotine-induced catecholamine secretion (Wagner et al., 1999).

In chromaffin cells, large conductance calcium- and voltage-dependent potassium channels (BK for Big K⁺) are uniquely positioned to influence features of electrical excitability (Solaro et al., 1995; Lingle et al., 1996; Lovell and McCobb, 2001). Glucocorticoids have been shown to regulate BK channel sensitivity to phosphatase activity in pituitary-related cells, however this regulatory effect requires changes in protein synthesis (Tian et al., 2001b; Tian et al., 2001a). In contrast, BK channels expressed in smooth muscle can be modulated immediately by 17 β -estradiol (Valverde et al., 1999; Dick et al., 2001) and dehydroepiandrosterone (DHEA; Farrukh et al., 1998; Peng et al., 1999). To our knowledge similar modulation of BK channels by corticosteroids has not been shown in chromaffin cells or any other cells. We demonstrate here that these steroids provide a proximate link through which the principle endocrine stress-response system, the hypothalamic-pituitary-adrenocortical (HPA) axis, can exert a rapid, fine-control of the adrenomedullary branch of the sympathetic response system. Heterogeneity in BK activation and inactivation gating shape the repertoire of CORT responses at cell and species levels.

MATERIALS AND METHODS

Chromaffin Cell Isolation and Culture

Bovine and rat chromaffin cells were isolated and cultured using procedures described by Lovell et al., (2000) and Lai and McCobb (2002). Briefly, bovine adrenal glands (Cudlin's Meat Mkt., Newfield, NY) were perfused for 30 min. at 20°C with a

Ca²⁺- and Mg²⁺-free Locke's buffer (recipe in mM: 154 NaCl, 5.6 KCl, 3.6 NaHCO₃, 5.6 Glucose, and 5mM HEPES at pH 7.4) followed by perfusion with a modified Ca²⁺- and Mg²⁺-free Locke's solution containing an additional 1X MEM vitamins (GIBCO), 1X MEM Amino Acids (GIBCO), Na-Pyruvate (11g/L), L-Glutamine (0.2M), penicillin (80U/ml), streptomycin (80μg/ml), nystatin (0.8%), collagenase B (1mg/ml; Boehringer Mannheim) and 0.0875% bovine serum albumin (BSA) for 1 hour at 37°C. For perfusion steps an O₂ and CO₂ (Carbogen 41%/4.92%) mix was continuously bubbled into solution. Following digestion, medullary tissue was manually removed from the adrenal, minced, and filtered through a plastic mesh strainer (13mm pore size). Tissue was placed in a second saline containing collagenase B (see above) for 1 hour at 37°C, filtered through cheesecloth, and washed several times using pellet centrifugation (120 x g for 10 minutes) and a modified Locke's with 0.5% added BSA. Isolated chromaffin cells were filtered through a 70μM nylon mesh filter (Falcon 2350, Fisher Scientific, Pittsburgh, PA), resuspended in pre-warmed cell culture medium (GIBCO; RPMI 1640 with 10% horse serum, 5% fetal calf serum, 2U/ml penicillin-G, 2μg/ml streptomycin sulfate, 100U/ml nystatin). 100μl aliquots of cells were then plated either into the center of ~15 mm glue rings in 35 mm plastic dishes (Falcon 3001) coated with collagen (Vitrogen, Collagen Corporation, Carlsbad, CA; 0.6 mg/ml in ddH₂O), or onto the glass bottom of a 35mm plastic dish (Plastek Cultureware) coated with 0.1% poly-D-lysine (Sigma) in water. Cell cultures were maintained at 37°C in a 5% CO₂ atmosphere and used for several days.

Electrophysiological Methods

Single channel and macroscopic currents were recorded using standard patch clamp recording techniques (Hamill et al., 1981; Sakmann and Neher, 1985). Borosilicate glass patch electrodes (World Precision Instruments, Sarasota, FL;

inside/outside 1.5/ 1.12 mm; 3-6 M Ω) were pulled and coated with Sylgard 184 (Dow Corning, Midland, OH) to decrease capacitance. Voltage clamp and current clamp electrophysiology data were collected as described previously (Lovell et al., 2000; Lovell and McCobb, 2001) using a List EPC-7 or EPC-9 patch clamp amplifier and standard clamp protocols designed with Pulse software (Heka Elektronik, Lambrecht, Germany) for the Macintosh G3. Data was acquired and digitized at 20 kHz for voltage-clamp data and 10 kHz for current-clamp traces. Offline analysis of clamp data was performed using custom software written for Igor Pro (Wavemetrics, Lake Oswego, OR).

All experiments were conducted at room temperature, 20°C. As described previously (Lovell et al., 2000), seals with resistances of 3-6 G Ω were obtained, following which 0 Ca²⁺ saline was perfused onto the cell for excision of inside-out patches. Solution exchange was accomplished with a seven-barrel gravity fed perfusion pipette.

Current clamp recordings were made using the perforated patch technique as described by Lovell and McCobb (2001). Briefly, high resistance seals were achieved as described above and recordings were made when the apparent input resistance had dropped to between 50-150 M Ω . For some cells, a small holding current was used to maintain cell resting potentials between -65 and -75 mV. To verify that the perforated patch was not ruptured during recording, input resistance was monitored during deliberate rupture at the end of the recording. For excitability measurements, a series of 2-second current pulses of increasing strength was used to elicit repetitive firing. The magnitude of current and serial increment of steps was adjusted by trial and error to fit the varying input resistances of the cells.

Solutions

For inside-out patches, symmetrical K^+ solutions were used to eliminate a potassium driving force and allow any DC offset to be cancelled at zero mV. The usual pipette and bath saline solutions contained (in mM): 160 KCl, 10 HEPES, 1 HEDTA, 0.0375 CaOH, pH adjusted with KOH to 7.2 to make ~ 500 nM free $[Ca^{2+}]$. The free $[Ca^{2+}]$ was calculated using MaxChelator software (WebMaxC v2.10; Bers et al., 1994). Zero Ca^{2+} solution contained an additional 5 mM EGTA (Sigma). When pharmacology experiments were performed, iberiotoxin (IBTX; 10 nM; Alomone Labs, Jerusalem, Israel), was added to the bath perfused salines and pH adjusted to 7.2. Dexamethasone and corticosterone (Sigma) were dissolved in 100% dimethylsulfoxide (DMSO), stored in small aliquots at -20° C, and added fresh to recording salines as needed. In previous experiments, inclusion of DMSO in the recording saline did not appreciably alter BK channel activity. The osmolarity of salines was measured by dew point osmometer and adjusted to 300 osmM.

For current clamp recordings, the bath solution contained the following (in mM): 145 NaCl, 5 KCl, 10 HEPES, 2 $CaCl_2$, 1 $MgCl_2$, pH adjusted to 7.4 with 2M NaOH. The technique used for obtaining perforated patches has been described elsewhere (Lovell and McCobb, 2001). Briefly, the tips of patch electrodes (2-4 $M\Omega$) were first filled with whole-cell saline containing (in mM): 140 KCl, 5 $MgCl_2$, 10 EGTA, 10 HEPES, pH 7.4. The electrode barrel was then back-filled with a solution containing 20 μ l of fresh stock amphotericin B (6mg/ 100 μ l DMSO; GIBCO) and 40 μ l of stock Pluronic acid F-127 (2.5 mg/ 100 μ l DMSO; Molecular Probes) added to 1ml of whole-cell recording saline. To maintain perforating efficacy, fresh aliquots were used during each hour of recording.

Data Collection and Statistical Analysis

Single and multi-channel currents were linear leak subtracted as previously described (Lovell and McCobb, 2001). The fraction of inactivated current (BK_i/BK_{total}) was estimated by calculating a ratio of the BK current at 350 ms as a function of the estimated peak BK current. The calcium-/voltage-dependence of activation (G-V) and inactivation (H_{inf}) were evaluated by measuring peak amplitudes at increasing voltage steps of 20 mV, converting these to conductances by dividing out the driving force, and fitting G-V and H_{inf} curves to a single term Boltzmann of the form:

$$G_{Vm} = G_{max} / (1 + \exp((V_m - V_{0.5})/ s))$$

with parameters for maximum conductance (G_{max}), voltage of half-activation ($V_{0.5}$), and slope (s ; the steepness of the voltage-dependence of activation, in mV/e-fold change in voltage). Effects of steroids on currents were normalized by dividing currents after exposure by currents before. Natural logs of fractional changes were taken for averaging and statistical comparisons, to avoid the confounding effects of averaging unbounded values for increases with values for decreases bounded between 0 and 1. Means were compared using a standard student t-test that assumed either equal or unequal variances as appropriate ($\alpha = 0.05$). Statistical outcomes on non-transformed data did not alter conclusions drawn.

RESULTS

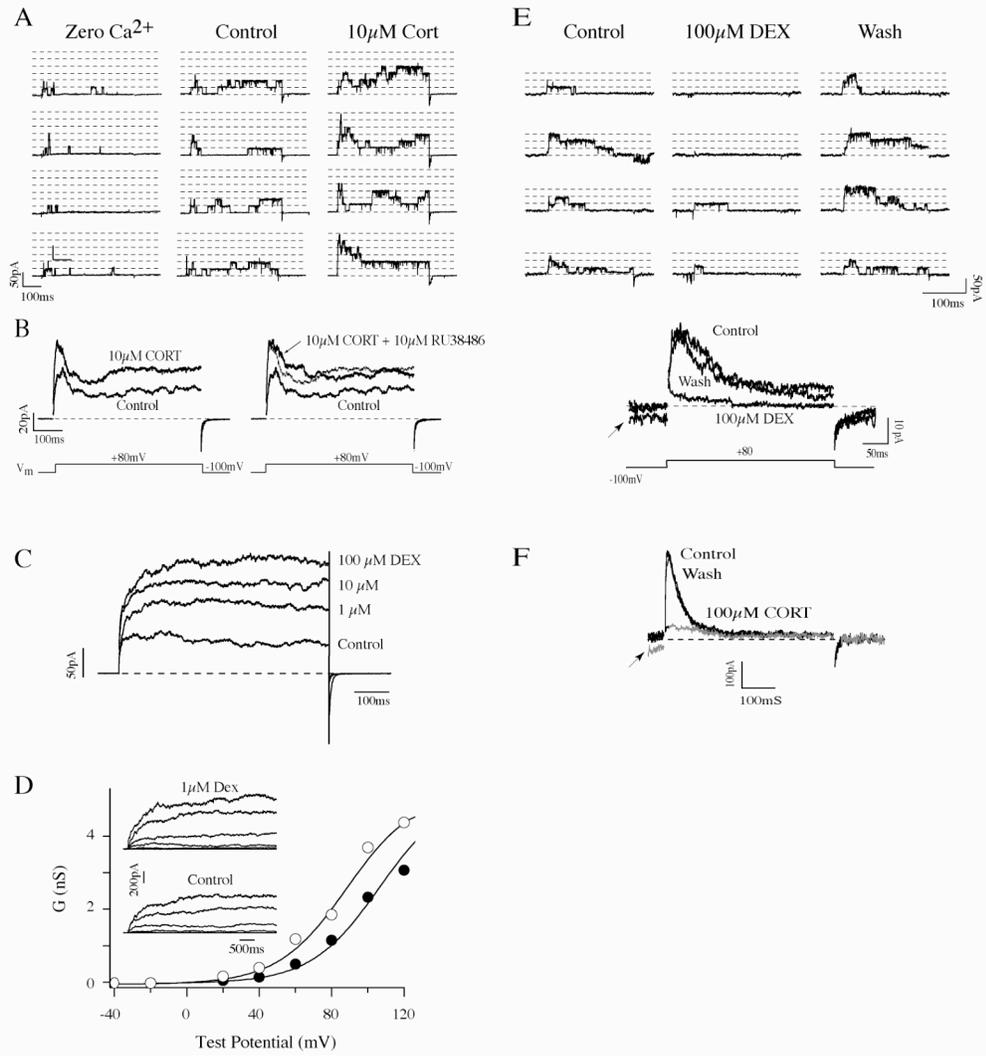
Rapid Modulation of Chromaffin BK Channel Function by Corticosteroids

In inside-out patch recordings from bovine or rat adrenal chromaffin cells, BK channels overshadow any other voltage-gated potassium channels when a low micromolar Ca^{2+} concentration is present on both sides of the patch and K^+ is the only

permeant cation (Lovell et al., 2000; Solaro et al., 1995). This has been confirmed by pharmacological block with 1 mM TEA⁺, and with iberiotoxin and charybdotoxin. In the bovine patch in Figure 1, a small number of channels were activated by 350ms voltage-steps to +80mV from a holding potential of -100mV (V_{hold}), including both inactivating and non-inactivating BK channels (BK_i and BK_s; Lovell et al., 2000; Solaro et al). Such patches typically exhibit very little other channel activity in the absence or presence of Ca²⁺, with the exception of infrequent, very brief openings in the 50-75 pS range, or channels too small to resolve at this gain. The BK channels seen here open with a low probability when exposed to zero-Ca²⁺ solution buffered with EGTA. In the center and right panels, the perfusion solution contains 500 nM Ca²⁺ and 0.1% DMSO, the vehicle used to dissolve steroids. Addition of 10 μ M cortisol resulted in an immediate increase in the number of channel opening events, with no observable change in the types of channel events observed. No significant change in the amplitude of single channel currents was noted, making it unlikely that effects on more macroscopic or ensemble-averaged currents described below resulted from a change in single channel conductance, as has been suggested for the effect of estradiol-like tamoxifen (Dick and Sanders, 2001; Dick et al., 2001).

The modulation of BK current by CORT is very rapid; a sharp increase in channel activity is detectable within seconds of exposure (Figure 1A). Similar results were obtained with cortisol in 6 of 8 patches tested. In 6 of 7 patches tested, current was increased by simultaneous exposure to 10 μ M CORT and 10 μ M RU38486 (Figure 1B). The latter is a selective antagonist of the cytosolic glucocorticoid receptor (GR, also known as GR-II, to distinguish it from the mineralocorticoid receptor, MR or GR-I). These observations and the fact that many isolated, continuously-perfused patches responded to CORT or DEX (to which MR is not sensitive) argue strongly that these steroid effects are mediated via a novel, membrane-associated receptor, or on the

Figure 1. Glucocorticoids modulate BK channel gating in chromaffin cells. A) Cortisol (10 μM) increases BK channel opening events elicited by a step to +80mV from a holding potential of -100mV in an inside-out patch from a bovine chromaffin cell. Channel openings with a conductance of approximately 280pS occur infrequently during a step to -80 mV in the absence of Ca^{2+} . 'Control' solution contains 500nM Ca^{2+} plus 0.1% DMSO, the vehicle used to dissolve steroids. B) Cortisol produced an immediate (< 4 sec after switching perfusion lines) increase in openings of both rapidly inactivating (BK_i) and sustained (BK_s) channels, as evidenced by the ensemble averages of 30 traces. Concurrent application of RU38486 (10 μM), an antagonist of the classic cytoplasmic glucocorticoid receptor (GR α), does not reduce the effect of CORT on the same patch. C) Dose-dependent response to dexamethasone (DEX), a synthetic glucocorticoid and selective GR β agonist. This patch contains only sustained (BK_s). D) 1 μM DEX produced a strong negative shift in the voltage-dependence of activation in a bovine patch similar to that shown in C. Peak conductance measurements of the currents in the inset were plotted against the appropriate conditioning potentials (G-V plot) and fit to a standard Boltzmann equation. In contrast to their effects on bovine cells, DEX (E) or CORT (F) sometimes profoundly inhibited BK current at +80 mV in rat chromaffin cells. The effect of either was readily reversed by washing. Reduced channel activity during the test step was often accompanied by increased channel activity at the holding potential (arrows).



channel directly.

In the top panel of Figure 1C, a multi-channel patch containing predominantly non-inactivating BK channels showed nearly a 70% increase in amplitude in response to application of the synthetic glucocorticoid, dexamethasone (DEX) at a concentration of $1\mu\text{M}$. Increasing DEX to 10 and then $100\mu\text{M}$ led to further increases to 130 and 200% above control. This progressive effect of increasing doses was typical of patches exposed to multiple concentrations. DEX effects could be partially reversed with a brief washout, though reversal of the sustained component was typically less efficacious. Figure 1D illustrates the negative shift in the voltage-dependence of activation of BK channels by DEX. Further evidence for a negative shift, as opposed to an increase in the maximum achievable P_o , or the number of channels participating in the current, is provided by currents in rat chromaffin patches, where the plot of conductance as a function of test potential (G-V curve) typically plateaus in a more negative range, allowing more accurate determination of G_{max} and the half-activation point ($V_{0.5}$; Figure 3B and C).

Glucocorticoid Effects on BK Channels in Rat Chromaffin Cells

Starting at the high end of the concentration range, we made the seemingly paradoxical observation that $100\mu\text{M}$ DEX or CORT tended to have an effect on rat patches that was the opposite of that seen on bovine patches, profoundly inhibiting BK channel activity (Figure 1E and F). This effect was usually largely reversed by washing, and was repeatable. Of 18 rat patches tested at $100\mu\text{M}$, a net decrease in current was observed in 14 (Figure 2B). By contrast, only 3 of 21 bovine patches showed a decrease in peak current at this DEX concentration, whereas 16 showed an increase. As explained in the Methods section, drug responses were averaged after natural log-transformation of fractional changes in current. Thus in rat, $100\mu\text{M}$ DEX

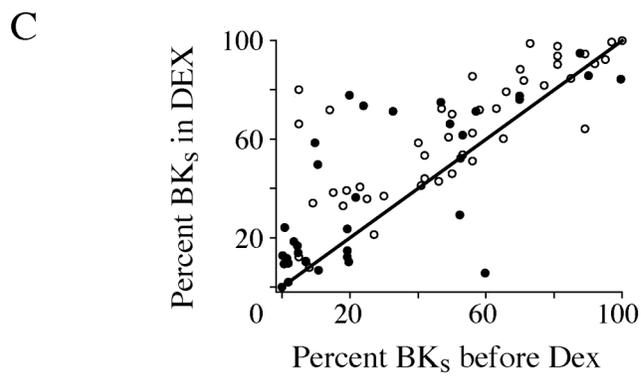
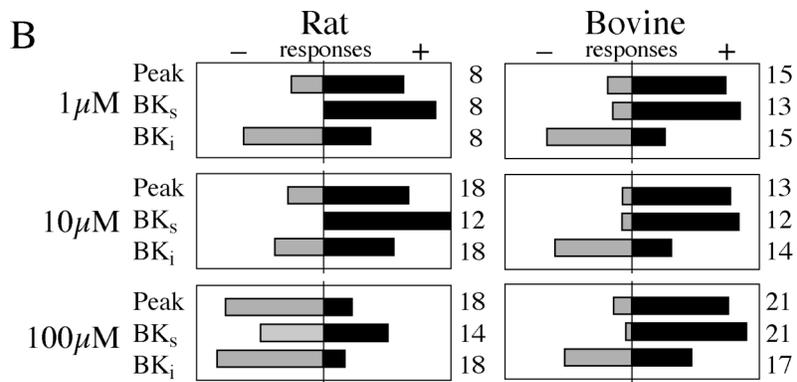
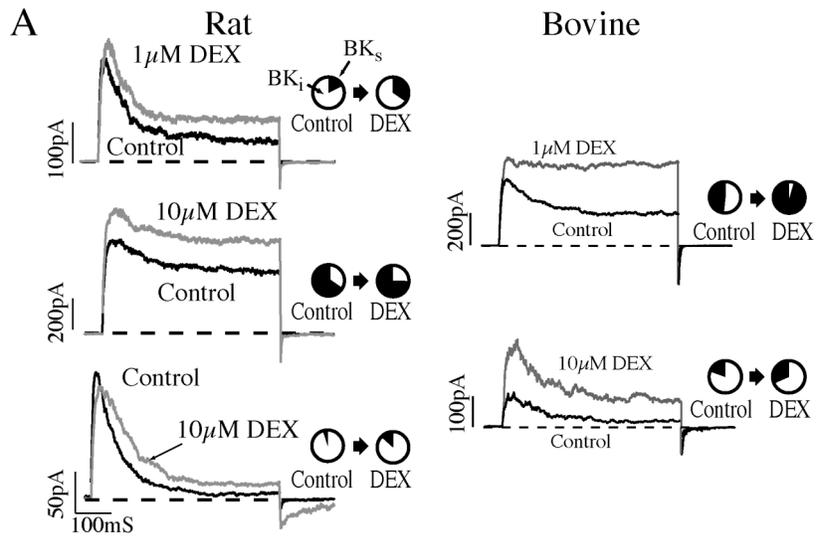
reduced peak current amplitude by an average of 57.1%, whereas it increased peak current in bovine by 47.7%.

1 and 10 μM concentrations of DEX had more varied and complex effects. In the majority of patches representing both species, DEX increased peak BK current (38 out of 54 patches, see Figure 1B for a breakdown). Average peak currents in 1 and 10 μM DEX were increased by 35.7 and 29.6% in bovine, and 8.9 and 39.8% in rat. However, the quantitative variation was sufficient that net responses did not differ statistically between doses. Changes in peak current amplitudes in the negative direction, as well as in the positive direction, were typically at least partially reversed by washout, and qualitatively reproduced upon reintroduction of DEX.

BK currents of both species are very heterogeneous with respect to the rate and voltage range of activation, and the rate, extent, and the voltage range of inactivation. Though both species exhibit cells covering the full range from BK_s to BK_i channels exclusively, bovine currents are much more likely to be of the former type, or to have a mix of channels with a resultant macroscopic or ensemble current that is slowly and/or incompletely inactivating (Lovell, et al., 2000; Lovell, et al., 2001). Moreover, a relatively negative voltage-dependence of activation correlates with a greater proportion of BK_i channels, and faster inactivation, across patches of both species (Lovell, et al., 2000).

Differential responses of inactivating and sustained subcomponents of BK current can explain much of the variation within and between species; subdividing revealed a more consistent pattern of DEX responses. In the present study, we subdivided currents from both species before and after DEX: inactivation was virtually complete well before 350 ms, thus the current remaining at that point was taken as the BK_s current, with the difference between it and the total representing BK_i current. In absolute terms, the BK_s component of current in bovine patches was increased by

Figure 2. Glucocorticoids at lower concentrations typically increased peak BK current in rat chromaffin cells, though substantial variability was seen at all concentrations in both species. Importantly, sustained (BK_s) and inactivating (BK_i) components were differentially affected. In A, representative traces from rat (left) and bovine (right panel) are shown. Pie diagrams (right of traces) illustrate the relative proportion of the BK subcomponents. Note the tendency of BK_s and BK_i components to increase and decrease, respectively, regardless of species, net effect on peak current, or relative sizes of the two components prior to DEX. B) Summary of direction of change in absolute size of peak current and two subcomponents following exposure to 3 DEX concentrations. Black bars represent that proportion of patches showing increases, and grey bars that showing decreases, with total numbers of patches tallied for each category given on the right. Note that some patches lacked one or the other of the two subcomponents. C) The relative proportion of the peak current that was non-inactivating was much more likely to increase with DEX exposure for both rat (filled circles) and bovine patches (unfilled circles). The diagonal line represents no change.

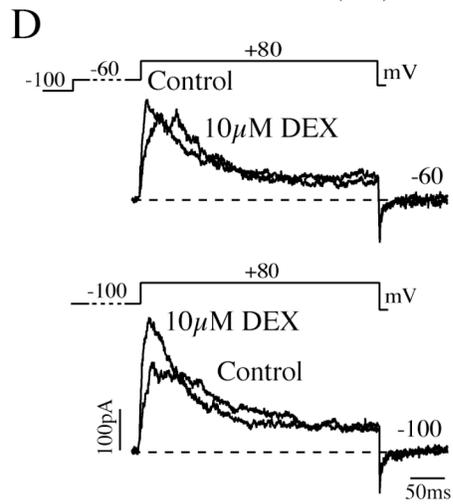
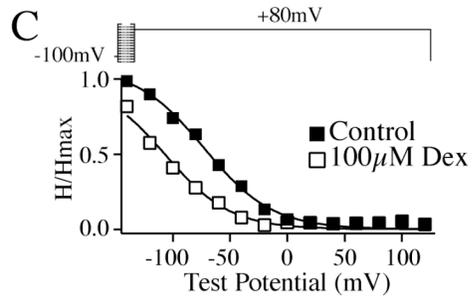
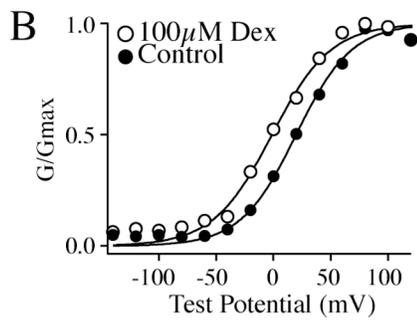
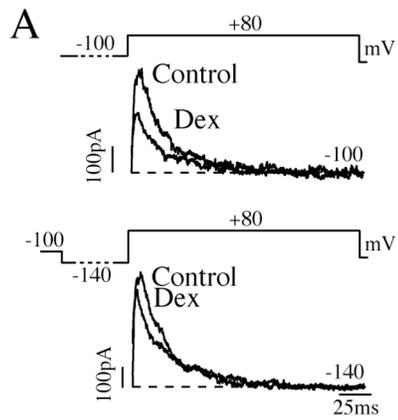


DEX in 42 of 48 cases, while it was reduced in 2 and unaffected in 4. Rat BK_s current was increased in 22 of 34 patches having a sustained component, reduced in 8, and unaffected in 4 (Figure 2). 5 of the 8 reductions were in 100 μ M DEX. Compared with currents from the same patches before DEX, BK_s currents after 1, 10, and 100 μ M DEX, respectively, exhibited an average increase in amplitude of 187, 269, and 5.4% for rat and 84, 102, and 101% for bovine. In contrast to the BK_s component, the absolute amplitude of the BK_i component was reduced in 28 of 45 and 23 of 41 bovine and rat patches, respectively. As illustrated in Figure 3C, the proportion of BK_s current was much more often increased than decreased by DEX.

Experiments with very negative “prepulse” potentials preceding BK activation steps further demonstrate the confounding effects introduced by BK channel inactivation. Inactivation, a process conferred by one or more accessory ‘ β ’ subunits interacting with the *Slo* gene-encoded α subunit, is voltage-dependent. Inactivation more rapidly follows activation (elicited by a depolarizing voltage step) at more depolarized step potentials, deinactivation is faster at more negative return potentials, and the number of channels inactivated at a ‘steady state’ holding potential is a sigmoidal function of that potential.

In the top panel of Figure 3A, the membrane potential was stepped from -100 mV to +80 mV before and after application of 100 μ M DEX. DEX reduced peak current amplitude dramatically. However, when the membrane potential was stepped briefly to -140 before stepping to +80, we observed that 1) the peak current was slightly greater (610 pA vs. 550 pA, or about 10%), demonstrating that some channels remain inactivated even at a steady state of -100mV, and 2) the negative effect of DEX was virtually eliminated. With a series of test potentials stepping from -140mV, it was clear that DEX, even at 100 μ M on rat cells, shifted the half-activation voltage strongly in the negative direction (by ~25mV), and without affecting the maximum

Figure 3. Negative effects of DEX on BK currents are best explained by concurrent negative shifts in the voltage-dependence of activation and inactivation. A) Rat BK currents showing a more negative response to 10 μ M DEX from a prepulse potential of -100 mV (top traces) than from a prepulse potential of -140 mV (bottom traces). B and C) Conductance-voltage (G-V) and steady state inactivation (h_{∞}) curves from a similar patch, illustrating the leftward shifts of roughly 20 and 40 mV, respectively, with exposure to 100 μ m DEX. Left shifting the inactivation curve dramatically reduces the channels available for activation from -100 mV. D) Opposite effects of DEX on a single patch, and involving a more physiological range of voltages. From -60 mV, DEX slowed channel activation kinetics and reduced peak current, whereas from -100 mV, DEX increased activation and inactivation rates and increased peak current. This suggests that the more rapidly activating channel subset is inactivated at -60 mV in DEX, while a slower-gating subset is activated to a greater extent.



activatable current (G_{\max} ; Fig. 3B). Furthermore, as seen in the bottom panel of 3C, a full series of steps to +80mV from prepulse potentials as low as -140mV revealed that DEX shifted the voltage-dependence of inactivation dramatically; in this case by about 40mV. This clearly indicates that the apparent negative effect of DEX is explained by a reduction in the number of channels available for activation from starting potentials positive to -140mV. There is no compensatory increase in channels activated during the test step to +80mV, despite the negative shift in the G-V curve, because the plateau had been reached even without the DEX. While in bovine cells we could not conclusively rule out that DEX acted to increase G_{\max} independently of the shift in the G-V curve because the curve was shifted too far right to reach saturation, the consistent observation in rat cells that G_{\max} was not increased provides strong support for a common mechanism of action involving a negative shift in channel open probability.

In the experiment shown in 3D, DEX has a small negative effect on peak amplitude when the current is elicited from -60 mV, though when it is elicited from -100mV it has a strong positive effect. Because the range of voltages spanned in this case encompasses that of probable physiological “resting membrane potential”, the opposite effects of DEX on one patch argues strongly that the impact of DEX on channel function in vivo will depend critically on the starting membrane potential, as it interacts with channel gating parameters.

In summary, the combination of inherent complexity in BK gating and the intra- and inter-species heterogeneity in various aspects of this gating, as observed in chromaffin cells from two species, ensures a complex landscape upon which glucocorticoids can have quite varied effects. The more frequent occurrence of inhibitory effects of DEX on rat cells than bovine cells, and the detailed patterns of current changes in cells of different BK channel compositions (particularly with

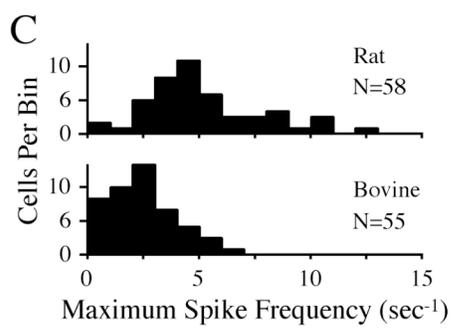
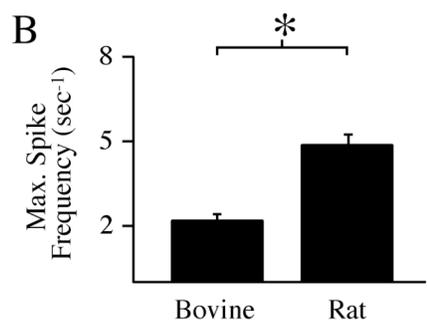
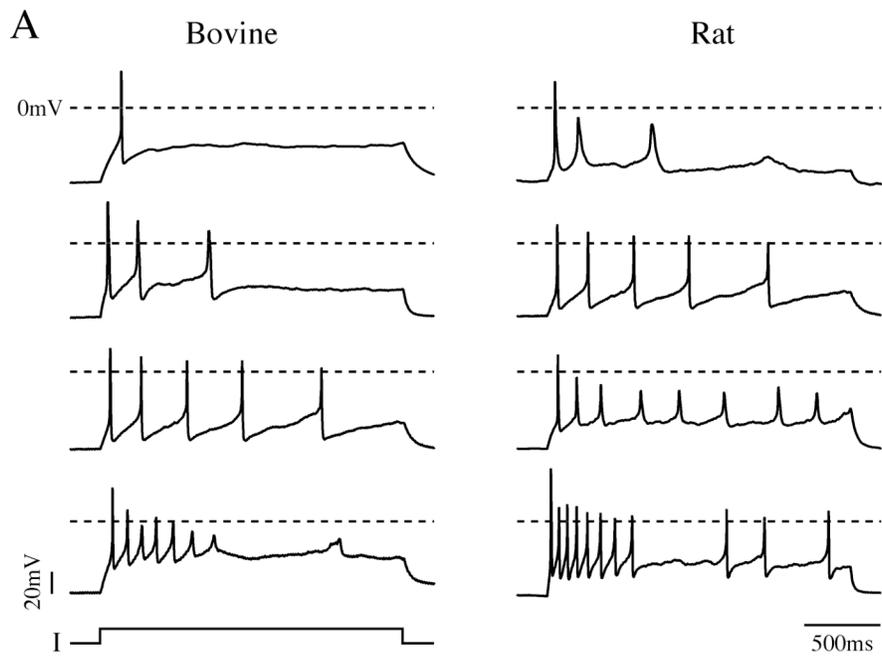
respect to inactivation), are consistent with a relatively simple effect of glucocorticoids in facilitating the activation (and inactivation) of BK channels at more negative potentials.

Bovine Chromaffin Cells Tend to Fire at Lower Frequencies Than Rat Cells

Before studying the effects of steroids on action potential generation, we characterized responses to current injection using perforated-patch current clamp recording in the absence of glucocorticoids. Prior rat-bovine comparisons of BK gating in voltage clamp revealed striking differences (Lovell et al., 2000), thus we characterized chromaffin cell excitability in the two species in parallel.

Trains of action potentials (APs) could be elicited reliably by 2-second supra-threshold depolarizing current injections in bovine and rat cells (Figure 4). For uniformity in our quantitative comparisons, a small holding current was applied, where necessary, to maintain membrane potentials between -65 and -70 mV, thereby minimizing variation in pre-existing Na^+ or Ca^{2+} channel inactivation. Cell to cell variation in input resistance was controlled for by altering the initial amplitude and serial increment of the injected current. Solaro et al., (1995) and Lovell et al., (2001) have shown previously that incremental increases in the amount of depolarizing current injected increases the number of action potentials fired (or average frequency) up to a peak, after which further increases in current produced fewer and fewer spikes. We were therefore careful to increment currents finely enough, and over a wide enough range, to determine the point of peak response accurately. Because studies have suggested that Ca^{2+} action potentials, relatively broad action potentials that do not necessarily cross the zero mV axis, produce calcium transients that elicit catecholamine exocytosis (as do Na^+ action potentials; Artalejo et al., 1994; López et al., 1994), they were counted (after Lovell et al., 2001).

Figure 4. In the absence of glucocorticoids, features of chromaffin cell repetitive firing differ markedly between species. A) Trains of action potentials (APs) elicited from 4 representative bovine and rat chromaffin cells with 2-second depolarizing current injections delivered using perforated patch recording methods. These spike trains illustrate the wide range of spiking abilities in both species. B) On average, fewer than half as many spikes per 2-second depolarizing pulse could be evoked from bovine cells at optimum pulse size, as compared with rat cells ($P < 0.0001$, $N = 55$). Asterisk indicates significance using Student's T-test. C, Frequency distributions of maximum spike frequencies that could be elicited from individual chromaffin cells.

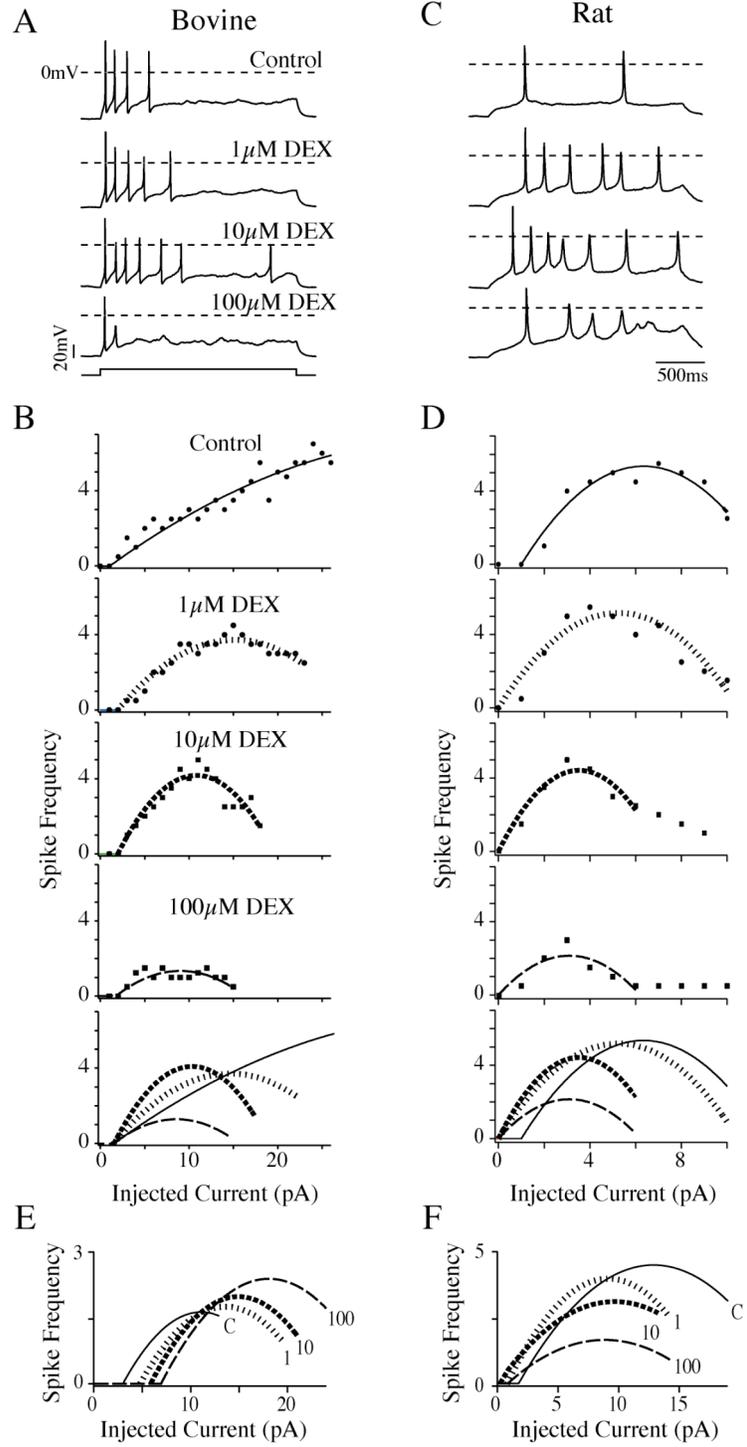


Action potential response patterns of bovine and rat chromaffin cells varied over a wide range. While it was not uncommon for bovine cells to respond with a single action potential over a wide range of inputs, most were able to vary their response with current amplitude. Examples of maximal responses to graded series of inputs are shown in Figure 4. Relationships between input current and response frequency were roughly parabolic in virtually all cases. Examples are shown in Figure 5. Under the parameters defined above, rat cells were consistently found to fire many more spikes at the peak of the curve than bovine cells (Figure 4) in the absence of steroids. On average, the number of spikes that could be elicited during a 2-sec pulse was 4.49 ± 0.39 (Mean \pm SEM; $n = 55$) for bovine cells, as compared with 9.96 ± 0.67 for rat cells (Figure 5B; $n = 58$; $P < 0.0001$). The frequency distributions in Figure 4C illustrate characteristic differences between the rat and bovine chromaffin cell populations.

Corticosteroids Enhance and Depress Features of Excitability in Bovine and Rat Chromaffin Cells

Using the techniques described above to assess excitability, multiple series of AP trains were elicited from bovine and rat chromaffin cells before and after exposure to corticosteroid or DEX for approximately 2 minutes. The bovine traces presented in Figure 5A illustrate the effects of various concentrations of DEX on repetitive firing elicited by an intermediate intensity current step of 9pA. At this stimulus intensity, the cell fired 4 distinct spikes in control saline (Figure 5A; *top trace*). Relatively low concentrations of DEX (1 and 10 μ M) favored improved firing of 5 and 7 spikes respectively. In contrast, 100 μ M DEX dramatically reduced the ability of this cell to sustain firing. Figure 5B shows the frequency of spikes elicited from the same cell in response to representative series of test current pulses (frequency values represent the

Figure 5. Corticosteroids alter chromaffin cell responses to stimulation. A and C) Trains of action potentials (APs) recorded from one representative bovine and one rat cell illustrating the effects of varying concentrations of DEX or CORT on spikes elicited by 2-sec current pulses (of 9pA and 2pA, respectively). B and D) Stimulus-response plots constructed by fitting three term polynomials to plots of spike frequencies elicited by a broad range of stimulus intensities. Fits are overlain in the bottom plots of B and D, for easy comparison. For the bovine cell in B, both 1 and 10 μ m substantially increased the number of spikes elicited by stimuli in the lower end of the amplitude range, while also decreasing the number elicited in the upper range. 100 μ M had little effect in the lower end of the range, but clearly suppressed firing in the upper range. Steroid effects on rat cells were very similar in most respects. For the cell in D, responses to stimuli were also augmented and suppressed in the low and high stimulus input ranges, respectively. In this particular case, all three concentrations (of corticosterone in this case) shifted the threshold for spiking towards smaller stimuli. E and F) Overlain fits like those in B and D are shown for an additional bovine and rat cell, respectively. In the bovine cell, higher doses of DEX progressively raised the maximum number of spikes that could be elicited from the cell. For the rat cell in F, as well as D, 10 μ m did not produce a bigger effect than 1 μ m, as was typically seen in the bovine cells.



number of spikes elicited by the two second steps, divided by 2 seconds). To better characterize the responses, we fit stimulus-response plots to a standard three term polynomial function using a Levenburg-Marquardt least-squares search algorithm. These parabola-shaped fits typically approximated the characteristic shape and dynamics of AP generation adequately over most of the range of input values. The bottom panels of 5B overlay the fits obtained in the plots above them, for easier comparison. For the bovine cell shown, it is clear that the lower concentrations of DEX improved spike generation in the lower range of injected current amplitudes, though at higher amplitudes response frequencies reach the maximum and decline faster. This predicts that cortisol in vivo will trigger chromaffin cells to respond more vigorously to inputs over a significant range, while at the same time potentially reducing responses in a range of higher amplitude inputs, and potentially capping the maximum obtainable frequency at a lower level than possible in the absence of the steroid. Both suppressive and capping effects are dramatically evident on application of $100\mu\text{M}$ DEX in this experiment.

In 10 of 12 bovine cells subjected to the full range of steps, action potential firing was enhanced by DEX at some range of stimulus intensity, whereas it was unaffected in one, and decreased in another. In the case illustrated in 5E progressive increases were seen 1, 10, and $100\mu\text{M}$ DEX concentrations. Similar results were obtained in 4 additional cells exposed to DEX or CORT. In the other 5 cells, response frequencies were increased in absolute terms by DEX at low concentrations, whereas $100\mu\text{M}$ DEX had a suppressive effect similar to that in 5B. In these cells, lower DEX concentrations also had the effect of decreasing the range of input intensities to which the cell would respond.

Rat chromaffin cells were consistent in exhibiting complex responses to glucocorticoids not unlike those in most of the bovine cells described above.

However, 1 or 10 μ M DEX or corticosterone in all cases increased the number of action potentials that could be elicited with low stimulus strengths (N = 6). In some cases there was also a decrease in the minimum current needed to elicit action potentials (e.g., 5D and F). The traces in 5C show responses of a rat cell to 2pA current pulses in various DEX concentrations. In DEX at 1, 10, and even 100 μ M, the same stimulus elicited multiple spikes. 1 and 10 μ m DEX also enhanced responses to several larger inputs. In rat cells, the effect of 10 μ m DEX was typically no bigger than that to 1 μ M. As observed in most bovine cells, all DEX concentrations shifted the peak of the frequency response curve towards lower values, substantially decreasing below control levels the number of spikes elicited by larger inputs. The cell in 5F exhibited a pattern of responses similar to that in 5D.

DISCUSSION

Immediate effects of glucocorticoids on chromaffin cells have been reported only for nicotinic acetylcholine receptor activation and catecholamine secretion (Inoue and Kuriyama, 1989; Wagner et al., 1999). We report that physiological concentrations of glucocorticoids have immediate effects on BK-type K⁺ channels in chromaffin cells, and that these impact directly on action potential firing. With this discovery, we add to the short list of steroids, including 17 β -estradiol and dehydroepiandrosterone (DHEA), known to acutely modulate BK channels (Valverde et al., 1999; Farrukh et al., 1998). Whether BK channels in any of the many other tissues in which they are expressed are influenced by glucocorticoids will be of interest to determine.

Steroid Effects on BK Currents in Voltage Clamp Mode

The opposite effects of high glucocorticoid concentrations on rat and bovine BK channels presented an initial paradox. However, further analysis suggested a common mechanism, in which activation gating of channels in both species is enhanced by exposure to the steroids in a roughly dose-dependent manner, while the inhibition is an important consequence of facilitated channel inactivation. In the many records where individual channel events were easily measured, no change in single channel conductance was observed. The possibility that new channels could be recruited from an otherwise inactive pool of channels cannot be excluded in some cases, because G_{\max} was out of range (too positive) in control saline. Nevertheless, in those in which G_{\max} was determined, the full curve was shifted left substantially, with no significant change in G_{\max} or in the steepness of the voltage-dependence.

Accompanying the negative shift in the G-V curve in the case depicted in Figure 3C was a robust negative shift ($\sim 40\text{mV}$) in the curve defining the voltage-dependence of inactivation at steady state (the h_{∞} curve). Such a shift can dramatically reduce the number of channels available for activation, an effect that directly counteracts the effect of shifting the G-V curve left, and leads to an underestimation of activation-promoting effects of glucocorticoids. Even in control saline, a substantial number of BK channels were inactivated at $V_{\text{hold}} = -100\text{mV}$ in some cells, and a few at -120 and even -140 mV, ($[\text{Ca}^{2+}]_i = 500\text{nM}$). It is not surprising therefore, that a profound inhibition of current was observed at this holding potential in patches with predominantly inactivating BK channels, nor by extension, that a reduction was seen in the inactivating fraction of the current in many additional patches from both species.

BK channels differ from 0 to four in the number of inactivation-conferring subunits associated with them (Wang et al., 2002). They also vary widely, within and

between cells, as well as between species, in the voltage dependence of activation and inactivation gating, reflecting diverse factors such as subunit and splice variant composition, phosphorylation state, redox state and other unknowns. By differentially altering the subset of a heterogeneous population of channels that respond to a stimulus, different concentrations of steroids are likely to have markedly non-linear dose-dependent effects. Moreover, patch-to-patch heterogeneity can be expected to obscure any dose-dependency in averaged voltage-clamp data. In our experiments, dose-sensitivity was evident in individual patches and in current clamp recordings, despite the increasingly negative effects of higher doses seen particularly in averaged rat patches.

Based on comparisons between patches from bovine, rat, and hypophysectomized rat chromaffin cells, we suggested that BK_i channels tend to have a more negative voltage-dependence of activation than BK_s channels (Lovell et al., 2000, Lovell and McCobb, 2001). This must derive in part from the negative shift in the voltage-dependence and kinetics of activation conferred by inactivation-conferring β subunits (Wallner et al., 1999; Xia et al., 1999; Wang et al., 2002). One consequence is that steroid exposure will increase the relative representation of slowly activating, and slowly inactivating and non-inactivating channels. This view explains kinetic changes seen in many patches (see Figures 2 and 3).

There may be a closer link between steroid sensitivity and β subunits. Rapid effects on isolated patches, and failure of classical GR antagonists to block them, suggest a membrane-associated receptor. Steroids can interact with members of several super-families of membrane proteins, including G-protein coupled receptors, ligand-gated ion channels, and voltage-gated channels (Evans, 2000; Falkenstein et al., 2000, Wehling, 1999; Moore, 1999; Makara and Haller, 2001). The discovery that estradiol modulates smooth muscle BK channel gating by interacting with the Slo β 1

subunit makes related members of the Slo- β family particularly attractive candidate glucocorticoid receptors.

Steroid Effects on Cellular Excitability

Chromaffin BK channels are very prominent, and contribute heavily to shaping intrinsic excitability. Rapid BK activation minimizes action potential duration and augments the brief afterhyperpolarization. These effects minimize Na⁺ channel entry into an inactivated state and optimize deinactivation, thus preparing the cell to fire again (Figure 4, above; Lovell and McCobb, 2001; Lingle et al., 1996). Our data suggest that glucocorticoid augmentation of BK channel activation will increase the number of spikes elicited by a wide range of stimulus intensities *in vivo*, and thus augment catecholamine secretion.

The effect of CORT on BK channel inactivation raises the provocative hypothesis that CORT may, under some circumstances, suppress firing. We report negative effects of even lower concentrations of CORT or DEX, including 1) reduction in the number of action potentials that could be elicited at peak, and 2) narrowing of the range of stimulus strengths that effectively elicit spikes.

The strongest arguments that the aforementioned effects will have consequences *in vivo* come from species comparisons. Both species' channels are heterogeneous, however, 1) rat cells have more inactivating BK channels than their bovine equivalents, and 2) rat channels tend to activate at more negative voltages and more rapidly at equivalent voltages (Lovell et al., 2000). In the present study, we report that rat cells are substantially more effective at repetitive firing. While channels other than BK channels probably enter into the species difference, the general picture is consistent with the idea that greater ease of activation of rat BK channels outweighs the potentially negative effect of easier BK inactivation, in the absence of steroids.

However, suppressive effects of steroids on firing were clearly more pronounced in rat cells. The maximum number of action potentials that could be elicited was reduced more, and reduced by lower concentrations, whereas even 100 μ M DEX could raise the maximal spiking frequency in bovine cells. The range of stimulus strengths that could effectively elicit spikes was, though shifted to a lower intensity range, also more consistently narrowed in rat cells at all concentrations.

Are in vivo firing and secretion suppressed at the peak of large episodic swings in rat (or bovine) CORT levels, which may exceed 100 μ M within the rat adrenal medulla (Betito, et al, 1992, 1994)? 5- to 10-fold higher levels make suppression more likely in rats than cows (el-Nouty et al., 1978; Koehl et al., 1999; Manzanares et al., 1999; Veissier et al., 1999; Viau et al., 1999). Bovine chromaffin cells generally reside lower on the scale of responsiveness, but have more room for increase. Rat cells, comparatively speaking, may be on chronic high alert. While speculative, a damping effect on potential “runaway firing” may be offered by BK channel inactivation. This could serve to pace, or restrain, catecholamine output to protect against too rapid exhaustion of stores, especially under intense stress.

Moderating effects of corticosteroids at high concentrations or stimulus intensities are not incompatible with a facilitatory role at lower concentrations or stimulus intensities; glucocorticoids could exert important modulatory effects at both ends of the firing range. Relative emphasis probably differs between species. From another vantage point, the relative lack of inactivation in bovine BK channels allows more channels to be open at rest. The low input resistance will then make the cells harder to bring to threshold (with the converse true in rat cells). In this context, BK channels would be playing an “anti-excitatory” role, and glucocorticoids, by shifting inactivation of rat channels in the negative direction, would help ensure that the negative effects of BK channels can still be overridden. Roles in threshold regulation

of BK or CORT are not necessarily incompatible with roles in regulation of repetitive firing, but would apply in a different range of voltage (or $[Ca^{2+}]_i$).

It seems likely that HPA-driven diurnal and stress-related increases in CORT synthesis will rapidly prime epinephrine-secreting chromaffin cells to respond more efficaciously to sympathetic stimulation than they would otherwise. Given the gating complexity of BK channels and their relationship to excitability, we suggest that a capping or suppressive effect is also likely to accrue in the high end of the response range. How moment-to-moment changes in CORT affect cellular excitability and catecholamine output *in vivo* remains an open and challenging question. Species comparisons will continue to help elucidate the electrophysiological, molecular, and ultimately, the adaptive significance of acute BK channel interactions with stress steroids.

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REFERENCES

- Artalejo CR, Adams ME, Fox AP (1994) Three types of Ca^{2+} channel trigger secretion with different efficacies in chromaffin cells. *Nature* 367: 72-76.
- Behrens R, Nolting A, Reimann F, Schwarz M, Waldschütz R, Pongs O (2000) hKCNMB3 and hKCNMB4, cloning and characterization of two members of the large-conductance calcium-activated potassium channel β -subunit family. *FEBS Letters* 474: 99-106.
- Bers D, Patton C, Nuccitelli R (1994) A practical guide to the preparation of Ca^{2+} buffers. *Methods Cell Biol* 40:3-29.
- Betito K, Diorio J, Meaney MJ, Boksa P (1992) Adrenal phenylethanolamine-N-methyltransferase induction in relation to glucocorticoid receptor dynamics: evidence that acute exposure to high cortisol levels is sufficient to induce the enzyme. *J Neurochem* 58: 1853-1862.
- Betito K, Mitchell JB, Bhatnagar S, Boksa P, Meaney MJ (1994) Regulation of the adrenomedullary catecholaminergic system after mild, acute stress. *Am J Physiol* 267: R212-R220.
- Cameron JS, Dryer SE (2000) BK-Type K(Ca) channels in two parasympathetic cell types: differences in kinetic properties and developmental expression. *J Neurophysiol* 84:2767-76.
- Dick GM, Rossow CF, Smirnov S, Horowitz B, Sanders KM (2001) Tamoxifen activates smooth muscle BK channels through the regulatory $\beta 1$ subunit. *J Biol Chem* 276:34594-34599.
- Dick GM, Sanders KM (2001) (Xeno)estrogen sensitivity of smooth muscle BK channels conferred by the regulatory beta1 subunit: a study of beta1 knockout mice. *J Biol Chem* 276(48): 44835-44840.
- el-Nouty FD, Elbanna IM, Johnson HD (1978) Effect of adrenocorticotrophic hormone on plasma glucocorticoids and antidiuretic hormone of cattle exposed to 20 and 33 C°. *J Dairy Sci* 61:189-196
- Falkenstein E, Tillmann H-C, Christ M, Feuring M, Wehling M (2000) Multiple actions of steroid hormones- a focus on rapid, nongenomic effects. *Pharmacol Rev* 52:513-555.
- Farrukh IS, Peng W, Orlinska U, Hoidal JR (1998) Effect of dehydroepiandrosterone on hypoxic pulmonary vasoconstriction: a Ca^{2+} -activated K^{+} -channel opener. *Am J Physiol* 274: L186-195.

- Fuller LZ, Lu C, McMahon DG, Lindemann MD, Jorgensen MS, Rau SW, Sisken JE, Jackson BA (1997) Stimulus-secretion coupling in porcine adrenal chromaffin cells: effect of dexamethasone. *J Neurosci Res* 49(4): 416-424.
- Fuller L, Lu C, McMahon D, Alaudin E, Jorgensen M, Rau S, Sisken J, Jackson B (1997) Effect of dexamethasone on voltage-gated Ca²⁺ channels and cytosolic Ca²⁺ in rat chromaffin cells. *Neuroreport* 8(5): 1169-1172.
- Hall SK, Armstrong DL (2000) Conditional and unconditional inhibition of calcium-activated potassium channels by reversible protein phosphorylation. *J Biol Chem* 275(6): 3749-54.
- Hamill OP, Marty A, Neher E, Sakmann B, Sigworth FJ (1981) Improved patch-clamp techniques for high-resolution current recording from cells and cell-free membrane patches. *Pflügers Arch* 391:85-100.
- Hodel A (2001) Effects of glucocorticoids on adrenal chromaffin cells. *J Neuroendocrinol* 13(2): 216-220.
- Inoue M, Kuriyama H (1989) Glucocorticoids inhibit acetylcholine-induced current in chromaffin cells. *Am J Physiol* 257: C906-C912.
- Koehl M, Darnaudery M, Dulluc J, Van Reeth O, Le Moal M, Maccari S (1999) Prenatal stress alters circadian activity of hypothalamo-pituitary-adrenal axis and hippocampal corticosteroid receptors in adult rats of both gender. *J Neurobiol* 40:302-315.
- Lai GJ, McCobb DP (2002) Opposing actions of adrenal androgens and glucocorticoids on alternative splicing of Slo potassium channels in bovine chromaffin cells. *Proc Natl Acad Sci U S A* 99(11): 7722-7727.
- Levitan ES, Takimoto K (1998) Dynamic regulation of K⁺ channel gene expression in differentiated cells. *J Neurobiol* 37(1): 60-68.
- Lingle CJ, Solaro CR, Prakriya M, Ding JP (1996) Calcium-activated potassium channels in adrenal chromaffin cells. In: *Ion channels* (Narahashi T, ed, pp 261-301. New York: Plenum.
- López MG, Villarroya M, Lara B, Sierra RM, Albillos A, García G, Gandía L (1994) Q- and L-type Ca²⁺ channels dominate the control of secretion in bovine chromaffin cells. *FEBS Lett* 349:331-337.

- Lovell PV, James DG, McCobb DP (2000) Bovine versus rat chromaffin cells: big differences in BK potassium channel properties. *J Neurophysiol* 83: 3277-3286.
- Lovell PV, McCobb DP (2001) Pituitary control of BK potassium channel function and intrinsic firing properties of adrenal chromaffin cells. *J Neurosci* 21(10): 3429-3442.
- Makara GB, Haller J (2001) Non-genomic effects of glucocorticoids in the neural system. Evidence, mechanisms and implications. *Prog Neurobiol.* 65(4): 367-390.
- Manzanares J, Corchero J, Fuentes JA (1999) Opioid and cannabinoid receptor-mediated regulation of the increase in adrenocorticotropin hormone and corticosterone plasma concentrations induced by central administration of delta(9)-tetrahydrocannabinol in rats. *Brain Res* 839:173-179.
- McCobb DP, Fowler NL, Featherstone T, Lingle CJ, Saito M, Krause JE, Salkoff L (1995) A human calcium-activated potassium channel gene expressed in vascular smooth muscle. *Am J Physiol.* 269: H767-77.
- McEwen BS (1991) Our changing ideas about steroid effects on an ever-changing brain. *The Neurosciences* 8: 497-507.
- Moore FJ, Evans SJ (1999) Steroid hormones use non-genomic mechanisms to control brain functions and behaviors: a review of evidence. *Brain Behav Evol* 54: 41-50.
- Nilius B, Droogmans G (2001) Ion channels and their functional role in vascular endothelium. *Physiol Rev.* 2001 81(4): 1415-1459.
- Pedarzani P, Kulik A, Muller M, Ballanyi K, Stocker M (2000) Molecular determinants of Ca²⁺-dependent K⁺ channel function in rat dorsal vagal neurones. *J Physiol* 52: 283-290.
- Peng W, Hoidal JR, Farrukh IS (1999) Role of a novel KCa opener in regulating K⁺ channels of hypoxic human pulmonary vascular cells. *Am J Respir Cell Mol Biol* 20: 737-745.
- Ramanathan K, Michael TH, Fuchs PA (2000) Beta subunits modulate alternatively spliced, large conductance, calcium-activated potassium channels of avian hair cells. *J Neurosci* 20: 1675-1684.
- Sakmann B, Neher E (1985) *Single-channel recording*, 2nd ed. New York, NY: Plenum Press

- Sapolsky RM, Romero LM, Munck AU (2000) How do glucocorticoids influence stress responses? Integrating permissive, suppressive, stimulatory, and preparative actions. *Endocr Rev* 21:55-89.
- Shipston MJ, Duncan RR, Clark AG, Antoni FA, Tian L (1999) Molecular components of large conductance calcium-activated potassium (BK) channels in mouse pituitary corticotropes. *Mol Endocrinol* 13(10): 1728-1737.
- Shipston MJ (2001) Alternative splicing of potassium channels: a dynamic switch of cellular excitability. *Trends Cell Biol* 11(9): 353-358.
- Solaro CR, Prakriya M, Ding JP, Lingle CJ (1995) Inactivating and noninactivating Ca^{2+} - and voltage-dependent K^+ current in rat adrenal chromaffin cells. *J Neurosci* 15: 6110-6123.
- Stachowiak MK, Rigual RJ, Lee PHK, Viveros OH, Hong JS (1988) Regulation of tyrosine hydroxylase and phenylethanolamine N-methyltransferase mRNA levels in the sympathoadrenal system by the pituitary-adrenocortical axis. *Brain Res Mol Brain Res* 3:275-286.
- Sun D, Huang A, Koller A, Kaley G (2001) Endothelial K(Ca) channels mediate flow-dependent dilation of arterioles of skeletal muscle and mesentery. *Microvasc Res* 61(2): 179-186.
- Tank AW, Ham L, Curella P (1986) Induction of tyrosine hydroxylase by cyclic AMP and glucocorticoids in a rat pheochromocytoma cell line: effects of inducing agents alone or in combination on enzyme levels and rate of synthesis of tyrosine hydroxylase. *Mol Pharmacol* 30: 486-496.
- Takimoto K, Li D, Nerbonne JM, Levitan ES (1997) Distribution, splicing and glucocorticoid-induced expression of cardiac alpha 1C and alpha 1D voltage-gated Ca^{2+} channel mRNAs. *J Mol Cell Cardiol* 29(11): 3035-3042.
- Tian L, Hammond MS, Florance H, Antoni FA, Shipston MJ (2001a) Alternative splicing determines sensitivity of murine calcium-activated potassium channels to glucocorticoids. *J Physiol* 537:57-68.
- Tian L, Duncan RR, Hammond MS, Coghill LS, Wen H, Rusinova R, Clark AG, Levitan IB, Shipston MJ (2001b) Alternative splicing switches potassium channel sensitivity to protein phosphorylation. *J Biol Chem* 276:7717-7720.
- Valverde MA, Rojas P, Amigo J, Cosmelli D, Orío P, Bahamonde MI, Mann GE, Vergara C, Latorre R (1999) Acute activation of maxi-K channels (hsl α) by estradiol binding to the β subunit. *Science* 285:1929-1931.

- Van Goor F, Li YX, Stojilkovic SS (2001) Paradoxical role of large-conductance calcium-activated K⁺ (BK) channels in controlling action potential-driven Ca²⁺ entry in anterior pituitary cells. *J Neurosci* 21(16): 5902-15.
- Veissier I, van Reenen CG, Andanson S, Leushuis IE (1999) Adrenocorticotrophic hormone and cortisol in calves after corticotropin-releasing hormone. *J Anim Sci* 77:2047-2053.
- Viau V, Chu A, Soriano L, Dallman MF (1999) Independent and overlapping effects of corticosterone and testosterone on corticotropin-releasing hormone and arginine vasopressin mRNA expression in the paraventricular nucleus of the hypothalamus and stress-induced adrenocorticotrophic hormone release. *J Neurosci* 19:6684-6693.
- Wagner PG, Jorgensen MS, Arden WA, Jackson BA (1999) Stimulus-secretion coupling in porcine adrenal chromaffin cells: acute effects of glucocorticoids. *J Neurosci Research* 57: 643-650.
- Wallner M, Meera P, Toro L (1999) Molecular basis of fast inactivation in voltage and Ca²⁺-activated K⁺ channels: a transmembrane β -subunit homolog. *Proc Natl Acad Sci USA* 96:4137-4142.
- Wang YW, Ding JP, Xia XM, Lingle CJ (2002) Consequences of the stoichiometry of Slo1 alpha and auxiliary beta subunits on functional properties of large-conductance Ca²⁺-activated K⁺ channels. *J Neurosci* 22:1550-1561.
- Warner SG, Koch RO, Koschak A, Trieb M, Garcia ML, Kaczorowski GJ, Knaus HG. (1999) High-conductance calcium-activated potassium channels in rat brain: pharmacology, distribution, and subunit composition. *Biochemistry* 38:5392-5400
- Wehling M (1997) Specific, nongenomic actions of steroid hormones. *Ann Rev Physiol* 59: 563-593.
- Xia X-M, Ding JP, Lingle CJ (1999) Molecular basis for the inactivation of Ca²⁺- and voltage-dependent BK channels in adrenal chromaffin cells and rat insulinoma tumor cells. *J Neurosci* 19:5255-5264.
- Xie J, McCobb DP (1998) Control of alternative splicing of potassium channels stress hormones. *Science* 280:443-446.

CHAPTER 3

β 2 AND β 4 SUBUNITS OF BK CHANNELS CONFER DIFFERENTIAL SENSITIVITY TO ACUTE MODULATION BY STEROID HORMONES*

Membrane-associated receptors for rapid, steroidal neuromodulation remain elusive. Estradiol has been reported to facilitate activation of voltage- and Ca^{2+} -dependent 'BK' potassium channels encoded by *Slo*, if associated with β 1 subunits. We demonstrate here that 1) multiple members of the β family confer sensitivity to multiple steroids on BK channels, 2) that β subunits differentiate between steroids, and 3) that different β s have distinct relative preferences for particular steroids. Expressed in HEK 293 cells, inside-out patches with channels composed of *Slo*- α alone showed no steroid sensitivity. Cells expressing $\alpha\beta$ 4 exhibited potent, rapid, reversible, and dose-dependent potentiation by corticosterone (CORT, a glucocorticoid), and were potentiated to a lesser degree by other sex and stress steroids. In contrast, $\alpha\beta$ 2 channels were potentiated more strongly by dehydroepiandrosterone (DHEA, an enigmatic, stress-related, 'adrenal androgen'), and to a lesser extent by CORT, estradiol, testosterone, and DHEA-S. Cholesterol had no effect on any BK channel compositions tested. Conductance-voltage plots of channels composed of α plus β 2 or β 4 subunits were shifted in the negative direction by steroids, indicating greater activation at negative voltages. Thus our results argue that the variety of *Slo*- β subunit coexpression patterns occurring in vivo expands the repertoire of *Slo* channel gating in yet another dimension not fully appreciated,

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rendering BK gating responsive to dynamic fluctuations in a multiple of steroid hormones.

INTRODUCTION

While some immediate, non-genomic effects of steroid hormones on electrophysiological function have been known for 50 years, many rapid effects are still being discovered, as potential players are identified and characterized (Wehling, 1997; Falkenstein et al., 2000; Makara and Haller, 2001). Ion channels, G-protein-coupled receptors, phospholipases, and protein kinases are modulated acutely, but primary receptors and transducing intermediates are mostly unknown.

Big-conductance calcium- and voltage-activated potassium (BK) channels are prominent in nerve, muscle, endocrine and exocrine cells. Their functions vary widely with context. In smooth muscle they counteract depolarization and contraction. In presynaptic nerve terminals they can limit secretion by speeding rapid repolarization. Rapid repolarization and brief afterhyperpolarization mediated by BK current can increase or decrease neuronal repetitive firing rates. These channels can also make interesting and sometimes paradoxical contributions to firing patterns, affecting threshold, spike frequency adaptation, and burst firing patterns (Brayden and Nelson, 1992; Lingle, 1996; Vergara et al., 1998; Fettiplace and Fuchs, 1999; Jin et al., 2000; Van Goor et al., 2001; McCobb, 2004). BK channels have a pore formed from a tetramer of α -subunits encoded by the *Slo* gene. β subunits encoded by at least 4 related genes differentially co-occur with *Slo*, and have distinctive and sometimes dramatic effects on channel gating (Orio et al., 2002). β 1, prominent in smooth muscle, shifts BK activation to more negative voltages for a given Ca^{2+} concentration, while slowing activation (McManus et al., 1995; Dworetzky et al., 1996). β 2 negatively shifts activation, but also confers rapid inactivation through a 'ball-and-

chain' mechanism (Wallner et al., 1999; Xia et al., 1999; Uebele et al., 2000). One splice variant of $\beta 3$ confers extremely rapid but incomplete inactivation (Xia et al., 2000). $\beta 4$, prevalent in brain, increases the steepness of Ca^{2+} sensitivity and dramatically slows activation and deactivation kinetics (Behrens et al., 2000; Brenner et al., 2000). Temporal lobe seizures reported in $\beta 4$ knockout mice show that $\beta 4$'s effects are critical for damping excessive firing frequencies in granule cells of the dentate gyrus (Brenner et al., 2005).

Several steroids modulate BK channels. Estradiol, testosterone, and bile salts, (steroidal anions) can relax smooth muscle by potentiating BK channels (Deenadayalu et al., 2001; Rosenfeld et al., 2001; Dopico et al., 2002; Salom et al., 2002). We have recently shown that adrenal glucocorticoids cortisol and corticosterone (together abbreviated CORT), and their synthetic analog dexamethasone, facilitate BK activation in adrenal chromaffin cells, promoting rapid action potential repolarization, repetitive firing, and presumably augmenting catecholamine secretion under stress (Lovell et al., 2004). Similar effects of CORT on pituitary corticotrope- and somatotrope-like cell lines have also been reported (Huang et al., 2005). Whether CORT modulates BK channels in hippocampus, pituitary, or other native tissues controlling stress responses is not yet known.

DHEA (dehydroepiandrosterone) is another steroid that has been proposed to regulate BK channels in vivo. DHEA, with its sulfated form DHEA-S, is the most abundant, but perhaps least understood, steroid hormone. Produced in the adrenal cortex and elsewhere, including the brain, serum DHEA levels are increased by stress in humans (Oberbeck et al., 1998; Kroboth et al., 1999; Zinder and Dar, 1999). An age-related decline in serum levels has prompted widespread therapeutic use, despite very limited understanding of its natural role(s) or mechanisms of action (Vermeulen, 1995; Wolkowitz et al., 1997; Allolio and Arlt, 2002). There is evidence for

neuroprotective effects of DHEA in hippocampus (Kimonides et al., 1998; Bastianetto et al., 1999). DHEA or DHEA-S modulates CNS GABA_A receptors and calcium channels (Majewska et al., 1990; French-Mullen and Spence, 1991). Importantly, BK channel activation in pulmonary vascular smooth muscle is acutely potentiated by DHEA, reducing vasoconstriction under hypoxia (Farrukh et al., 1998; Peng et al., 1999).

Coexpression of $\beta 1$ and *Slo* in *Xenopus* oocytes or HEK-293 cells confers both estradiol binding and channel facilitation (Valverde et al., 1999; Behrens et al., 2000). The response mimics that of BK channels in vascular smooth muscle cells. Here we test the possibility that other related β subunits mediate modulation of BK channels by other steroids. We report that $\beta 4$, prominent in hippocampus and other brain regions (Brenner et al., 2000), confers particular sensitivity to CORT. On the other hand, inactivation-conferring $\beta 2$, expressed in lung, adrenal medulla, and brain, confers preferential sensitivity to DHEA. We submit that distinct, multi-steroidal interaction profiles add another nuance to β subunit-related diversification of BK channel function in vivo.

MATERIALS AND METHODS

Plasmid Constructs and Expression

A human BK channel α -subunit cDNA (*hSlo*; accession no. U13913) was excised as a HindIII-SalI fragment and cloned into the expression vector pcDNA3.1 (Invitrogen) under the control of a CMV promoter. The human BK channel $\beta 1$ subunit cDNA (accession no. NM004137) was excised with BamHI, and NotI and subcloned into pcDNA3.1. The human $\beta 2$ subunit cDNA (from American Type Culture Collection; accession no. AF099137) was excised with HindIII, and XhoI and subcloned into pcDNA3.1. $\beta 2_{NI}$, a non-inactivating $\beta 2$ mutant lacking the inactivation-

conferring amino terminal residues FIW in positions 2 - 4 (Xia et al., 2003) was created by incorporating a HindIII site followed by an initial methionine and residues 5-9 into the forward primer, and XhoI in the reverse primer, and amplifying from the full length cDNA using a proof-reading polymerase (Pfu). The amplicon was then digested with HindIII and XhoI, gel purified and cloned into the corresponding sites in pcDNA3.1(+). Rat $\beta 4$ subunit was amplified by reverse-transcription PCR from rat adrenal RNA, and TA cloned into pCR2.1, excised with HindIII and XhoI, and subcloned into pcDNA3.1. Directional orientation and sequence of the inserts was determined by DNA sequencing at the BioResource Center (Cornell University).

HEK-293 cells (ATCC: CRL-1573) were transiently transfected using lipofectamine 2000 (Gibco) following the manufacturer's protocols. Briefly, HEK cells were grown to 70-80% confluency in DMEM (Dulbecco's modified Eagle medium) + 2mM glutamine supplemented with 10% fetal bovine serum. The cells were grown at 37°C in a humidified atmosphere of 5% CO₂ and 95% air. After DNA-lipid complexes were allowed to form, cells were transfected with either cDNAs encoding *Slo- α* alone (1 μ g DNA) or cotransfected with cDNAs for *Slo- α* and *Slo- β* subunits in a 1:1 ratio. An additional plasmid containing a modified green fluorescent protein (pBFP-N1; Clontech, Palo Alto, CA) was also cotransfected into the cells for identification. Cells were plated on 35 mm culture dishes (Falcon) coated with collagen (0.53 mg/mL; Vitrogen, Palo Alto, CA) and lifted with a solution containing in mM: 0.5 EDTA, 38 sucrose, 34 D-glucose, 156 NaCl, 5 KCl, 4 NaHCO₃, 9 HEPES, pH 7.2. Transfected cells were patched between 2 and 5 days after re-plating.

Chromaffin Cell Isolation and Culture

Rat chromaffin cells were isolated and cultured as described previously (Lovell et al., 2000). Briefly, Sprague Dawley rats (Charles River Laboratories, Wilmington,

MA) were anesthetized with CO₂ and euthanized by cervical spinal dislocation. Adrenomedullary tissue was dissected from surrounding cortical tissue, minced, washed in sterile saline, and incubated for 60 minutes in collagenase B (Boehringer Mannheim, Indianapolis, IN; 1.5 mg/ml; pH 7.0), at 37°C. Tissue was washed in Ca²⁺- and Mg²⁺-free saline, and incubated for 30 min. at 37°C in trypsin (Gibco; 0.125% in Hank's Ca²⁺ and Mg²⁺-free saline). Chromaffin cells were triturated through fire-polished pipettes and plated in sterile culture medium (Gibco; RPMI 1640 with 10% horse serum, 5% fetal calf serum, 2 U/ml penicillin-G, 2 µg/ml streptomycin sulfate, 100 U/ml nystatin) in glass-bottom dishes coated with Poly-D-Lysine (Sigma, St. Louis, MO; 0.01% in ddH₂O). Cell cultures were maintained in a 5% CO₂ atmosphere at 37°C for 1-4 days.

Electrophysiological Methods

Single channel and macroscopic currents were recorded in the inside-out configuration. Patch electrodes (3-5 MΩ) were pulled from borosilicate glass and coated with silicone elastomer (Sylgard 184; Dow Corning, Midland, OH). Data were collected using a List EPC-9 patch clamp amplifier (Heka Elektronik, Lambrecht, Germany) Bessel filtered at 10 kHz, and stored on a Power Macintosh G3 using Pulse 8.5 software (Heka Elektronik, Lambrecht, Germany). Offline analysis was done with custom software written for Igor Pro (Wavemetrics, Lake Oswego, OR).

Experiments were conducted at 20-22°C. After attaining GΩ seals, calcium-free saline was perfused on the cell and inside-out patches were pulled. Exchange of solutions was accomplished with eight computer-controlled, gravity-fed lines converging near the tip of a large perfusion pipette.

For inside-out patches, standard patch clamp recording techniques were used. HEK cells were typically held at -80 mV and stepped to +80 mV for 450 ms after a

prepulse to -140 mV for 450 ms, with a 2 sec interval between sweeps. For the determination of the voltage dependence of BK channels, a series of increasing voltage steps was applied in 20 mV increments from -140 mV to $+140$ mV for 350 ms. For the voltage dependence of inactivation, the steady state current after activation was measured by a second voltage step to $+80$ mV for 350 ms.

Solutions

To eliminate a potassium driving force and allow DC offset to be cancelled at 0 mV, symmetrical K^+ solutions were used. The pipette saline and perfusion solutions were comprised of (in mM): 160 KCl, 10 HEPES, 1 HEDTA, 0.0375 CaOH, pH was adjusted with KOH to 7.2 to make ~ 500 nM $- 1$ μ M free $[Ca^{2+}]$. Calculations of free $[Ca^{2+}]$ were made through MaxChelator software (WebMaxC v2.10; Bers et al., 1994). Zero- Ca^{2+} solution contained 5 mM EGTA (Sigma). Rodent ringers contained (in mM): 145 NaCl, 5 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 10 HEPES, pH 7.2. The osmolarity of all solutions before the addition of steroids was measured by dew point osmometry and adjusted to 300 ± 10 mosM with ddH₂O or NaCl. Steroids were dissolved in 100% dimethyl-sulfoxide (DMSO), stored in small aliquots, and added fresh to recording salines. DMSO vehicle in the control saline (0.1%, same concentration as steroid solution) did not alter BK channel activity.

Data and Statistical Analysis

Single and multi-channel currents were linear leak subtracted and ensemble currents fit using a Levenberg-Marquardt search algorithm to estimate kinetic parameters. Percent changes in steroids were measured by comparing the maximum peak amplitudes of the current traces before and after treatment with steroid. Ensemble currents were usually averages of 15-30 individual current traces taken 30-60 seconds

after switching perfusion lines to allow patches to stabilize. Patches where BK current was not stable after 3 minutes were excluded from analysis. Patches used in analysis showed stable BK currents throughout the experiment (refer to Figs. 2B & 4B). Dose response curves were generated with mostly patches exposed to only one concentration of steroid. In a few cases, patches were included where complete washout was observed following the previous application of a lower concentration of steroid.

The voltage-dependence of activation (G-V) was determined by measuring either peak current activation, or peak amplitudes of tail currents, and dividing by the respective driving force. Plotted values of conductance (G) as a function of test potential (V_m) were fit to a single term Boltzmann function of the form:

$$G = G_{\max} / (1 + \exp((V_m - V_{0.5})/ s))$$

with parameters for maximum conductance (G_{\max}), voltage of half-activation ($V_{1/2}$), and slope (s; the steepness of the voltage-dependence of activation, in mV/e-fold change in voltage). Median peak current amplitudes were compared using the Kruskal-Wallis nonparametric test. ANOVA was conducted on rank transformed data. Tukey's conservative pairwise comparisons, with a family error rate (combined P value) of 0.05, were then made to determine which categories differed. Inactivation and deactivation time constants, $V_{1/2}$ measurements, and rat chromaffin cell peak current amplitudes were compared using a Student's T-test ($p < 0.05$).

RNA Extraction and RT-PCR

Total RNA was harvested from quick-frozen tissues using the Qiagen RNeasy kit. 2 μ g RNA was added to each standard 20 μ l reverse transcription reaction (Allolio

and Arlt, 2002) with Superscript II reverse transcriptase (GibcoBRL) and 10 μ M oligo-(dT). 1.5 μ l of the RT product was transferred to each 30 μ l PCR reaction with Taq DNA polymerase). Primers for rat β 2 (r β 2s 5' AATCACACTGCTGCGCTCATA CAT 3' and r β 2as 5' TTCTGTGTGGTAGAGGAGGAGC 3') gave a predicted product of 319bp (accession no. AF209747). Primers for β 4 (r β 41s 5' CGGCTCG GCTTGTTCCCTCA 3', r β 42as 5' GCTGGTGCTGGTCGCTGT 3') gave a predicted product of 265bp (Accession no. AF207992). Following 3 minutes at 95°C, 30 cycles were run with 30 s at 95° C and 55° C, and 45 s at 72° C. 6 μ l of PCR product was then run on a 2% agarose gel. Ethidium bromide stained gels were UV trans-illuminated and images captured with a Cohu camera and an LG-3 digitizer, controlled using modified NIH image software (Scion, Frederick, MD).

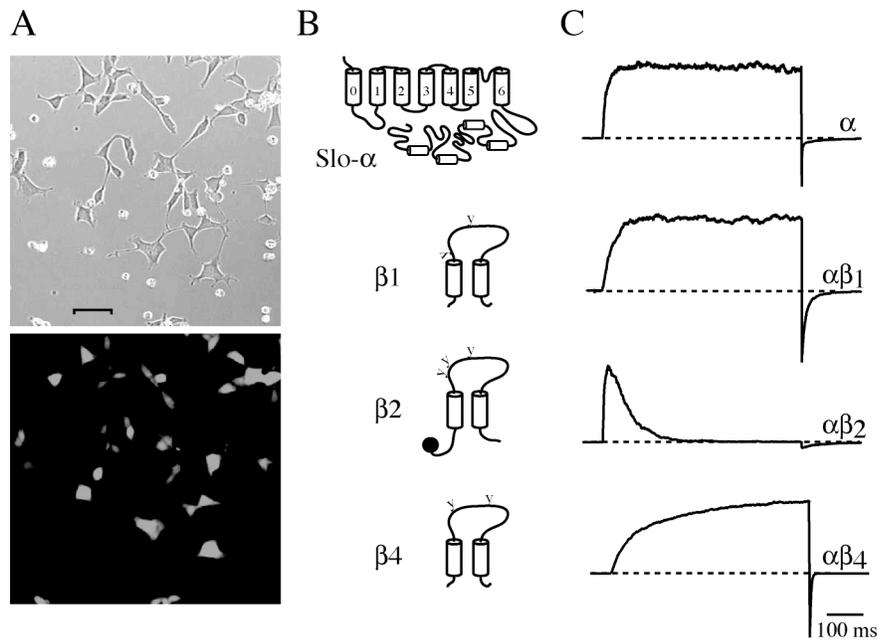
RESULTS

HEK-293 cells were transiently transfected with cDNA plasmids encoding *hSlo- α* alone or in combination with β 1, β 2, or β 4 cDNAs, in addition to GFP. Inside-out patches were pulled from green-fluorescing cells after 48-72 hours (Fig. 1). Representative ensemble-averaged current traces obtained in the presence of 500 nM $[Ca^{2+}]_i$ show fast activating and deactivating BK current from cells expressing the α *Slo* subunit alone. Ca^{2+} and voltage-sensitivity and kinetics were altered by coexpression with β subunits. Thus β 1 and β 4 slow the activation and the deactivation kinetics of the channel, and rapid inactivation is conferred by coexpression with β 2.

β 4 Confers Sensitivity to Corticosterone on Slo Channels

Inside-out patches with varying numbers of BK channels were pulled from HEK 293 cells expressing *Slo- α* and β 4 subunits. In control saline (500 nM $[Ca^{2+}]_i$),

Figure 1. Heterologous expression of *Slo- α* and β subunits in HEK-293 cells. A) HEK cells transfected with α and $\beta 2$ subunits and a modified-GFP protein, viewed under bright-field (top panel; scale bar = 50 μm) and fluorescence (bottom). B) Schematic representation of the membrane topology of the *Slo- α* and β -subunits. C) Representative current traces from patches containing BK channels with and without β -subunits. Currents were elicited by stepping the membrane voltage to +80 mV from a prepulse potential of -140 mV in the presence of 500 nM $[\text{Ca}^{2+}]_i$. *Slo- α* subunits alone produced fast activating and deactivating outward currents that showed no apparent inactivation during the 450 ms voltage-step (top trace). The $\beta 1$ -subunit ($\alpha\beta 1$) slowed both activation and deactivation kinetics (second trace). Cells expressing $\alpha\beta 2$ subunits conferred rapid and complete inactivation upon BK channels (third trace). Currents elicited from $\alpha\beta 4$ subunits characteristically activated and deactivated more slowly than *Slo- α* alone (bottom trace).

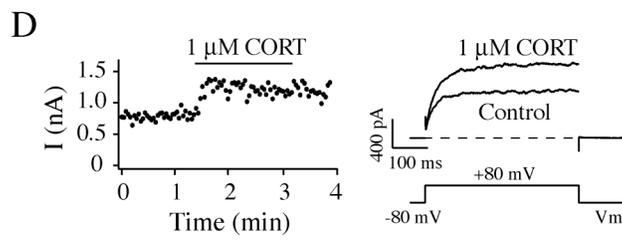
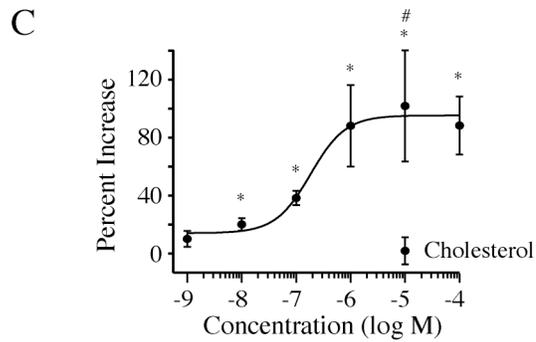
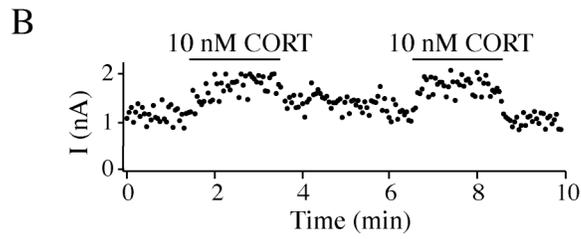
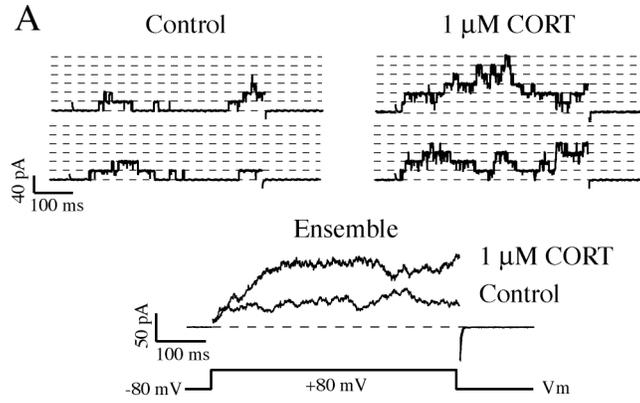


large-conductance (~ 300 pS) openings were often discernible in individual sweeps at +80 mV (450 ms duration; Fig. 2A). BK currents were allowed to stabilize for 3-5 minutes before application of steroid. Ensemble traces best illustrate the average relative increase in current amplitude produced by the steroid. Channel opening events increased in number within tens of seconds after the application of steroid with concentrations as little as 10 nM CORT (Fig. 2B). Though responses at 10 nM were not observed for every patch, washoff and repeat application in specific cases provided compelling proof of the effect at this low dose. Effects at 100 nM, while still variable, were statistically significant on average, as described further below. The potentiation did not diminish appreciably with time over 30 minutes or more of continuous exposure, and could be at least partially reversed by washout, typically fully at lower concentrations. 1 μ M CORT had minimal effect on α alone, as detailed below.

CORT effects could not be explained by a change in single channel conductance. Single channel current amplitude at +80 mV in control saline and 1 μ M CORT averaged 25.2 ± 0.5 pA and 24.4 ± 0.7 pA, respectively ($n = 9$ each; $p = 0.4$ students t-test). Reversibility was observed to be more complete at lower concentrations of CORT: at 0.01, 0.1, and 1 μ M, cases of complete washout of steroid effect (100%) were observed with an average washout of $81.8 \pm 8.0\%$ ($n = 13$), $81.6 \pm 24.9\%$ ($n = 7$), and $63.7 \pm 10.6\%$ ($n = 7$). At concentrations of 10 and 100 μ M CORT, an average washout of $53.1 \pm 8.6\%$ ($n = 12$) and $63.0 \pm 6.8\%$ ($n = 15$) was observed. To rule out effects of switching between perfusion lines, switches between two lines with control saline were performed. This had no significant effect on peak current amplitudes (mean change = $1.7 \pm 1.2\%$, $n = 16$, data not shown).

CORT was found to increase BK current measured at test potentials of +80 mV in a roughly dose dependent manner in inside-out patches expressing $\alpha\beta 4$ channels (Fig. 2C). Mean (\pm SEM) increases in current elicited with steps to +80 mV

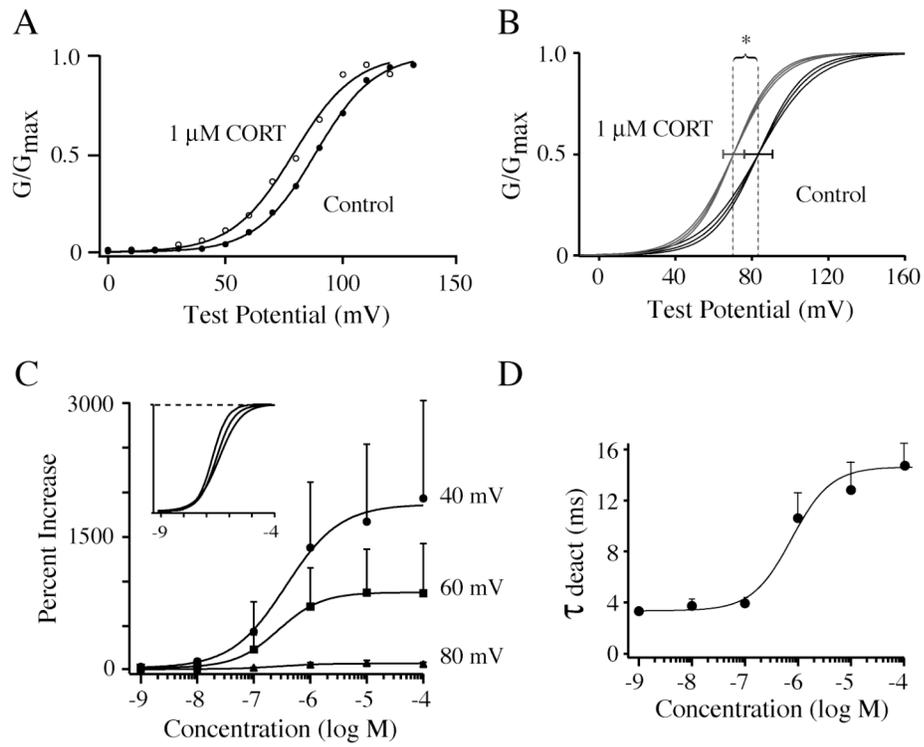
Figure 2. Rapid modulation of $\alpha\beta 4$ channels by corticosterone. A) In an inside-out patch, containing at least 6 channels, BK currents were activated by a test pulse to +80 mV from a holding potential of -80 mV. Application of 1 μM corticosterone (CORT) to the patch rapidly increased the number of BK channels open during successive sweeps. Ensemble averages of 30 such sweeps are shown below. B) Timescale plot from an inside-out patch showing the acute potentiation of BK current (within 30 seconds) at 10 nM CORT, the reproducibility, and the reversibility of the steroid effect. Each point on the time scale represents a running average of 5 trace samples. C) Dose-dependent increases are evident in different concentrations of CORT covering 6 orders of magnitude (mean \pm SEM). Current amplitudes were measured from tails at -80 following a test potential of +80 mV. A significant increase compared with perfusion of control saline (* $p < 0.001$, Kruskal-Wallis test) was seen at concentrations of 0.01, 0.1, 1, 10 and 100 μM CORT. Effects at 1, 10 and 100 μM were significantly greater than at 0.001 and 0.01 μM CORT. 10 μM CORT had a significantly greater effect on $\alpha\beta 4$ channels than 10 μM cholesterol, a steroid precursor ($p < 0.05$, students t-test, denoted by #). The dose response curve was fit by a Hill equation with a Hill coefficient of 1.4, and a half maximal point of 0.182 μM . D) Timescale plot from whole-cell patch configuration showing the acute potentiation of BK current at 1 μM CORT (left panel). BK current elicited in whole-cell mode by a test potential to +80 mV is potentiated after the application of 1 μM CORT (right panel).



were measured for CORT concentrations covering 6 orders of magnitude. Increases were $10.2 \pm 5.5\%$ ($n = 10$), $20.2 \pm 4.3\%$ ($n = 21$), $38.4 \pm 4.9\%$ ($n = 15$), $88.2 \pm 28.2\%$ ($n = 18$), $101.9 \pm 38.4\%$ ($n = 23$), and $88.4 \pm 20.0\%$ ($n = 21$), for 0.001, 0.01, 0.1, 1, 10, and 100 μM CORT respectively. Responses to 0.01, 0.1, 1, 10, and 100 μM CORT were significantly different than switching between control solutions. Additionally, 1, 10 and 100 μM were significantly different than 0.001 and 0.01 μM CORT (One Way ANOVA, F statistic with 6 and 122 degrees of freedom for between treatment and within treatment variation, $F_{6,122} = 20.13$; $p < 0.001$, means compared with Tukey's pairwise post-hoc comparisons, family error rate of 0.05). 10 μM cholesterol, a steroid precursor, had a significantly smaller effect on $\alpha\beta 4$ channels than 10 μM CORT ($2.0 \pm 9.3\%$, $n = 5$, $p < 0.05$ students t-test). The dose response curve was fit by a Hill equation with a Hill coefficient of 1.4, and a half maximal effect at 182 nM. Under the whole-cell recording configuration 1 μM CORT also potentiated $\alpha\beta 4$ BK channels within seconds of application (Fig. 2D, left panel). In the example shown (Fig. 2D, right panel), 1 μM CORT potentiated BK current by 57.3%. In whole-cell mode, the average potentiation by 1 μM CORT was $52.3 \pm 5.1\%$ ($n = 2$).

For a subset of patches, the effects of steroids were tested with a full series of test potentials (Fig. 3A), revealing that steroids produce a simple parallel shift of the G-V curve in the negative direction. In the example shown in Fig. 3A, 1 μM CORT shifted $V_{1/2}$ by -10.0 mV. On average $V_{1/2}$ was shifted by -13.0 ± 2.8 mV by 1 μM CORT (Fig. 3B, $n = 4$; note $[\text{Ca}^{2+}]_i = 1 \mu\text{M}$ in these experiments). The average $V_{1/2}$ was significantly different in control conditions than in 1 μM CORT ($V_{1/2} = 83.4 \pm 7.3$ mV and 70.4 ± 5.5 mV in control and in 1 μM CORT, $n = 4$ and 4, respectively, $p = .019$, students t-test). At other concentrations, $V_{1/2}$ was also shifted to more negative potentials. At 10 μM and 100 μM CORT (with $[\text{Ca}^{2+}]_i = 500$ nM), $V_{1/2}$ was shifted on average by -10.8 ± 0.8 mV and by -17.4 ± 4.1 mV, $n = 5$ and 4, respectively).

Figure 3. CORT shifts the voltage dependence of activation and slows deactivation. A) For patches containing $\alpha\beta 4$ channels, test potentials were stepped in 10 mV increments in control saline and in the presence of 1 μM CORT. Conductance-voltage (G-V) plots were generated from tail currents, and fit with a Boltzman function. In 1 μM CORT a shift of -10 mV was seen for the half activation voltage ($V_{1/2}$) in one particular patch. B) On average, 1 μM CORT produced a -13.0 mV shift in $V_{1/2}$. Under control conditions, $V_{1/2}$ (83.4 ± 7.3 mV, $n = 4$) was significantly different than $V_{1/2}$ in 1 μM CORT (70.4 ± 5.5 mV, $n = 4$, * $p < 0.05$, Students t-test). C) For a subset of patches, dose response curves showed higher percent increases at lower test potentials (+40 mV and +60 mV). When each dose response curve was fit with a Hill function (upper left), Hill coefficients were approximately 1.0, and half maximal response values ranged from 183 nM to 367 nM. D) Higher concentrations of CORT also slowed the deactivation kinetics of $\alpha\beta 4$ channels. At a concentration of 1 μM CORT, the average time constant of deactivation at a test potential of +80 mV was increased significantly ($p < 0.01$, Students t-test).



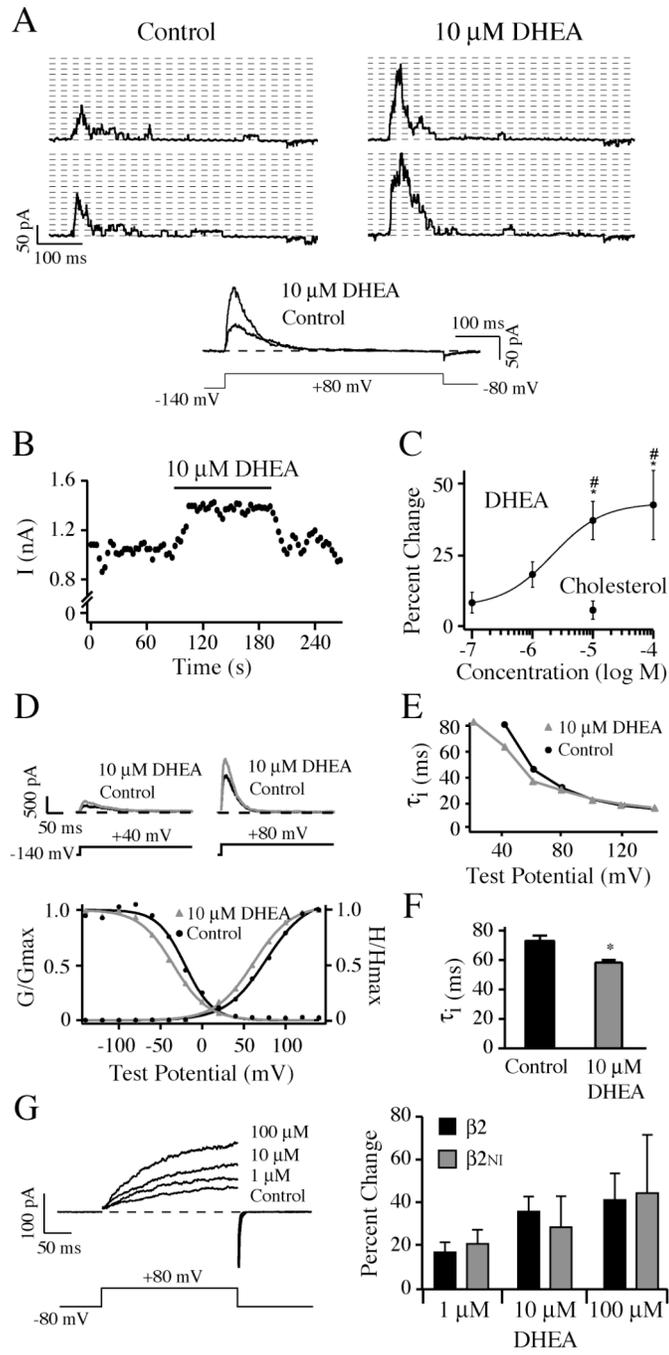
For one set of patches, the effects of CORT were measured at +40, 60, and 80 mV, and mean percentage increases computed over the 6 orders of concentrations (Fig. 3C). Despite wide variation related to the aforementioned complications, mean dose responses at +40, 60, and 80 were each fit well by a Hill equation, yielding three curves with strikingly similar Hill coefficients of approximately 1.0, and half-maximal response values of 183 nM, 278 nM, and 360 nM (for +40, +60, and +80 mV respectively).

CORT also reduced the rate of current deactivation (τ_{deact} estimated by a single exponential fit to the tail current). On average, the time constant of deactivation at a test potential of +80 mV was increased significantly from 3.3 ± 0.3 ms in control saline to 10.6 ± 2 ms in 1 μM CORT ($n = 15$ and 8, respectively, $p = 0.008$; Fig. 3D). This effect was observed at several CORT concentrations, and several test potentials. The dose-response of this effect could also be well approximated by a Hill function with a coefficient of 1.0 and a half-maximal point below 500 nM.

$\beta 2$ Confers Sensitivity to DHEA on Slo Channels

Inside-out patches with varying numbers of channels were pulled from cells expressing Slo α plus $\beta 2$ subunits. In control saline (500 nM $[\text{Ca}^{2+}]_i$), large-conductance (~ 260 pS) openings were often discernible in individual sweeps at +80 mV (450 ms duration) after a brief prepulse to -140 mV to relieve all inactivation (Fig. 4A). Ensemble averages of 25 traces show the average relative increase in current amplitude produced by the steroid. As with CORT on $\beta 4$ channels, channel openings increased in number within tens of seconds after application of 10 μM DHEA (Fig. 4B). Mean responses (\pm SEM) were measured at concentrations covering 4 orders of magnitude (Fig. 4C). The average increase at 100 nM DHEA was not significant, $7.9 \pm 3.6\%$ ($n = 7$). However, 1 μM DHEA significantly increased current,

Figure 4. Rapid modulation of $\alpha\beta 2$ channels by dehydroepiandrosterone (DHEA). A) In an inside-out patch containing at least 14 channels, steps to +80 mV activates rapidly- and completely-inactivating BK channels. Treatment of the patch with 10 μM DHEA increased the average number of channels open during successive sweeps. Ensemble averages of 25 such sweeps are shown below. B) A timescale plot shows that 10 μM DHEA rapidly potentiates BK channel opening within 10 seconds of application. Each point on the timescale represents a running average of 3 points. C) Exposure of BK channels to DHEA resulted in dose-dependent increases in mean current amplitude (\pm SEM), expressed as a percentage of pretreatment amplitude for respective patches. * denotes significant difference from vehicle perfusion experiments, # denotes significant difference from 1 μM result shown. ($n = 7, 17, 18,$ and 11 for 0.1, 1, 10, and 100 μM DHEA, respectively, $p < 0.001$). The curve has a Hill coefficient of ~ 1.0 and a half-maximal dose at $\sim 2 \mu\text{M}$. D) Representative traces from one patch with steps to +40 or +80 mV in the presence of 10 μM DHEA (top panel). DHEA shifts the voltage-dependence of BK channel activation and inactivation to more negative voltages (bottom panel). Conductance-voltage (G-V) plots were generated from traces as in top panel. Steady-state inactivation (H/H_{Max}) plots were generated from steps to +80 mV from varying inactivating-prepulse voltages of 350 ms duration. E) At lower test potentials, inactivation time constants (τ_i) obtained with single exponential fits were smaller in DHEA than control saline. F) The mean time constant of inactivation at +40 mV was significantly decreased by 10 μM DHEA ($n = 6; p = 0.01$). G) Traces from a single patch expressing the non-inactivating mutant form of $\beta 2$, showing dose-dependent increases in current in response to 1, 10, and 100 μM DHEA (left panel). Currents produced by $\alpha\beta 2_{\text{NI}}$ channels were potentiated to the same extent by 1, 10, and 100 μM DHEA as $\alpha\beta 2$ channels (no significant difference, right panel).

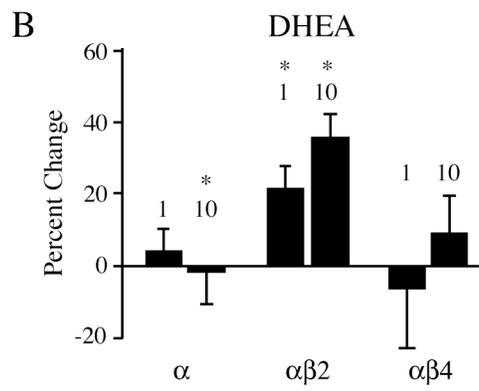
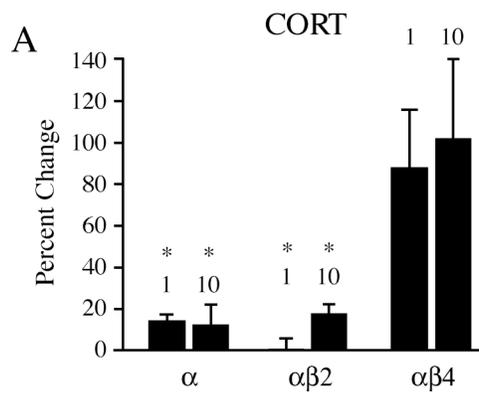


as compared with control saline, by an average of $17.9 \pm 4.5\%$ ($n = 17$). 10 and 100 μM DHEA increased current by $36.9 \pm 6.8\%$ and $42.5 \pm 12.2\%$, respectively ($n = 18$ and 11; $F_{4,64} = 19.18$; $p < 0.001$). Responses at 10 and 100 μM were significantly greater than at 1 μM , but not significantly different from each other. The dose-response curve was fit with a Hill equation having a coefficient of 1.03 and a half-maximal concentration at 2.2 μM . Responses at all concentrations were quite variable in size among patches. However, in individual patches, higher concentrations typically produced bigger responses (e.g., Fig. 4G).

Conductance-voltage (G-V) plots were constructed from peak amplitudes of currents elicited by an incremental series of depolarizing steps before and after application of one or more concentrations of DHEA (Fig. 4D, bottom panel). In the example in 4D, 10 μM DHEA shifted $V_{1/2}$ by -15.1 mV. Similar shifts were seen in an additional 5 cases, with an average $V_{1/2}$ shift of -14.3 ± 3.5 mV. 10 μM DHEA also produced a negative shift in the voltage-dependence of steady-state inactivation (V_{inact} ; Fig. 4D bottom panel). In this example, V_{inact} was shifted -13.9 mV. The average shift was -10.5 ± 1.9 mV ($n = 3$). In all patches, DHEA increased the rate of inactivation at relatively negative voltages. The rate was sharply voltage-dependent, particularly at potentials in the range of +20 to +60 mV (Fig. 4E; τ estimated from a single-exponential fit to the current decay during steps from the -140 mV prepulse). At +40 mV, control traces had an average τ of 72.9 ± 3.8 mV, whereas in 10 μM DHEA the average was 58.3 ± 1.9 ms ($n = 6$ patches; $p = 0.01$, Student's t-test; Fig. 4F).

To rule out any possible confounding effects of inactivation on apparent steroid sensitivity we removed the inactivation domain. Deletion of the amino terminal residues FIW in positions 2 - 4 from the $\beta 2$ construct as detailed in methods and reported previously (Xia et al., 2003) completely eliminated functional inactivation. The effects of DHEA on non-inactivating $\alpha\beta 2_{\text{NI}}$ BK current were very similar to those

Figure 5. Different β subunits show different sensitivities to steroids. A) At 1 μM CORT, BK currents elicited at +80 mV, from $\alpha\beta4$ channels ($n = 18$) showed significantly greater potentiation than currents from $\alpha\beta2$ channels ($n = 5$) or α channels ($n = 3$). Potentiation of BK current at 10 μM CORT was also significantly greater for $\alpha\beta4$ channels ($n = 23$) than $\alpha\beta2$ ($n = 16$) and α -only ($n = 7$, * $p < 0.01$, Kruskal-Wallis test). B) For 1 μM DHEA, $\alpha\beta2$ channels ($n = 12$) were more responsive than α -only ($n = 5$) or $\alpha\beta4$ channels ($n = 6$, $\alpha\beta2$ and $\alpha\beta4$ differed significantly, * $p < 0.05$). $\alpha\beta2$ channels ($n = 19$) were significantly more sensitive to 10 μM DHEA than α -only ($n = 12$) and $\alpha\beta4$ ($n = 15$, * $p < 0.001$, Kruskal-Wallis test).



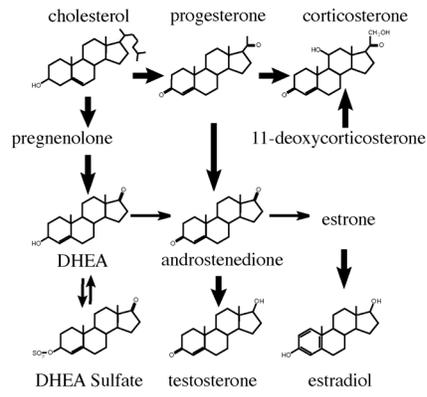
seen with the fully intact $\beta 2$ construct (Fig. 4G). From inside-out patches, at 1 μM DHEA, peak current at +80 mV was increased by $17.9 \pm 4.5\%$ ($n = 17$) for $\alpha\beta 2$ channels, and $21.9 \pm 6.3\%$ ($n = 12$) for $\alpha\beta 2_{\text{NI}}$ channels ($p = 0.60$, Students t-test). At 10 μM DHEA, peak current was increased by $37.0 \pm 6.8\%$ ($n = 13$) for $\alpha\beta 2$ channels, and $29.8 \pm 14.2\%$ ($n = 7$) for $\alpha\beta 2_{\text{NI}}$ channels ($p = 0.61$, Students t-test). At 100 μM DHEA peak current was increased by $42.5 \pm 12.2\%$ ($n = 8$) for $\alpha\beta 2$ channels, and $45.7 \pm 27.1\%$ ($n = 7$) for $\alpha\beta 2_{\text{NI}}$ channels ($p = 0.91$, Students t-test).

β Subunits Confer Distinct Steroid Sensitivities

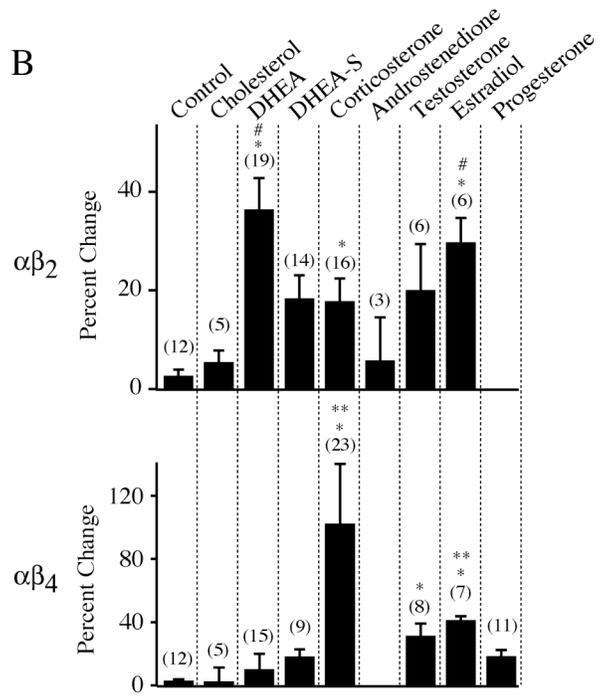
Steroid responses were different for different channel subunit compositions. With a test potential of +80 mV, the application of 1 or 10 μM CORT on HEK cells expressing only the *Slo*- α subunit had little effect on BK currents. On average, 1 or 10 μM CORT altered current amplitude in α channels by $14.3 \pm 3.2\%$ and $12.4 \pm 9.8\%$ and ($n = 3$ and 7, respectively). $\alpha\beta 2$ channels (both the full construct and non-inactivating construct) were typically potentiated by 1 and 10 μM CORT ($0.2 \pm 5.5\%$, $n = 5$ and $17.6 \pm 4.8\%$, $n = 16$, respectively), but to a lesser extent than $\alpha\beta 4$ channels ($88.2 \pm 28.2\%$, $n = 18$ for 1 μM CORT, and $101.9 \pm 38.4\%$, $n = 23$ for 10 μM CORT, Fig. 5A). Cells expressing $\alpha\beta 4$ channels were significantly more responsive to 1 μM CORT than α -only or $\alpha\beta 2$ channels ($F_{2,23} = 16.46$, $p < 0.01$). At 10 μM CORT $\alpha\beta 4$ channels responded significantly more than $\alpha\beta 2$ or α -only channels ($F_{2,43} = 8.21$, $p < 0.01$, Fig. 5A). The application of 1 or 10 μM DHEA also had little effect on α channels, current was altered by $4.6 \pm 6.2\%$ ($n = 5$) for 1 μM DHEA, and $-2.1 \pm 8.8\%$ ($n = 12$) for 10 μM DHEA (Fig. 5B). Patches expressing $\alpha\beta 2$ channels were potentiated to a greater extent by 1 or 10 μM DHEA ($22.2 \pm 6.2\%$, $n = 12$, and $36.3 \pm 6.5\%$, $n = 19$, respectively) than patches expressing $\alpha\beta 4$ channels ($-6.5 \pm 16.5\%$, $n = 6$ for 1 μM DHEA, and $9.6 \pm 10.4\%$, $n = 15$ for 10 μM DHEA, Fig. 5B). At 1 μM

Figure 6. Multiple steroids modulate *Slo* $\alpha\beta 2$ and $\alpha\beta 4$ channels. A) Flow chart illustrating the synthesis relationships between steroids used in this study. B) Bar graphs of cells expressing $\alpha\beta 2$ or $\alpha\beta 4$ channels, showing mean (\pm SEM) peak current amplitudes expressed as a percentage of pretreatment amplitudes, after exposure to 10 μ M steroid. (*n* shown in parentheses, * denotes significant difference from responses to control saline, # denotes significant difference from cholesterol, ** denotes significant difference from cholesterol and DHEA, $p < 0.001$, Kruskal Wallis test).

A



B



DHEA, $\alpha\beta 2$ channels were significantly more sensitive to DHEA than $\alpha\beta 4$ channels ($F_{2,20} = 4.82, p < 0.05$). At 10 μM DHEA $\alpha\beta 2$ channels responded significantly more than $\alpha\beta 4$ or α -only channels ($F_{2,43} = 12.71, p < 0.01$, Fig. 5B).

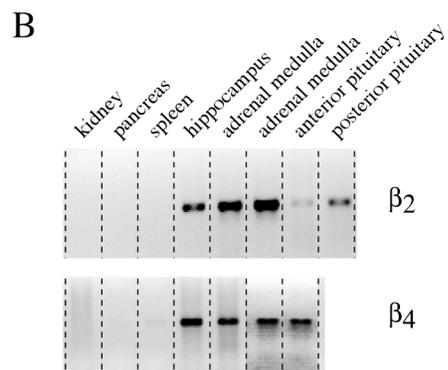
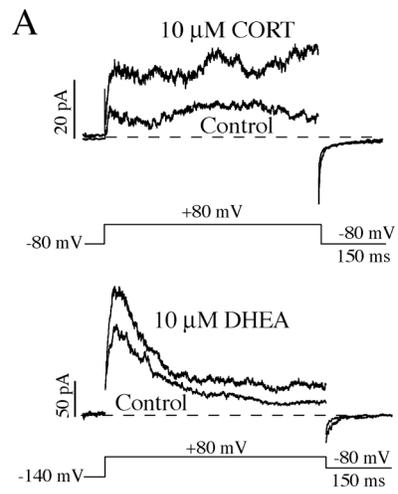
$\beta 2$ and $\beta 4$ Confer Sensitivity to Multiple Steroids

We examined the possibility that several other related steroids might also modulate BK channels (Fig. 6A). For $\alpha\beta 2$ channels, mean percentage increases in peak current amplitude at 10 μM were $5.3 \pm 2.5\%$ for cholesterol, $36.3 \pm 6.5\%$ for DHEA, $18.2 \pm 4.9\%$ for DHEA-S, $17.6 \pm 4.8\%$ for corticosterone, $5.6 \pm 8.9\%$ for androstenedione, $19.9 \pm 9.5\%$ for testosterone, and $29.6 \pm 5.1\%$ for 17β -estradiol (Fig. 6B top panel; $n = 5, 19, 14, 16, 3, 6$, and 6 patches, respectively). Effects of DHEA, CORT, and estradiol were significantly different from control saline. In addition, the effects of DHEA and estradiol were significantly different than cholesterol ($F_{7,73} = 6.42; p < 0.001$). For $\alpha\beta 4$ channels, mean percentage increases in 10 μM steroid were $2.0 \pm 9.3\%$ for cholesterol, $9.6 \pm 10.4\%$ for DHEA, $17.7 \pm 4.9\%$ for DHEA-S, $101.9 \pm 38.4\%$ for corticosterone, $18.1 \pm 4.3\%$ for progesterone, $30.9 \pm 8.3\%$ for testosterone, and $40.6 \pm 3.2\%$ for 17β -estradiol (Fig. 6B bottom panel; $n = 5, 15, 9, 23, 11, 8$, and 7 patches, respectively). Effects of CORT, testosterone, and estradiol were significantly different from control saline, effects of DHEA and CORT were significantly different from each other, and effects of estradiol and CORT were significantly different from 10 μM cholesterol ($F_{7,82} = 7.89; p < 0.001$).

CORT and DHEA Affect Chromaffin Cell BK Channels

We have recently shown that glucocorticoids modulate BK gating and chromaffin cell excitability in rat and bovine chromaffin cells (Lovell et al., 2004). The gating effects of CORT and DHEA on heterologously expressed channels

Figure 7. Voltage-dependent activation of native BK channels in chromaffin cells is potentiated by 10 μ M CORT and 10 μ M DHEA. BK currents were elicited by voltage steps to +80 mV in the presence of 500 nM $[Ca^{2+}]_i$. A) Current from a bovine chromaffin cell was increased by 103.2% by 10 μ M CORT in the example shown, and by an average of $48.5 \pm 10.8\%$ ($n = 6$) (top panel). 10 μ M DHEA potentiated BK current from rat chromaffin cells by 40.2% in the example shown, and by an average of $42.5 \pm 13.2\%$ ($n = 6$, bottom panel). B) RT-PCR analysis reveals robust expression of $\beta 2$ and $\beta 4$ subunit mRNA in rat adrenal medulla. $\beta 2$ and $\beta 4$ were both expressed in hippocampus. $\beta 2$ was expressed primarily in the posterior pituitary; weak expression in anterior pituitary may reflect imperfect separation from posterior pituitary, however the assay is not quantitatively accurate. $\beta 4$ expressed strongly in anterior pituitary. The results are representative of tissue samples from at least 4 rats.



described above are very similar. Inside-out patches were pulled from bovine chromaffin cells and stepped from -80 mV to $+80$ mV. BK currents were recorded before and after application of 10 μ M CORT (Fig. 7A). 10 μ M CORT caused a rapid increase in BK current, in the trace shown, a 103.2% increase. An average increase (\pm SEM) of $48.5 \pm 10.8\%$ was observed in 5 additional patches. While full characterization of net effects of DHEA on native channel gating, and its consequences, remain to be investigated, we have made an initial test to determine whether DHEA has any effect on rat chromaffin BK channels. In this study, complications from inactivation were minimized by stepping for 450 ms to -140 mV prior to the test step to $+80$ mV, as was done with HEK-293 patches. The magnitude of ensemble current from inside-out patches from dissociated cells was robustly increased by DHEA. Peak current in this case (Fig. 7A; bottom panel) was increased by 40.2% . Current was increased similarly in 6 of 6 patches (mean increase of $42.5 \pm 13.2\%$). Both inactivating and sustained components of the current were increased similarly, compared to control experiments where patches were reperfused with control saline ($p < 0.05$, Student's t-test).

RT-PCR primers were used to test for expression of *Slo* $\beta 2$ and $\beta 4$ mRNA in adrenal medulla and a small sample of other tissues. Both were robustly represented in RNA acutely extracted from medullary tissue (Fig. 7B). Both were expressed in hippocampus and both anterior and posterior pituitary, though $\beta 2$ signal was weak in anterior pituitary, possibly reflecting contamination from posterior tissue. As discussed below, the results are consistent with the hypothesis that these β subunits participate in transducing DHEA responses of native chromaffin BK channels.

DISCUSSION

Our findings, taken with the observations that estradiol interacts with $\beta 1$ and $\beta 4$ subunits to potentiate BK channel gating (Valverde et al., 1999; Behrens et al., 2000; Dick and Sanders, 2001), suggest that all products of the family of mammalian *Slo*- β subunit genes should be considered candidate receptors for rapid BK modulation by multiple steroids. We show that physiologically relevant concentrations of corticosterone facilitate gating of BK channels in HEK-293 cells comprised of α plus $\beta 4$ subunits, acting within tens of seconds of application to cell-free inside-out patches, and under whole-cell conditions. CORT acts in a rapid and reversible manner, ruling out transcriptional mechanisms. Its effects were dose-dependent, and consistent with a Hill coefficient of approximately 1.0 (no co-operativity between effector sites) and a half-maximal dose below 400 nM. Percentage increases in current amplitude depend on test potential, reflecting the fact that CORT acts primarily by shifting the voltage-dependence of gating in the negative direction, under conditions of constant $[Ca^{2+}]_i$. CORT's effects on $\alpha\beta 4$ channels were significantly different than on BK channels with $\alpha\beta 2$ -encoded subunits, demonstrating that β subunits differentiate between steroids. The difference was not an artifact of $\alpha\beta 2$ channel inactivation; a non-inactivating mutant $\beta 2$ also conferred only weak sensitivity to CORT. While $\alpha\beta 2$ channels did not respond as strongly to any of the steroids yet tested, robust, rapid, and dose-dependent potentiation was elicited by exposure to DHEA, an androgen-related steroid produced in large quantities by the adrenal cortex. The DHEA sensitivity conferred by $\alpha\beta 2$ was significantly greater than that conferred by $\alpha\beta 4$. Both channel types showed modest potentiation by several other steroids, as tested with test potentials of +80 mV, but no response to the common precursor steroid cholesterol. BK channels comprised of only α subunits were completely insensitive to any steroids at all levels tested.

The results indicate that the previously known modifying effects of the various β subunits on BK gating seen in the absence of steroids must represent only part of their biological significance; the shared ability of β subunits to confer sensitivity to changes in steroid concentrations broadens their potential relevance to cellular excitability in many contexts. Moreover, while β subunits tend to react to more than one steroid, they clearly discriminate between steroids; different members of the family confer distinct profiles of relative affinity for specific steroids. This heterogeneity implies a greater dynamic repertoire of BK functional nuances than previously postulated, warranting further investigation *in vivo*.

Increasing concentrations of CORT (from 10^{-9} to 10^{-4} M) and DHEA (from 10^{-7} to 10^{-4} M) had progressively stronger effects, suggesting a wide dynamic range of steroid modulation, encompassing physiologically relevant concentrations, as discussed below. Nevertheless, variability in the percentage increase in current was substantial at all concentrations. Some of this can be attributed to the stochastic nature of BK channel activity, given their relatively small number in patches and large unit-conductance, and the irregular bursting and mode-shifting behaviors for which BK channels are known. Variation in steroid sensitivity *per se* also probably arises from variation in the number of β subunits associated with each *Slo- α* tetramer (1 to 4); variation in this stoichiometry is suggested by variation in the kinetics of inactivation, a property also conferred by $\beta 2$ (Wang et al., 2002). We observed a modest positive correlation between the DHEA effect and the rate of inactivation (reciprocal of the inactivation time constant). Additional heterogeneity almost certainly derives from unknowns in both α and β functional status, including phosphorylation, glycosylation, redox state, and accessory protein associations (Gong et al., 2000; Tang et al., 2001; Jin et al., 2002a; Jin et al., 2002b; McCobb, 2004).

Previous research has shown that BK channel activation by DHEA or estrogens was not mediated by the production of cAMP and cGMP, or by the activation of cyclic nucleotide dependent protein kinases (Farrukh et al., 1998; Peng et al., 1999; Dick and Sanders, 2001). The demonstration that expression of $\beta 1$ by itself confers estradiol binding on either oocytes or HEK-293 cells argued that estradiol-binding capacity is inherent in the β structure (Valverde et al., 1999). Most of our experiments were conducted on inside-out patches (to control $[Ca^{2+}]_i$), steroids are highly lipophilic and can easily pass through the membrane. However, application of CORT to the outside of the cell in whole-cell experiments also resulted in rapid potentiation of $\alpha\beta 4$ BK channels. Thus our results are not inconsistent with action at binding sites in extracellular domains of the β -subunits, as has been suggested for estradiol, xenoestrogens, and fatty acids (Valverde et al., 1999; Dick et al., 2001; Clarke et al., 2002). However the steroids could also act at a site that depends on the presence of both α and β (Korovkina et al., 2004), or to an intermediate membrane protein that interacts with the β subunit to confer a conformational change in the channel (Dick and Sanders, 2001). To formally eliminate the possibility that the subunit induces a steroid binding capacity that is extrinsic to the subunit itself, direct binding of steroids to purified β subunits must be demonstrated. The observation that similar but distinct binding-specificities to multiple steroids are conferred by different β 's, which have 21-43% amino acid identity (Wallner et al., 1999; Xia et al., 1999; Behrens et al., 2000; Brenner et al., 2000; Uebele et al., 2000), further supports the idea that binding *per se* is inherent in the subunits. Several segments of conserved residues interspersed with numerous non-conserved regions in the extracellular domains of the $\beta 2$ and $\beta 4$ subunits could be responsible for overlapping but distinct steroid sensitivities. Cross-comparisons and targeted mutagenesis experiments with

these gene products should elucidate relationships between steroid and protein structural and functional interactions.

The proximity of chromaffin cells to the source of adrenal steroid synthesis exposes them to at least episodic pulses of steroids at much higher concentrations than in systemic blood. Systemic CORT levels may not greatly exceed 1 μM , but adrenal blood concentrations may reach concentrations as high as 100 μM (Betito et al., 1992; Betito et al., 1994). Androgen levels in adrenal blood are not known, but systemic DHEA and DHEA-S levels are on the order of 10 nM and 10 μM , respectively (Baulieu, 1996). BK channels shape the intrinsic excitability of chromaffin cells, particularly repetitive firing properties, and thus shape catecholamine secretory responses to input (Lingle, 1996; Lovell and McCobb, 2001; Lovell et al., 2004). In addition to data presented here, molecular and functional expression studies suggest that $\beta 2$ (Solaro and Lingle, 1992; Xia et al., 1999; Behrens et al., 2000; Ding and Lingle, 2002) and $\beta 4$ (Behrens et al., 2000; Brenner et al., 2000) are expressed in chromaffin cells. Channel inactivation very similar to that conferred by $\beta 2$ is seen in both rat and bovine cells, though viewed across the respective chromaffin populations, there are striking species differences (Lovell et al., 2000). Thus inactivation is more common, and tends to be faster and more complete in rat than bovine cells. We hypothesize that $\beta 2$ and $\beta 4$ subunits constitute at least part of the mechanism for both DHEA and corticosterone effects on chromaffin BK channels (Lovell et al., 2004). It seems probable that differential expression within one gland, between individuals, and between species, also confers differential stress-related tuning on cell excitability.

DHEA (50 μM) acutely facilitates BK channel activation in patches from ferret pulmonary vascular smooth muscle cells by shifting the voltage-dependence of activation 20-25 mV in the negative direction (Farrukh et al., 1998; Peng et al., 1999). In addition to $\beta 1$, $\beta 2$ is strongly expressed in rat lung, if perhaps less strongly in

human (Wallner et al., 1999; Xia et al., 1999; Behrens et al., 2000). $\beta 4$ is apparently expressed in human lung as well (Behrens et al., 2000). Given the similarities between the ferret lung and our patch clamp results, we postulate that $\beta 2$ and/or $\beta 4$ are involved, and speculate that stress-evoked rises in DHEA promote vasodilation to facilitate oxygen uptake.

Roles played by glucocorticoids in stress responses have been studied in some detail. The ability of CORT to both, reduce neuronal firing rate in celiac ganglion cells, and enhance firing rate in cardiovascular neurons located in the rostral ventrolateral medulla (Hua and Chen, 1989; Rong et al., 1999) demonstrates the importance of rapid steroid modulation in neuronal excitability. Most recently, the acute application of dexamethasone has been shown to increase BK channel activity in pituitary GH₃ and AtT-20 cells, and reduce the firing of action potentials in GH₃ cells (Huang et al., 2005). However, rapid modulation of BK channels and repetitive firing properties of adrenomedullary chromaffin cell by steroids synthesized in the adrenal cortex had not been considered until recently (Lovell et al., 2004). The “strategic” significance of DHEA and adrenal androgens in relation to stress responses or other aspects of physiology remains enigmatic. Chronic effects of DHEA on alternative splicing of the *Slo*- α subunit at the ‘STREX’ site in chromaffin cells were recently reported to be directly opposite those of glucocorticoids (Lai and McCobb, 2002). In contrast, acute effects of the two classes of steroids on BK gating appear to run in parallel (Lovell et al., 2004). This sort of multidimensional complexity of steroid functionality (elegantly reviewed by Sapolsky et al., 2000) ultimately bears on the complexities of organismal survival. Differential expression of *Slo*- β subunits has already been suggested to spawn species-differences in nuanced BK gating and cell excitability (Solaro et al., 1995; Lovell et al., 2000; Wang et al., 2002). We suggest that differences in the multi-steroidal sensitivity conferred by the β variants allow for

the fine tuning of BK dynamics by different steroids, and provide yet another level of flexibility to the coupling between excitatory input to the adrenal medulla and catecholamine output.

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REFERENCES

- Allolio B, Arlt W (2002) DHEA treatment: myth or reality? *Trends Endocrinol Metab* 13:288.
- Bastianetto S, Ramassamy C, Poirier J, Quirion R (1999) Dehydroepiandrosterone (DHEA) protects hippocampal cells from oxidative stress-induced damage. *Brain Res Mol Brain Res* 66:35-41.
- Baulieu EE (1996) Dehydroepiandrosterone (DHEA): a fountain of youth? *J Clin Endocrinol Metab* 81:3147-3151.
- Behrens R, Nolting A, Reimann F, Schwarz M, Waldschutz R, Pongs O (2000) hKCNMB3 and hKCNMB4, cloning and characterization of two members of the large-conductance calcium-activated potassium channel beta subunit family. *FEBS Lett* 474:99-106.
- Betito K, Diorio J, Meaney MJ, Boksa P (1992) Adrenal phenylethanolamine N-methyltransferase induction in relation to glucocorticoid receptor dynamics: evidence that acute exposure to high cortisol levels is sufficient to induce the enzyme. *J Neurochem* 58:1853-1862.
- Betito K, Mitchell JB, Bhatnagar S, Boksa P, Meaney MJ (1994) Regulation of the adrenomedullary catecholaminergic system after mild, acute stress. *Am J Physiol* 267:R212-220.
- Brayden JE, Nelson MT (1992) Regulation of arterial tone by activation of calcium-dependent potassium channels. *Science* 256:532-535.
- Brenner R, Jegla TJ, Wickenden A, Liu Y, Aldrich RW (2000) Cloning and functional characterization of novel large conductance calcium-activated potassium channel beta subunits, hKCNMB3 and hKCNMB4. *J Biol Chem* 275:6453-6461.
- Brenner R, Chen QH, Vilaythong A, Toney GM, Noebels JL, Aldrich RW (2005) BK channel beta4 subunit reduces dentate gyrus excitability and protects against temporal lobe seizures. *Nat Neurosci* 8:1752-1759.
- Clarke AL, Petrou S, Walsh JV, Jr., Singer JJ (2002) Modulation of BK(Ca) channel activity by fatty acids: structural requirements and mechanism of action. *Am J Physiol Cell Physiol* 283:C1441-1453.
- Deenadayalu VP, White RE, Stallone JN, Gao X, Garcia AJ (2001) Testosterone relaxes coronary arteries by opening the large-conductance, calcium-activated potassium channel. *Am J Physiol Heart Circ Physiol* 281:H1720-1727.

- Dick GM, Sanders KM (2001) (Xeno)estrogen sensitivity of smooth muscle BK channels conferred by the regulatory beta1 subunit: a study of beta1 knockout mice. *J Biol Chem* 276:44835-44840.
- Dick GM, Rossow CF, Smirnov S, Horowitz B, Sanders KM (2001) Tamoxifen activates smooth muscle BK channels through the regulatory beta 1 subunit. *J Biol Chem* 276:34594-34599.
- Ding JP, Lingle CJ (2002) Steady-state and closed-state inactivation properties of inactivating BK channels. *Biophys J* 82:2448-2465.
- Dopico AM, Walsh JV, Jr., Singer JJ (2002) Natural bile acids and synthetic analogues modulate large conductance Ca²⁺-activated K⁺ (BKCa) channel activity in smooth muscle cells. *J Gen Physiol* 119:251-273.
- Dworetzky SI, Boissard CG, Lum-Ragan JT, McKay MC, Post-Munson DJ, Trojnacki JT, Chang CP, Gribkoff VK (1996) Phenotypic alteration of a human BK (hSlo) channel by hSlobeta subunit coexpression: changes in blocker sensitivity, activation/relaxation and inactivation kinetics, and protein kinase A modulation. *J Neurosci* 16:4543-4550.
- Falkenstein E, Tillmann HC, Christ M, Feuring M, Wehling M (2000) Multiple actions of steroid hormones--a focus on rapid, nongenomic effects. *Pharmacol Rev* 52:513-556.
- Farrukh IS, Peng W, Orlinska U, Hoidal JR (1998) Effect of dehydroepiandrosterone on hypoxic pulmonary vasoconstriction: a Ca²⁺-activated K⁺-channel opener. *Am J Physiol* 274:L186-195.
- Fettiplace R, Fuchs PA (1999) Mechanisms of hair cell tuning. *Annu Rev Physiol* 61:809-834.
- Ffrench-Mullen JM, Spence KT (1991) Neurosteroids block Ca²⁺ channel current in freshly isolated hippocampal CA1 neurons. *Eur J Pharmacol* 202:269-272.
- Gong L, Gao TM, Huang H, Tong Z (2000) Redox modulation of large conductance calcium-activated potassium channels in CA1 pyramidal neurons from adult rat hippocampus. *Neurosci Lett* 286:191-194.
- Hua SY, Chen YZ (1989) Membrane receptor-mediated electrophysiological effects of glucocorticoid on mammalian neurons. *Endocrinology* 124:687-691.
- Huang MH, So EC, Liu YC, Wu SN (2005) Glucocorticoids stimulate the activity of large-conductance Ca²⁺-activated K⁺ channels in pituitary GH(3) and AtT-20 cells via a non-genomic mechanism. *Steroids*.

- Jin P, Weiger TM, Levitan IB (2002a) Reciprocal modulation between the alpha and beta 4 subunits of hSlo calcium-dependent potassium channels. *J Biol Chem* 277:43724-43729.
- Jin P, Weiger TM, Wu Y, Levitan IB (2002b) Phosphorylation-dependent functional coupling of hSlo calcium-dependent potassium channel and its hbeta 4 subunit. *J Biol Chem* 277:10014-10020.
- Jin W, Sugaya A, Tsuda T, Ohguchi H, Sugaya E (2000) Relationship between large conductance calcium-activated potassium channel and bursting activity. *Brain Res* 860:21-28.
- Kimonides VG, Khatibi NH, Svendsen CN, Sofroniew MV, Herbert J (1998) Dehydroepiandrosterone (DHEA) and DHEA-sulfate (DHEAS) protect hippocampal neurons against excitatory amino acid-induced neurotoxicity. *Proc Natl Acad Sci U S A* 95:1852-1857.
- Korovkina VP, Brainard AM, Ismail P, Schmidt TJ, England SK (2004) Estradiol Binding to Maxi-K Channels Induces Their Down-regulation via Proteasomal Degradation. *J Biol Chem* 279:1217-1223.
- Kroboth PD, Salek FS, Pittenger AL, Fabian TJ, Frye RF (1999) DHEA and DHEA-S: a review. *J Clin Pharmacol* 39:327-348.
- Lai GJ, McCobb DP (2002) Opposing actions of adrenal androgens and glucocorticoids on alternative splicing of Slo potassium channels in bovine chromaffin cells. *Proc Natl Acad Sci U S A* 99:7722-7727.
- Lingle CJ, Solaro, C.R., Prakriya, M., Ding, J.P. (1996) Calcium-activated potassium channels in adrenal chromaffin cells. In: *Ion Channels* (Narahashi T, ed), pp 261-301. New York: Plenum.
- Lovell PV, McCobb DP (2001) Pituitary control of BK potassium channel function and intrinsic firing properties of adrenal chromaffin cells. *J Neurosci* 21:3429-3442.
- Lovell PV, James DG, McCobb DP (2000) Bovine versus rat adrenal chromaffin cells: big differences in BK potassium channel properties. *J Neurophysiol* 83:3277-3286.
- Lovell PV, King JT, McCobb DP (2004) Acute modulation of adrenal chromaffin cell BK channel gating and cell excitability by glucocorticoids. *J Neurophysiol* 91:561-570.
- Majewska MD, Demirgoren S, Spivak CE, London ED (1990) The neurosteroid dehydroepiandrosterone sulfate is an allosteric antagonist of the GABAA receptor. *Brain Res* 526:143-146.

- Makara GB, Haller J (2001) Non-genomic effects of glucocorticoids in the neural system. Evidence, mechanisms and implications. *Prog Neurobiol* 65:367-390.
- McCobb D (2004) Towards a natural history of calcium-activated potassium channels. In: *Advances in molecular and cell biology: Molecular and cellular insights to ion channel biology* (Maue RA, ed), pp 51-73. Amsterdam: Elsevier Science.
- McManus OB, Helms LM, Pallanck L, Ganetzky B, Swanson R, Leonard RJ (1995) Functional role of the beta subunit of high conductance calcium-activated potassium channels. *Neuron* 14:645-650.
- Oberbeck R, Benschop RJ, Jacobs R, Hosch W, Jetschmann JU, Schurmeyer TH, Schmidt RE, Schedlowski M (1998) Endocrine mechanisms of stress-induced DHEA-secretion. *J Endocrinol Invest* 21:148-153.
- Orio P, Rojas P, Ferreira G, Latorre R (2002) New Disguises for an Old Channel: MaxiK Channel beta-Subunits. *News Physiol Sci* 17:156-161.
- Peng W, Hoidal JR, Farrukh IS (1999) Role of a novel KCa opener in regulating K⁺ channels of hypoxic human pulmonary vascular cells. *Am J Respir Cell Mol Biol* 20:737-745.
- Rong W, Wang W, Yuan W, Chen Y (1999) Rapid effects of corticosterone on cardiovascular neurons in the rostral ventrolateral medulla of rats. *Brain Res* 815:51-59.
- Rosenfeld CR, Cornfield DN, Roy T (2001) Ca²⁺-activated K⁺ channels modulate basal and E(2)-beta-induced rises in uterine blood flow in ovine pregnancy. *Am J Physiol Heart Circ Physiol* 281:H422-431.
- Salom JB, Burguete MC, Perez-Asensio FJ, Centeno JM, Torregrosa G, Alborch E (2002) Acute relaxant effects of 17-beta-estradiol through non-genomic mechanisms in rabbit carotid artery. *Steroids* 67:339-346.
- Sapolsky RM, Romero LM, Munck AU (2000) How do glucocorticoids influence stress responses? Integrating permissive, suppressive, stimulatory, and preparative actions. *Endocr Rev* 21:55-89.
- Solaro CR, Lingle CJ (1992) Trypsin-sensitive rapid inactivation of a calcium-activated potassium channel. *Science* 257:1694-1698.
- Solaro CR, Prakriya M, Ding JP, Lingle CJ (1995) Inactivating and noninactivating Ca²⁺- and voltage-dependent K⁺ current in rat adrenal chromaffin cells. *J Neurosci* 15:6110-6123.

- Tang XD, Daggett H, Hanner M, Garcia ML, McManus OB, Brot N, Weissbach H, Heinemann SH, Hoshi T (2001) Oxidative regulation of large conductance calcium-activated potassium channels. *J Gen Physiol* 117:253-274.
- Uebele VN, Lagrutta A, Wade T, Figueroa DJ, Liu Y, McKenna E, Austin CP, Bennett PB, Swanson R (2000) Cloning and functional expression of two families of beta-subunits of the large conductance calcium-activated K⁺ channel. *J Biol Chem* 275:23211-23218.
- Valverde MA, Rojas P, Amigo J, Cosmelli D, Orio P, Bahamonde MI, Mann GE, Vergara C, Latorre R (1999) Acute activation of Maxi-K channels (hSlo) by estradiol binding to the beta subunit. *Science* 285:1929-1931.
- Van Goor F, Li YX, Stojilkovic SS (2001) Paradoxical role of large-conductance calcium-activated K⁺ (BK) channels in controlling action potential-driven Ca²⁺ entry in anterior pituitary cells. *J Neurosci* 21:5902-5915.
- Vergara C, Latorre R, Marrion NV, Adelman JP (1998) Calcium-activated potassium channels. *Curr Opin Neurobiol* 8:321-329.
- Vermeulen A (1995) Dehydroepiandrosterone sulfate and aging. *Ann N Y Acad Sci* 774:121-127.
- Wallner M, Meera P, Toro L (1999) Molecular basis of fast inactivation in voltage and Ca²⁺-activated K⁺ channels: a transmembrane beta-subunit homolog. *Proc Natl Acad Sci U S A* 96:4137-4142.
- Wang YW, Ding JP, Xia XM, Lingle CJ (2002) Consequences of the stoichiometry of Slo1 alpha and auxiliary beta subunits on functional properties of large-conductance Ca²⁺-activated K⁺ channels. *J Neurosci* 22:1550-1561.
- Wehling M (1997) Specific, nongenomic actions of steroid hormones. *Annu Rev Physiol* 59:365-393.
- Wolkowitz OM, Reus VI, Roberts E, Manfredi F, Chan T, Raum WJ, Ormiston S, Johnson R, Canick J, Brizendine L, Weingartner H (1997) Dehydroepiandrosterone (DHEA) treatment of depression. *Biol Psychiatry* 41:311-318.
- Xia XM, Ding JP, Lingle CJ (1999) Molecular basis for the inactivation of Ca²⁺- and voltage-dependent BK channels in adrenal chromaffin cells and rat insulinoma tumor cells. *J Neurosci* 19:5255-5264.
- Xia XM, Ding JP, Lingle CJ (2003) Inactivation of BK channels by the NH₂ terminus of the beta2 auxiliary subunit: an essential role of a terminal peptide segment of three hydrophobic residues. *J Gen Physiol* 121:125-148.

Xia XM, Ding JP, Zeng XH, Duan KL, Lingle CJ (2000) Rectification and rapid activation at low Ca^{2+} of Ca^{2+} -activated, voltage-dependent BK currents: consequences of rapid inactivation by a novel beta subunit. *J Neurosci* 20:4890-4903.

Zinder O, Dar DE (1999) Neuroactive steroids: their mechanism of action and their function in the stress response. *Acta Physiol Scand* 167:181-188.

CHAPTER 4

CHARACTERIZATION AND MODULATION OF K⁺ CHANNELS IN ANTERIOR PITUITARY CORTICOTROPES

The secretion of ACTH from anterior pituitary corticotropes is a vital step in the activation of the stress response. Potassium ion channels are key determinants of cellular excitability and secretion in neuroendocrine cells. Here we report the first characterization of BK channels from identified “real” anterior pituitary corticotropes. Perforated patch, whole cell and inside out patch experiments with specific ion channel blockers reveal BK channels (with conductance of ~300 pS) as the prominent voltage-gated K⁺ channel, accounting for over 50% of the peak outward K⁺ current. Other Ca²⁺ sensitive K⁺ channels, including SK and IK channels, were also detected. Corticotrope electrical excitability was also examined in this study. Spontaneous plateau bursting action potentials were observed in the majority of corticotropes tested. Application of TEA or paxilline altered the firing of patterns of corticotropes, implicating BK channels as important regulators of excitability. Burst duration was increased with the application of the secretagogue CRH and the stress steroid corticosterone. The modulation of K⁺ channels in corticotropes could elucidate their role in the regulation of the stress response.

INTRODUCTION

An overactive stress response has detrimental consequences on physical and mental health in our society. The hypothalamic pituitary adrenal (HPA) – axis directs the body’s response to external stressors. Corticotrope cells in the anterior pituitary are central players in the feed-forward and feedback networks that regulate the magnitude and duration of the stress response. They secrete adrenocorticotrophic hormone

(ACTH) in response to corticotropin releasing hormone (CRH) produced by the hypothalamus. ACTH triggers the release of stress steroids (cortisol in humans and corticosterone in rodents, together abbreviated CORT) from the adrenal cortex. Extensive research in corticotrope-like cells has examined the biochemistry and regulation of ACTH release. CRH stimulates ACTH release through mechanistic steps including: stimulating adenylate cyclase, activating protein kinase A, depolarizing membrane potential increasing action potential firing, and increasing $[Ca^{2+}]_i$; (Labrie et al., 1982; Aguilera et al., 1983; Abou-Samra et al., 1987; Childs et al., 1987; Guerineau et al., 1991; Kuryshev et al., 1996). However, the electrophysiological characterization of corticotropes remains incomplete. Corticotropes comprise about 10-15% of the cells in the anterior pituitary (Horvath and Kovacs, 1994). Selection and sorting of corticotropes can be achieved through a complex counter flow centrifugation method (Childs et al., 1988). Such difficulty in isolating pituitary cell types has lead the majority of corticotrope research to focus on the mouse pituitary tumor AtT20 cell line (Woods et al., 1992). Determining the ionic currents present in non-tumor corticotropes is key for understanding the role of excitability in regulating ACTH secretion.

Large conductance Ca^{2+} and voltage activated potassium (BK) channels are prominent players in neuroendocrine cellular excitability (Solaro et al., 1995; Vergara et al., 1998; Lovell et al., 2000). Their large single channel conductance allows for rapid repolarization and brief afterhyperpolarization of the membrane potential. BK channels have been examined in cultured anterior pituitary cells, such as melanotropes, somatotropes, gonadotropes and lactotropes (Kehl and Wong, 1996; Van Goor et al., 2001a; Van Goor et al., 2001b) but not in cultured corticotropes. The majority of information regarding BK channels in corticotropes stems from the AtT20 cell line (Woods et al., 1992). In AtT20 cells, BK channels participate in the

regulation of ACTH release by glucocorticoids (Shipston et al., 1996; Lim et al., 1998). Furthermore, the steroid dexamethasone (a synthetic glucocorticoid) regulates the phosphorylation of BK channels present in AtT20 cells (Shipston et al., 1996) (Tian et al., 1998) (Shipston et al., 1999) (Tian et al., 2001). BK channels are important regulators of cellular excitability and their characterization in corticotropes could further elucidate their importance in the stress response.

Corticotropes are capable of firing action potentials (Guerineau et al., 1991), but their excitability has not been aggressively studied *in vitro*. The majority of research has focused on the firing properties of other cells located in the anterior pituitary (i.e. gonadotropes, somatotropes, and lactotropes). Anterior pituitary cells can fire tall single action potential spikes and plateau bursts of action potentials. BK channels in somatotropes contribute to the spontaneous plateau bursting activity by limiting the activity of delayed rectifier K⁺ channels. Gonadotropes predominately fire tall single action potentials due to a low expression of BK channels, but are capable of firing plateau bursts through the activation of SK channels (Van Goor et al., 2001a; Stojilkovic et al., 2005). It is thought that plateau bursting controls hormone secretion in anterior pituitary cells. Whether BK channels also contribute to cellular excitability in corticotropes has not been explored. To address the issues of excitability and the presence of various K⁺ channels we have used a transgenic mouse line with GFP labeled corticotropes to facilitate the first characterization of “real” non-tumor corticotropes.

In the present study, we determined the types of different K⁺ channels present in corticotrope membranes by using the K⁺ channel blockers, 4-aminopyridine, clotrimazole, TRAM-34, paxilline, and apamin. We found that the Ca²⁺ sensitive K⁺ channels BK, SK and IK channels are present in corticotropes, with BK channels

being the most prominent. Additionally we examined corticotrope excitability and the role that BK channels play in shaping the firing properties of corticotropes.

MATERIALS AND METHODS

Corticotrope Isolation and Cell Culture

Corticotropes were isolated and cultured from transgenic POMC-GFP C57/BL6 mice (courtesy of Jeff Friedman, Rockefeller University). Transgenic mice were generated by using a bacterial artificial chromosome expressing topaz GFP under the transcriptional control of POMC 5' regulatory gene sequence (Pinto et al., 2004). Mice were anesthetized with CO₂ and sacrificed by cervical spinal dislocation. The anterior lobes of the pituitary gland were removed from the whole pituitary, minced and placed in a solution of Locke's buffer (in mM: 154 NaCl, 5.6 KCl, 5 HEPES, 3.6 NaHCO₃, and 5.6 glucose, at pH 7.4) with collagenase B (1.5 mg/ml; Boehringer Mannheim, Indianapolis, IN) and incubated at 37°C with gentle agitation. After 10 minutes the tissue was washed several times in Locke's buffer, transferred to a Locke's solution with DNase (1 mg/ml; Sigma, St. Louis, MO), and incubated for an additional 5 minutes at 37°C. Pituitaries were then triturated through fire polished pipettes and plated on 0.02% poly-D-lysine coated glass cover-slips. Cultures were maintained in a humidified 5% CO₂ incubator at 37°C in sterile culture medium (Gibco; DMEM with 10% FBS, 2 U/ml penicillin-G, 2 µg/ml streptomycin sulfate). Cells were used between 1-5 days in culture.

Immunocytochemistry

Dissociated corticotropes were washed several times in a PBS solution containing bovine serum albumin (BSA; 0.1% Life Technologies) and fixed in 4% paraformaldehyde solution (PFA: 4% in PBS) for 60 min at room temperature. Cells

were washed a second time in PBS-BSA and permeabilized with a Triton X-100 solution (0.2% Triton X-100 in PBS; Fisher Scientific) for 10 min at room temperature. Cells were then incubated overnight with a primary antibody directed against ACTH (1:1000 dilution in PBS-BSA). The next day, corticotrope cultures were washed several times with PBS and incubated for 60 min with fluorophore conjugated Texas Red (1:1000 dilution in PBS, Vector Laboratories, Burlingame, CA) and anti-GFP Alexa Fluor 488 conjugated (1:500 dilution in PBS, Invitrogen) secondary antibodies. Images were captured with MetaMorph (MDS, Toronto, CA) at the Cornell Microscopy and Imaging Facility, and further processed by ImageJ (NIH, Bethesda, Md) (Abramoff et al., 2004).

Electrophysiology

Electrophysiological measurements were carried out in inside-out, perforated patch, or whole cell configurations using standard patch clamp techniques (Hamill et al., 1981). Patch electrodes (3-5 M Ω) were pulled from borosilicate glass (World Precision Instruments, Sarasota, FL) and coated with silicone elastomer (Sylgard 184, Dow Corning, Midland, OH). Data were collected using a List EPC-9 patch clamp amplifier (Heka Electronik, Lambrecht, Germany), Bessel filtered at 10 kHz and stored on a Power Macintosh G3 using Pulse 8.5 software (Heka Electronik, Lambrecht, Germany). Offline analysis was conducted with custom software written for Igor Pro (Wavemetrics, Lake Oswego, OR).

Experiments were conducted at 20-22°C. For inside-out patches, after attaining a gigaohm seal, calcium free saline was perfused on the cell and inside-out patches were pulled. For perforated patch recordings, a series resistance < 40 M Ω was reached within 10-30 min after formation of a gigaohm seal (seal resistance > 2 G Ω) and remained stable for up to 1 hour. For some cells, a small holding current was used to

maintain cell resting potentials between -60 and -65 mV. For measurements of electrical activity, most cells were not injected with any depolarizing current and fired spontaneous action potentials. Some cells required a series of 2 sec depolarizing current pulses of increasing strength to elicit spikes or plateau bursting. An average membrane capacitance (C_m) of 9.6 ± 0.7 pF was recorded for corticotropes. Solution exchange was achieved through five computer controlled, gravity-fed lines converging near the tip of a large perfusion pipette. Corticotropes were typically held at -60 mV and stepped to +60 mV for 450 ms after a prepulse to -140 mV for 200 ms, with 8 s intervals between sweeps. For the determination of the voltage dependence of K^+ channels, a series of increasing voltage steps was applied in 10 mV increments from -140 to +100 mV. For the voltage dependence of inactivation, the steady state current after activation was determined by a two pulse protocol consisting of a series of increasing voltage steps applied in 10 mV increments from -140 to 100 mV followed by another voltage step to +80 mV for 350 ms.

Solutions

For inside-out patches, symmetrical K^+ solutions were used to eliminate potassium driving force and allow DC offset to be cancelled at 0 mV. The pipette and bath saline solutions contained (in mM): 160 KCl, 10 HEPES, 1 MgCl₂, 1 HEDTA, 0.188 CaCl₂, pH adjusted to 7.2 with KOH to obtain approximately 5 μ M free [Ca²⁺]. The free [Ca²⁺] was calculated with MaxChelator software (WebMaxC v2.1) (Bers et al., 1994). Zero-Ca²⁺ solution contained an additional 5 mM EGTA (Sigma). For experiments in the perforated patch and whole cell mode, the pipette solution contained (in mM): 140 KCl, 10 HEPES, 10 EGTA, 5MgCl, pH 7.4. The bath solution contained (in mM): 145 NaCl, 5 KCl, 2 CaCl₂, 1 MgCl, 10 HEPES, pH 7.2. To obtain perforated patches, the tips of each electrode were backfilled with pipette solution, and

then the barrel was filled with a previously prepared solution containing 20 μ l of fresh stock amphotericin B (6 mg/100 μ l DMSO; Sigma) and 20 μ l of stock Pluronic acid F-127 (2.5 mg/ 100 μ l DMSO; Sigma), and 3 ml of pipette recording saline. Pipette solution containing amphotericin B was refreshed every hour. For all pharmacology experiments, drugs were diluted from a stock solution and added to bath perfused saline. Paxilline (pax), apamin, clotrimazole, 4-aminopyridine (4-AP), and tetraethylammonium (TEA) were all purchased from Sigma, and dissolved in 100% DMSO (for paxilline and clotrimazole) or saline solution (for 4-AP, TEA and apamin), made into stock solutions (10 mM for paxilline, clotrimazole, 4-AP, and TEA, and 500 μ M for apamin) and stored at -20°C until the day of experiment. The inclusion of the vehicle DMSO did not alter K⁺ channel activity. The osmolarity of the saline solutions was measured by a dew point osmometer and adjusted to 300 OsmM.

Data and Analysis

Potassium currents were linear leak subtracted using a Levenberg-Marquardt search algorithm to estimate kinetic parameters. Percent block by various drugs was measured by comparing the maximum peak amplitudes (or the sustained current amplitudes at the end of the voltage step) before and after drug treatment. Data are presented as the mean \pm SEM. The voltage dependence of activation (G-V) and voltage dependence of steady state inactivation (H_{inf}) were determined by measuring peak current and dividing by the respective driving force. Plotted values of conductance (G) as a function of test potential (V_m) or steady state inactivation (H_{inf}) as a function of test potential (V_m) were fit to a single Boltzmann function of the form:

$$G_{V_m} = G_{max} / (1 + \exp((V_m - V_{1/2}) / s))$$

with parameters for maximum conductance (G_{\max}), voltage of half activation ($V_{1/2}$), and slope (s ; the steepness of the voltage dependence of activation, in mV/e-fold change in voltage).

For voltage clamp experiments, ensemble currents were generated from averages of 10-15 individual traces. For current clamp experiments, burst duration was calculated as the length of time that a burst lasted at a given threshold (-50 mV or 55 mV). Instantaneous frequency was calculated as the reciprocal of the interburst interval (time between the start of each burst at a given threshold). Sliding box running averages were calculated for 5 individual points over the duration of the trace.

RESULTS

Anterior pituitary corticotropes were isolated from POMC-GFP transgenic mice, and subsequent experiments were conducted on green-fluorescing cells. Post translational processing of the POMC protein yields different hormones ACTH, α -melanocyte stimulating hormone (α MSH), endorphins, and lipotrophin (Jacobson and Drouin, 1994). Antibody staining revealed that most cells (~95%) expressing GFP predominately express ACTH. Figure 1A is a brightfield micrograph showing dissociated anterior pituitary cells. Localization of ACTH and GFP was accomplished by fluorescent labeling of ACTH with mouse anti-ACTH and labeling of GFP was achieved with Anti-GFP alexa 488. Fig. 1B show GFP staining in green, and Fig. 1C show ACTH staining after using primary antibody anti-ACTH and secondary antibody anti mouse Texas Red. A merged image of both the green and red channels is shown in Fig 1D.

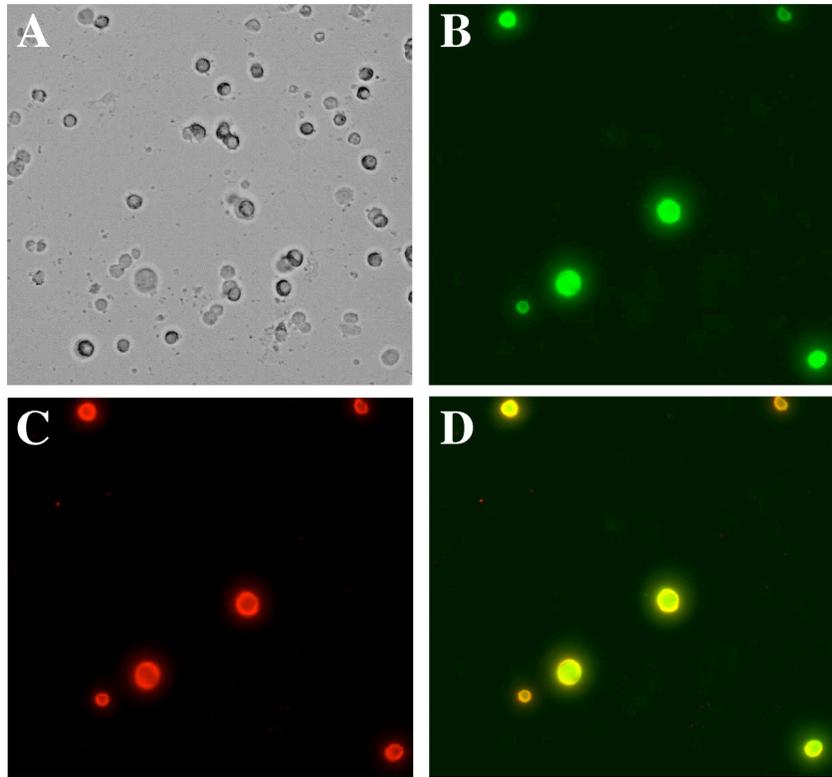


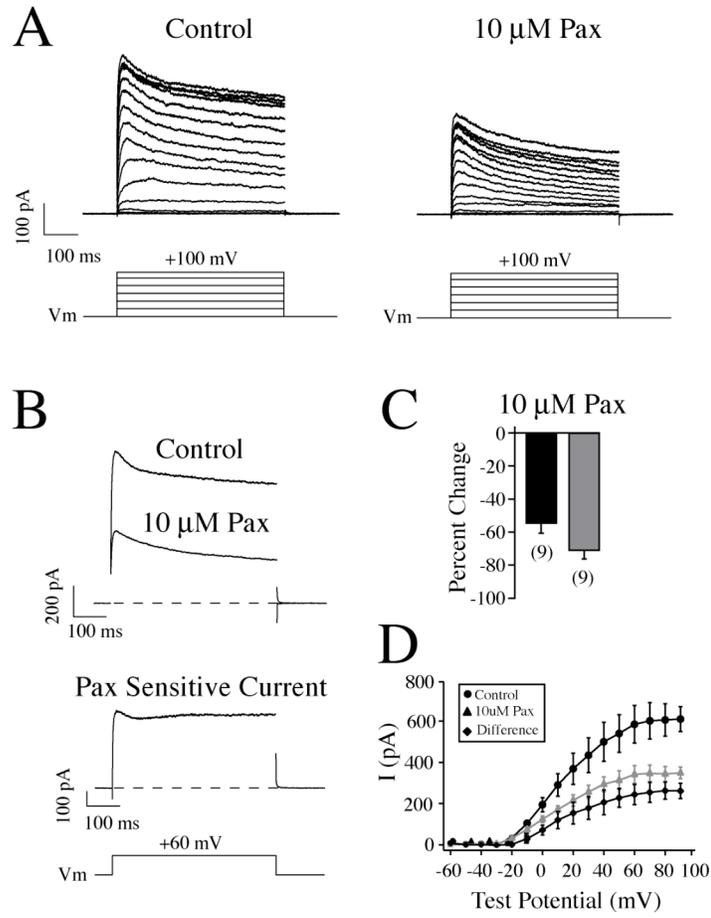
Figure 1. Primary cultures of non-tumor anterior pituitary corticotropes from POMC-GFP transgenic mice. A) Dissociated anterior pituitary cells viewed under bright-field optics. B) Immunofluorescence image of pituitary cells stained with Anti-GFP Alexa 488. C) Pituitary cells stained for the corticotrope secreting hormone ACTH. D) Merged immunofluorescent image overlaying the GFP positive and ACTH positive cells.

BK Channels are Prominent in Corticotropes

Perforated patch and whole cell recordings conducted on green-fluorescing pituitary corticotropes revealed that K^+ channels sensitive to the toxin paxilline represented the most prominent K^+ channel. Outward K^+ currents were elicited by a series of voltage steps from a holding potential of -60 mV to +100 mV in 10mV increments (Fig. 2A- left panel). Bath application of the BK channel blocker paxilline (10 μ M) (Knaus et al., 1994; Sanchez and McManus, 1996) reduced K^+ currents substantially (Fig. 2A- right panel). A single voltage step to +60 mV from a holding potential of -60 mV was used to characterize the contribution of BK channels to the overall K^+ current. Subtracting the paxilline sensitive current from the overall K^+ current revealed the BK current present in corticotropes (Fig. 2B). The average percent block of total K^+ current by paxilline was $54.9 \pm 5.8\%$ ($n = 9$) measured from the peak amplitude, and $71.2 \pm 5.0 \%$ for the sustained current component ($n = 9$, Fig. 2C). For a subset of corticotropes, the current was measured with a full series of test potentials from -60 mV to 100 mV (Fig. 2D). The resulting current-voltage relationship is shown ($n = 3$). The difference current plot represents the subtraction of the total outward K^+ current (control) and the remaining current after application of 10 μ M paxilline (Fig. 2D). By using the BK specific blocker, paxilline, we were able to dissect out the BK component of the overall outward K^+ current.

Inside-out patches with varying numbers of channels were pulled from corticotropes and examined for BK channels. In control saline (5 μ M $[Ca^{2+}]_i$), current openings were often discernable in individual sweeps at +60 mV (450 ms duration; Fig. 3A). Currents were allowed to stabilize 3-5 min before application of drugs. In the example shown in Fig. 3A, 1 μ M paxilline blocked the majority of the outward K^+ current (94.5%), indicating the presence of BK channels in this patch. Single channel conductance was measured to be \sim 300 pS for the paxilline sensitive channels.

Figure 2. Characterization of BK current from anterior corticotropes. A) Perforated patch recordings of outward K^+ current activated by voltage steps from -60 mV to +100 mV. Application of 10 μ M paxilline reduces overall outward K^+ current. B) Outward K^+ current elicited by a voltage step to +60 mV from a holding potential of -60 mV. K^+ current was reduced by the addition of 10 μ M paxilline to the bath. Subtracting the paxilline blocked current trace from the control current trace results in the BK current measured from this cell. C) Average decrease of peak K^+ current (black bars, $n = 9$) and sustained K^+ current (grey bars, $n = 9$) after the application of 10 μ M paxilline. D) Current-voltage (I-V) plots were constructed from current traces elicited by a series of voltage steps from -60 mV to +100 mV in 10 mV increments. The difference IV curve (\blacklozenge) was calculated by subtracting the paxilline sensitive current from the control current at each test potential.



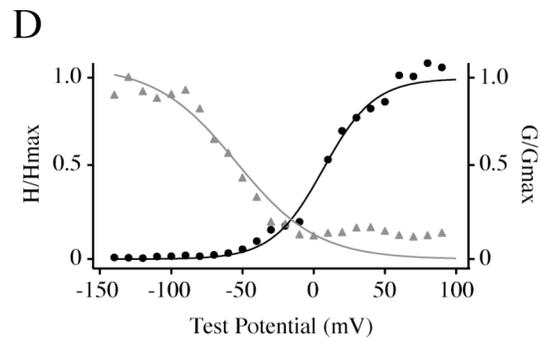
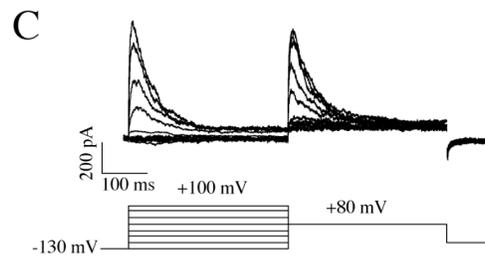
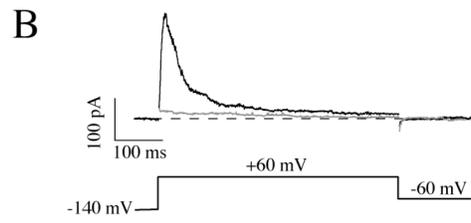
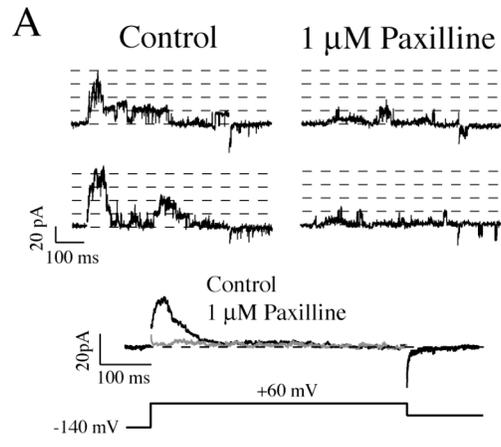
Ensemble traces best show the average relative decrease (89.4%) in the amplitude of the current conferred by paxilline (shown below the single channel records represents an average of 15 traces). Exposure to a zero Ca^{2+} saline was found to decrease K^+ current measured at a test potential to +60 mV. An inside out patch is shown in Fig. 3B, under control conditions, a voltage step to +60 mV elicited a large outward current, and after exposure to a zero Ca^{2+} saline (buffered with EGTA, grey line) the outward current is completely abolished indicating the presence of Ca^{2+} sensitive K^+ current. Currents from an inside-out patch were elicited with a series of voltage steps (Fig. 3C) to determine the voltage dependence of steady state inactivation and voltage dependence of activation. In the inside-out patch example in Fig. 3D, H_{inf} and G-V curves were constructed from current peak amplitudes and fitted with a Boltzman function, yielding a value for V_{inact} (voltage at half inactivation) of -54.2 mV and $V_{1/2}$ (voltage at half activation) of 6.8 mV. An additional cell produced similar H_{inf} and GV curves with a V_{inact} of -54.9 mV and $V_{1/2}$ of 16.6 mV.

In summary, K^+ current from inside out patches blocked with paxilline and a zero Ca^{2+} saline solution further support the presence of BK channels in anterior pituitary corticotropes.

Corticotropes Contain Other K^+ Channels

Other pharmacological agents were used to help identify other K^+ channels present in anterior pituitary corticotropes (Fig. 4). Apamin was used to test for the presence of small conductance Ca^{2+} -activated K^+ (SK) channels (Blatz and Magleby, 1986), clotrimazole (CLT) was used to test for intermediate Ca^{2+} -activated K^+ (IK) channels (Wulff et al., 2000), and 4-aminopyridine (4-AP) was used to determine the presence of A-type K^+ ion channels. Apamin (500 nM) on average, blocked $11.2 \pm 5.3\%$ of the peak current and $17.6 \pm 7.4\%$ of the sustained current ($n = 3$, for peak and

Figure 3. Characterization of BK channels in inside-out patches. A) In an inside-out patch, containing ≥ 4 channels. BK currents were activated by a test pulse to +60 mV from a prepulse potential of -140 mV. Application of 1 μ M paxilline to the patch rapidly decreased the number of channels open during successive sweeps. Ensemble averages of 15 sweeps are shown below. B) Exposure of an inside out patch to a zero Ca^{2+} solution rapidly decreased the activity of K^+ channels. C) Representative current traces from an inside out patch elicited by a two step protocol, with initial voltage steps from -130 mV to +100 mV and a second voltage step to +80 mV. D) Conductance-voltage (GV) plots were generated from traces in C. Steady state inactivation (H_{inf}) plots were generated from the second step to +80 mV after the varying prepulse potentials shown in C.



sustained). Clotrimazole (1 μ M) on average, blocked 24.2 ± 2.2 % of the peak current and 41.1 ± 3.8 % of the sustained current (n = 4, for peak and sustained). On average, 4-AP (5 mM) blocked 65.5 ± 3.1 % and 63.6 ± 2.3 % of the peak and sustained current, respectively (n = 3 for each). 10 mM 4-AP on average blocked 76.1 ± 3.2 % of the peak and 72.8 ± 2.7 % of the sustained current. Recent studies suggest that CLT at concentrations ≥ 1 μ M is not selective for IK channels only and has some effects blocking voltage sensitive K⁺ channels (Wulff et al., 2000). A more potent blocker of IK channels is TRAM-34, which is derived from clotrimazole (Wulff et al., 2001), and more studies are needed to clarify the contribution of IK channels to the overall K⁺ current and the selectivity of IK blockers to BK channels. 4-aminopyridine was initially used in this study to characterize A-type K⁺ channels, but 4-AP can also block BK channels at concentrations of 1 mM (Khan et al., 1997). Additional studies are needed to clarify the selectivity of high concentrations (> 1mM) of 4-AP on BK channel block.

Corticotropes Display a Variety of Firing Patterns

Our studies show that corticotropes display a variety of firing patterns. Under whole-cell conditions, slow membrane potential oscillations (spontaneous plateau bursting with no current injection), with average durations that lasted seconds were observed in 57% of the cells that were examined (n = 14). Plateau potentials typically consisted of multiple small amplitude fast spikes during the duration of the burst. Of the spontaneously active cells, one fired tall single spike action potentials with a duration of several milliseconds (Fig. 5A). Other corticotropes displayed plateau bursting along with tall single spike action potentials (Fig. 5B; 3 of 8 cells). Another subset of corticotropes fired plateau bursts of action potentials only (Figs. 5C and 5D; 5 of 8). The burst duration of spontaneously active corticotropes could be shortened

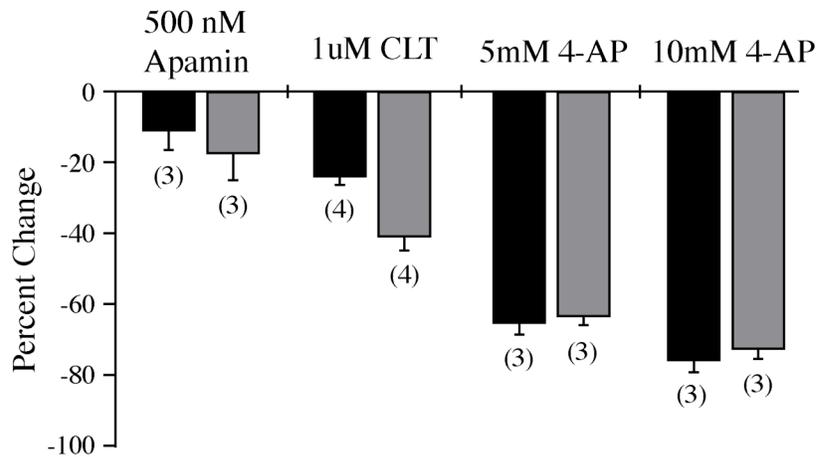


Figure 4. Different K^+ channels are present in anterior corticotropes. Outward K^+ current was elicited in the perforated patch mode with voltage steps to +60 mV. Average percent changes for the drugs apamin, 4-AP, and clotrimazole are shown for the peak current (black bars) and for the sustained current (grey bars). Sample sizes are shown in parentheses below each bar.

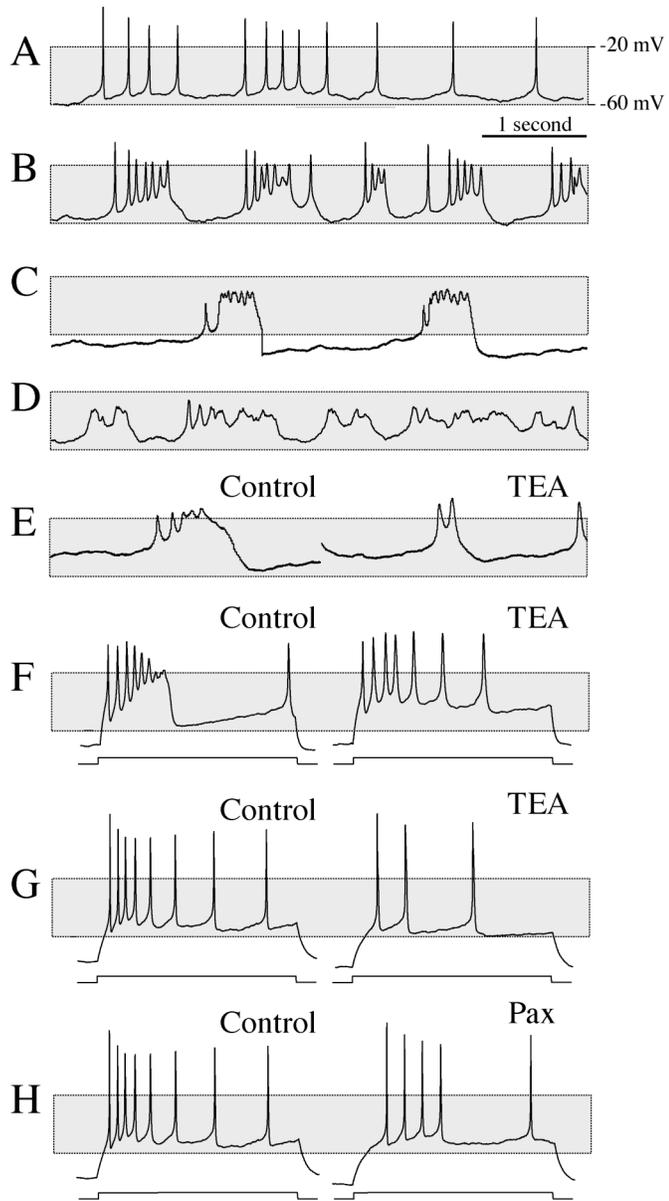
with 1 mM tetraethylammonium (TEA; Fig. 5E) a potent blocker of BK channels at low concentrations (Lovell and McCobb, 2001). Another subset of corticotropes were not spontaneously active, and required current injection to elicit action potentials (3 of 14 cells). In the example in Fig. 5F, 14 pA of injected current elicited a burst of action potentials. After the application of 1 mM TEA to the bath, the plateau bursting behavior was turned into single tall action potentials, implicating the role of BK channels in plateau bursting. Other cells when injected with current displayed single action potentials. Fig. 5G-H show a corticotrope injected with 6 pA of current and the resulting action potentials. After the application of 1 mM TEA or 10 μ M paxilline, the number of action potentials was reduced. These experiments indicate that BK channels play prominent roles in the excitability of corticotropes as shown in chromaffin cells and somatotropes (Solaro et al., 1995; Lovell and McCobb, 2001; Stojilkovic et al., 2005).

In summary, out of a total of 14 cells tested, 8 were spontaneously active, 3 required current injection to elicit action potentials, and 3 cells were inexcitable (data not shown). This variety in the excitability profiles may be an indication for the need of in vivo cell to cell interactions or constant hormone activation for corticotrope excitability to occur, and need to be addressed in future studies.

The Activity of Corticotropes is Acutely Modulated by Hormones

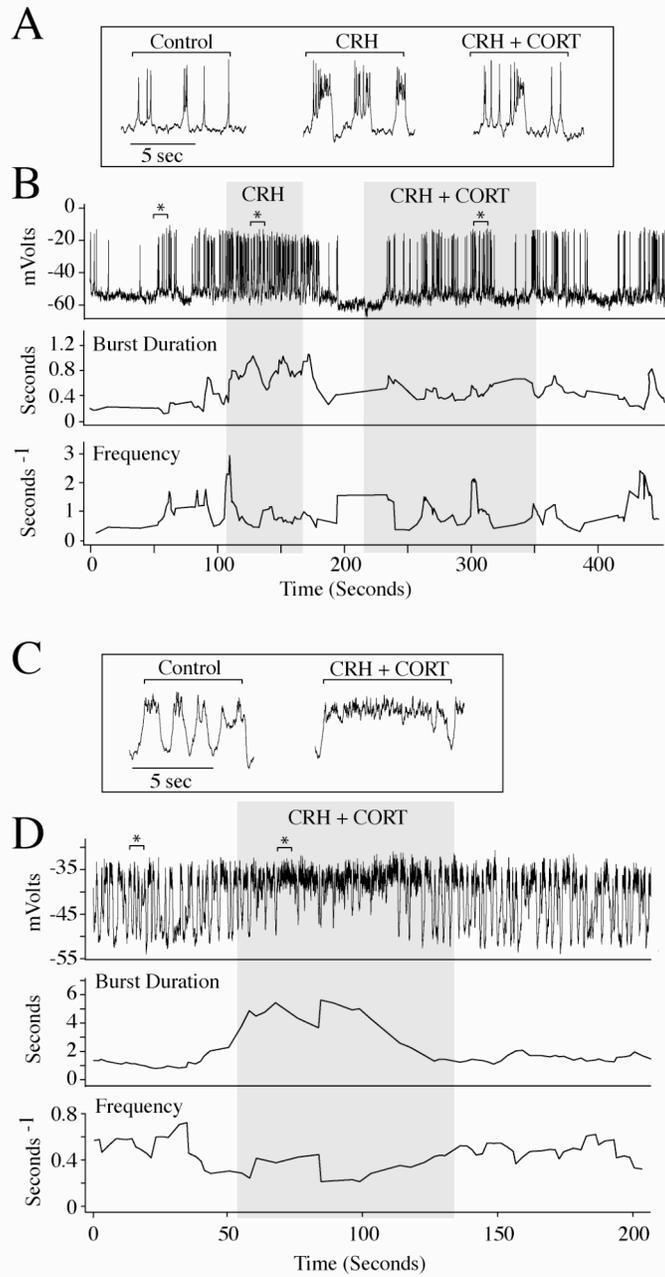
Spontaneous bursts were recorded from corticotropes using the conventional whole cell technique. Stable recordings could be made from individual cells for 20-30 min. To reduce variability in recordings, a small holding current was applied, when necessary, to maintain membrane potentials between -60 and -65 mV. Burst duration and shape were altered by the application of the natural secretagogue CRH (10 nM), and the application of 10 nM CRH + 10 μ M CORT to the bath (Fig. 6A; 3 out of 6

Figure 5. Anterior corticotropes exhibit a wide range of firing patterns. A-D) Membrane potential oscillations were recorded in the whole cell mode from corticotropes. Trains of tall single action potentials and slower plateau bursts could be observed from spontaneously active corticotropes (0 pA current injection). E) Application of 1 mM TEA to a spontaneously active cell reduces the duration of plateau bursts. F) Depolarizing current of 14 pA was injected for 2 sec to elicit a burst of action potentials. Application of 1 mM TEA altered the bursting behavior. G-H) A different cell was injected with 6 pA of depolarizing current to elicit a train of action potentials. The application of 1 mM TEA or 10 μ M paxilline reduced the number of action potentials during a 2 sec depolarizing pulse. The grey band represents a voltage range of 40 mV.



cells). Example bursts in Fig. 6A taken from a longer record of bursting activity (from the brackets shown in Fig. 6B; top panel). The spontaneous bursting activity along with the average burst duration and the instantaneous frequency are all plotted on the same timescale for easy comparison (Fig. 6B & 6D). Each burst duration was measured and a running sliding box average of 5 burst durations was plotted. The instantaneous frequency plot is a representation of a running average of 5 individual frequencies. After application of CRH, burst durations decreased during washout, and then slightly increased in response to the application of CRH + CORT. An additional example of bursting activity is shown in Fig. 6C & 6D, which showed a more pronounced effect of CRH + CORT on burst duration. The application of 10 nM CRH + 10 μ M CORT prolonged the average burst duration (Fig. 6D; middle panel) and slightly decreases the instantaneous frequency (Fig. 6D; bottom panel) when compared to control. The variability of the response to CRH + CORT in the two cells shown may indicate the importance of prior exposure to hormones (application of CRH first then CRH + CORT), and can be resolved with a longer wash between applications. Also further studies are needed to examine whether the modulation by CORT is separate from the CRH effect. Some cells were exposed to CORT only (data not shown) and no change in burst duration was observed, but application of CRH and CORT together have a synergistic effect on burst duration (seen in Fig. 6C and 6D). Examining each effect separately will lead to a better understanding of rapid steroid modulation on excitability and hormone release in anterior pituitary corticotropes.

Figure 6. Corticotrope excitability is modulated by hormones. A) Representative voltage traces from a spontaneously active cell, under whole cell patch clamp mode. Action potential bursts were modulated when the cell was exposed to 10 nM CRH (a natural secretagogue) and 10 nM CRH + 10 μ M CORT. Traces are expanded time traces from the longer record of activity below (denoted by brackets). B) Plots of the membrane potential, burst duration and instantaneous frequency before and during the exposure to different hormones. All are plotted on the same timescale for easy comparison. Duration of hormone exposure is represented with a grey box. Burst durations were measured at a threshold of -50 mV, and are plotted as a running average of 5 durations. The frequency of bursts is plotted as a running average of 5 individual frequencies. C) Representative voltage traces from a different cell exposed only to 10 nM CRH + 10 μ M CORT. D) An expanded plot of the bursting activity, average burst duration and average frequency in control saline and after exposure to 10 nM CRH and 10 μ M CORT.



DISCUSSION

The mouse pituitary corticotrope tumor AtT20 cell line has been a key system in studying the physiology and regulation of anterior pituitary corticotropes. The dissociation of corticotropes is difficult and AtT20 cells provide an easy alternative to study. This study is the first characterization of the complement of K^+ ion channels present in non-tumor “real” corticotropes. The isolation and characterization of corticotropes was facilitated in this study by the use of a transgenic mouse line, where GFP is under the control of the POMC promoter. Our results agree with previous research from AtT20 cells showing that BK channels are prominent in anterior pituitary corticotropes and play a vital role in shaping the firing properties and the secretion of ACTH. In the pituitary, the POMC gene is expressed primarily by anterior pituitary corticotropes as well as intermediate lobe melanotropes (Jacobson and Drouin, 1994). During our dissociation we tried to exclude any cells from the intermediate lobe. We show that the majority of GFP expressing cells dissociated from fresh anterior pituitary glands express the main hormone secreted by corticotropes, ACTH (Fig. 1). The majority of the outward K^+ current from corticotropes is sensitive to the drug paxilline (Fig. 2), which is a potent blocker of BK channels. In inside-out patches, paxilline was observed to block the majority of K^+ current obtained from corticotropes, and the unitary conductance of channels blocked was measured to be ~ 300 pS (Fig. 3). Additionally, inside-out patches revealed that the majority of K^+ channels present in corticotropes are Ca^{2+} sensitive (Fig. 3B), and exhibited a rapid inactivation phenotype characteristic of BK channels expressing the $\beta 2$ - subunit (Xia et al., 1999). The ensemble records of the K^+ current in inside out patches have an average inactivation time constant of 51.5 ± 6.0 ms. Other Ca^{2+} sensitive K^+ channels were also detected in corticotropes with the use of various pharmacological agents. The presence of SK and IK channels were detected with the drugs apamin and

clotrimazole (Fig. 4). Additionally, the blocker 4-AP was used to test for the presence of A-type K^+ channels. Previous research in AtT20 cells has identified A-type K^+ channels, and SK channels (Pennington et al., 1994). Our study is the first to show that non-tumor corticotropes also contain an array of different K^+ channels. From inside-out experiments, we estimate approximately 90% of the outward K^+ current is sensitive to Ca^{2+} . However, perforated patch experiments show at least a 65% block of outward K^+ current by 5 mM 4-AP. It has been shown that 1 mM 4-AP blocks BK channels in uterine myocytes (Khan et al., 1997). The concentrations of 5 mM and 10 mM 4-AP could non-selectively block BK, IK, and SK channels. Detailed experiments examining the cross reactivity of 4-AP with BK, IK, and SK channels need to be conducted to interpret our results.

The membrane excitability of corticotropes has not been extensively studied. In the current study, we have recorded action potential bursts from primary cultured corticotropes. The spontaneous plateau bursting action potentials observed in this study are similar to those seen in other studies of non-tumor corticotropes (Kuryshv et al., 1995, 1996). Additionally, corticotropes show plateau bursting similar to that observed in anterior pituitary somatotropes and lactotropes (Van Goor et al., 2001a; Stojilkovic et al., 2005). However, our recordings show that corticotropes can display different firing patterns (Fig. 5). The majority (57%) of the corticotropes displayed slow membrane potential oscillations (spontaneous plateau bursting with no current injection), with average durations that lasted seconds. However, some corticotropes displayed different spontaneous firing patterns. Specific blockers for BK channels altered the firing pattern of corticotropes. Application of 1 mM TEA to spontaneously active corticotropes shortened the burst duration, and in a different cell converted bursting behavior into tonically firing behavior. The application of 10 μ M paxilline also decreased the number of action potentials elicited by a 2 sec depolarizing current

pulse (Fig. 5). This implicates BK channels having a prominent role in repetitive firing properties in anterior corticotropes, which has been previously shown in chromaffin cells and somatotropes (Solaro et al., 1995; Lovell and McCobb, 2001; Stojilkovic et al., 2005).

Differences in the expression of ion channels could account for the differences in the patterns of action potentials observed in corticotropes. Prior studies have found that somatotropes and lactotropes both have high expression of BK channels while gonadotropes have low expression levels (Stojilkovic et al., 2005). In their model they propose that the activation of BK channels by the influx of Ca^{2+} through voltage gated Ca^{2+} channels truncates the amplitude of the initial spike and limits the activation of delayed rectifier K^+ channels, leading to the generation of plateau bursting (Van Goor et al., 2001a). BK channels then deactivate quickly to allow for the generation of the sustained plateau potential. BK channels could play a similar role in corticotropes. Differences in BK channel expression and association with β -subunits could also account for the variability seen in the firing patterns of corticotropes. It should be noted that the amount of total K^+ current contributed by SK and IK current was not fully characterized in this study. SK and IK currents are voltage insensitive and additional studies examining their activation by Ca^{2+} should be performed to clarify their role in shaping the properties of membrane excitability.

The natural secretagogue CRH prolongs the burst duration of spontaneously active corticotropes (Fig. 6). A combination of CORT and CRH also prolongs the burst duration when compared with control traces. The prolonged burst duration could drive an increase in Ca^{2+} influx, as suggested by previous research (Kuryshv et al., 1995, 1996). We have observed an acute effect of CORT on membrane excitability that may help to enhance ACTH secretion. Glucocorticoids have been reported to have genomic effects on different ion channels found in AtT20 cells. Thus, they block the

PKA mediated inhibition of BK channels (Tian et al., 2001), as well as inhibit stimulated, as opposed to, basal ACTH secretion by limiting Ca^{2+} influx through Ca^{2+} channels (Antoni et al., 1992). They also enhance the A-type K^+ current through a mechanism requiring protein synthesis (Pennington et al., 1994). Glucocorticoids have recently been shown to increase BK channel activity in AtT20 cells through a non-genomic mechanism (Huang et al., 2006). Molecular studies have suggested that BK α -subunits do not couple to BK β -subunits in AtT20 cells (Shipston et al., 1999). However, the mouse pituitary as a whole exhibits expression of β -subunits (Behrens et al., 2000; Chatterjee et al., 2007), and it is unknown whether β -subunits are associated with BK channels in native mouse corticotropes. However, the BK inactivation described here suggest that BK β 2-subunits are abundantly expressed. We have previously shown that the rapid modulation of adrenal chromaffin cell excitability by steroid hormones is attributed to the presence of BK β 2 and β 4-subunits (Lovell et al., 2004; King et al., 2006). Furthermore, β 2 and β 4-subunits expressed in the whole pituitary are upregulated in response to social stress (Chatterjee et al., 2007). Further studies are needed to determine whether acute modulation of BK channels by steroid hormone has a role in the regulation of ACTH release.

We postulate that steroid hormones released during the stress response can have acute (non-genomic) effects on native corticotropes in the anterior pituitary altering ionic current, excitability, and hormone secretion, as well as the more studied genomic actions. BK channels are present in pituitary corticotrope tumor cells, and our study confirms their presence in native corticotropes. Moreover, the regulation of excitability in native corticotropes is not completely understood. Steroid hormones have well-established genomic effects on each component of the HPA axis. The role that steroids may play in the acute modulation of each component will aid our knowledge of cellular mechanisms in action during the stress response.

REFERENCES

- Abou-Samra AB, Catt KJ, Aguilera G (1987) Calcium-dependent control of corticotropin release in rat anterior pituitary cell cultures. *Endocrinology* 121:965-971.
- Abramoff MD, Magelhaes PJ, Ram SJ (2004) Image Processing with ImageJ. *Biophotonics International* 11:36-42.
- Aguilera G, Harwood JP, Wilson JX, Morell J, Brown JH, Catt KJ (1983) Mechanisms of action of corticotropin-releasing factor and other regulators of corticotropin release in rat pituitary cells. *J Biol Chem* 258:8039-8045.
- Antoni FA, Hoyland J, Woods MD, Mason WT (1992) Glucocorticoid inhibition of stimulus-evoked adrenocorticotrophin release caused by suppression of intracellular calcium signals. *J Endocrinol* 133:R13-16.
- Behrens R, Nolting A, Reimann F, Schwarz M, Waldschutz R, Pongs O (2000) hKCNMB3 and hKCNMB4, cloning and characterization of two members of the large-conductance calcium-activated potassium channel beta subunit family. *FEBS Lett* 474:99-106.
- Bers DM, Patton CW, Nuccitelli R (1994) A practical guide to the preparation of Ca²⁺ buffers. *Methods Cell Biol* 40:3-29.
- Blatz AL, Magleby KL (1986) Single apamin-blocked Ca-activated K⁺ channels of small conductance in cultured rat skeletal muscle. *Nature* 323:718-720.
- Chatterjee O, Taylor LA, Ahmed S, Nagaraj S, Hall JJ, Finckbeiner SM, Chan PS, Suda N, King JT, Zeeman ML, McCobb DP (2007) Social stress alters expression of BK potassium channel subunits in mouse adrenal and pituitary glands. In: Cornell University.
- Childs GV, Marchetti C, Brown AM (1987) Involvement of sodium channels and two types of calcium channels in the regulation of adrenocorticotropin release. *Endocrinology* 120:2059-2069.
- Childs GV, Lloyd JM, Rougeau D, Unabia G (1988) Enrichment of corticotropes by counterflow centrifugation. *Endocrinology* 123:2885-2895.
- Guerineau N, Corcuff JB, Tabarin A, Mollard P (1991) Spontaneous and corticotropin-releasing factor-induced cytosolic calcium transients in corticotrophs. *Endocrinology* 129:409-420.
- Hamill OP, Marty A, Neher E, Sakmann B, Sigworth FJ (1981) Improved patch-clamp techniques for high-resolution current recording from cells and cell-free membrane patches. *Pflugers Arch* 391:85-100.

- Horvath E, Kovacs K (1994) Morphology of Adenohypophysial Cells and Pituitary Adenomas. In: The Pituitary Gland, Second Edition Edition (Imura H, ed), pp 29-62. New York: Raven Press.
- Huang MH, So EC, Liu YC, Wu SN (2006) Glucocorticoids stimulate the activity of large-conductance Ca^{2+} -activated K^{+} channels in pituitary GH3 and AtT-20 cells via a non-genomic mechanism. *Steroids* 71:129-140.
- Jacobson L, Drouin J (1994) Regulation of Proopiomelanocortin Gene Transcription. In: The Pituitary Gland, Second Edition Edition (Imura H, ed), pp 117-138. New York: Raven Press.
- Kehl SJ, Wong K (1996) Large-conductance calcium-activated potassium channels of cultured rat melanotrophs. *J Membr Biol* 150:219-230.
- Khan RN, Smith SK, Morrison JJ, Ashford ML (1997) Ca^{2+} dependence and pharmacology of large-conductance K^{+} channels in nonlabor and labor human uterine myocytes. *Am J Physiol* 273:C1721-1731.
- King JT, Lovell PV, Rishniw M, Kotlikoff MI, Zeeman ML, McCobb DP (2006) Beta2 and beta4 subunits of BK channels confer differential sensitivity to acute modulation by steroid hormones. *J Neurophysiol* 95:2878-2888.
- Knaus HG, McManus OB, Lee SH, Schmalhofer WA, Garcia-Calvo M, Helms LM, Sanchez M, Giangiacomo K, Reuben JP, Smith AB, 3rd, et al. (1994) Tremorgenic indole alkaloids potently inhibit smooth muscle high-conductance calcium-activated potassium channels. *Biochemistry* 33:5819-5828.
- Kuryshv YA, Childs GV, Ritchie AK (1995) Corticotropin-releasing hormone stimulation of Ca^{2+} entry in corticotropes is partially dependent on protein kinase A. *Endocrinology* 136:3925-3935.
- Kuryshv YA, Childs GV, Ritchie AK (1996) Corticotropin-releasing hormone stimulates Ca^{2+} entry through L- and P-type Ca^{2+} channels in rat corticotropes. *Endocrinology* 137:2269-2277.
- Labrie F, Veilleux R, Lefevre G, Coy DH, Sueiras-Diaz J, Schally AV (1982) Corticotropin-releasing factor stimulates accumulation of adenosine 3', 5'-monophosphate in rat pituitary corticotrophs. *Science* 216:1007-1008.
- Lim MC, Shipston MJ, Antoni FA (1998) Depolarization counteracts glucocorticoid inhibition of adenohypophysial corticotroph cells. *Br J Pharmacol* 124:1735-1743.
- Lovell PV, McCobb DP (2001) Pituitary control of BK potassium channel function and intrinsic firing properties of adrenal chromaffin cells. *J Neurosci* 21:3429-3442.

- Lovell PV, James DG, McCobb DP (2000) Bovine versus rat adrenal chromaffin cells: big differences in BK potassium channel properties. *J Neurophysiol* 83:3277-3286.
- Lovell PV, King JT, McCobb DP (2004) Acute modulation of adrenal chromaffin cell BK channel gating and cell excitability by glucocorticoids. *J Neurophysiol* 91:561-570.
- Pennington AJ, Kelly JS, Antoni FA (1994) Selective enhancement of an A type potassium current by dexamethasone in a corticotroph cell line. *J Neuroendocrinol* 6:305-315.
- Pinto S, Roseberry AG, Liu H, Diano S, Shanabrough M, Cai X, Friedman JM, Horvath TL (2004) Rapid rewiring of arcuate nucleus feeding circuits by leptin. *Science* 304:110-115.
- Sanchez M, McManus OB (1996) Paxilline inhibition of the alpha-subunit of the high-conductance calcium-activated potassium channel. *Neuropharmacology* 35:963-968.
- Shipston MJ, Kelly JS, Antoni FA (1996) Glucocorticoids block protein kinase A inhibition of calcium-activated potassium channels. *J Biol Chem* 271:9197-9200.
- Shipston MJ, Duncan RR, Clark AG, Antoni FA, Tian L (1999) Molecular components of large conductance calcium-activated potassium (BK) channels in mouse pituitary corticotropes. *Mol Endocrinol* 13:1728-1737.
- Solaro CR, Prakriya M, Ding JP, Lingle CJ (1995) Inactivating and noninactivating Ca²⁺- and voltage-dependent K⁺ current in rat adrenal chromaffin cells. *J Neurosci* 15:6110-6123.
- Stojilkovic SS, Zemkova H, Van Goor F (2005) Biophysical basis of pituitary cell type-specific Ca²⁺ signaling-secretion coupling. *Trends Endocrinol Metab* 16:152-159.
- Tian L, Knaus HG, Shipston MJ (1998) Glucocorticoid regulation of calcium-activated potassium channels mediated by serine/threonine protein phosphatase. *J Biol Chem* 273:13531-13536.
- Tian L, Duncan RR, Hammond MS, Coghill LS, Wen H, Rusinova R, Clark AG, Levitan IB, Shipston MJ (2001) Alternative splicing switches potassium channel sensitivity to protein phosphorylation. *J Biol Chem* 276:7717-7720.
- Van Goor F, Li YX, Stojilkovic SS (2001a) Paradoxical role of large-conductance calcium-activated K⁺ (BK) channels in controlling action potential-driven Ca²⁺ entry in anterior pituitary cells. *J Neurosci* 21:5902-5915.

- Van Goor F, Zivadinovic D, Stojilkovic SS (2001b) Differential expression of ionic channels in rat anterior pituitary cells. *Mol Endocrinol* 15:1222-1236.
- Vergara C, Latorre R, Marrion NV, Adelman JP (1998) Calcium-activated potassium channels. *Curr Opin Neurobiol* 8:321-329.
- Woods MD, Shipston MJ, Mullens EL, Antoni FA (1992) Pituitary corticotrope tumor (AtT20) cells as a model system for the study of early inhibition by glucocorticoids. *Endocrinology* 131:2873-2880.
- Wulff H, Gutman GA, Cahalan MD, Chandy KG (2001) Delineation of the clotrimazole/TRAM-34 binding site on the intermediate conductance calcium-activated potassium channel, IKCa1. *J Biol Chem* 276:32040-32045.
- Wulff H, Miller MJ, Hansel W, Grissmer S, Cahalan MD, Chandy KG (2000) Design of a potent and selective inhibitor of the intermediate-conductance Ca²⁺-activated K⁺ channel, IKCa1: a potential immunosuppressant. *Proc Natl Acad Sci U S A* 97:8151-8156.
- Xia XM, Ding JP, Lingle CJ (1999) Molecular basis for the inactivation of Ca²⁺- and voltage-dependent BK channels in adrenal chromaffin cells and rat insulinoma tumor cells. *J Neurosci* 19:5255-5264.