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University of Massachusetts Medical School

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MODULATION OF NEUROPEPTIDE RELEASE VIA
VOLTAGE-DEPENDENT AND -INDEPENDENT SIGNALING IN
ISOLATED NEUROHYPOPHYSIAL TERMINALS

A Dissertation Presented

By

Cristina M. Velázquez-Marrero

Submitted to the Faculty of the University of Massachusetts Graduate
School of Biomedical Sciences, Worcester in partial fulfillment of the
requirements for the degree of:

DOCTOR OF PHILOSOPHY

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Neuroscience

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- ▶ Valérie De Crescenzo, Ronghua ZhuGe, Cristina Velázquez-Marrero, Lawrence M. Lifshitz, Edward Custer, Jeffrey Carmichael, F. Anthony Lai, Richard A. Tuft, Kevin E. Fogarty, José R. Lemos and John V. Walsh, Jr (2004). Ca^{2+} syntillas, miniature Ca^{2+} release events in terminals of hypothalamic neurons, are increased in frequency by depolarization in the absence of Ca^{2+} influx. *J. Neurosci.*, Feb 2004; 24: 1226 - 1235.
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Alejandro, Catalina, and Eliana

Who always inspire me

to

keep moving forward...

Abstract

This thesis details my examination of several mechanisms for modulation of neuropeptide release via voltage-dependent and voltage-independent intraterminal signaling in isolated neurohypophysial terminals. The first part of this work characterizes depolarization-induced neuropeptide release in the absence of extracellular calcium. The goal of this project was to examine the relationship between depolarization-induced release of intracellular calcium stores and depolarization-secretion coupling of neuropeptides. We demonstrate that depolarization in the absence of extracellular calcium induced by either High K^+ or electrical stimulation induces a rise in $[Ca^{2+}]_i$ and subsequent neuropeptide release from Hypothalamic Neurohypophysial System (HNS) terminals. A portion of extracellular calcium-independent neuropeptide release is due to intraterminal calcium, but the remaining depolarization-induced release may be due to calcium-independent voltage-dependent (CIVD) release (Zhang and Zhou, 2002; Zhang et al., 2004; Yang et al., 2005). Nevertheless, our results clearly show that extracellular calcium is not necessary for depolarization-induced neuropeptide secretion from these CNS terminals.

In addition, I investigated the role of internal calcium stores in mediating μ -opioid inhibition of voltage-gated calcium channels (VGCCs). Inhibition of VGCCs via μ -opioid agonists has been shown to reduce neuropeptide release in response to High K^+ stimulation of isolated terminals (Bicknell et al., 1985b; Russell et al., 1993; van Wimersma Greidanus and van de Heijning, 1993; Munro et al., 1994; Ortiz-Miranda et al., 2003; Russell et al., 2003; Ortiz-Miranda et al., 2005). My findings show μ -opioid

inhibition, of VGCC and High K^+ -mediated rise in $[Ca^{2+}]_i$, are via a voltage-independent diffusible second-messenger targeting release of calcium from ryanodine-sensitive stores, possibly mediated via the cyclic ADP ribose signaling pathway.

Furthermore, I detail a different intracellular messenger pathway mediating the κ -opioid inhibition of VGCC and High K^+ -mediated rise in $[Ca^{2+}]_i$. In contrast to the μ -opioid inhibition, κ -receptor activation is coupled to a voltage-dependent membrane-delimited pathway. Inhibition of neuropeptide release via both endogenous and exogenous κ -opioid agonists has been extensively studied (Bicknell et al., 1985a; Nordmann et al., 1986a; Wammack and Racke, 1988; Munro et al., 1994; Ingram et al., 1996; Rusin et al., 1997a). My investigation shows that the κ -inhibition of VGCC is voltage-dependent and is furthermore, relieved within the context of a physiological burst of action potentials (APs). This physiologically-evoked, activity-dependent modulation of VGCC and subsequent release, represents an important mechanism for short-term synaptic plasticity at the level of the terminals. Given the ubiquitous nature of voltage-dependent G-protein signaling in the CNS, our results may prove important in understanding modulatory effects of specific bursting patterns throughout the CNS.

In the last 30 years the neurohypophysial system has proven to be an excellent system to study the complexities of depolarization-secretion coupling (DSC). There have been many advances in our understanding of the underlying mechanisms involved and their physiological implications. The current work focuses on two important features of DSC; voltage and calcium. Although in many ways these two are intrinsically linked through VGCC activation, we have found that in isolated HNS terminals that is not always the case. We have further found that when voltage and calcium influx are linked during DSC,

modulation by opioids may or may not be linked to activity-dependent relief depending on the opioid receptor activated. This finding has important implications in neuropeptide release during patterned stimulation *in vivo*. As I will discuss further, many factors play into the complexities of the regulatory mechanisms involving release. As investigations into this remarkable field continue, I hope to have contributed a valuable piece to the puzzle.

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Chapter I

Introduction

Hypothalamic Neurohypophysial system

Magnocellular neurosecretory cells (MNC) project axons to the neurohypophysis where both oxytocin (OT) and arginine vasopressin (AVP) are released for systemic circulation. Oxytocin (OT) has been shown to be important during parturition and essential for milk-let down during lactation in females, in males it stimulates contractions of the reproductive tract aiding sperm ejaculation (Insel, 1992; Russell et al., 2003; Leng et al., 2005; Thackare et al., 2006) (Fig. 1.1). OT also has an emerging role as a natriuretic agent (Lemos et al., 2002). Behavioral studies have shown the role of oxytocin (OT) in modulating stress, pair-bond formation, parental behavior, social recognition, and important aspects of attachment (Insel, 1992; Zingg, 1996; Choleris et al., 2003; Razzoli et al., 2003; Bartz and McInnes, 2007; Jin et al., 2007; Campbell, 2008). Arginine vasopressin (AVP) plays a vital role in systemic sodium and water homeostasis as an antidiuretic (Gruber and Eskridge, 1986; Bourque and Oliet, 1997), it is also a potent vasoconstrictor (Altura and Altura, 1977; Russ et al., 1992) and, like OT, has a role in affiliation behaviors (Lohmeier, 2003; Caldwell et al., 2008). Various endogenous excitatory and inhibitory inputs project onto the magnocellular neurosecretory system at various levels affecting release of both oxytocin and vasopressin. Autocrine and paracrine effects of AVP at the dendrites and cell bodies have shown activity-dependent modulation (Niermann et al., 2001; Mueller et al., 2005; Bull et al., 2006; Brown et al., 2007). Other compounds co-released with both neuropeptides also exert modulatory effects on somatodendritic excitability such as; ATP (Troade and Thirion, 2002; Bull et al., 2006), and endogenous opioids (Leng et al., 1985; Brown et al., 2007). At the terminals, characteristic electrical input from OT and AVP cell bodies is associated with

optimum excitation-secretion coupling of their specific neurohormones. OT neurons are characterized by a high-frequency discharge, which occurs during suckling, triggering pulsatile release of OT, while AVP neurons are characterized by their asynchronous phasic activity (bursting) during maintained AVP release (Armstrong and Sladek, 1982; Poulain and Wakerly, 1982; Andrew and Dudek, 1983). Autocrine and paracrine modulation by AVP and OT seems unlikely at the Neurohypophysis given there are no known OT receptors in the terminals and the presence of AVP receptors is still debated (Dashwood and Robinson, 1988; Hatton et al., 1992). However, substances co-released with either/or AVP and OT, such as endogenous opioids (Iversen et al., 1980; Lightman et al., 1983; Bicknell et al., 1985b; Nordmann et al., 1986b; Bicknell et al., 1988; Zhao et al., 1988a; Sumner et al., 1990; Leng et al., 1994; Ortiz-Miranda et al., 2003), and ATP (Lemos and Wang, 2000a; Sladek and Kapoor, 2001; Troadec and Thirion, 2002; Wang et al., 2002a; Knott et al., 2005; Song and Sladek, 2005; Knott et al., 2007) are known to have significant modulatory effects on VGCC and DSC. The intraterminal mechanisms mediating these effects on VGCC and subsequent stimulus-secretion coupling are yet to be understood.

Patterns of electrical stimulation inducing optimum neuropeptide release

As mentioned above, the optimum firing pattern for eliciting AVP and OT release are different (Fig. 1.2). Optimum AVP release is stimulated by an asynchronous phasic bursting with periods of firing and silence from 20-40 sec. with an average mean frequency of 10-20 Hz (Wakerley et al., 1978; Armstrong and Sladek, 1982; Cazalis et al., 1985; Gainer et al., 1986; Richard et al., 1997). OT discharge is preceded by a high

frequency burst pattern approx. 10-77 Hz for 0.9-4.7 sec. with a burst latency of 13.3 sec. (Lincoln and Wakerley, 1974; Armstrong and Sladek, 1982; Poulain and Wakerly, 1982; Andrew and Dudek, 1983). Research exploring the possible mechanisms that connect the burst patterning to optimal release have been ongoing. Starting in 1971, action potentials were determined to be the mechanisms via which electrical impulses were transmitted from magnocellular neurons to the Neurohypophysis eliciting neuropeptide release (Dreifuss et al., 1971). These studies showed for the first time, that the total number of stimuli applied and the frequency of stimulation were both important for optimum release. Future studies confirmed these findings and further found other important parameters for eliciting release such as burst frequency, AP broadening, burst duration and interburst interval (Dutton and Dyball, 1979; Shaw et al., 1984; Jackson et al., 1991b; Seward et al., 1995). In the neurohypophysis, as in other systems, action potentials undergo a gradual broadening when elicited repetitively. The degree and rate of broadening is dependent on firing frequency (Gainer et al., 1986; Bourque, 1990; Jackson et al., 1991b). It was first believed that the phenomenon of AP broadening, which leads to an increased rise in $[Ca^{2+}]_i$ per AP, was responsible for neuropeptide release facilitation in response to higher frequency stimulation. However, this could not be the complete answer, given that parameters for maximal broadening at the somata did not correlate with firing rates evoking maximal release from HNS terminals (Andrew, 1986; Jackson et al., 1991b). Furthermore, after eliminating frequency dependent changes in AP duration there was still a residual frequency dependent rise in $[Ca^{2+}]_i$ (Jackson et al., 1991b). Other considerations, such as axonal cable properties (Nordmann and Stuenkel, 1986), changes in $[K^+]_o$ accompanying high-frequency stimulation (Leng et al., 1988a),

and endogenous opioid modulation (Bicknell, 1988; Bondy et al., 1988; Wammack and Racke, 1988) emerged as important factors in the efficiency of patterned stimulation *in vivo*. Studies done in the intact Neurohypophysis further showed that known intrinsic changes in intracellular concentrations of calcium and potassium during action potential activity, modulate biophysical properties of voltage-gated channels contributing to frequency-dependent facilitation of neuropeptide release (Marrero and Lemos, 2005). It is now known that activity-dependent changes, play a vital role in translating electrical activity to individual terminals and modulate the spatial spread of excitation within a field of HNS axons (Bourque, 1991; Marrero and Lemos, 2005; Bull et al., 2006). The activation of VGCC during action potential activity represents an important link in establishing a connection between stimulus and secretion. Therefore, one of the aims of my current work is to understand the activity-dependent modulation of VGCC via opioid receptor activation in isolated HNS terminals.

Intraterminal Ca²⁺ stores in the Neurohypophysis

Initial studies of the role of internal calcium stores in isolated Neurohypophysial terminals during DSC were controversial (Salzberg et al., 1985; Stuenkel, 1991, 1994). The lack of documented effects of either caffeine or ryanodine on depolarization-induced release (Stuenkel, 1994) suggested there were no ryanodine-sensitive stores present in the Neurohypophysial terminals. However recently, spontaneous small ryanodine-sensitive Ca²⁺ release events from isolated terminals have been characterized (De Crescenzo et al., 2004b; De Crescenzo et al., 2006). The spontaneous Ca²⁺ release events were termed syntillas, and are both ryanodine- and voltage-sensitive. Syntillas result from the

activation of both RyR type-1 and type-2. Presumably, the type-1 RyR confers the voltage-sensitivity via direct interaction with VGCC, as documented in the skeletal muscle (De Crescenzo et al., 2006). Similar spontaneous release events were later discovered in chromaffin cells with similar ryanodine sensitivity although no voltage-dependence (ZhuGe et al., 2006). Interestingly there is only RyR type-2 and type-3 in chromaffin cells, supporting the hypothesis that type-1 RyR in the neurohypophysial terminals are linked to voltage-dependence. Both of these subclasses of ryanodine receptors may act on distinct pools of calcium stores in terminals modulating intraterminal Ca^{2+} -sensitive processes in distinct spatio/temporal configurations triggered by Ca^{2+} , cADPr, and/or voltage. Ongoing studies suggest that these ryanodine receptors are located in the granules and are involved in DSC modulation (McNally et al., 2006; 2007).

Other HNS intraterminal stores are also present such as, the inositol- 1,4,5-trisphosphate (IP_3) calcium stores (Sabatier et al., 2004; Ludwig and Leng, 2006; Leng et al., 2008). Studies on isolated HNS permeabilized terminals, treatment with IP_3 induced a 242% rise above baseline AVP release (Cazalis et al., 1987a) indicating IP_3 stores not only exist in terminals but are capable of participating in release. Immunogold labeling with electromagnetic imaging (EM) has confirmed the existence of IP_3 receptors in HNS terminals (B. Salzberg personal communication). In magnocellular oxytocin neurons, IP_3 stores have been further shown to contribute to OT release in the absence of extracellular calcium (Richard et al., 1997). Both ryanodine and IP_3 intraterminal calcium stores pose interesting questions regarding their physiological role during DSC, which is currently the focus of intense research.

On the other side of the intraterminal calcium equation, several calcium extrusion mechanisms exist to regulate $[Ca^{2+}]_i$ (Fig. 1.3). Regulating changes in intraterminal calcium due to either calcium release from stores or calcium influx, are endogenous buffering mechanisms such as intracellular Ca^{2+} binding proteins (Stuenkel, 1994), Ca^{2+} extrusion through the Ca^{2+} pump in the plasma membrane (Sasaki et al., 2005), Ca^{2+} uptake by mitochondria (Stuenkel, 1994; Sasaki et al., 2005) and K^+ -dependent Na^+/Ca^{2+} exchanger (Lee et al., 2002). Therefore, as in other systems, HNS intraterminal calcium is tightly regulated due to its important role in eliciting and modulating DSC.

Ca²⁺ channels contributing to neuropeptide release from isolated terminals

Initial characterization of the L-type voltage-gated calcium channel (VGCC) in isolated terminals was first published in 1989 (Lemos and Nowycky, 1989a). Studies on neuropeptide release in response to a depolarizing stimulus had already demonstrated the importance of VGCC during DSC (Nordmann et al., 1982; Dayanithi et al., 1988). Further research on the characterization of VGCC in isolated terminals found the presence of N-type, P/Q-type and R-type in the Neurohypophysis (Dayanithi et al., 1988; Salzberg and Obaid, 1988; Lemos and Nowycky, 1989b; Obaid et al., 1989; Fatatis et al., 1992; Wang et al., 1993b; Wang et al., 1999a). Interestingly, OT containing terminals express L-, N-, and R-type VGCC while AVP containing terminals express L-, N-, and Q-type VGCC (Wang et al., 1991, 1992; Wang et al., 1997a; Wang et al., 1999b).

The relative contributions of each VGCC to release of either OT or AVP were measured in response to High K^+ -induced release of neuropeptide and analyzed with a radioimmunoassay (Wang et al., 1997a; Wang et al., 1999b). Results showed approx.

59% of either OT or AVP release was via activation of the L- and N-type calcium channel, Q-type represented approx 24% of AVP release and R-type approx. 35 % of OT release. Interestingly, in these studies there was a component of High K^+ -induced release that was not blocked by any of the VGCC blockers.

G-protein modulation of calcium channels

Regulation of VGCC by G-proteins (guanine nucleotide-binding proteins) begins with the activation of G-protein coupled receptors (GPCR). GPCRs interact with G-proteins, which are membrane-associated proteins, composed of three subunits, α , β , and γ . Activation of the GPCR leads to a conformational change in the G-protein resulting in two important events. First, the $G\alpha$ subunit exchanges GDP for GTP, followed by the dissociation of the $G\alpha$ with the $\beta\gamma$ -subunits. Both the $G\alpha$ -GTP and the $G\beta\gamma$ are active signaling complexes with several possible downstream effectors. G-protein signaling is terminated by the intrinsic alpha-subunit GTPase activity, catalyzing the hydrolysis of bound GTP to GDP + P_i . This is accelerated *in situ* by regulators of G-protein signaling (RGS) proteins, acting as GTPase-activating proteins (GAPs). Hydrolysis of the GTP promotes the reassembly of the inactive $G\alpha$ -GDP and $G\beta\gamma$ trimer.

Several different subtypes of α , β , and γ subunits can form each of the G-protein trimers. More than twenty $G\alpha$ subunits have been identified in mammalian systems (Offermanns et al., 1997). These are classified into five groups according to their downstream effectors (Tedford and Zamponi, 2006). Activation of adenylyl cyclase is targeted by G_{α_s} and inhibition is mediated by G_{α_i} , which include G_{α_o} and G_{α_z} (McCudden

et al., 2005). The $G_{\alpha q}$ is associated to activation of phospholipase C_{β} (Rhee, 2001). The G_{β} subunits have five known subtypes and twelve subtypes for the G_{γ} (Davies, 2002; Tedford and Zamponi, 2006). Differences in subtypes can be genetic or post-translational modifications resulting in >1,000 possible combinations of G_{α} , G_{β} , and G_{γ} trimers. However, constraints on cellular and subcellular expression, as well as, thermodynamic binding compatibility limit the number of heterotrimeric complexes that actually exist (Connor and Christie, 1999; Davies, 2002; Tedford and Zamponi, 2006).

Trimeric G-proteins primarily involved in the modulation of voltage-gated calcium channels are composed of $G_{\alpha i}$, $G_{\alpha o}$, or $G_{\alpha q}$ (Brown and Sihra, 2008). Inhibitory effects have been reported to mediate the presynaptic inhibitory effects on VGCC of many neurotransmitters such as muscarinic receptors (Brody et al., 1997; Brody and Yue, 2000), adrenoreceptors (McFadzean et al., 1989; Silinsky, 2004), opioid receptors (Rusin and Moises, 1995; Rusin et al., 1997b; Ortiz-Miranda et al., 2003), and GABAB receptors (Pfrieger et al., 1994). The $G_{\alpha(i/o)}$ $\beta\gamma$ -associated subunits ubiquitously act via direct binding to VGCC known as a voltage-dependent membrane-delimited pathway, resulting in a shift in gating kinetics from “willing” to “reluctant” and thus, reduced Ca^{2+} influx during DSC (Fig. 1.4). The G-protein voltage-dependent inhibition of VGCC can be reversed by strong membrane depolarizations triggering dissociation of the $G_{\beta\gamma}$ subunits from the VGCC, thus relieving inhibition of the current. Different $G_{\beta\gamma}$ subunit complexes have been studied to quantify their ability to mediate this type of voltage-dependent inhibition on specific types of VGCC (Garcia et al., 1998; Arnot et al., 2000; Ruiz-Velasco and Ikeda, 2000). These studies show a range of complex inhibitory interactions between the $G_{\beta\gamma}$ subunit complexes and VGCC, which strongly depend on

experimental conditions. However, results suggest N-type voltage-dependent inhibition is best mediated by the $G_{\beta_1\gamma_2}$ and $G_{\beta_3\gamma_2}$ complexes and the P/Q-type by the $G_{\beta_4\gamma_2}$ (Arnot et al., 2000). The G_{α_q} has been reported to inhibit VGCC through activation of target diffusible second-messenger pathways (phospholipase-C PLC, and adenylate cyclase, AC) by the GTP-bound α -subunits (Aantaa et al., 1995; Zhou et al., 2007; Brown and Sihra, 2008). This type of inhibition cannot be reversed by strong membrane depolarizations and is thus referred to as voltage-independent G-protein modulation. Membrane-delimited voltage-dependent pathways and diffusible second messenger, voltage-independent pathways, are not mutually exclusive. For example, the muscarinic M1 (Meza et al., 1999; Kammermeier et al., 2000) and neurokinin 1 (Meza et al., 2007) receptors both inhibit the N-type VGCC via a voltage-independent G_{α_q} mediated pathway concurrent with $G_{\beta\gamma}$ membrane-delimited voltage-dependent interaction. Many factors contribute to the overall complexity of GPCR signaling. The signaling complexities expand the possibilities for modulation tailoring calcium influx in response to a particular physiological context. This results in an orchestrated response for optimum neuropeptide release.

Mechanisms of G-protein mediated opioid effects

Opioid receptors are part of the G-protein coupled receptor (GPCR) family I subfamily IV (Davies, 2002). This classification is based on similarities within the GPCR amino acid sequence and the chemical nature of their ligands as determined by molecular cloning studies and genome data analysis. The three main types of opioid receptors (using the classic Greek nomenclature) are μ , κ and δ . A fourth opioid receptor (opioid receptor

like 1), with over 60% homology with the three previously cloned opioid receptors, has also been isolated (New and Wong, 2002). ORL1 is activated by nociceptin, however, it has a very different pharmacological profile than the other three receptors. The IUPHAR Nomenclature Committee has formally renamed opioid receptors μ OP or MOP, δ OP or DOP and κ OP or KOP, and NOP (for ORL1), OP standing for opioid peptide family, although the classic Greek nomenclature for the three main receptors is still extensively used (Dhawan et al., 1996; Davies, 2002).

Opioid receptors have been found all over the peripheral and central nervous system and most notably modulate behaviors such as, stress, pain, reward and motivation (Anton, 1996; Zadina et al., 1999; Fricchione and Stefano, 2005; Fichna et al., 2007) amongst others. They are activated by several endogenous opioid agonists derived from proopiomelanocortin, proenkephalin and prodynorphin (Davies, 2002). Like other GPCRs, opioid receptors mediate receptor signaling via activation of heterotrimeric G-proteins. They are all capable of interacting with the pertussis toxin-sensitive G-protein alpha-subunits $G_{\alpha i1}$, $G_{\alpha i2}$, $G_{\alpha i3}$, $G_{\alpha o1}$, $G_{\alpha o2}$ and the pertussis toxin-insensitive $G_{\alpha z}$ and $G_{\alpha 16}$ (Connor and Christie, 1999; Brown and Sihra, 2008). The predominant effector pathway being inhibition of adenylyl cyclase through pertussis toxin-sensitive G-proteins, most likely of the $G_{\alpha i}/G_{\alpha o}$ class coupled to inwardly rectifying K^+ channels or voltage-gated Ca^{2+} channels (North and Williams, 1985; Pirois et al., 1996; Hawes et al., 2000). It is generally accepted that interaction between any given opioid receptor and its effector is dictated more by availability of G-protein subtypes within the cell or presynaptic site, rather than the requirement for a specific coupling between an opioid receptor and G-protein subtype.

G-protein targeting of ionic currents involved in modulation of neurotransmitter release are primarily of the G_o , G_q , and G_s subtypes (Brown and Sihra, 2008). The G_s alpha subunit is not known to mediate any opioid receptor effects unlike the G_o and G_q . These alpha G-proteins are further associated with their respective β , γ subunits. The β , γ subunits can directly associate to the voltage-gated Ca^{2+} channel α_1 pore forming complex, resulting in a gating mode shift from “willing” to “reluctant” resulting in the reduction of Ca^{2+} influx through the channel. This is also known as voltage-dependent inhibition mediated by a membrane-delimited signaling pathway (Zamponi and Snutch, 1998; Endoh, 2004; Brown and Sihra, 2008).

Furthermore, opioid receptor activation of G-proteins can initiate intracellular signaling cascades involving diffusible second-messengers such as, adenylyl cyclase (Meng et al., 1993; Fukuda et al., 1994; Piroo et al., 1995; Law et al., 2000; Kaminski, 2004), phospholipase C (Johnson et al., 1994; Spencer et al., 1997; Law et al., 2000; Kaminski, 2004), and mitogen-activated protein kinases ERK1 and ERK2 (Fukuda et al., 1996; Li and Chang, 1996; Law et al., 2000; Kim et al., 2006). Mobilization of internal calcium in response to activation of opioid receptors has been well documented in both neuronal and non-neuronal systems (for review; Samways and Henderson, 2006). Typically a rise in $[Ca^{2+}]_i$ results from the activation of a G_{α_q} -coupled opioid receptor activating an inositol phosphate signaling pathway leading to release of $[Ca^{2+}]_i$ from IP_3 stores. Ryanodine-sensitive calcium stores have also been shown to be targets of G-protein opioid activation. Although the mechanism is not known, evidence suggests it is either via a calcium-induced calcium release mechanism (CICR), as a secondary effect of release from IP_3 stores, or through activation of the cyclic ADP ribose (cADPR) signaling

pathway. For example, activation of the μ -opioid receptor in isolated mouse astrocytes elicited a release of $[Ca^{2+}]_i$ blocked by the ryanodine-sensitive antagonist, dantrolene (Hauser et al., 1996). In isolated rat ventricular myocytes the rise in $[Ca^{2+}]_i$ due to κ -receptor activation was blocked when ryanodine-sensitive stores were depleted with ryanodine pretreatment (Tai et al., 1992). Opioid receptor-mediated elevations of $[Ca^{2+}]_i$ may target multiple Ca^{2+} -dependent processes important in modulating vesicular mobilization, priming and/or exocytosis during stimulus-evoked release. Currently, we are proposing a diffusible second messenger pathway for the μ -opioid receptor activation in isolated terminals of the Neurohypophysis, initiating release of intraterminal Ca^{2+} from ryanodine-sensitive stores via cyclic ADP ribose (cADPR) signaling pathway mediating the inhibition of VGCC and subsequent release (Fig.1.5).

Opioid modulation of VGCC in Hypothalamic Neurohypophysial system

In the Neurohypophysis, like in other systems, Ca^{2+} influx through activation of presynaptic high threshold voltage-gated calcium channels is essential to induce large bulk neuropeptide release (Cazalis et al., 1987b; Giovannucci and Stuenkel, 1997; Wang et al., 1997a; Wang et al., 1999b). Endogenous opioid inputs to the magnocellular neurosecretory system (See Fig. 1.6) which potentially target VGCC include enkephalins from the pars intermedia (Rossier et al., 1980), endomorphin from the arcuate nucleus (Chen et al., 2004; Hui et al., 2006), met-enkephalin and dynorphin A, co-released with OT and AVP respectively (Bondy et al., 1988; Leng et al., 1994). Studies showed that in the HNS, both AVP and OT are inhibited during electrically stimulated release by opioid agonists (Clarke et al., 1979; Bicknell and Leng, 1981; Clarke et al., 1981; Bicknell et al.,

1985b; Nordmann et al., 1986a; Bondy et al., 1988). Furthermore, a pure preparation of isolated terminals from the Neurohypophysis also showed a reduction in depolarization-induced release of both OT and AVP in response to both μ - (Zhao et al., 1988b; Leng et al., 1992; Russell et al., 1995a; Ortiz-Miranda et al., 2003; Ortiz-Miranda et al., 2005) and κ -opioid (Bicknell et al., 1988; Bondy et al., 1988; Zhao et al., 1988a; Rusin et al., 1997b) receptor activation. This strongly suggests that opioid inhibition of neuropeptide release is acting directly on the terminal receptors and not on surrounding pituicytes (Zhao et al., 1988c). This inhibition has been shown to target specifically, high-threshold voltage-gated calcium channels (Rusin et al., 1997b; Ortiz-Miranda et al., 2003; Ortiz-Miranda et al., 2005). Unlike μ -opioid inhibition, κ -inhibition may target both OT and AVP release differently depending on the specific κ -receptor agonist (Zhao et al., 1988a). A mechanism for the net inhibition of macro calcium currents in response to either μ - or κ -opioid inhibition is being currently proposed (See Chapter VI).

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Figure 1.1

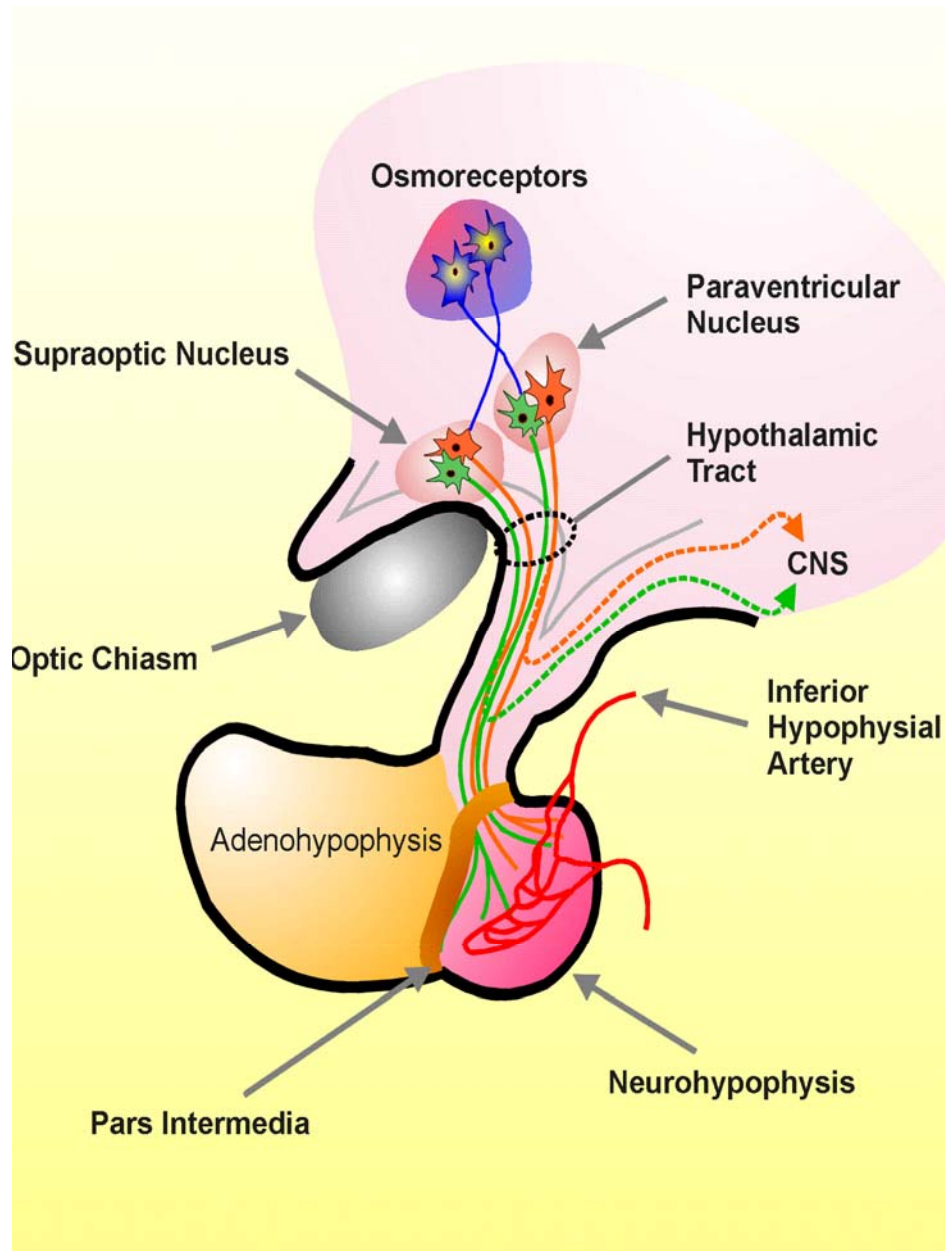


Figure 1.1 The hypothalamo-neurohypophysial system contains magnocellular neurosecretory cells which send projections down to the neurohypophysis. Their terminals secrete arginine-vasopressin (AVP) and oxytocin (OT) into the capillary bed. Figure from Lemos, 2002.

Figure 1.2

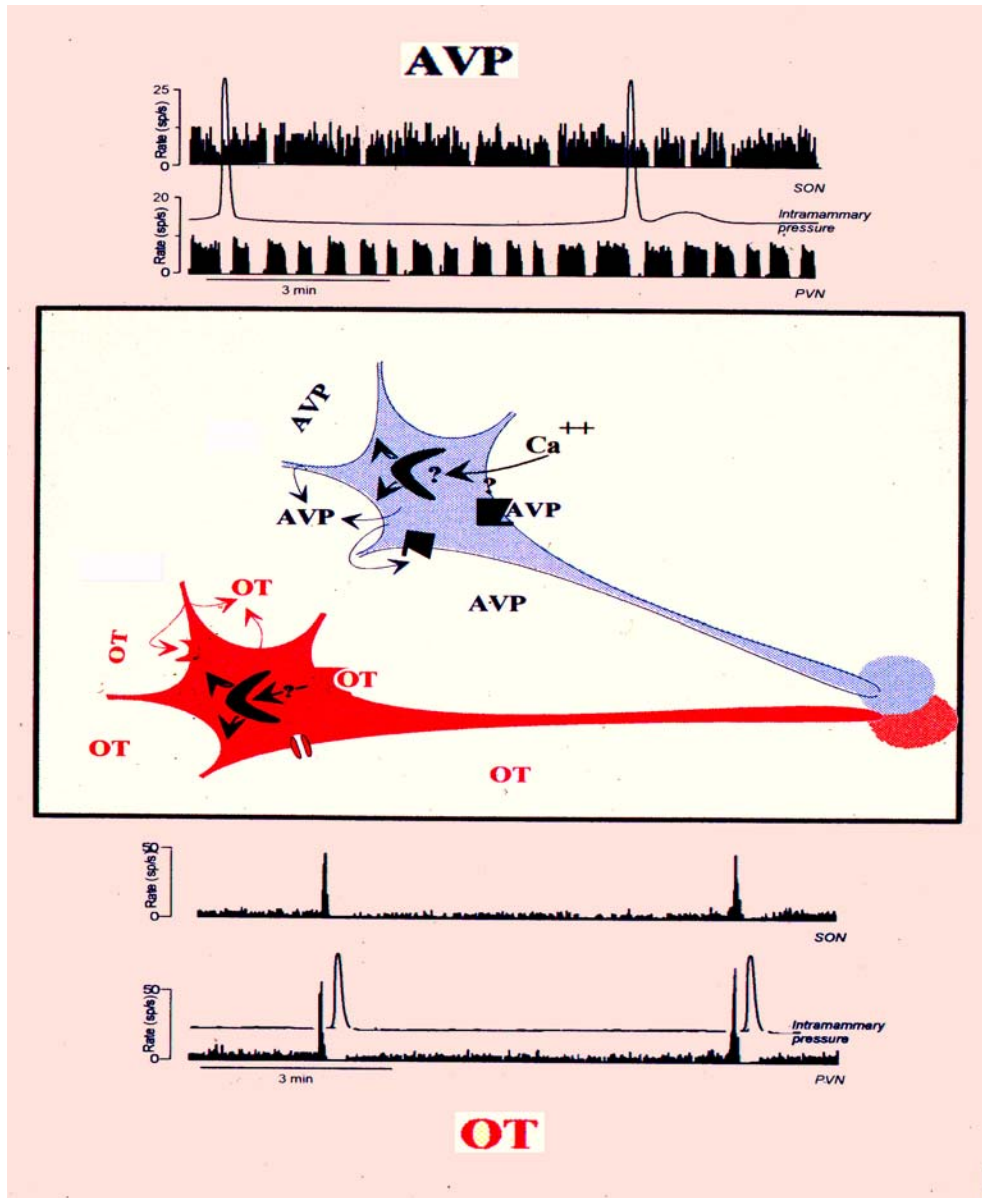


Figure 1.2 **Burst Frequency is important in Neuropeptide Secretion.** AVP

and OT release are elicited through action potentials which travel down from the cell bodies (middle box). Both AVP and OT secretion is determined by the frequency of firing in bursts of action potentials. Bottom traces show OT secretion is optimized with firing frequencies between 65 and 100 Hz. Top traces show secretion of AVP from the neurohypophysis is optimized by short phasic bursts of action potentials with a mean intraburst frequency around 10 -20 Hz. Figure adapted from Dr. Govindan Dayanithi (U. Montpellier II, France).

Figure 1.3

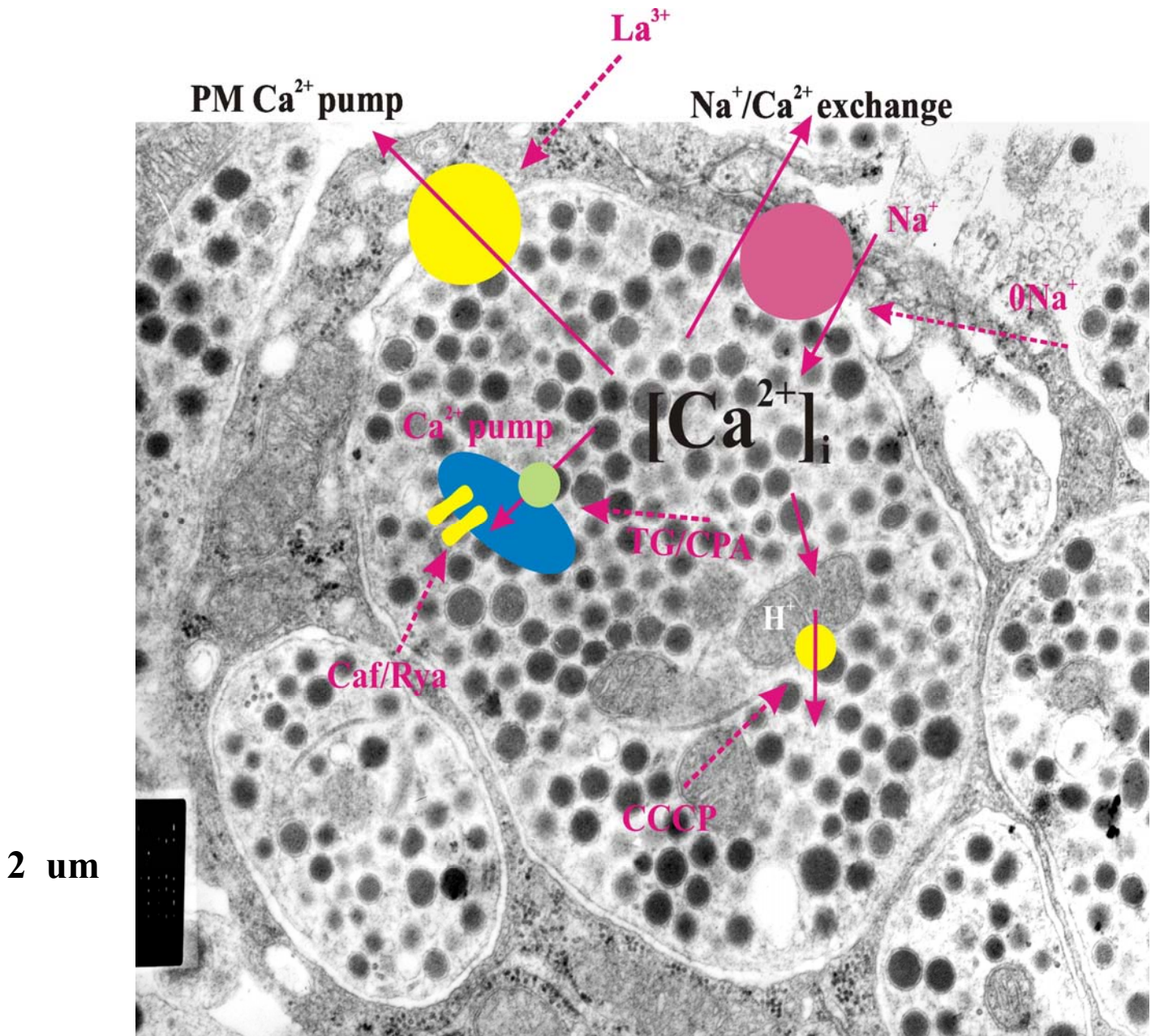


Figure 1.3 Potential intracellular calcium stores. An EM of individual terminals in the neurohypophysis shows organelles such as the mitochondria as well as secretory granules containing AVP or OT. Outlined are potential intracellular calcium stores, and calcium extrusion mechanisms known to be present in the HNS terminals, as well as pharmacological agents that block each of these. Figure from Sasaki et al., 2005.

Figure 1.4

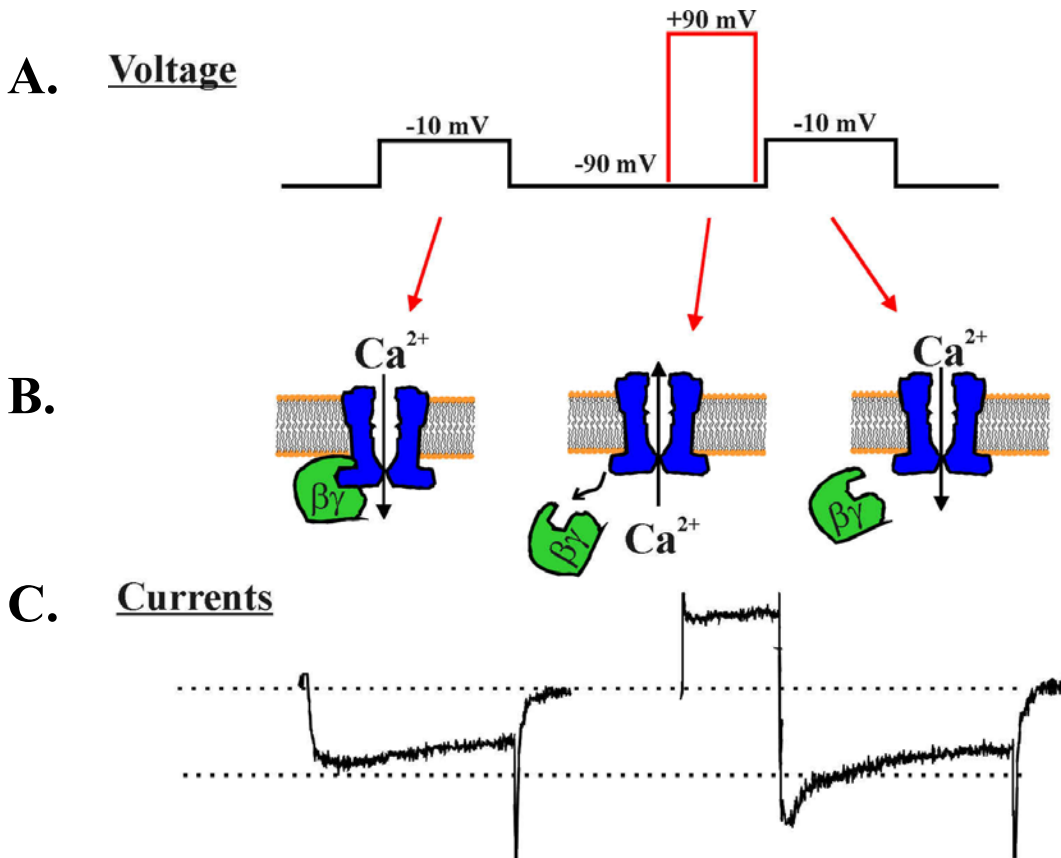


Figure 1.4 Depolarizing Pre-Pulse Protocol. **A.** Voltage protocols designed for the application of a depolarizing Pre-Pulse (DPP). **B.** The DPP was designed to knock-off the G $\beta\gamma$ subunit from direct inhibitory association to the cytoplasmic link I-II region of the VGCC. **C.** Dissociation of the G $\beta\gamma$ subunits relieves VGCC inhibition allowing increased calcium influx through the channel. Figure inspired by C.F. Barrett, Ph.D thesis (2001).

Figure 1.5

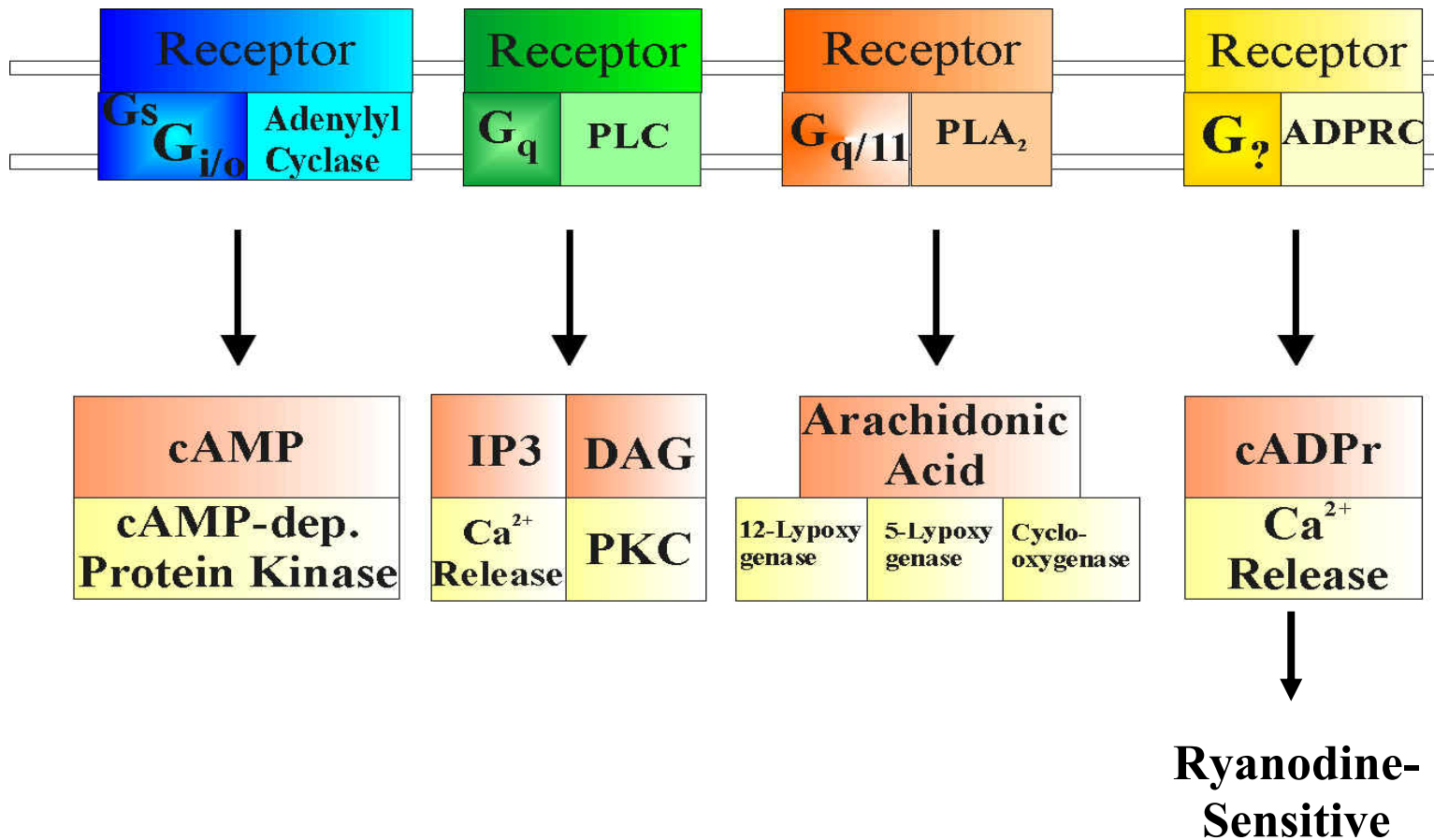


Figure 1.5. Membrane diffusible second-messenger signaling. Classic $G\alpha$ coupling with different diffusible second-messenger systems. G_s and $G_{i/o}$ are typically associated with the activation or inhibition, respectively, of adenylyl cyclase leading to changes in cAMP intracellular concentrations. G_q activation is characterized by activation of the phospholipase C pathway which activates IP₃ receptors liberating internal calcium from IP₃ calcium stores, diacylglycerol and protein kinase C. $G_{q/11}$ has been shown to activate the phospholipase A₂ pathway. A $G\alpha$ protein mediates activation of the ADP ribose cyclase initiating production of cADPr and release of intracellular calcium from ryanodine-sensitive stores. Figure modified from Sigma Signal Transduction Catalogue (2004).

Figure 1.6

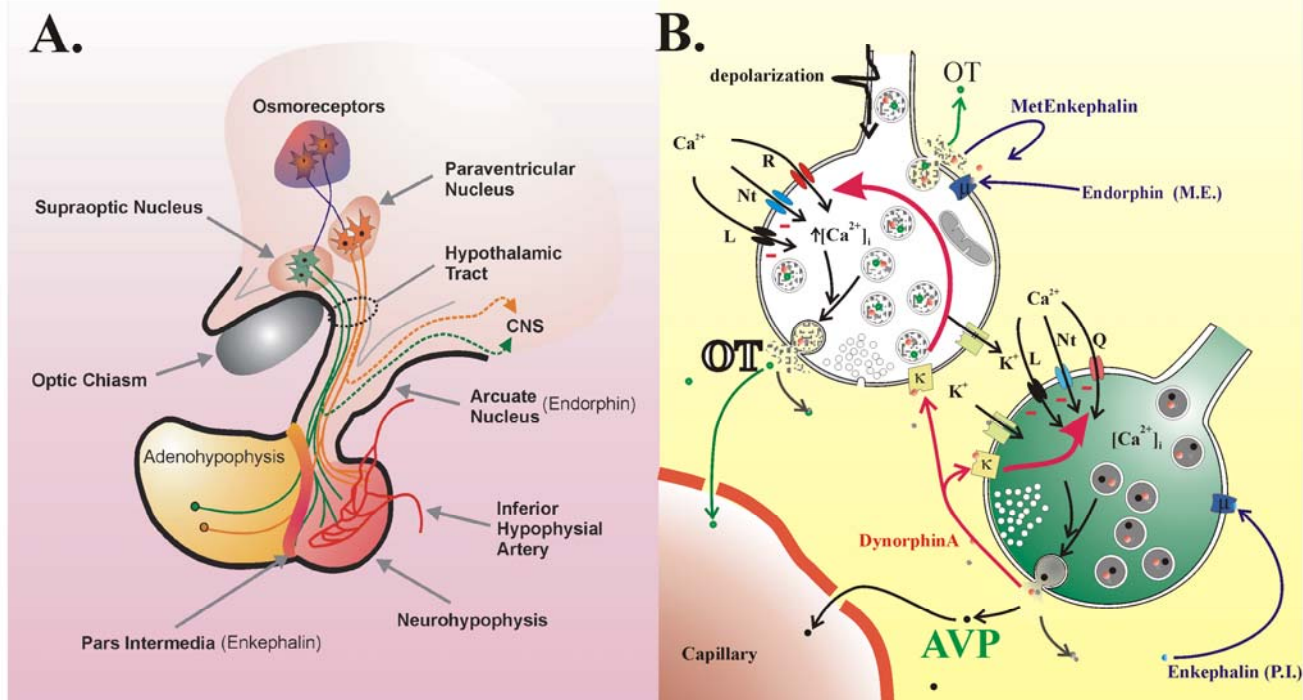


Figure 1.6 The hypothalamo-neurohypophysial system contains magnocellular neurosecretory cells which send projections down to the neurohypophysis. Endogenous opioid inputs to the magnocellular neurosecretory system which potentially target VCGG include enkephalins from the pars intermedia (Rossier et al., 1980), endomorphin from the arcuate nucleus (Chen et al., 2004; Hui et al., 2006), met-enkephalin (Leng et al., 1994) and dyanorphin A co-released with OT and AVP respectively (Bondy, 1988) (A). Diagram of individual terminals in the neurohypophysis shows the presence of both κ - and μ -opioid receptors and the respective voltage-gated calcium channels (VGCC) present in each terminal subtype. Also shown are organelles such as the mitochondria as well as secretory granules containing AVP or OT. Arrows indicate the release of AVP (co-released with Dyanorphin A) and OT (co-released with MetEnkephalin) along with endogenous opioid input from other systems and following κ -receptor activation, intraterminal arrows indicate VGCC targets (B). Modified and/or adapted from Lemos et al., 2002, and Ortiz-Miranda, unpublished.

Chapter II

Extracellular calcium is not necessary for all depolarization-induced neuropeptide secretion from CNS terminals

Extracellular calcium is not necessary for all depolarization-induced neuropeptide secretion from CNS terminals

Cristina Velázquez-Marrero, Edward E. Custer, Valérie De Crescenzo, Kevin E. Fogarty, John V. Walsh, Jr., and José R. Lemos

Depolarization-secretion (DSC) coupling is thought to be exclusively dependent on extracellular calcium (Douglas and Poisner, 1964; Katz, 1969). Competing theories (Parnas and Parnas, 1986; Zhang and Zhou, 2002; Khanin et al., 2006), however, hypothesize that voltage may be a key factor in initiating fast release. Recently, voltage-activated ryanodine-sensitive Ca^{2+} release events (“syntillas”) have been described in neurohypophysial terminals (De Crescenzo et al., 2004a; De Crescenzo et al., 2006). Could mobilization of Ca^{2+} from intracellular stores by depolarization trigger neuropeptide release? We now demonstrate that depolarization in the absence of extracellular calcium induced by either High K^+ or electrical stimulation can give rise to neuropeptide release from Hypothalamic Neurohypophysial System (HNS) terminals. Agents that block voltage-gated calcium channels (VGCCs) did not block this depolarization-induced release. To help determine whether intraterminal calcium was involved in neuropeptide release in response to High K^+ in $0 \text{ mM} = [\text{Ca}^{2+}]_o$, increases in intracellular calcium were buffered by pre-incubating with Bapta-AM. This resulted in over 50% inhibition of release by both High K^+ stimulation in $0 \text{ mM} [\text{Ca}^{2+}]_o$, as well as in normal extracellular Ca^{2+} . The remaining intraterminal calcium-independent neuropeptide release may be due to calcium-independent voltage-dependent (CIVD) release (Zhang and Zhou, 2002; Zhang et al., 2004; Yang et al., 2005). Given the existence of ryanodine- and voltage-sensitive Ca^{2+} stores in HSN terminals (De Crescenzo et al., 2004b; De Crescenzo et al.,

2006) we tested whether these stores could elicit neuropeptide release. Interestingly, caffeine (3-20 mM) and other RyR agonists, such as ryanodine (10 μ M), and imperatoxin A (10 and 100 nM), elicited peptide release from populations of isolated HNS terminals in the absence of extracellular calcium. These agonists also elicited increases in intraterminal calcium as did High K^+ in 0 mM $[Ca^{2+}]_o$. However, in the absence of $[Ca^{2+}]_o$ caffeine- but not High K^+ -evoked release of neuropeptide was partially inhibited by 100 μ M ryanodine. This strongly suggested that ryanodine-sensitive voltage-activated Ca^{2+} stores are not involved in High K^+ -evoked (in 0 mM $[Ca^{2+}]_o$) release. Nevertheless, our results clearly show that extracellular calcium is not necessary for all depolarization-induced neuropeptide secretion from these CNS terminals.

INTRODUCTION

Calcium influx through voltage-gated calcium channels (VGCC) has had a central role in neurotransmitter release since studies first identified its importance in presynaptic function and exocytosis (Douglas and Poisner, 1964; Katz, 1969; Llinas, 1977; Silinsky, 1985; Wojtowicz and Atwood, 1986; Augustine et al., 1987; Zucker, 1993; Berridge, 1998; Branchaw et al., 1998; Kasai et al., 1999; Atwood, 2006). In contrast, Ca^{2+} release from internal stores in nerve terminals is poorly understood and somewhat controversial. Nevertheless, mounting evidence suggests that internal Ca^{2+} stores contribute to presynaptic function. Ca^{2+} influx during action potentials triggers Ca^{2+} -induced calcium release (CICR) from ryanodine-sensitive stores (Narita et al., 2000) and caffeine suppression of glutamate release can regulate synaptic transmission from retinal rod photoreceptors (Krizaj et al., 1999). Furthermore, Ryanodine-sensitive calcium release and Ca^{2+} /calmodulin-dependent kinase II (CaMKII) have been shown to be essential for post-tetanic potentiation of neuropeptide secretion (Shakiryanova et al., 2007).

The Hypothalamic-Neurohypophysial system (HNS) has proven to be a useful model system to study depolarization-secretion coupling (Wang et al., 1993a; Lemos, 2002). The peptide hormones arginine-vasopressin (AVP) and oxytocin (OT) are released from nerve terminals of the magnocellular neurons (MCN) of the neurohypophysis. Although it had been previously reported that there were no caffeine-sensitive stores in the neurohypophysis (Stuenkel, 1994), recent work (De Crescenzo et al., 2004a) has identified voltage-sensitive Ca-sparks (“syntillas”) which emanate from ryanodine-sensitive stores. In the HNS, High K^{+}

and electrical-stimulation induces peptide release from the HNS, which is not completely inhibited by VGCC blockers, such as toxins (Wang et al., 1999a; Wang et al., 2002a) or $\text{Ni}^{2+}/\text{Cd}^{2+}$ (Cazalis et al., 1987b; Stuenkel and Nordmann, 1993b; Turner and Stuenkel, 1998). However, there has been no demonstration of a mechanism whereby depolarization, in the absence of Ca^{2+} influx, causes neuropeptide release from terminals through release of calcium from internal stores. Here we demonstrate that release of both AVP and OT is increased by depolarization in the absence of calcium influx through VGCCs. However, unexpectedly, this release is due, in part, to intraterminal calcium increases from ryanodine-insensitive stores. To the best of our knowledge, this is the first direct demonstration of voltage-dependent yet extracellular calcium-independent neuropeptide release elicited in part by calcium stores within nerve terminals.

METHODS

Assay for peptide release

The experiments were conducted on freshly dissociated Neurohypophysial NH nerve terminals of male Swiss Webster adult (6-8 weeks) mice (Taconic Farms, USA) unless otherwise stated. The mice were cervically dislocated and decapitated (as approved by the University of Massachusetts Medical School protocol A-1135), the brain was removed and the pituitary then excised. The isolated neurohypophysis was homogenized in a solution containing (mM); sucrose, 270; Tris-Hepes, 10 (pH 7.25); EGTA 0.2. The homogenate was centrifuged at 100xg for 2 min. and the resulting pellet was centrifuged at 2400 x g for 6 min. The final pellet contains highly purified nerve terminals. The isolated nerve terminals were loaded onto filters (0.45 mm Acrodisc, Gelman Scientific, Ann Arbor, MI, USA) and perfused at 37°C with Locke's solution (Cazalis et al., 1987c). The modified Locke's solution contains (mM): NaCl, 100; CaCl₂, 0.0022; KCl, 5; *N*-methyl-D-glucamine (NMG)-Cl₂, 45; MgCl₂, 1; glucose, 10; HEPES, 10 (pH 7.4). Ca²⁺ free solution contained (mM): NaCl, 100; EGTA, 0.002; KCl, 5; *N*-methyl-D-glucamine (NMG)-Cl₂, 45; MgCl₂, 1; glucose, 10; HEPES, 10 (pH 7.4), and gave a calculated free [Ca²⁺] of zero. The Ca²⁺ free solution was further tested using a Ca²⁺ probe which determined the Ca²⁺ concentration in the solution to be below 10 nM, its lowest sensitivity reading. Fractions of perfusate were collected at 4 min. intervals and release was evoked by a 30-45 min. exposure to Locke's with High K⁺ (45 mM NMG-Cl exchanged for 45 mM KCl) or ryanodine agonists, e.g. 20 mM caffeine (Sigma). A specific and sensitive enzyme-linked immunoassay (ELISA: Assay Designs, Inc.; Ann Arbor, MI) was used to determine the content of AVP and/or OT for individual terminals

isolated and collected as described above. The results are given as AVP or OT release per fraction measured. In all cases, data are reported as mean \pm SEM; n being the number of terminals or loaded filters. Statistical analysis of differences were made with paired t-tests, with $p \leq 0.05$ considered significant.

Calcium imaging

Freshly dissociated nerve terminals (Nordmann et al., 1987) prepared from adult Swiss Webster mice (De Crescenzo et al., 2004a) were incubated with 2.5 μ M Fura-2 AM for 45 min. at 37°C and thoroughly washed with Normal Locke's solution. Normal Locke's contained (mM): 145 NaCl, 5 KCl, 10 Hepes, 10 Glucose, 1 MgCl₂ and 2.2 CaCl₂, pH 7.4. Ca²⁺ free bath solution contained (mM): 145 NaCl, 5 KCl, 10 Hepes, 10 Glucose, 0.0002 EGTA, 1 MgCl₂, pH 7.4, and gave a calculated free [Ca²⁺] of zero. The Ca²⁺ free bath solution was further tested using a Ca²⁺ probe which determined the Ca²⁺ concentration in the solution to be below 10 nM, its lowest sensitivity reading. Cytosolic [Ca²⁺] was determined with ratiometric indicator fura-2 AM loaded terminals and calibrated utilizing an *in-vitro* calibration kit (Invitrogen, Carlsbad CA). This was performed according to the method of (Grynkiewicz et al., 1985) with an assumed Ca²⁺-fura 2 KD of 200 nM, as previously described (Becker et al., 1989). Resting values for global cytosolic [Ca²⁺] in the presence and absence of extracellular Ca²⁺ were 73.3 \pm 6.9 nM ($n = 12$) and 46.2 \pm 7.5 nM ($n = 8$), respectively, and these values demonstrated a statistically significant difference ($p < 0.05$). In all cases, data are reported as mean \pm SEM; n being the number of terminals. Statistical analyses of differences were made with paired t-tests, with $p < 0.05$ considered significant.

Fluorescence images using Fura-2 AM as a calcium indicator were viewed with a Nikon Diaphot TMD microscope, using a Zeiss Plan- NEOFLUAR 100X oil immersion lens, and fitted with a Photometrics SenSys CCD camera. The camera was interfaced to the inverted microscope adapted with a Chroma 71000A Fura2 filter cube. The terminals were excited using a Xenon arc lamp within a Lambda DG4 high-speed filter changer (Sutter Instruments Incorporated, Novato, CA) with the appropriate filters (340 and 380 nm wavelengths). Intraterminal emission of Fura-2 Ca^{2+} indicator was gathered at 510 nm wavelength. Fluorescent images were acquired and processed with Axon Imaging Workbench 2.1 software (Axon Instruments, Foster City, CA).

Capacitance Measurements

Freshly dissociated terminals (Nordmann et al., 1987) from adult Swiss Webster mice were plated in Normal Locke's solution with 1.2 mM CaCl_2 . Tight seal "whole terminal" recordings were obtained using the perforated-patch configuration described above. The pipettes resistance ranged from 5-8 M Ω . Perforation of the terminals' membrane was obtained by adding 30 μM amphotericin B (SIGMA) to the pipette solution containing (mM): 145 Cs-gluconate, 15 CsCl, 5 NaCl, 2 MgCl_2 , 7 Glucose, 10 HEPES pH 7.3. The bath solution contained (mM): 145 NaCl, 5 KCl, 1 MgCl_2 , 10 HEPES, 10 Glucose, 1.2 CaCl_2 or 0.2 EGTA, pH 7.5. Capacitance measurements were obtained using the piecewise-linear method (Knott et al., 2007). The changes in capacitance induced by a depolarizing pulse (750 ms duration) were measured 1 second after cessation of stimulus, in order to avoid interference of stimulus "end-tail" effects. These stimulus-induced capacitance changes were measured for isolated terminals (perforated-patch) using the piece-wise method (Neher and

Marty, 1982; Lindau and Neher, 1988; Gillis, 1995). Briefly, the method consists in applying a sinusoidal voltage of low amplitude to the sample (in order to avoid voltage-elicited channel currents) and obtaining the phase shift of the resultant sinusoidal current. Changes in this phase shift (“locked-in”) are used in a formula (computer software that emulates a lock-in amplifier) for the determination of the capacitance changes (i.e., the capacitance that would cause such change of phase shift). The method is sensitive to very small changes in capacitance and, in practice, large baseline capacitance and resistance (series) transients must be compensated (i.e., null) before measuring any small capacitance change. In this particular case the parameters used were a sine wave of 1000 Hz at ± 25 mV (about holding potential), with the program reporting a capacitance averaged for every 30 points (24 μ s sampling rate). The current was filtered at a bandwidth of 5000 Hz.

RESULTS

Depolarization- induced release of both neuropeptide and $[Ca^{2+}]_i$:

To investigate whether depolarization-induced release occurs in the absence of extracellular calcium we first monitored hormone release in response to 50 mM KCl (High K^+) challenges in both normal calcium (2.2 mM) Lockes (NL) and calcium-free Lockes buffered with 2 mM EGTA. In NL, AVP hormone release increases to $576 \pm 140\%$ above baseline upon High K^+ challenge. In 0 mM $[Ca^{2+}]_o$, High K^+ is still able to increase AVP

release $223 \pm 31\%$ above baseline (Fig. 2.1A). High K^+ -induced neuropeptide release in the absence of extracellular calcium represents 39% of the release observed in 2.2 mM $[Ca^{2+}]_o$. Previous experiments had shown that High K^+ induced release occurred with 2.2 mM $[Ca^{2+}]_o$ even in the presence of all known voltage-gated Ca-channel (VGCC) blockers (Wang et al., 1997b; Wang et al., 2002a). This suggested that a fraction of the High K^+ -induced release was VGCC-independent. Furthermore, we now demonstrate that High K^+ -induced $[Ca^{2+}]_i$ rise is only partially blocked by 100 μM Ni^{2+}/Cd^{2+} (Fig.2.2), well characterized non-specific VGCC blockers (Salzberg et al., 1983; Cazalis et al., 1987b; Keja et al., 1991; Stuenkel and Nordmann, 1993b; Fisher and Bourque, 1995; Wang et al., 1999b; Luther and Tasker, 2000; Wang et al., 2002b; Marrero and Lemos, 2003). To test whether the release observed with High K^+ in 0 mM $[Ca^{2+}]_o$ was also VGCC-independent we applied these VGCC blockers, Ni^{2+}/Cd^{2+} at 100 μM (Fig. 2.3A&B). Changes in AVP release were quantified as percent release above baseline (Fig. 2.3B). Results show High K^+ in 0 mM $[Ca^{2+}]_o = 112.9 \pm 15.2\%$, similar to High K^+ in 0 mM $[Ca^{2+}]_o$ with 100 μM $Ni^{2+}/Cd^{2+} = 120.9 \pm 26.8\%$. Furthermore, High K^+ in 2.2 mM $[Ca^{2+}]_o$ was $249.7 \pm 58\%$, but High K^+ in 2.2 mM $[Ca^{2+}]_o$ with 100 μM Ni^{2+}/Cd^{2+} was only $85 \pm 19.1\%$. No statistical significant difference was observed between High K^+ -induced release of AVP with and without these calcium channel blockers in the absence of extracellular calcium. There was a statistically significant difference ($p < 0.03$), however, between High K^+ in 2.2 mM $[Ca^{2+}]_o$ with and without 100 μM Ni^{2+}/Cd^{2+} . Furthermore, all treatments with 100 μM Ni^{2+}/Cd^{2+} (with or without $[Ca^{2+}]_o$) and High K^+ in the absence of $[Ca^{2+}]_o$, were not statistically different ($p > 0.05$). This suggests a common

VGCC-independent component to depolarization-induced release in the presence and absence of $[Ca^{2+}]_o$.

Importantly, capacitance measurements on individual HNS terminals in 0 mM $[Ca^{2+}]_o$ show an increase in capacitance of 24.2 ± 4 fF following a 750 ms square pulse stimulus from -80 mV to 0 mV (Fig. 2.1B). This represents a $22.3 \pm 3.6\%$ rise in capacitance in comparison to the same electrical stimulation under normal calcium conditions. The electrical stimulation was chosen for its ability to elicit maximum calcium currents (see inset in Fig. 2.1B) and substantial capacitance increase of 106.6 ± 8.8 fF in 1.2 mM $[Ca^{2+}]_o$ conditions (Fig. 2.1B). Calcium currents under the same 0 mM $[Ca^{2+}]_o$ conditions were completely blocked (Fig. 2.1B inset). Peak capacitance measurements in the absence of extracellular calcium were statistically significantly different from controls ($p < 0.002$; $n = 4$). Therefore, our results demonstrate there is release of neuropeptide from both a population and from individual terminals in response to depolarizing stimuli in the absence of $[Ca^{2+}]_o$.

Changes in $[Ca^{2+}]_i$ during depolarization in the absence of $[Ca^{2+}]_o$:

To investigate whether High K^+ depolarization-induced release in the absence of extracellular calcium is associated with intraterminal calcium changes, we monitored intracellular calcium concentrations in response to a High K^+ challenge. Calcium imaging using fura-2 AM confirmed a cytosolic rise in $[Ca^{2+}]_i$ in response to the High K^+ challenge in 0 mM $[Ca^{2+}]_o$ equivalent to a $150 \pm 50\%$ rise above baseline (Fig. 2.4A) equivalent to 84.2 ± 17.0 nM; ($n = 4$) change in baseline $[Ca^{2+}]_i$. In the presence of 100 μ M ryanodine the change in baseline $[Ca^{2+}]_i = 22.8 \pm 7.2$ nM; ($n = 4$) in response to High K^+ challenge in 0 mM $[Ca^{2+}]_o$ which represents a 73% inhibition by the ryanodine antagonist (Fig. 2.4B). This correlates

well with previously reported measurements of 100 μM ryanodine inhibition of global $[\text{Ca}^{2+}]_i$ rise in response to 400 ms duration depolarizing pulses of 20-40 mV (De Crescenzo et al., 2004a). Therefore, depolarization initiates a rise in intracellular calcium in the absence of extracellular calcium, presumably via release of Ca^{2+} from an intracellular store, possibly ryanodine-sensitive. This leads to an important question. Is the rise in $[\text{Ca}^{2+}]_i$ necessary for depolarization-induced release in the absence of extracellular calcium?

Buffering of $[\text{Ca}^{2+}]_i$ during depolarization- induced release of both neuropeptide and $[\text{Ca}^{2+}]_i$ in the absence of $[\text{Ca}^{2+}]_o$:

To determine if the rise in $[\text{Ca}^{2+}]_i$ in response to High K^+ is necessary for High K^+ - induced release of neuropeptide in 0 mM $[\text{Ca}^{2+}]_o$ we buffered changes in $[\text{Ca}^{2+}]_i$ using Bapta-AM. High K^+ -induced release of neuropeptide in 0 mM $[\text{Ca}^{2+}]_o$ is partially inhibited by preincubation with the calcium chelator, Bapta-AM at 26 μM (Fig. 2.5). High K^+ in 2.2 mM $[\text{Ca}^{2+}]_o$ was inhibited to $47 \pm 5\%$ of control (without Bapta-AM preincubation) and High K^+ in 0 mM $[\text{Ca}^{2+}]_o$ was reduced to $59 \pm 5\%$ of control (without Bapta-AM preincubation) (Fig. 2.5C). Therefore, approx. half of High K^+ in 0 $[\text{Ca}^{2+}]_o$ induced neuropeptide release is due directly to intraterminal calcium. Furthermore, rises in intraterminal calcium were blocked by $98 \pm 4\%$ in High K^+ in 0 mM $[\text{Ca}^{2+}]_o$ and inhibited by $79 \pm 8\%$ when challenged with High K^+ in 2.2 mM $[\text{Ca}^{2+}]_o$ (Fig. 2.6). HNS terminals pre-incubated with 26 μM Bapta-AM demonstrated no statistically significant ($p > 0.05$) rise of $[\text{Ca}^{2+}]_i$ in response to High K^+ in 0 mM $[\text{Ca}^{2+}]_o$ with respect to baseline $[\text{Ca}^{2+}]_i$. There was, however, a statistically significant difference between the 26 μM Bapta-AM pre-incubated terminals in NL in response to High K^+ ($p < 0.05$). Presumably influx of Ca^{2+} through VGCC when in NL may saturate Bapta's

ability to buffer $[Ca^{2+}]_i$. This however, is clearly not the case when in 0 mM $[Ca^{2+}]_o$. Therefore, persistent release of neuropeptide in response to High K^+ after pre-incubation with Bapta-AM in 0 mM $[Ca^{2+}]_o$, strongly suggests there is a calcium-independent yet voltage-dependent component to depolarization-induced extracellular Ca^{2+} -independent release (ECIR). Furthermore, 41% of the depolarization-induced ECIR is intraterminal Ca^{2+} -dependent. The question becomes whether the intraterminal Ca^{2+} -dependent portion of neuropeptide release is due to Ca^{2+} released from ryanodine- and voltage-sensitive stores.

Effects of ryanodine agonists on neuropeptide release:

Since release of $[Ca^{2+}]_i$ from intraterminal stores is partially responsible for depolarization-induced ECIR, we tested whether release from ryanodine-sensitive stores could elicit neuropeptide release in the absence of $[Ca^{2+}]_o$. Caffeine is a xanthine that releases $[Ca^{2+}]_i$ from intracellular stores by increasing the affinity of the ryanodine receptor for cytoplasmic Ca^{2+} (Pozzan et al., 1994; Hernandez-Cruz et al., 1995). In isolated neurohypophysial terminals, application of caffeine evokes the release of calcium from intracellular stores (De Crescenzo et al., 2004b). This release is typically observed as a transient global increase in $[Ca^{2+}]_i$ reminiscent of that observed with High K^+ in 0 mM $[Ca^{2+}]_o$ (Fig. 2.5A). Figure 2.7B quantifies the average change in $[Ca^{2+}]_i$ in response to 20 mM caffeine = 20.2 ± 3.1 nM; (n=7), representing a 33% rise from baseline. The High K^+ in 0 mM $[Ca^{2+}]_o$ challenge elicited a 24.2 ± 1.9 nM; (n=7), change in $[Ca^{2+}]_i$ which represents a 40% rise from baseline (Fig. 2.7B). The average changes in $[Ca^{2+}]_i$ for the 20 mM caffeine as compared to the High K^+ in 0 mM $[Ca^{2+}]_o$ challenge were not statistically different ($p >$

0.05). However, both challenges were statistically different from baseline $[Ca^{2+}]_i$ values with $p < 3.43e^{-5}$.

Application of 20 mM caffeine evokes concentration-dependent hormone release from a population of HNS terminals (Fig.2.8). Furthermore, at the single terminal level, a 20 mM caffeine in 0 mM $[Ca^{2+}]_o$ challenge results in a rise in membrane capacitance of 103.0 ± 45.6 fF, indicative of exocytotic release. This response is not dependent on VGCCs since 100 μ M Ni^{2+}/Cd^{2+} had no effect on caffeine-evoked hormone release (Fig. 2.3B).

Repetitive challenges with 20 mM Caffeine in calcium free Normal Locke's did not diminish the $[Ca^{2+}]_i$ rises nor the hormone release response (Fig. 2.9) in 0 mM $[Ca^{2+}]_o$. This allowed for cross-comparisons between experimental approaches regardless of technical differences. Four 30 second applications of 20 mM caffeine, 1 min. apart increased neuropeptide release: 98%, 78%, 86%, and 97 % above baseline, respectively. No differences were observed between the release evoked by the first and last application of 20 mM caffeine. Prolonged application (7 min.) of 20 mM caffeine also elicited a sustained response. This further confirmed that the internal calcium stores in the isolated terminals are very difficult to deplete.

Caffeine is by no means the only RyR agonist capable of eliciting hormone release from neurohypophysial terminals. The concentration-dependent response described above extends to Imperatoxin A (Table 2.1), a highly specific RyR agonist (Valdivia et al., 1992). In our hands, Imperatoxin A is capable of inducing both a rise in intracellular calcium and an increase in AVP release at concentrations as low as 0.1 nM. The activation properties of Imperatoxin A during its association to the RyR are known to be concentration-dependent ($EC_{50}=10$ nM), with a fast onset and fully reversible (el-Hayek et al., 1995). Imperatoxin A

showed a similar concentration-dependent response in its ability to evoke both a rise in intraterminal calcium and release of neuropeptide (Table 2.1). Furthermore, application of ryanodine at a 10 μM concentration, also elicited increases in both $[\text{Ca}^{2+}]_i$ and basal hormone release (Table 2.1). Therefore, release of $[\text{Ca}^{2+}]_i$ from ryanodine-sensitive stores is capable of triggering neuropeptide release from HNS terminals.

Ryanodine-sensitive Ca^{2+} stores do not make a significant contribution to voltage-dependent release in the absence of $[\text{Ca}^{2+}]_o$

In some types of cells, ryanodine at higher concentrations (100 μM) can block caffeine-induced release of Ca^{2+} from ryanodine-sensitive intracellular stores (McPherson et al., 1991). Furthermore, caffeine has been shown to cause release of calcium from ryanodine-insensitive stores (Schmid et al., 1990; McNulty and Taylor, 1993; Orkand and Thomas, 1995). Therefore, to determine if the calcium released from ryanodine-sensitive stores is responsible for the intraterminal Ca^{2+} -dependent portion of depolarization-induced ECIR, we tested the effects of an antagonist concentration of ryanodine on the caffeine- and High K^+ -induced neuropeptide release seen in the absence of extracellular calcium. Caffeine- but not High K^+ -evoked (in 0 mM $[\text{Ca}^{2+}]_o$) release of neuropeptide was partially inhibited, by $40 \pm 14\%$, in the presence of 100 μM ryanodine (Fig. 2.10B). Previous reports (De Crescenzo et al., 2004a) have shown that 100 μM ryanodine inhibited the caffeine-induced rise in syntilla frequencies to a similar extent. However, High K^+ -evoked (in 0 mM $[\text{Ca}^{2+}]_o$) neuropeptide release was not statistically different ($p > 0.05$) from High K^+ -evoked (in 0 mM $[\text{Ca}^{2+}]_o$) neuropeptide release in the presence of 100 μM ryanodine (Fig. 2.10B). Interestingly, we have shown that application of 100 μM ryanodine was able to inhibit approximately 75% of

the elevation of $[Ca^{2+}]_i$ due to a High K^+ challenge in 0 mM $[Ca^{2+}]_o$ (Fig. 2.4B). However, we must conclude that release of $[Ca^{2+}]_i$ from voltage- and ryanodine-sensitive stores does not significantly contribute to neuropeptide release in response to High K^+ in 0 mM $[Ca^{2+}]_o$.

DISCUSSION

Our studies show that depolarization, in the absence of external calcium, elicits release of neuropeptide from both individual and populations of HNS terminals. This release of peptide hormone was triggered, at least partially, by release of calcium from intraterminal stores. These findings broaden our view of potential sources of presynaptic calcium and subsequent dependence on extracellular calcium for depolarization-secretion coupling (DSC). The classical precept of DSC states that depolarization of the membrane due to an action potential opens voltage-gated calcium channels, allowing Ca^{2+} ions to flow in. The basis of the Calcium Hypothesis (CH) is that the post-stimulation rise in intracellular calcium, in the vicinity of sites of exocytosis, is the sole trigger for neurotransmitter release (Katz, 1969; Zucker, 1993). The subsequent removal of calcium from these sites terminates the process. Recently work has suggested that Ca^{2+} is required, yet insufficient for fast release to occur. This has led to an alternate Ca^{2+} -voltage hypothesis (CVH) (Parnas et al., 2002; Khanin et al., 2006) which proposes that membrane potential (*i.e.*, depolarization) is the key step in initiating depolarization-induced exocytosis, while repolarization controls its termination. We, and others, have observed that High K^+ and electrical stimuli induce an “extracellular calcium-independent” form of peptide release from the HNS, which is not

completely blocked by VGCC blockers, such as toxins (Wang et al., 1999a; Wang et al., 2002a) or $\text{Ni}^{2+}/\text{Cd}^{2+}$ (Stuenkel and Nordmann, 1993b; Turner and Stuenkel, 1998). This coupled with the recent characterization of voltage-dependent Ca^{2+} syntillas (De Crescenzo et al., 2004a; De Crescenzo et al., 2006) led to the question: could mobilization of intracellular calcium stores lead to the “extracellular calcium-independent” hormone release?

Depolarization-induced ECIR is not due to Ca^{2+} released from ryanodine-sensitive stores

Here we have demonstrated that in isolated HNS terminals depolarization-dependent release observed in 0 mM $[\text{Ca}^{2+}]_o$ is, at least partially, due to release of intracellular Ca^{2+} from calcium stores. To our surprise, part of this intracellular calcium-dependent release was shown to be ryanodine-insensitive. The remaining non-calcium yet voltage-dependent release could be due to changes in intracellular sodium, which has been shown to affect hormone release in this system (Stuenkel and Nordmann, 1993b). Alternatively a yet unknown, presynaptic inhibitory autoreceptor, as proposed by (Parnas and Parnas, 1994), could be responsible for exclusively voltage-dependent release. However, having shown that DSC occurs in the absence of extracellular calcium and independent of VGCCs supports the general premise of the Ca^{2+} -voltage hypothesis. Whether this process modulates or plays a key role in the initiation and/or the termination of physiological release during a burst of action potentials in the HNS remains to be proven.

Possible physiological role of ryanodine-sensitive stores during DSC

Large dense-core vesicle (LDCV) release is characterized by multiple steps involving populations of LDCVs in differing stages of exocytotic readiness (Horrigan and Bookman, 1994; Seward et al., 1995; Giovannucci and Stuenkel, 1997). Early experiments describe a

triphasic capacitance response to depolarizing stimuli of peptidergic nerve terminals characterized by a critical initial “threshold phase” which does not trigger hormone release (Seward et al., 1995). Giovannucci and Stuenkel (1997), suggested that the activation of unknown Ca^{2+} -dependent steps prepare the granules of the readily-releasable pool for secretion. Intraterminal calcium stores may act as pivotal modulators of the state of exocytotic readiness of any given terminal prior to and/or during electrical stimulation. Therefore, one prediction would be that experimentally elevating $[\text{Ca}^{2+}]_i$ via release of intraterminal calcium would meet the threshold calcium level requirement prior to DSC. This could effectively amplify the subsequent depolarization-secretion response by transiently maximizing the number of LDCV in a primed state for release. Such receptor-independent potentiation due to ryanodine-sensitive calcium stores has been recently reported in rat hippocampal area CA1 (Li et al., 2006). These studies showed that caffeine enhances this form of LTP. More recently, RyR and CaMKII have been shown to be essential for post-tetanic potentiation of neuropeptide secretion in *Drosophila* motor neuron terminals (Shakiryanova et al., 2007). In isolated HNS terminals, blocking ryanodine receptors using either 100 μM ryanodine or 8-Br cADPr, attenuated both the depolarization-induced release of OT and the rise in $[\text{Ca}^{2+}]_i$ due to High K^+ , with extraterminal calcium present (Jin et al., 2007). Although our current study shows that ryanodine receptor agonists stimulate basal release of neuropeptide, ryanodine-sensitive stores do not seem to play a significant role in depolarization-induced ECIR. Their contribution may be limited by how much intraterminal calcium is present in an extraterminal calcium free environment. Under physiological conditions amplification of a depolarization-induced release may require calcium influx from VGCC to trigger CICR, thus potentiating the neuropeptide release response.

Other possible stores of intraterminal Ca^{2+} in HNS terminals

Release from intracellular stores in response to High K^+ in calcium free medium may be triggered by release of calcium from inositol- 1,4,5-trisphosphate (IP_3) intracellular stores in the HNS (Sabatier et al., 2004; Ludwig and Leng, 2006). Our results support this possibility given there is a portion of the $[\text{Ca}^{2+}]_i$ rise due to High K^+ stimulation in $0 \text{ mM} = [\text{Ca}^{2+}]_o$ which is not blocked by antagonist concentrations of ryanodine. Furthermore, in permeabilized terminals, treatment with IP_3 induced a 242% rise above baseline of AVP release (Cazalis et al., 1987a) indicating IP_3 stores not only exist in terminals but are capable of participating in release. In magnocellular oxytocin neurons, IP_3 stores have also been shown to contribute to OT release in the absence of extracellular calcium (Richard et al., 1997). Other systems, such as Torpedo cholinergic synaptosomes, show increases in IP_3 production in response to a depolarizing stimulus (Carrasco et al., 1996). However, the exact mechanism involved in IP_3 production during DSC in cholinergic synaptosomes, is still unknown and seems to require the presence of extracellular calcium.

Physiological significance of depolarization-induced ECIR

Concurrently, voltage-dependent calcium-independent release could maintain the efficacy of neuropeptide release at the HNS terminals during high-frequency burst stimulation originating from the MNC neurons in the supraoptic and paraventricular nuclei. During high frequency bursts the interstitial space surrounding the HNS terminals *in situ* would be depleted of Ca^{2+} towards the latter part of the burst, conditions which do not favor calcium influx through VGCC. Zhang and Zhou (2002) have shown in DRG somata, that High K^+ -induced exocytosis occurred in the absence of a detectable $[\text{Ca}^{2+}]_i$ change. They ascribed this as direct evidence of the existence of calcium-independent but voltage-

dependent secretion (CIVDS). Our results demonstrate that both CIVDS and intraterminal calcium release can contribute to the release of neuropeptides from HNS terminals. This is the first time, to the best of our knowledge, that a CIVDS has been found at a peptidergic terminal (Zhang and Zhou, 2002; Zhang et al., 2004; Yang et al., 2005). An extracellular calcium-independent but voltage-dependent mechanism for release could explain the efficacy of release at the end of an HNS burst *in situ* when $[K^+]_o$ would depolarize the NH terminals, but $[Ca^{2+}]_o$ would normally be depleted within the NH interstitial space (Leng and Shibuki, 1987; Leng et al., 1988a; Marrero and Lemos, 2005).

Conclusion

In conclusion, our results indicate that depolarization-induced neuropeptide release is present in the absence of external calcium, and calcium release from ryanodine-insensitive internal stores is an important contributor to this release from NH terminals. We have further shown that in isolated HNS terminals, there is a component of depolarization-induced ECIR that is also independent of intraterminal Ca^{2+} . This strongly suggests that there is a CIVD mechanism in HNS presynaptic structures involved in DSC. Given the *in situ* physiological conditions of most presynaptic structures in the CNS, voltage-dependent and extraterminal calcium-independent neuropeptide release may be an important mechanism involved in DSC during high frequency stimulation at many CNS terminals.

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Figure 2.1

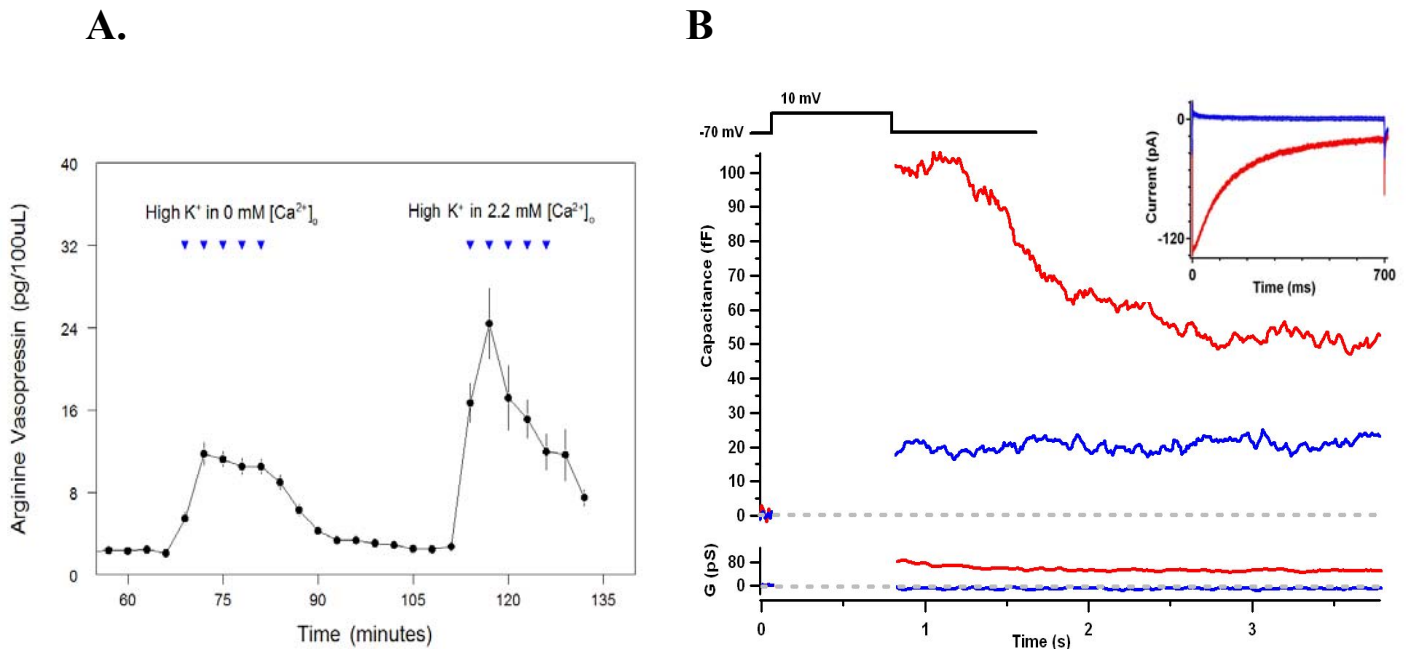


Fig. 2.1 Effect of calcium on depolarization-induced exocytosis. **A.** High K⁺-induced AVP neuropeptide release with and without [Ca²⁺]_o. Fractions collected every 4 min. **B.** Capacitance measurements of single terminal in the presence (red) and absence (blue) of extracellular calcium stimulated with a square pulse of 80 mV for a duration of 750 ms. Inset shows the currents obtained from the actual stimulus (same color indicators). The generalized conductance changes (“G”) are shown for reference. Dashed gray lines represent the zero-change baselines. Peak capacitance measurements in the absence of extracellular calcium were statistically significantly different from controls (p<0.002; n=4).

Figure 2.2

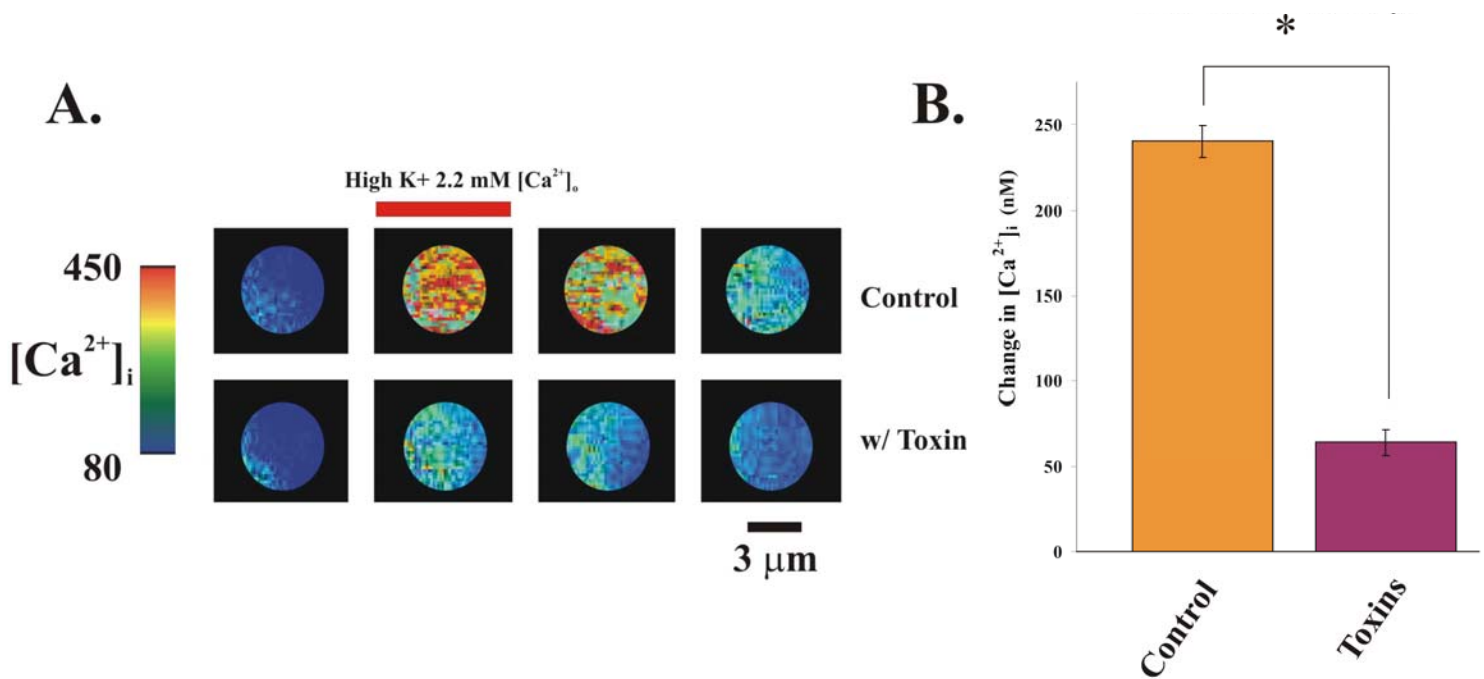


Figure 2.2 Intraterminal calcium rise in response to depolarization in the absence of calcium influx through VGCC. **A.** Images of Fura-2 AM loaded single terminal 4 seconds apart challenged with High K^+ in 2.2 mM $[Ca^{2+}]_o$ for 10 sec. with and without toxins specifically blocking VGCC: Nicardipine (1-2 mM), SNX 482 (20-30 nM), SNX 230 (100-200 nM), ω -conotoxin MVIIC (100 nM) to block L-, R-, N-, and Q-type calcium channels, respectively. Diameter of the terminal is approx. 4 μm . Control baseline $[Ca^{2+}]_i = 96.8 \pm 2.8$ nM and baseline $[Ca^{2+}]_i$ after incubation with toxins 99.4 ± 7.2 nM. **B.** Bar graph of change in $[Ca^{2+}]_i$ in response to High K^+ challenge in 2.2 mM $[Ca^{2+}]_o$ with and without incubation with VGCC blocker toxins (n=8 each). Asterisk (*) represents statistically significant difference ($p < 0.001$).

Figure 2.3

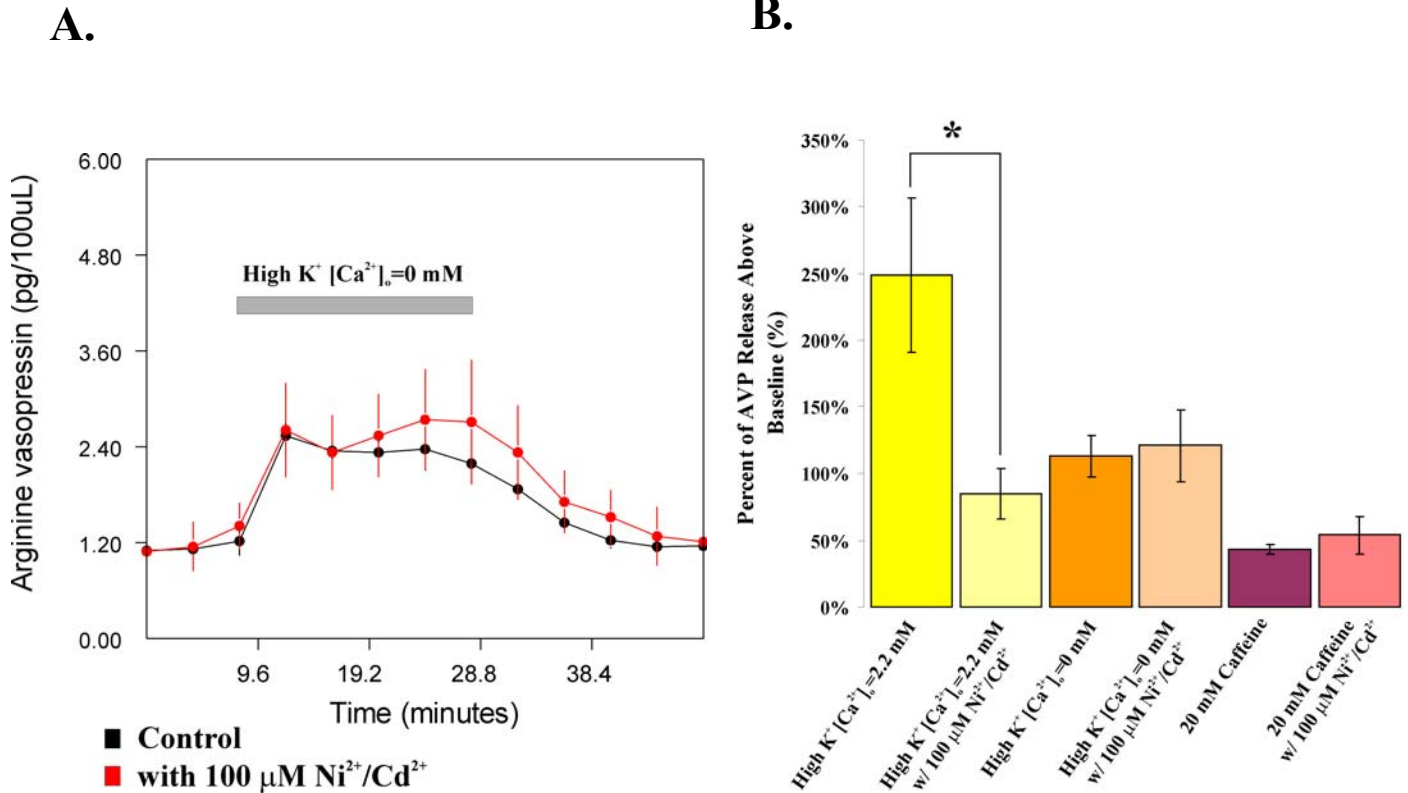


Figure 2.3 AVP release in response to High K⁺ in [Ca²⁺]_o=2.2 mM, High K⁺ in [Ca²⁺]_o=0 mM, and 20 mM caffeine with and without 100 μM Ni²⁺/Cd²⁺. **A.** Release of AVP from rats in response to High K⁺ in 0 mM [Ca²⁺]_o control (black: n=3) and in the presence of 100 μM Ni²⁺/Cd²⁺ (red: n=3). Fractions collected every 4 min. **B.** Bar Graph quantifying the effects of 100 μM Ni²⁺/Cd²⁺ on High K⁺ in 2.2 mM [Ca²⁺]_o, High K⁺ in [Ca²⁺]_o= 0 mM and 20 mM Caffeine in 0 mM=[Ca²⁺]_o. Asterisk (*) represents statistically significant difference (p<0.02).

Figure 2.4

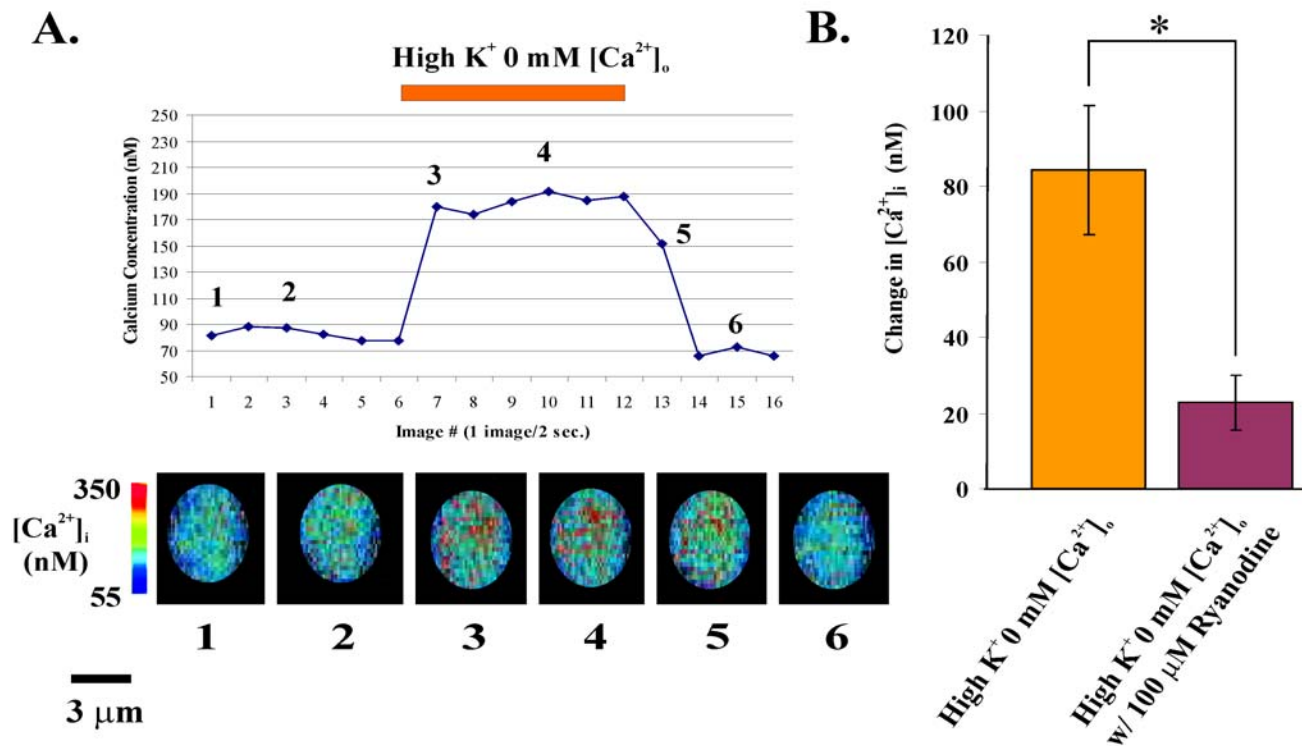


Figure 2.4 Calcium imaging in 0 mM $[Ca^{2+}]_o$ of High K^+ with and without 100 μ M ryanodine. **A.** Calcium Imaging using Fura-2 AM showing intracellular calcium rise in response to 50 mM High K^+ in the absence of extracellular calcium. Line trace points represent an average of intraterminal calcium concentration. Numbers correspond to designated time points. **B.** Bar graph of calcium rise as change in intraterminal calcium concentration (nM) of isolated terminals in 0 mM $[Ca^{2+}]_o$ challenged with 50 mM KCl: Control and with 100 μ M ryanodine. There was a statistically significant difference between Control High K^+ in 0 mM $[Ca^{2+}]_o$ and High K^+ in 0 mM $[Ca^{2+}]_o$ with 100 μ M ryanodine ($p=0.03$). There is a statistical difference from baseline ($p=0.02$) for both treatments. Average baseline $[Ca^{2+}]_i$ for all terminals = 74.9 ± 6.9 nM. Averages were calculated using the total number of points during the treatment and equal number of points for the baseline. Asterisk (*) represents statistically significant difference ($p<0.05$).

Figure 2.5

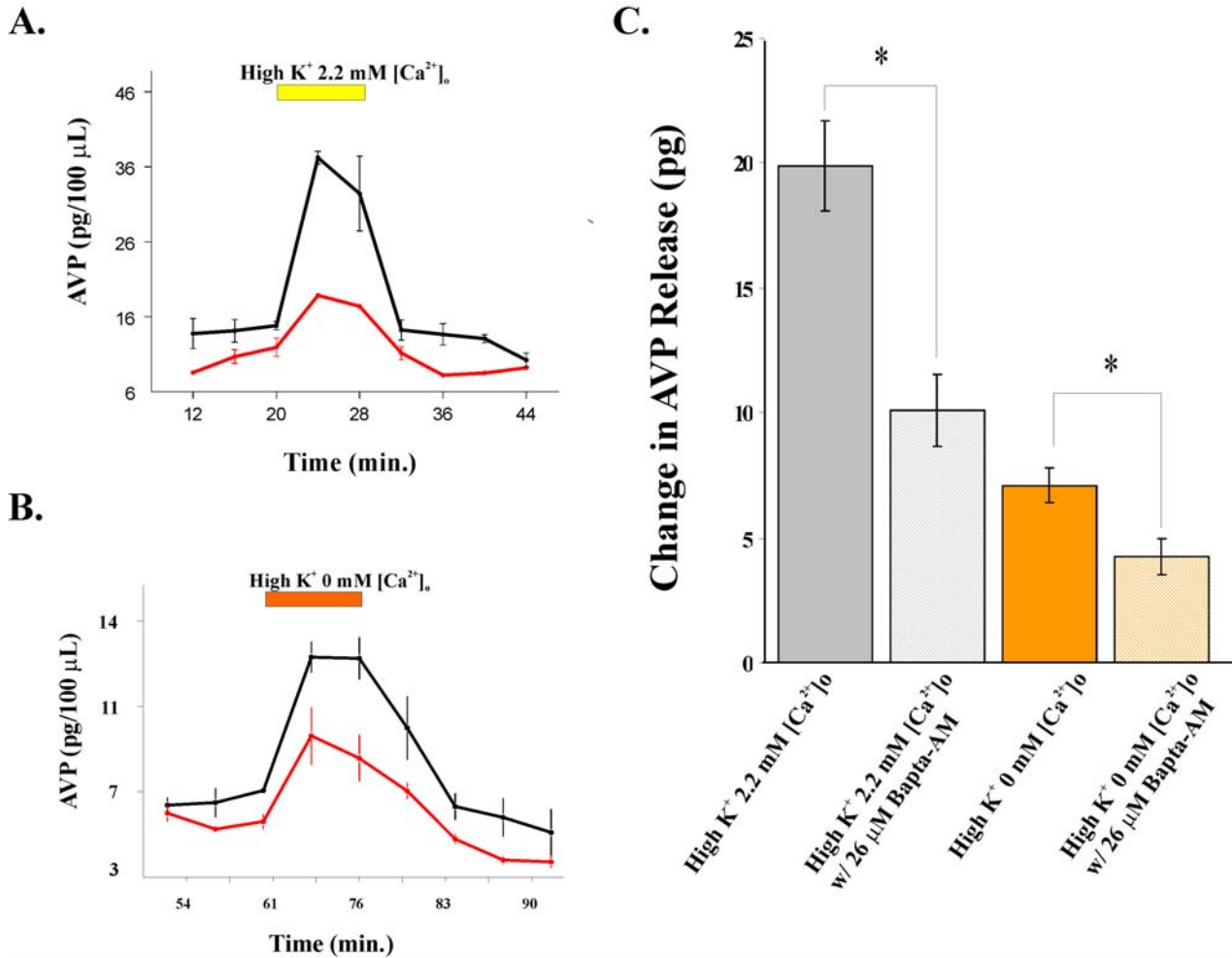


Figure 2.5 Inhibition of AVP release with Bapta-AM pre-incubation. **A.** AVP release induced by High K^+ in 2.2 mM $[Ca^{2+}]_o$ pre-incubated with 26 μ M Bapta-AM (red line) and pre-incubated in carrier alone as control. **B.** AVP release in High K^+ in 0 mM $[Ca^{2+}]_o$ pre-incubated with 26 μ M Bapta-AM (red line) and pre-incubated in carrier alone as control. **C.** Bar graph of Bapta-AM inhibition of High K^+ with and without extraterminal calcium (n=3 each). Averages were calculated using the total number of points during the treatment and equal number of points for the baseline. Asterisks (*) represent statistically significant differences ($p < 0.05$).

Figure 2.6

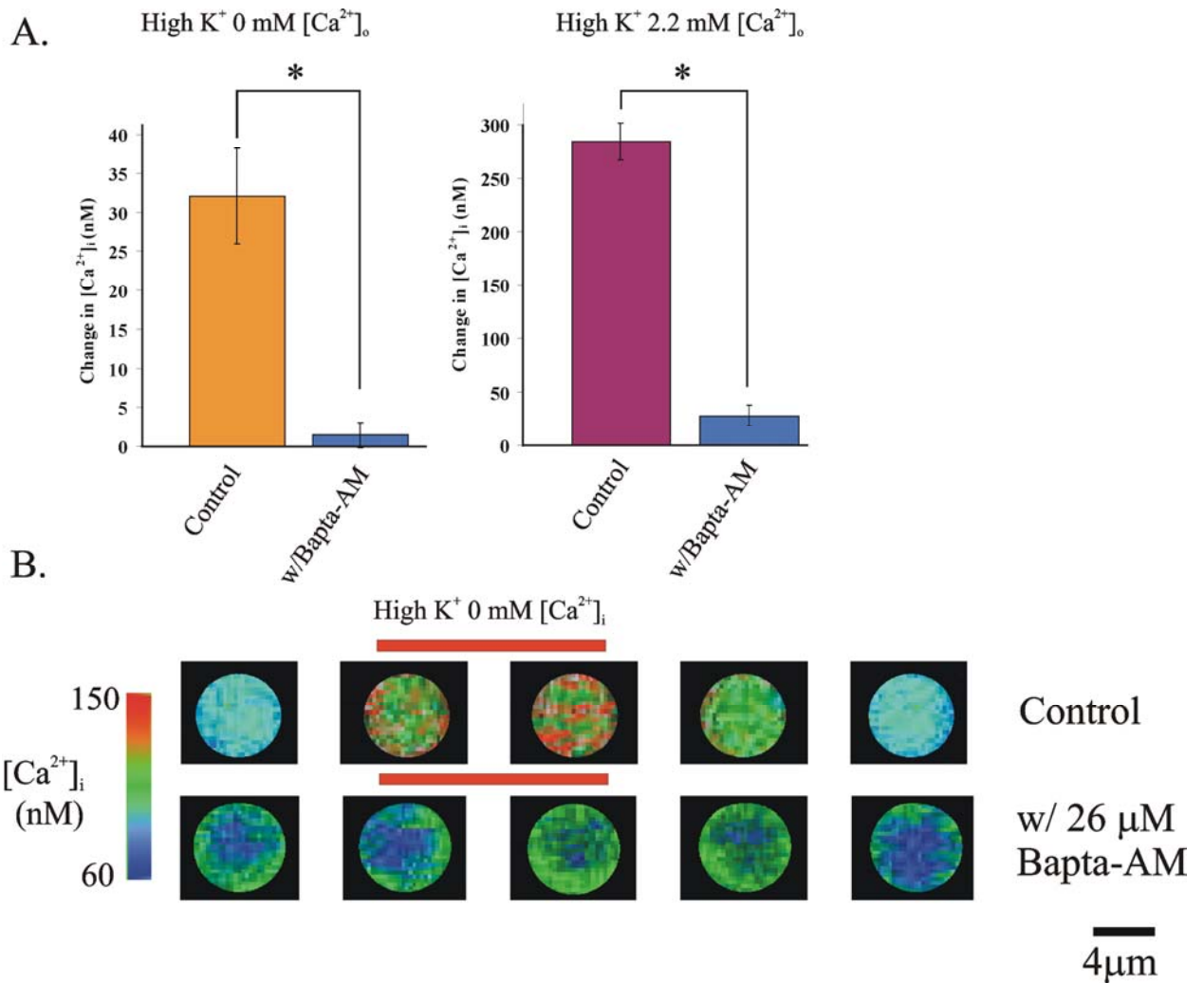


Figure 2.5 Intra-terminal Calcium rise in response to High K^+ with and without pre-incubation with Bapta-AM. **A.** Bar graph of change in $[Ca^{2+}]_i$ in response to High K^+ challenge in 0 mM $[Ca^{2+}]_o$ with and without pre-incubation with 26 μ M Bapta-AM ($n=6$) and with High K^+ challenge in 2.2 mM $[Ca^{2+}]_o$ with and without pre-incubation with 26 μ M Bapta-AM ($n=6$). High K^+ challenge in 0 mM $[Ca^{2+}]_o$ with pre-incubation with 26 μ M Bapta-AM is not statistically different from baseline. All other treatments are statistically different $p<0.01$ from baseline. **B.** Images of Fura-2 AM loaded single terminal 4 seconds apart challenged with High K^+ in 0 mM $[Ca^{2+}]_o$ for 10 sec. with and without pre-incubation with 26 μ M Bapta-AM. Diameter of the terminal is approximately 6 μ m. Control baseline $[Ca^{2+}]_i = 108.3 \pm 12.6$ nM and baseline $[Ca^{2+}]_i$ after pre-incubation with 26 μ M Bapta-AM = 98.6 ± 11.3 nM. Asterisks (*) represent statistically significant differences ($p<0.05$).

Figure 2.7

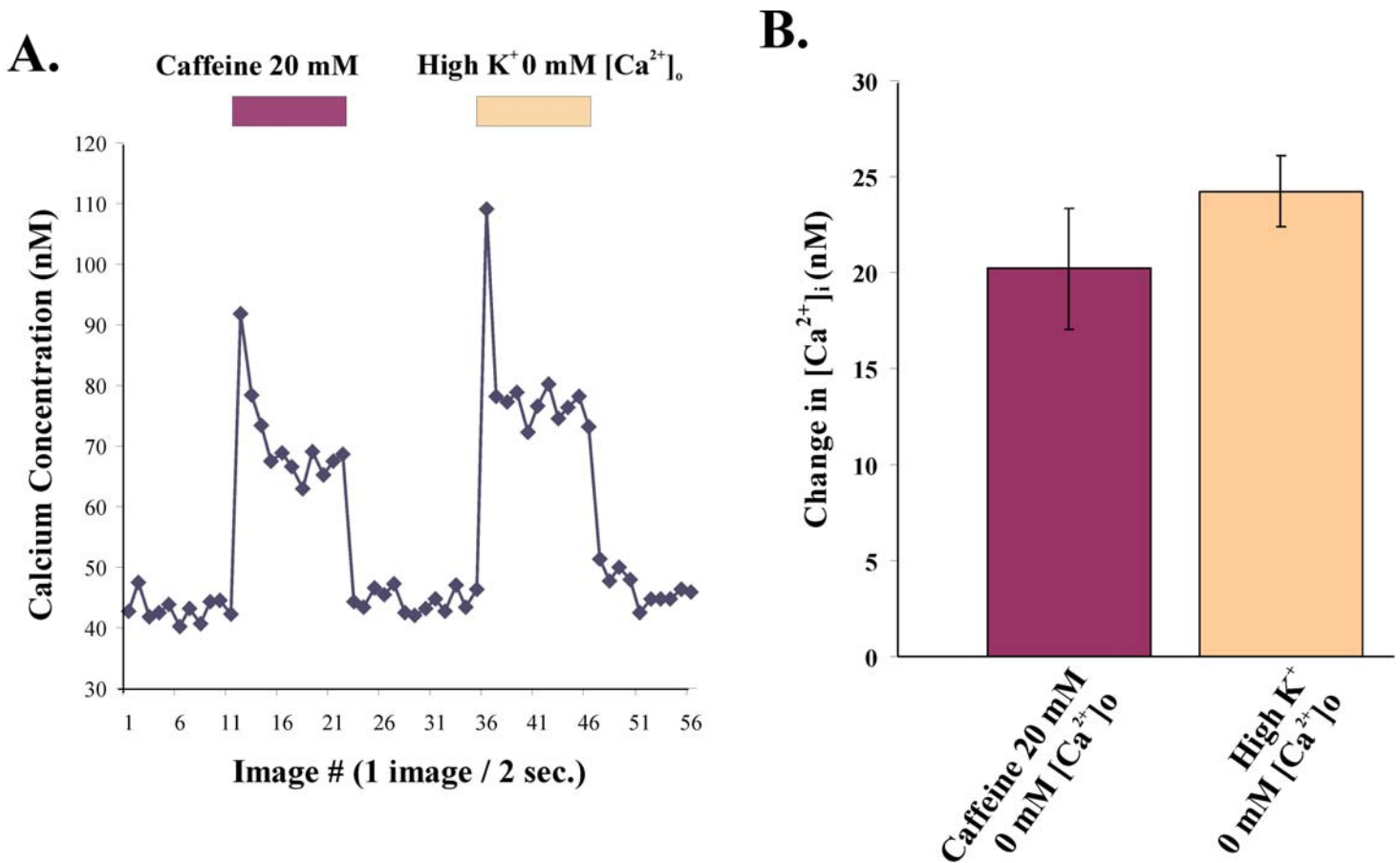


Figure 2.7 Calcium imaging in 0 mM [Ca²⁺]_o in response to High K⁺ and Caffeine. **A.** Trace representing [Ca²⁺]_i changes taken over time (1 image/ 2 sec.) from a single NH terminal. **B.** Bar graph of changes in intraterminal calcium in 0 mM [Ca²⁺]_o when microperfused with High K⁺ or 20 mM Caffeine (n=7 for each). All Averages are statistically significantly different ($p < 3.43 \times 10^{-5}$) as compared to baseline. Mean baseline [Ca²⁺]_i for all terminals = 60.4 ± 9.2 nM. Averages were calculated using the total number of points during the treatment and equal number of points for the baseline.

Figure 2.8

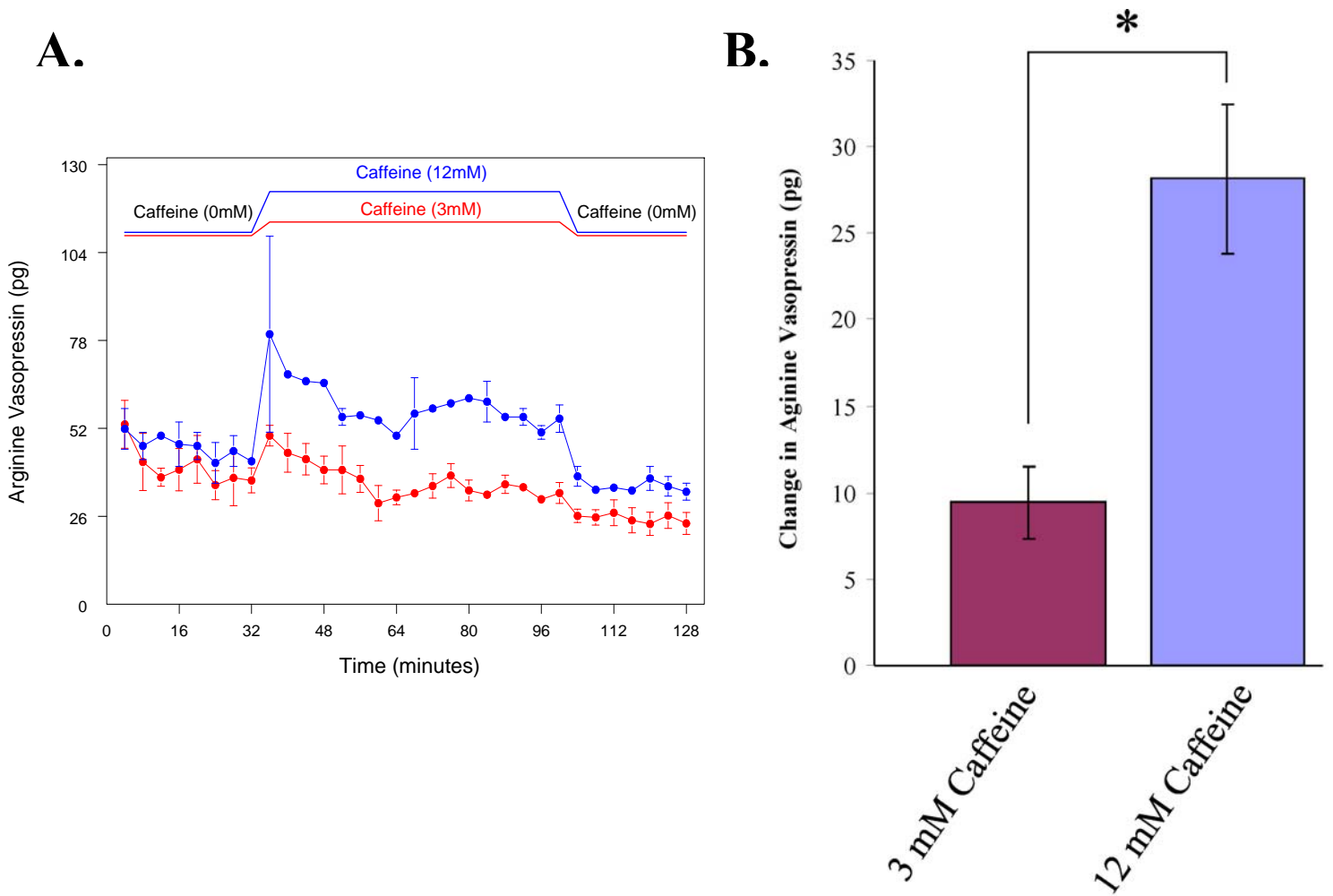


Figure 2.8 Caffeine-induced release of AVP. **A.** Release of arginine-vasopressin (AVP) is evoked in a dose-dependent manner by caffeine. All show similar kinetics of sustained release in the absence of extracellular calcium. Fractions collected every 4 min. **B.** Bar graph of Caffeine-evoked release of AVP at (mM): 3, and 12 (n=3 each) in the absence of extracellular calcium. Averages were calculated using the first three points during the treatment and equal number of points for the baseline. Asterisk (*) represents statistically significant difference (p<0.01).

Figure 2.9

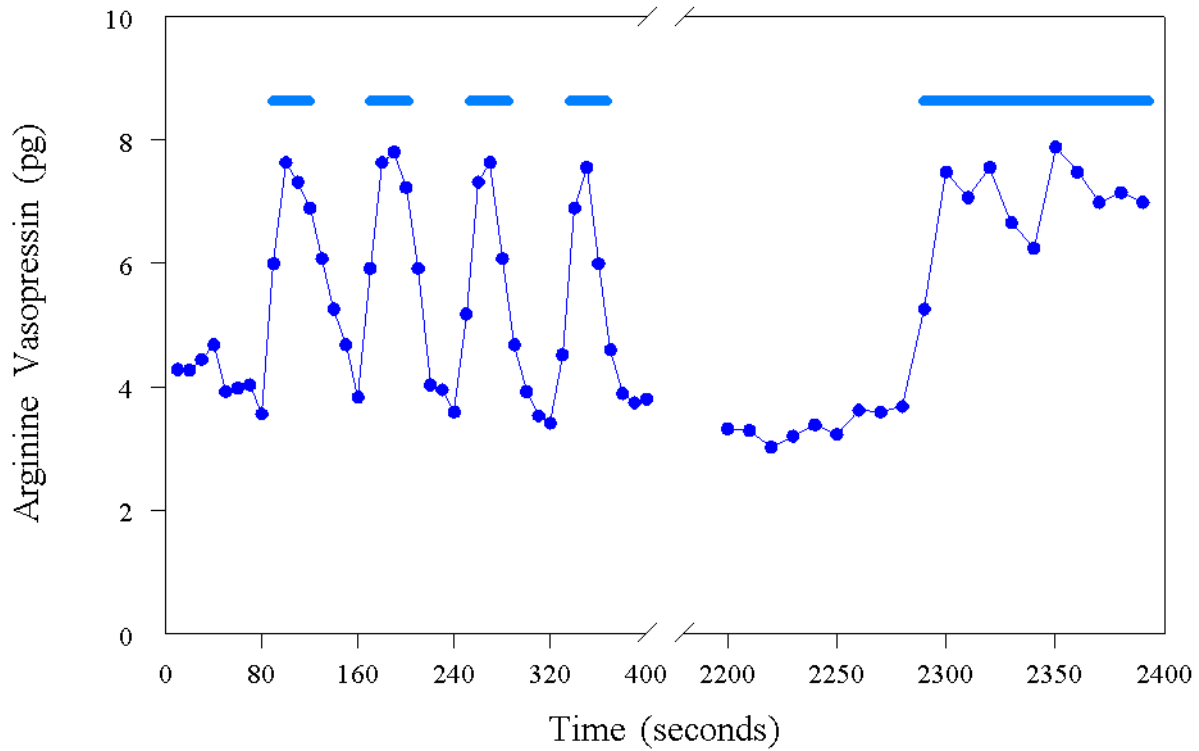


Figure 2.9 Repetitive stimulation with 20 mM Caffeine in 0 mM $[Ca^{2+}]_o$ evoked similar increases in AVP release. Fractions collected every 9 sec. Short blue bars represent 1 min duration, 20 mM Caffeine treatments. A more prolonged 7 min. application (long blue bar) evoked a sustained response throughout.

Table 2.1: Ryanodine receptor agonists trigger release of Arginine Vasopressin and $[Ca^{2+}]_i$.

AVP Neuropeptide Release				
<i>Ryanodine Agonist</i>	<i>Baseline (pg of AVP)</i>	<i>Change (pg of AVP)</i>	<i>Percent Change (%)</i>	<i>Number of lines</i>
Ryanodine 10 μM	14.44 \pm 0.56	6.31 \pm 1.28	43.69	3
Imperatoxin A 10 nM	9.11 \pm 1.69	1.75 \pm 0.32	19.21	3
Imperatoxin A 100 nM	5.25 \pm 0.22	5.17 \pm 0.78	98.48	5
Caffeine 20 mM	11.83 \pm 0.44	8.83 \pm 0.43	74.64	3
Calcium Imaging with Fura-2 AM				
<i>Ryanodine Agonist</i>	<i>Baseline (nM $[Ca^{2+}]_i$)</i>	<i>Change (nM $[Ca^{2+}]_i$)</i>	<i>Percent Change (%)</i>	<i>Number of terminals</i>
Ryanodine 10 μM	89.51 \pm 8.53	18.83 \pm 6.82	21.04	3
Imperatoxin A 10 nM	92.29 \pm 2.88	11.33 \pm 1.00	12.18	4
Imperatoxin A 100 nM	122.72 \pm 12.08	26.08 \pm 6.69	21.25	12
Caffeine 20 mM	60.42 \pm 9.24	20.18 \pm 3.12	33.40	7

Statistically different $p < 0.02$ from baseline for all ryanodine agonists.

Figure 2.10

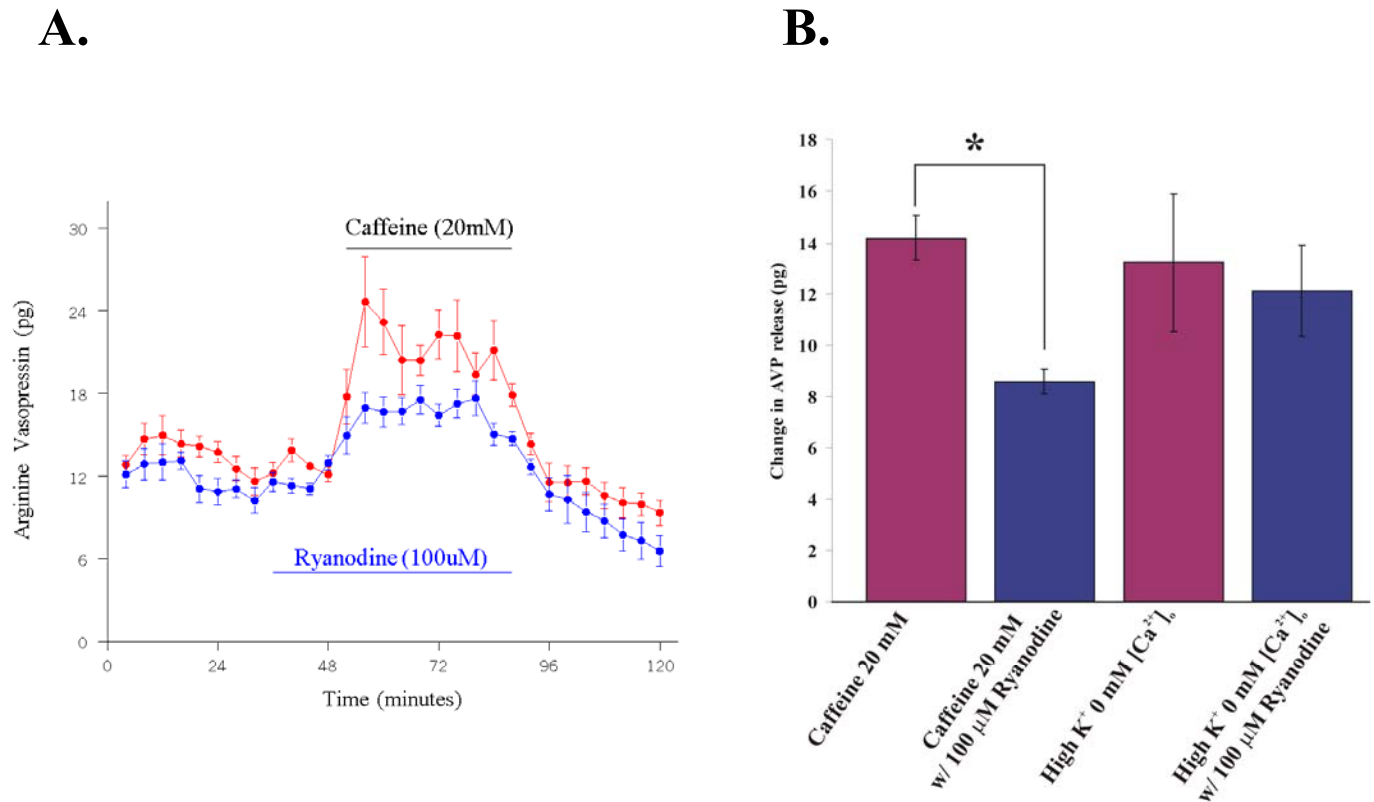


Figure 2.10 Ryanodine (100 μ M) effects on High K^+ - and Caffeine-induced AVP release in 0 mM $[Ca^{2+}]_o$. **A.** Caffeine (20mM) induced AVP release in the presence (red line) and absence (blue line) of 100 μ M Ryanodine. **B.** Bar graph of Caffeine (20mM) and High K^+ in 0 mM $[Ca^{2+}]_o$ induced AVP release in the absence and presence of 100 μ M Ryanodine, (n=3) and (n=6), respectively. Averages were calculated using the same number of points during the treatment as for the baseline. Asterisk (*) represents statistically significant difference (p<0.02).

Chapter III

Voltage-Dependent Opioid modulation of AP Waveform Elicited-Calcium Currents in Neurohypophysial Terminals

Voltage-Dependent Opioid modulation of AP Waveform Elicited- Calcium Currents in Neurohypophysial Terminals

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Abstract

Release of neurotransmitter is activated by influx of calcium (Katz and Miledi, 1968). Inhibition of Ca^{2+} channels results in less calcium current influx into the terminal and presumably a reduction in transmitter release. In the neurohypophysis, Ca^{2+} channel kinetics, and the associated Ca^{2+} influx, is primarily controlled by membrane voltage and can be modulated, in a voltage-dependent manner, by G-protein subunits interacting with voltage-gated calcium channels (VGCC). Voltage-dependent relief of G-protein inhibition of VGCC is achieved with either a depolarizing square pre-pulse or by action potential waveforms. Both protocols were tested in the presence and absence of opioid agonists targeting the μ - and κ -receptors. The κ -opioid VGCC inhibition is relieved by such pre-pulses, suggesting that this receptor is involved in a voltage-dependent membrane delimited pathway. In contrast, μ -opioid inhibition of VGCC is not relieved by such pre-pulses, indicating a voltage-independent diffusible second-messenger signaling pathway. Furthermore, relief of κ -opioid inhibition during a physiological AP burst stimulation indicates the possibility of activity-dependent modulation *in vivo*. Differences in the facilitation of Ca^{2+} channels due to specific G-protein modulation during a burst of action potentials may contribute to the fine-tuning of Ca^{2+} -dependent neurotransmitter release in other CNS synapses, as well.

Introduction

The hypothalamic neurohypophysial system (HNS) releases both oxytocin (OT) and vasopressin (AVP) neuropeptides into a capillary bed for systemic delivery. Action potentials from magnocellular neurons to the neurohypophysial terminals elicit secretion of both hormones by triggering the opening of voltage gated calcium channels (VGCC) leading to subsequent rise in intraterminal Ca^{2+} concentration (Bicknell, 1988; Lemos, 2002). Release from neurohypophysial terminals is very sensitive to intraterminal calcium (Dreifuss et al., 1971; Dreifuss and Nordmann, 1974; Bicknell, 1988; Berrino et al., 1989; Jackson et al., 1991a; Salzberg et al., 2000; Steffensen et al., 2002). Isolated neurohypophysial (NH) terminals show inhibition of VGCC in the presence of either μ -opioid agonists (Ortiz-Miranda et al., 2003; Ortiz-Miranda et al., 2005) or κ -opioid agonists (Rusin et al., 1997b), and inhibition of subsequent release of both oxytocin and vasopressin (Sumner et al., 1990; Kato et al., 1992; Russell et al., 1993). The signaling mechanism and modulatory importance of μ - and κ -receptor activation at these pre-synaptic terminals and subsequent VGCC inhibition is still not well understood, however.

Endogenous opioids, which are secreted from both the CNS and the Neurohypophysis, modulate both OT and AVP secretion from the magnocellular neurosecretory system (Douglas et al., 1995a; Elhamdani et al., 2000). However, modulation via μ - and κ -opioids is neither identical nor static and displays plasticity in response to changes in physiological status. Co-release of dynorphin, an endogenous κ -opioid agonist, with vasopressin from magnocellular neuron cell bodies and dendrites has been shown to facilitate activity-dependent modulation of vasopresinergic neurons

(Bourque et al., 1998; Brown and Bourque, 2004; Brown et al., 2004; Roper et al., 2004; Brown et al., 2006; Sabatier and Leng, 2007). κ -opioid receptors have also been found in isolated terminals of the Neurohypophysis (Hamon and Jouquey, 1990; Smith et al., 1993). In non-synaptic structures, activation of κ -opioid receptors reduces post-spike depolarizing after-potentials decreasing the spontaneous firing rate of magnocellular neurons *in-vitro* and subsequent transmitter release (Inenaga et al., 1994; Brown et al., 1999; Brown and Leng, 2000). Endogenous μ -opioid effects are associated with their predominant role in inhibition of oxytocin cells during pregnancy (Russell et al., 2003). The μ -opioid inhibition of the magnocellular neurosecretory becomes increasingly evident during pregnancy, but is subsequently interrupted prior to parturition allowing strong excitation of oxytocin cells and thus facilitates birth (Russell et al., 1995b; Russell et al., 2003).

Peak release efficiency for both oxytocin and vasopressin is achieved via specific bursting modes of activity of magnocellular neurons (Bicknell and Leng, 1981; Poulain and Wakerly, 1982). Oxytocin release is optimum during high frequency firing and vasopressin release is facilitated via an asynchronous bursting firing pattern. Frequency variations within a train of action potentials is a key component of both types of physiological bursts (Cazalis et al., 1985; Bicknell et al., 1988). Various possible explanations for this phenomenon have been proposed. Since repetitive firing produces broadening of action potentials, action potential broadening and subsequent buildup of residual calcium, have been proposed to explain the frequency dependence of both AVP and OT release (Leng and Shibuki, 1987; Muschol and Salzberg, 2000). However, a yet unexplained residual frequency-dependent facilitation of action potential-induced rise in

the intracellular Ca^{2+} concentration $[\text{Ca}^{2+}]_i$, unrelated to action potential broadening, remain to be explained (Jackson et al., 1991b). Furthermore, biophysical properties of the VGCC present in isolated terminals cannot account for the observed frequency-dependent facilitation (Wang et al., 1997a; Wang et al., 1999b; Lemos and Wang, 2000b). Persistent baseline release of AVP with its concurrent co-release of dynorphin (Bourque et al., 1998; Brown and Bourque, 2004; Brown et al., 2004; Roper et al., 2004; Brown et al., 2006; Sabatier and Leng, 2007), and its documented effects on action potential duration, post-spike excitability and reduced stimulated neuropeptide release (Inenaga et al., 1994; Brown et al., 1999; Brown and Leng, 2000), could represent a tonic inhibitory regulation of both OT and AVP release. We propose that voltage-dependent relief of tonic κ -opioid mediated inhibition of VGCC may help explain the importance of specific frequency-dependent bursting patterns in increasing efficacy of neuropeptide release.

Both the μ - and κ - receptors are G-protein coupled receptors can potentially mediate their inhibitory effects on VGCC through either a membrane-delimited or diffusible second-messenger pathway (Wilding et al., 1995; Kaneko et al., 1998; Soldo and Moises, 1998; Connor and Christie, 1999; Chen et al., 2000). A membrane-delimited pathway is described as G-protein activation leading to $\beta\gamma$ subunits directly associating with a voltage-gated calcium channel and typically inhibiting calcium currents. This association is described as voltage-dependent and characterized by a slowing of activation kinetics of the currents, as well as attenuation of the response when preceded by a depolarizing pre-pulse (Dolphin, 1996; Tedford and Zamponi, 2006). As the term implies, all signaling components are associated with the membrane. During classic whole-cell patch-clamp recordings, inhibition of VGCC via a G-protein membrane-

delimited pathway should be observable whether using classic or the perforated-patch configuration. In contrast, a voltage-independent inhibition typically involves signaling via a diffusible second-messenger and can be blocked by intraterminal dialysis as occurs during classic whole-cell patch-clamp in HNS terminals. This inhibition may only be observed in calcium currents recorded using the perforated-patch configuration of whole-cell patch clamp, as is the case for μ -opioid inhibition of calcium currents in the HNS (Ortiz-Miranda et al., 2003; Ortiz-Miranda et al., 2005) but not for κ -opioid inhibition (Rusin et al., 1997b).

The μ - and κ -opioid receptors are heterotrimeric G-protein coupled receptors, capable of interacting with either pertussis toxin-sensitive and pertussis toxin-insensitive G-proteins (Connor and Christie, 1999). N-ethylmaleimide (NEM), a sulfhydryl alkylating reagent, has been characterized as a blocker of specific G-protein ADP-ribosylation, which results in termination of downstream opioid receptor signaling initiated by agonist binding (Ueda et al., 1990; Ueda et al., 1996). Studies show that treating membranes with NEM abolishes signaling downstream of G-protein activation by the κ -opioid agonist U50488 and μ -receptor agonist DAMGO (Allgaier et al., 1989; Ueda et al., 1990; Ofri and Simon, 1992; Ueda et al., 1996).

G-protein membrane-delimited inhibition can be relieved by pre-pulse depolarization in a voltage-dependent manner. Similarly, voltage-dependent disinhibition of calcium currents via a simulated physiological burst of action potential waveforms (APWs) can induce activity-dependent synaptic facilitation underlying a form of short-term plasticity *in vivo*, which can presumably enhance neurotransmitter release (Currie and Fox, 2002; McDavid and Currie, 2006). The ability of action-potential-like

waveforms (APWs) to attenuate opioid-induced inhibition of VGCC has been shown in the NG108-15 cell line (Tosetti et al., 1999). Their results suggest that neuronal firing may relieve opioid inhibition of calcium currents in a frequency-dependent manner. In the present study we have looked at the voltage- and frequency-dependent inhibition of calcium currents by both the μ - and κ -opioid receptors in isolated HNS terminals. These calcium currents were elicited by both rectangular-pulses and APW stimulations and facilitation was induced utilizing varying rectangular depolarizing pre-pulses. Furthermore, we have studied relief of opioid inhibition on APW-elicited calcium currents during a simulated physiological burst of APWs.

Materials and Methods

Isolation of nerve endings:

Male Sprague-Dawley rats (Taconic Farms, Germantown, NY) weighing 200–250 g were sedated using CO₂ and immediately decapitated. The neurohypophysis was isolated as previously described (Lemos and Nordmann, 1986; Knott et al., 2002). Briefly, following removal of the anterior and intermediate lobes, the neurohypophysis was homogenized in 270 μ l of buffer at 37°C containing (in mM): 270 sucrose, 0.004 EGTA, 10 HEPES-Tris, buffered at pH 7.25; 298–302 mOsmol/L. The solution containing the homogenate was plated on a 35 mm petri dish and carefully washed in Low-calcium Locke's solution which consists of modified Normal Locke's (in mM): 145 NaCl, 2.5 KCl, 10 HEPES, 1.2 glucose, 0.8 CaCl₂, 0.4 MgCl₂, pH 7.4; 298–302 mOsmol/L.

Electrophysiological experiments:

The neurohypophysis was isolated and homogenized as previously described (Brethes et al., 1987; Brown et al., 2006). Current recordings were obtained using the perforated-patch configuration on isolated HNS terminals. Using an inverted microscope the terminals were identified visually by their characteristic appearance, spherical shape, lack of nuclei, and size (5–10 μm in diameter). The pipette solution consisted of (in mM): 145 Cs-gluconate, 15 CsCl, 2 MgCl_2 , 2 NaCl, 7 Glucose, 10 HEPES (pH 7.3), at 295 mOsm. Amphotericin B at a concentration of 30 μM (SIGMA) was added as a perforating agent. The bath solution consisted of (mM): 145 NaCl, 5 KCl, 1 MgCl_2 , 10 HEPES, 10 Glucose, 1.2 CaCl_2 , pH 7.5 (Normal Locke's solution). In all experiments TTX (100 nM) was added to the bath to block sodium influx via voltage-gated sodium channels. The pipette resistance was 5-8 $\text{M}\Omega$. Pipettes were made of thin borosilicate glass (Drummond Scientific Co., Broomall, PA, USA). After perforation the terminals were voltage-clamped at -80 mV. Depolarization was applied every 30 seconds to 0 mV for 250-300 ms. The preparation was either continually perfused, via a gravity driven perfusion, or left in a static non-perfused bath (as noted). Agonists and antagonists were either applied through the gravity driven perfusion system, or added to the static bath. All experiments were performed at room temperature (25°C). Data was acquired, stored and analyzed using a Pentium I computer (Gateway) and pClamp 7 (Axon Instruments, Foster City, CA). Currents were corrected online using an inverted P/4 protocol. All time constants (τ 's) were obtained using a single exponential curve fit (Igor, Wavemetrics) on inward calcium currents. For the rise times (activation), the fit was made between 2 ms

after start of stimulus (in order to avoid transient artifacts) and 2 ms before peak (since between this period and peak time the “shape” of the segment is not a simple exponential). For calcium currents elicited by action potential waveforms, the time of peak was measured from the beginning of the waveform to the maximum inward currents (in conditions where the sodium current was blocked).

Stimulus-induced (0.3 to 0.4 ms duration, 5-10 nA) action potentials (APs) were recorded from isolated neurohypophyseal terminals using the perforated-patch method and the fast current-clamp mode of an EPC9 amplifier (HEKA Instruments) in the absence of voltage-gated channel blockers. The APs were used as waveforms for voltage commands in voltage clamp mode on subsequent experiments with isolated terminals, also using the perforated-patch method. Action potentials from isolated terminals show frequency-dependent broadening (modulation). Inherent changes in rise time occur concomitant with AP broadening which are reflected in the APWs tested. Broadening was induced by repetitive stimulation (30 Hz or more, the plateau for broadening is reached at 10 Hz) at frequencies within known physiological conditions. Since action potentials were recorded independently, waveforms composed of bursts of modulated action potential (trains) were constructed artificially by joining successive action potentials (concatenating). In this manner, a burst consisting of gradually broadening action potentials (from initially-fastest to finally-slowest) was constructed having frequency characteristics within those observed physiologically.

Statistical comparisons.

In all cases, data are reported as mean \pm SEM; n being the number of terminals. Statistical analysis of difference was made with paired t-test, with $p < 0.05$ considered significant.

Results

G-protein mediated inhibition by U50488:

Ca²⁺ channel inhibition in isolated HNS terminals by κ -opioid receptor agonists, including U50488, has been previously shown using the “classic” whole-cell patch configuration (Rusin et al., 1997b) and now confirmed using the perforated-patch clamp mode (Fig. 3.1A). The perforated-patch configuration of the whole-cell patch-clamp method is typically utilized to prevent intracellular dialysis of diffusible components. The results show that current inhibition by κ -receptor agonists can be observed with (Rusin et al., 1997b) or without intraterminal dialysis of diffusible components. In contrast current inhibition by the μ -opioid agonist DAMGO can be observed only using the perforated-patch configuration (Ortiz-Miranda et al., 2003; Ortiz-Miranda et al., 2005).

In order to link μ - and κ -receptor inhibition of calcium currents to the activation of a G-protein coupled receptor by DAMGO (100 nM) and U50488 (100 nM), we tested both by pre-treating the terminals with 10 μ M NEM for 5 min. Functional studies of opioid receptor cysteine residues show that agonist binding to the receptor itself is not likely to be affected at 5-10 μ M concentrations of NEM (Ueda et al., 1996; Ehrlich et al., 1998). This concentration was sufficient to block all κ - and μ -opioid inhibition of

calcium currents (Fig. 3.1B-C) demonstrating that these responses are via a G-protein mediated pathway, potentially pertussis toxin-sensitive. Pertussis toxin could not be used given the necessity for long incubation periods that are incompatible with the lifetime of our isolated HNS preparation. Therefore, we have characterized the biophysical differences of the two pathways instead.

Voltage-dependent inhibition is also characterized by a slowing of current activation kinetics. Activation time constants were measured for rectangular pulse-elicited total calcium currents under control conditions and in the presence of either 100 nM U50488 or 100 nM DAMGO. Activation of total calcium currents includes contributions from VGCC such as the N-, L-, R- and Q-type (Wang et al., 1997c; Wang et al., 1999b). The time for an e-fold change (τ) of activation kinetics was measured in the presence of either κ - or μ -opioid agonists and divided by that of the control (no opioid). This was done in order to account for the variability of the measured time constants between samples (n=8). The ratio is expressed as a percentage difference from 100% (i.e., 0% difference = no change, negative % difference = faster time constant, and positive % difference = slower time constant). Results show a 75 ± 44.2 % difference for U50488 and only a 1.7 ± 11.3 % difference for DAMGO. Slower activation kinetics during κ -opioid inhibition of calcium currents concurs with the idea that κ -opioid inhibition of VGCC in the HNS terminals is mediated via a membrane-delimited pathway.

Voltage-dependent vs. Voltage-independent inhibition of VGCC via μ - and κ - opioid agonists:

Given the resistance of κ -opioid inhibition to intraterminal dialysis we hypothesized that κ - not μ -opioid inhibition would be voltage-dependent. We tested for voltage-dependent inhibition by using a depolarizing pre-pulse (DPP) protocol on rectangular pulse-elicited Ca^{2+} currents. When such calcium currents are preceded within 2-4 ms by a DPP consisting of a voltage jump from -80 mV to $+100$ mV with 30 ms duration, U50488 inhibition was completely blocked (Fig. 3.2A). DAMGO inhibition, however, was unaffected (Fig.3.2B). The depolarizing pre-pulse had no effect on control calcium currents without opioid treatment or during NEM treatment (data not shown). Given that short exposures of NEM may selectively block PTX-sensitive G-proteins, as observed in SCG neurons, it's important to note that PTX-sensitive G-proteins have been shown to mediate voltage-dependent, membrane-delimited processes (Zong et al., 1995; Yassin et al., 1996; Jeong et al., 1999). Therefore, results indicate that κ - but not μ -opioid inhibition is voltage-dependent.

DAMGO and U50488 inhibition of APW elicited calcium currents:

As mentioned above, endogenous opioids secreted from both the CNS and the Neurohypophysis, modulate both OT and AVP secretion from the magnocellular neurosecretory system (Douglas et al., 1995a; Elhamdani et al., 2000). However, calcium currents *in-situ* are not elicited by rectangular depolarizing pulses but by action potentials (APs). APW-elicited Ca^{2+} currents were thus studied to determine the effects of opioids on currents elicited by more physiologically relevant stimuli (See Methods). For tests

made with individual APs, four types of APs were chosen such that their degree of broadening would represent a sampling of the broadening span during a burst: from peak to half amplitude on the falling phase within a range of 1 to 4 ms (Fig. 3.3A). Peak calcium currents varied with longer duration APW (Fig.3.3B). The APW calcium currents were obtained using 100nM TTX to block all voltage-gated sodium channels and with Cs-glutamate in the pipette solution to block all K⁺ currents. The remaining currents were pharmacologically determined to be calcium currents by blocking them with 200 μM NiCl₂/CdCl₂ (Fig. 3.4). Inhibition with either κ- or μ-opioid agonists did not significantly change in relation to control currents of equal duration, regardless of the APW duration (Fig. 3.4C). Both U50488 (Fig. 3.5A) and DAMGO (Fig. 3.5B) inhibit APW-elicited calcium currents (APW duration was 4 ms from peak to half amplitude on the falling phase) to a similar extent as those elicited by rectangular pulses. Inhibition, expressed as percent of control current without opioid, by U50488 of APW currents (71 ± 5%) was similar to that of rectangular pulse-elicited currents (73 ± 12%). Inhibition by DAMGO of APW currents (77 ± 8%) was also similar to that of rectangular pulse-elicited currents (75 ± 9 %).

Voltage-dependent G-protein modulation of Ca²⁺ currents could be relieved by physiologically relevant electrical activity. We thus selected the broadest APW with a duration of 4 ms to measure all other effects on U50488 and DAMGO inhibition of APW elicited calcium currents. The criterion for selecting the broadest APW was based on using an APW that would be most likely at the end of a train of APs (Gainer et al., 1986; Leng and Shibuki, 1987; Jackson et al., 1991a; Branchaw et al., 1998; Muschol and Salzberg, 2000; Marrero and Lemos, 2005). Presumably, this APW would see a full

range of frequencies preceding it and the largest accumulation of endogenous opioids throughout a physiological burst (Bicknell et al., 1988; Bourque, 1991; Bourque et al., 1998).

To assess voltage-dependent relief of μ - and κ -opioid receptor inhibition of voltage-gated Ca^{2+} channels (VGCC) elicited with a more physiological stimulus, we have measured opioid effects on calcium currents elicited by action potential waveforms with and without a rectangular DPP. A DPP similar to that applied prior to the rectangular test pulse was applied prior to APW elicited currents using a 2 ms interpulse interval, a voltage jump to +100 mV (delta +180 mV) and duration of 30 ms (Fig. 3.6). All stimulations were measured as percent of control current without treatment. Ca^{2+} current inhibition by U50488 ($74.4 \pm 39\%$) disappeared after DPP ($97.6 \pm 5.2\%$) whereas Ca^{2+} current inhibition by DAMGO ($88.6 \pm 1.9\%$) increased ($75.4 \pm 6\%$ after DPP) (Fig. 3.6). The reduction in current during DAMGO treatment following DPP is likely due to voltage-dependent inactivation of VGCC. To establish optimum conditions for quantifying U50488 and DAMGO voltage-dependent inhibition, pre-pulse amplitude, duration and the interpulse interval were varied (Fig. 3.7). With a DPP prepulse interval of 2 ms and duration of 25 ms, DPP amplitude was varied from -70 mV (were -80 mV is no pre-pulse) to +130 mV (delta of 20 mV). Maximum relief of U50488 inhibition was achieved at +90 to +130 mV. DAMGO inhibition was at no point relieved and exhibits a downward trend indicative of voltage-dependent inactivation. Using fixed amplitude of +100 mV (delta of +180 mV) and a 2 ms pre-pulse interval, DPP duration was increased from 1 ms to a maximal duration of 104 ms (delta of 13 ms). No relief of U50488 inhibition occurred with a 1 ms duration DPP. However, following a 13 to 27 ms DPP,

inhibition by U50488 decreased and remained attenuated thereafter. In contrast, DAMGO inhibition was not attenuated for any of the same DPP durations. Pre-pulses to +100 mV (delta of +180 mV) were then used to examine the effect of increasing the interpulse interval from 2 to 8 msec. Maximum attenuation of U50488 inhibition was achieved at 2-4 ms interpulse intervals. These results were used to establish optimum prepulse parameters (and those used thereafter, unless otherwise stated) as follows: 2 ms initial interpulse interval, amplitude to +100 mV, 30 ms duration, all followed by the broadest APW. Opioid induced inhibition of APW-evoked currents was plotted as a percent of control current without opioid (Fig. 3.7). As observed for calcium currents elicited with a rectangular pulse, both U50488 ($71 \pm 5 \%$) and DAMGO ($52.8 \pm 9.5 \%$) inhibition of APW-elicited calcium currents were relieved with 10 μ M application of NEM ($96.1 \pm 3\%$ and $106 \pm 10.3 \%$, respectively) (Fig. 3.8). In contrast, control and NEM treated terminals showed no facilitation of APW calcium currents using identical DPP protocols.

Frequency-dependent relief of kappa opioid inhibition

Protocols of varying frequency were designed using 1ms duration rectangular prepulses, which, although similar to *in vivo* AP duration, were ineffective in relieving any inhibition (Fig. 3.9A-C). Currents decreased by DAMGO were further decreased progressively as DPP frequency increased (Fig. 3.9E). Similar voltage-dependent inactivation was observed with control and NEM treated calcium currents (data not shown). In contrast, inhibition by U50488 is attenuated (recovery to 100% of control) at 10 Hz (Fig. 3.9D,E). At 50 and 100 Hz the currents remain significantly different from DAMGO but exhibit a similar downward trend. This is probably due to voltage-

dependent inactivation and could reflect an inherent competing effect with DPP-induced relief of voltage-dependent inhibition.

As observed in the sample currents in fig. 3.9D, APW-elicited total calcium currents in the presence of U50488 show a change in activation kinetics. The time to peak of the APW-elicited calcium current was measured in the presence of either κ - or μ -opioid agonists and divided by the time to peak in its absence (control). This was done in order to account for the variability of the peak times between samples (n =8). The ratio was then expressed as a percentage difference from 100% (i.e., 0% difference = no change, negative % difference = decrease in time to peak, and positive % difference = increase in time to peak). Results show (% \pm SEM) 8.8 ± 2.9 % difference for U50488, and -3.3 ± 6.7 % difference for DAMGO. The change in activation kinetics of the APW-elicited current in the presence of U50-488 was smaller than those measured for rectangular pulse elicited currents. However, as noted by others (Park and Dunlap, 1998), APW-elicited currents have changing and comparatively brief activation phases which make kinetics more difficult to quantify and compare.

κ -opioid inhibition relieved by a train of action potentials

The effect of action-potential-like waveforms (APWs) on U50488 κ -opioid inhibition of voltage-gated Ca^{2+} channels was investigated using a train of APs (Fig. 3.10). The Long APW studied is present at the end of the train and compared in and out (individual APW) of the context of the burst. Burst-elicited example calcium currents under control (black) and opioid (color) conditions are presented in figure 3.11A and 3.11B. Single Long APW currents are presented under control (no opioid) and treated

(either 100 nM U50488 or 100 nM DAMGO) to show inhibition of the currents in the same terminal without the burst effects (Fig. 3.12C and 3.12D). In the presence of opioids, the currents from several individual APWs of different duration are compared to the currents obtained from the same APWs within the simulated burst (Fig. 3.12). This comparison was designed to examine the opioid inhibition in and out of the context of the burst as a percent of control (single APW of same duration without opioid). U50488 inhibition was significantly (* $p < 0.05$) relieved within the burst for APs shortly after the middle of the burst (AP #26, Fig. 3.12A). DAMGO inhibition was not relieved regardless of APW duration or AP position within the burst (Fig. 3.12B). The start of relief of U50488 inhibition was observed with 8 APs preceding the APW-elicited current, at an average frequency of 100 Hz.

Discussion

This is the first time, to the best of our knowledge, that κ -mediated inhibition of VGCC in the HNS has been shown to be voltage-dependent within a burst of APs. Given the importance and ubiquitous nature of κ -mediated inhibition of VGCC in HNS cell bodies, dendrites and in terminals, the mechanism of modulation of VGCC as key triggers for electrically evoked release is central to understanding some of the pivotal events in depolarization-secretion coupling. Furthermore, we have also shown that μ -opioid receptor inhibition is not voltage-dependent.

Intraterminal signaling of μ - and κ -opioid receptors in HNS terminals

The differences in mechanisms for μ - and κ -opioid receptor inhibition of VGCC have important physiological relevance. Oxytocin neurons are highly tuned to μ -receptor inhibition whereas AVP release is most sensitive to κ -receptor activation (Bicknell, 1985; van Wimersma Greidanus and van de Heijning, 1993; Russell et al., 1995a; Brown et al., 2000a; Russell et al., 2003; Ortiz-Miranda et al., 2005). In isolated terminals the inhibitory constraints of both μ - and κ -opioids are mirrored in the systemic output of both vasopressin and oxytocin in response to different physiological needs. The diffusible second-messenger pathway mediating μ -opioid inhibition is voltage-independent and therefore would remain relatively unaffected throughout the duration of an action potential burst (See Chapter IV). Therefore, attenuation of μ -opioid inhibition of Ca^{2+} currents must stem from either lack of agonist interaction with the receptor, receptor internalization/desensitization or changes in diffusible second-messenger signaling.

These are comparatively long-term changes in the magnocellular (MNC) neurons and HNS interactions in contrast to short-term relief of inhibition or facilitation observed during voltage-dependent κ -opioid inhibition. Given the necessary long-term inhibition of μ -receptors during pregnancy and, in contrast, the short-term synchronization of AVP activity for optimum release the transduction mechanisms of the two opioids are perfectly suited to modulate their specific neuropeptide target outputs in the system.

Frequency-dependent facilitation in HNS and the role of voltage-dependent relief of κ -receptor inhibition

Experiments performed on the isolated neurohypophysis have demonstrated that, within a certain range, the same number of pulses given at high frequency (50-100 Hz) induces the release of a larger amount of neuropeptide than when delivered at lower frequency (Dreifuss et al., 1971; Nordmann and Dreifuss, 1972). Oxytocin cells are known to fire within the 50-100 Hz range for periods of 0.5-2.5 sec. (Wang and Hatton, 2005) for optimum neuropeptide secretion (Nordmann and Stuenkel, 1986). AVP containing cells, upon physiological demands such as hemorrhage (Poulain et al., 1977) or dehydration (Arnauld et al., 1975) change their pattern of firing from slow or tonic to a higher frequency phasic firing pattern. The exact reason why specific frequency stimulations optimize neuropeptide secretion is still under intense study (Lemos and Wang, 2000b). However, we know that appropriate timing of electrical signals is important in conveying an accurate physiological response, as with learning and memory (D'Angelo and Rossi, 1998; Matsumoto and Okada, 2002; Debanne et al., 2003; Thivierge et al., 2007; Caporale and Dan, 2008). Hypothetically, integration of signaling

from various sources for optimum response at the presynaptic juncture may require a systemic inhibition relieved only by the appropriate timing of electrical input.

Voltage-dependent relief of κ -receptor inhibition *in-situ*

Here we propose that neuropeptide release is potentially tonically inhibited by endogenous κ -opioid modulation, until electrical stimuli from MCN facilitates voltage-dependent relief. Temporal integration of the appropriate signals from MCNs can result in high frequency bursting relieving VGCC from tonic voltage-dependent κ -opioid inhibition, thus facilitating optimum neuropeptide release (Dyball et al., 1988; Brody et al., 1997). This could also allow the terminals to prime release during the initial high frequency phase of the action potential burst, by mobilizing readily releasable pools of neurosecretory granules (Nowycky et al., 1998). Optimum release would result, during the latter part of the burst, from a combination of a larger population of immediately releasable granules, broader action potentials, and removal of voltage-dependent VGCC inhibition (Andrew, 1987; Leng et al., 1988b; Nowycky et al., 1998; Marrero and Lemos, 2005). Oxytocin release, would completely bypass any κ -opioid voltage-dependent inhibitory constraints on release given its much higher frequency range of stimulation.

Role of voltage-dependent inactivation

Our results show, that unlike rectangular DPPs of varying frequencies, a train of APWs is capable of relieving U50488 inhibition at frequencies higher than 10 Hz (Fig. 3.9). This may be due to APW pulses having comparatively slower activation kinetics than rectangular pulses making them capable of unmasking all of the facilitation while avoiding the competing accumulation of voltage-induced Ca^{2+} current inactivation. Voltage-dependent Ca^{2+} current inactivation occurs with all stimulations and could

possibly lead to underestimating U50488 facilitation by the DPP. The possibility that the $G_{\beta\gamma}$ subunits may actually accelerate inactivation of calcium currents during high-frequency trains of APW has been proposed (Patil et al., 1998). However, activation of endogenous G-proteins has been shown, so far, to reduce both Ca^{2+} -dependent, and voltage-dependent inactivation of calcium currents in different expression systems (Bourinet et al., 1996; McDavid and Currie, 2006). Although further studies are required to determine the nature of the frequency-dependent relief of inhibition, our results indicate there is a relationship between frequency and optimum relief from voltage-dependent κ -opioid inhibition. The optimum frequency-dependent relief is likely a function of both $G_{\beta\gamma}$ dissociation from and voltage-dependent inactivation of VGCC.

AP broadening in HNS terminals

Nerve terminals show a progressive broadening of their action potentials when elicited repetitively (i.e., within a burst). The degree and rate of broadening is dependent on firing frequency (Gainer et al., 1986; Bourque, 1990; Jackson et al., 1991b). We simulated burst broadening in our burst pattern and found that disinhibition of κ -receptor activation progressively occurs reaching a maximum during the latter part of the burst during the broadest APWs. This is consistent with studies done by Brody and colleagues (Brody et al., 1997) that show the extent of G-protein disinhibition of P/Q-type calcium channels in HEK cells increases linearly with the duration of the action potential waveform. Although further investigation is required to determine whether a specific type of APW or number of previous depolarizations and/or frequency is best suited for relief of voltage-dependent inhibition, we have shown that relief of κ -opioid inhibition of VGCC in the HNS is achieved by a physiological burst of action potentials.

Conclusion

The endogenous κ -opioid mediated inhibition of VGCC and its voltage-dependent modulation provides the potential for activity-dependent relief of inhibition during action potential trains. This physiologically-evoked, activity-dependent modulation of VGCC and subsequent release, represents an important mechanism for short-term synaptic plasticity at the level of the terminals. Given the ubiquitous nature of voltage-dependent G-protein signaling in the CNS, our results may prove important in understanding modulatory effects of specific bursting patterns throughout the CNS.

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Figure 3.1

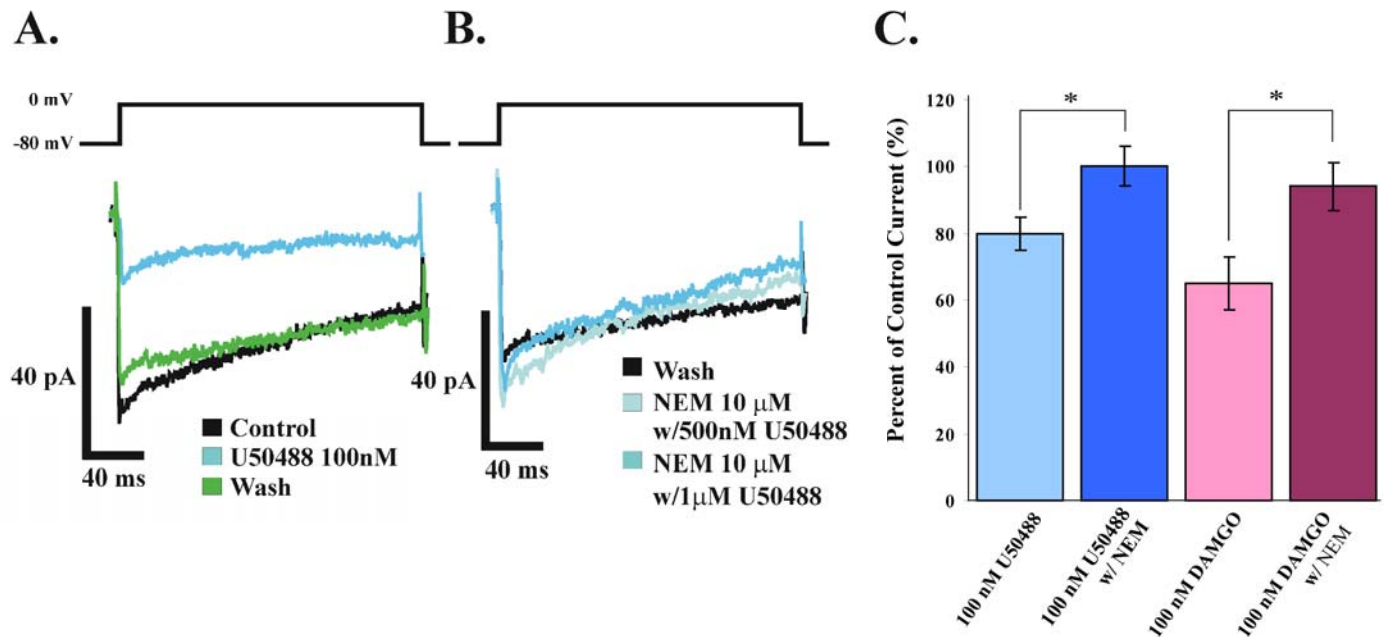


Figure 3.1 N-Ethylmaleimide blocks κ - and μ -receptor mediated inhibition of calcium currents. *A.* Calcium currents of single HNS terminal under control (black), 100 nM U50488 (blue) and wash (green) conditions using the perforated-patch configuration of the whole-cell patch-clamp technique. *B.* Same terminal under control (black) and 10 μ M NEM with 500 nM U50488 (blue) and 10 μ M NEM with 1 μ M U50488 (light blue) conditions. *C.* Bar Graph summarizing average effects of opioids on Ca^{2+} current (I_{Ca}) ($n=5$ U50488, $n=3$ DAMGO). All currents in the presence of NEM were not statistically different from their corresponding control ($p>0.05$) and all currents in the presence of opioid agonists without NEM were statistically different from their corresponding control. Asterisks (*) represent statistically significant differences, $p<0.05$.

Figure 3.2

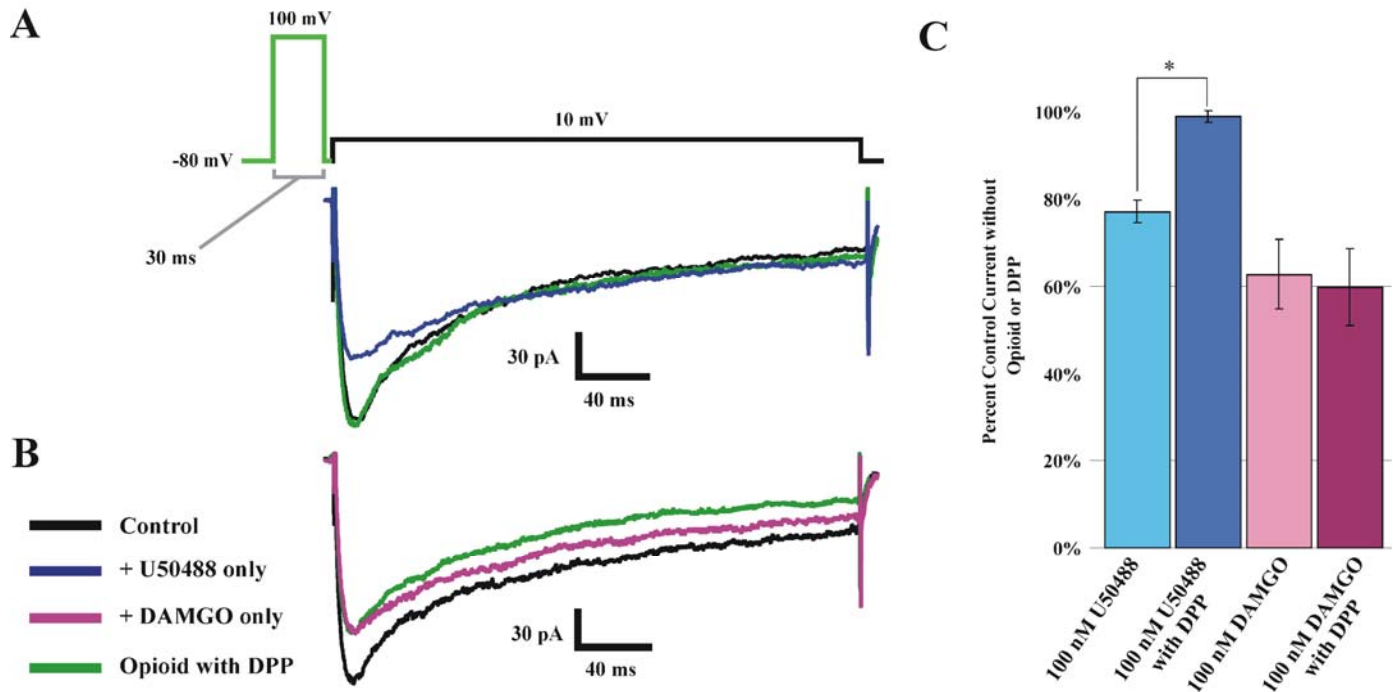


Figure 3.2 DPP relieves κ - but not μ -receptor mediated inhibition of calcium currents. *A.* HNS terminal calcium currents under control (black), with 100 nM U50488 (blue) and with a DPP in the presence of 100 nM U50488 (green) conditions. *B.* Calcium currents under control conditions (black), with 100 nM DAMGO (pink) and with a DPP in the presence of 100 nM DAMGO (green) conditions. *C.* Bar graph quantifying differences as percent control without opioid or DPP from rectangular pulse elicited calcium currents with 100 nM U50488, 100 nM U50488 with DPP, 100 nM DAMGO and 100 nM DAMGO with DPP. Asterisk (*) show statistical different (p=6.15e⁻⁵).

Figure 3.3

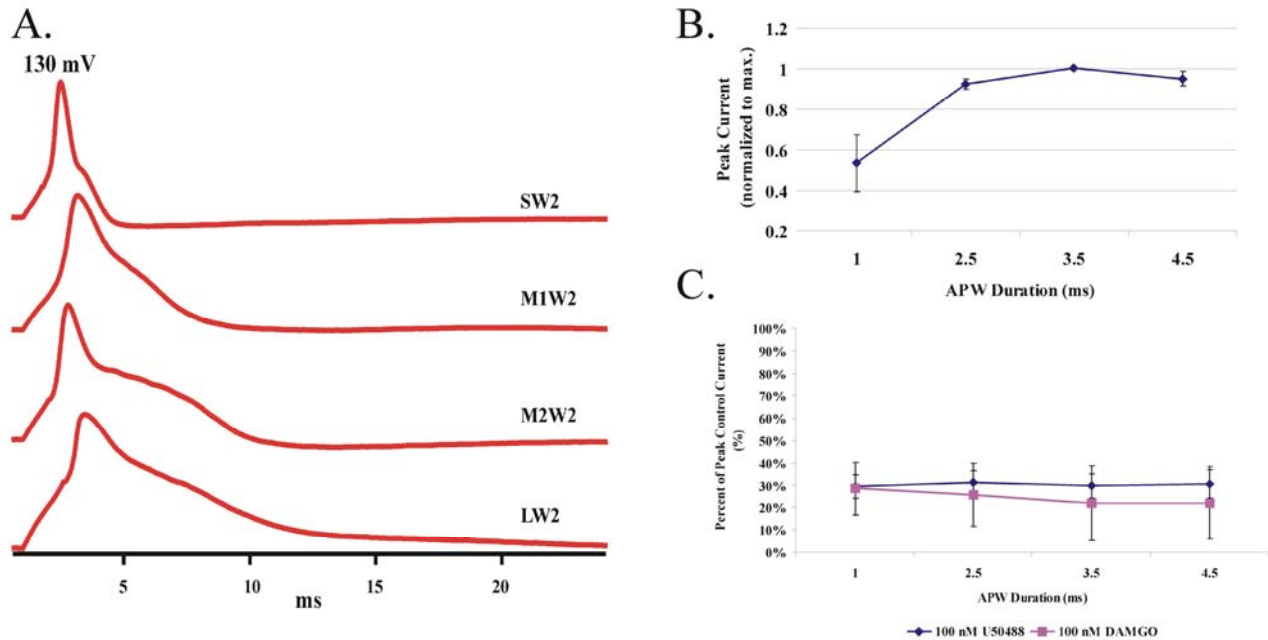


Figure 3.3 Action Potential Waveforms obtained from NH Terminal

Action Potentials.

A. Current waveform changes with action potential duration (measured from the peak to the half height of the falling phase) starting from the shortest recorded APW named SW2 with a duration of 1 ms followed by two intermediate waveforms M1W2 and M2W2 with durations of 2.5 and 3.5 ms respectively to the longest LW2 with a duration of 4.5 ms. APWs illustrated have an amplitude of 55 mV and rising phase of 3.2 msec. *B.* Peak Current normalized to maximum of APW-elicited calcium currents without treatment plotted as a function of action potential duration. *C.* Percent Peak control Ca^{2+} current with either κ - or μ -opioid agonist plotted as a function of APW duration. Data points represent means of 6 terminals.

Figure 3.4

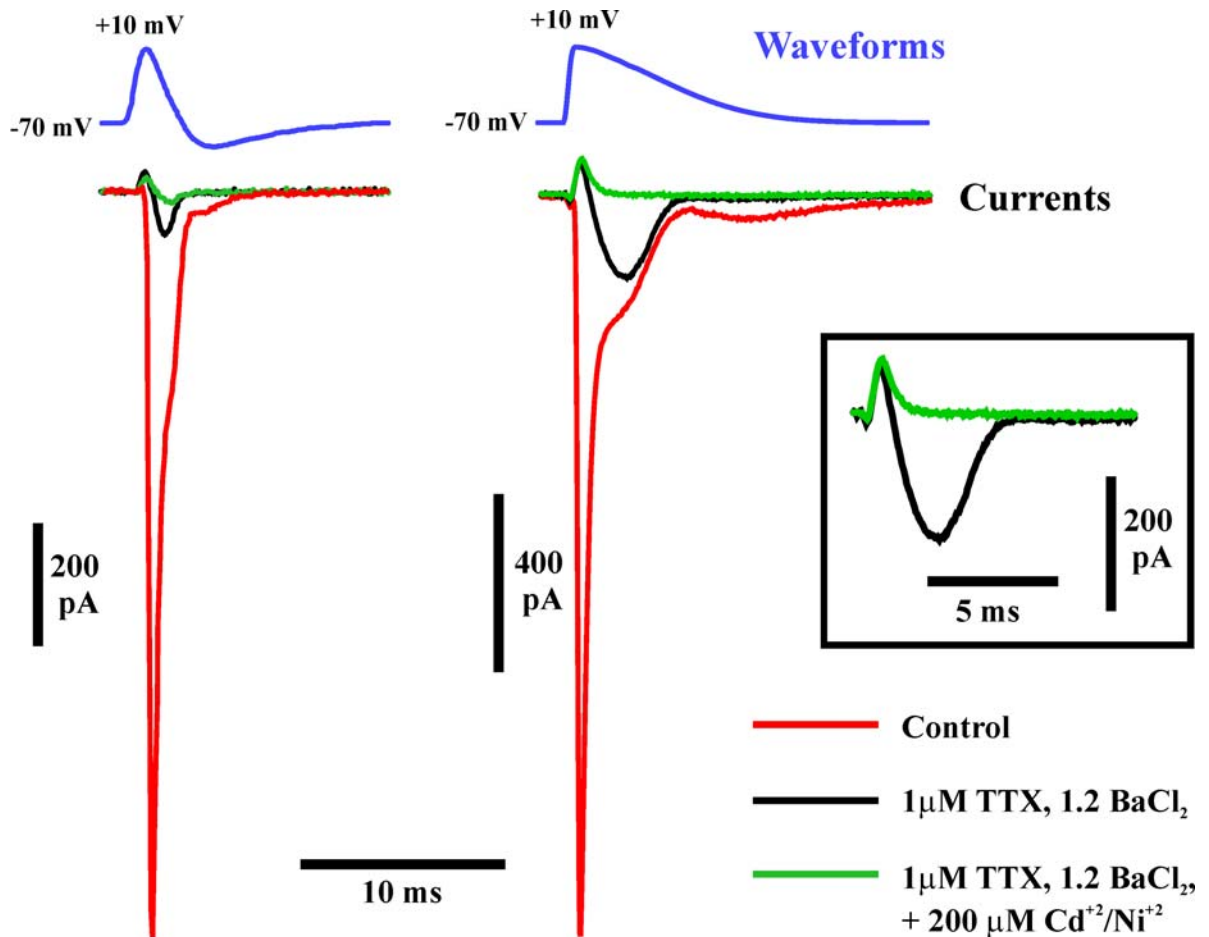


Figure 3.4 Currents elicited by Action Potential Waveforms. Currents elicited by single action potential waveforms (Waveforms, top blue traces). The internal solution contained excess Cs⁺ (145 mM) in order to block outward potassium current components. The APW's were artificially constructed to simulate action potentials for the same types and broadening (order of magnitude) as that observed in isolated terminals. The half-time (as measured from the top peak) was 0.85 and 4.6 ms (top left and right traces, respectively). Inset shows the elimination of elicited calcium currents by 200 μM Cd²⁺/Ni²⁺ (blow up of rightmost record).

Figure 3.5

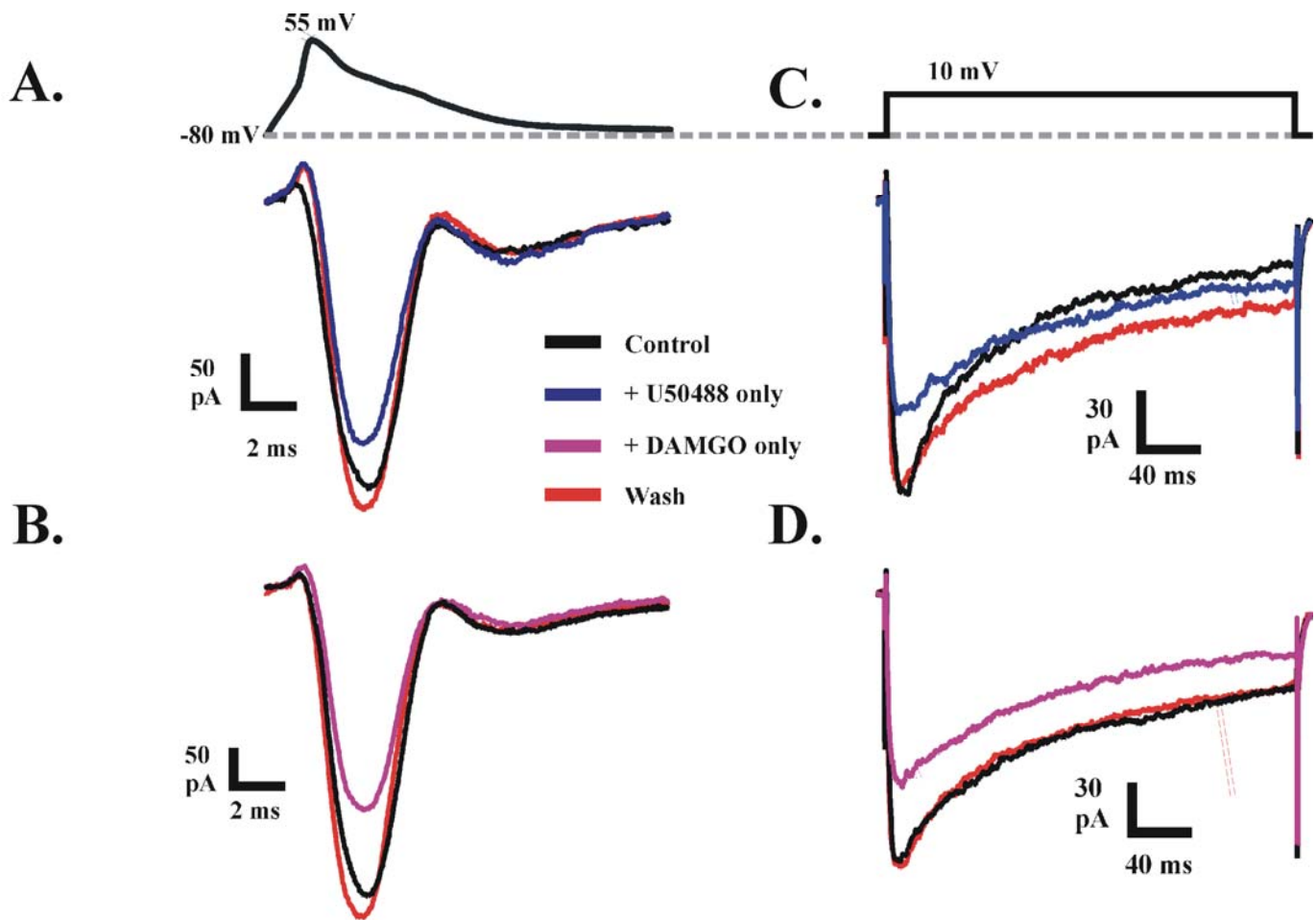


Figure 3.5 κ - and μ -opioid receptor activation similarly inhibits APW- and rectangular μ -opioid inhibition belong to the same terminal which is different from that used to test κ -opioid inhibition.

Figure 3.6

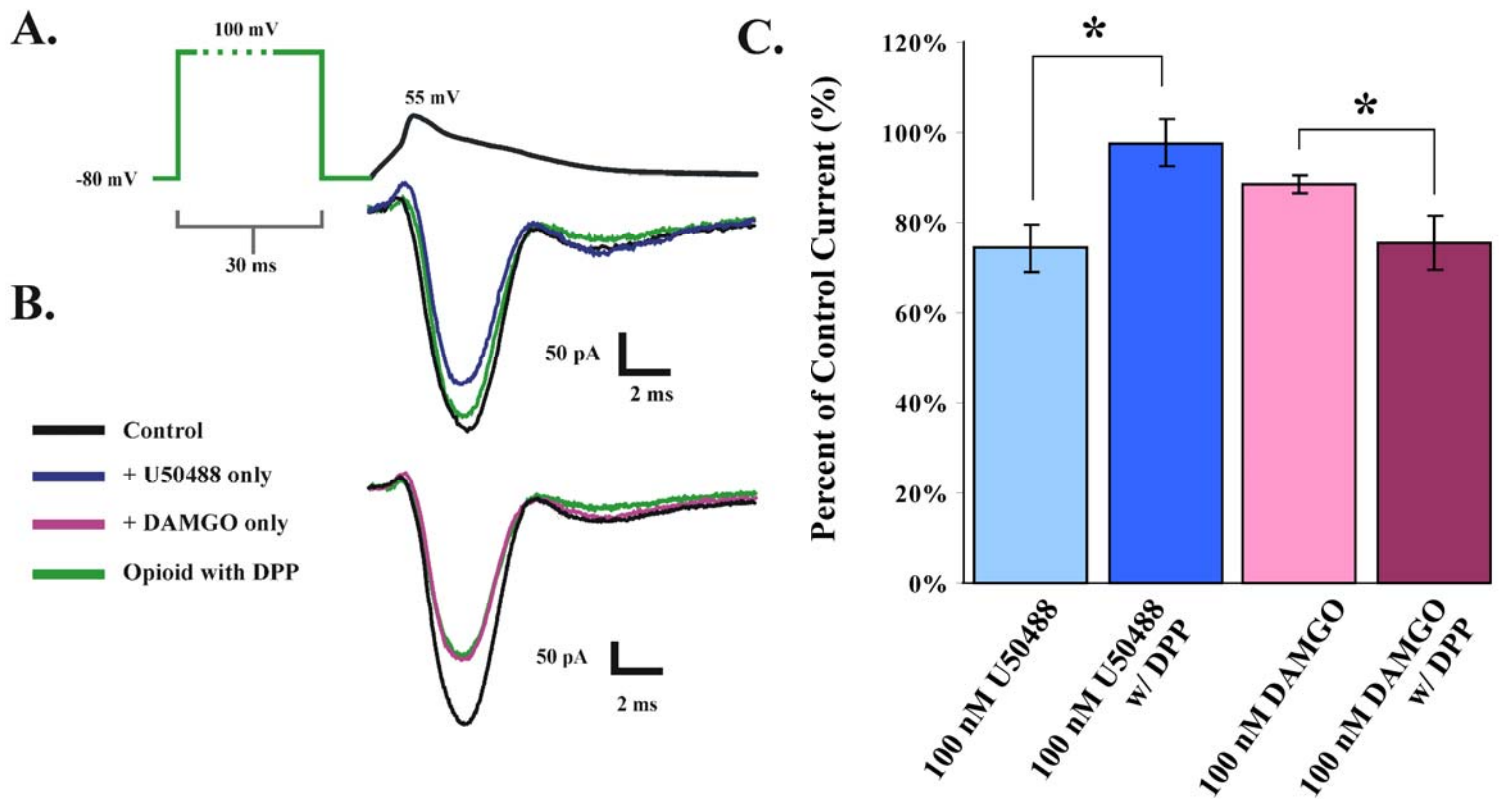


Figure 3.6 APW-elicited Calcium Currents are also relieved by DPP of κ - but not μ -opioid inhibition. Sample APW-elicited calcium currents in two different terminals with control (black), or in the presence of their respective opioid agonist, 100 nM κ -opioid agonist U50488 (blue) (A), and μ -opioid agonist DAMGO (pink) (B), after a depolarizing pre-pulse. C. Bar graph quantifying the effects of a DPP on κ - and μ -opioid inhibition of APW elicited calcium currents. Asterisks (*) represent statistically significant difference ($p < 0.03$).

Figure 3.7

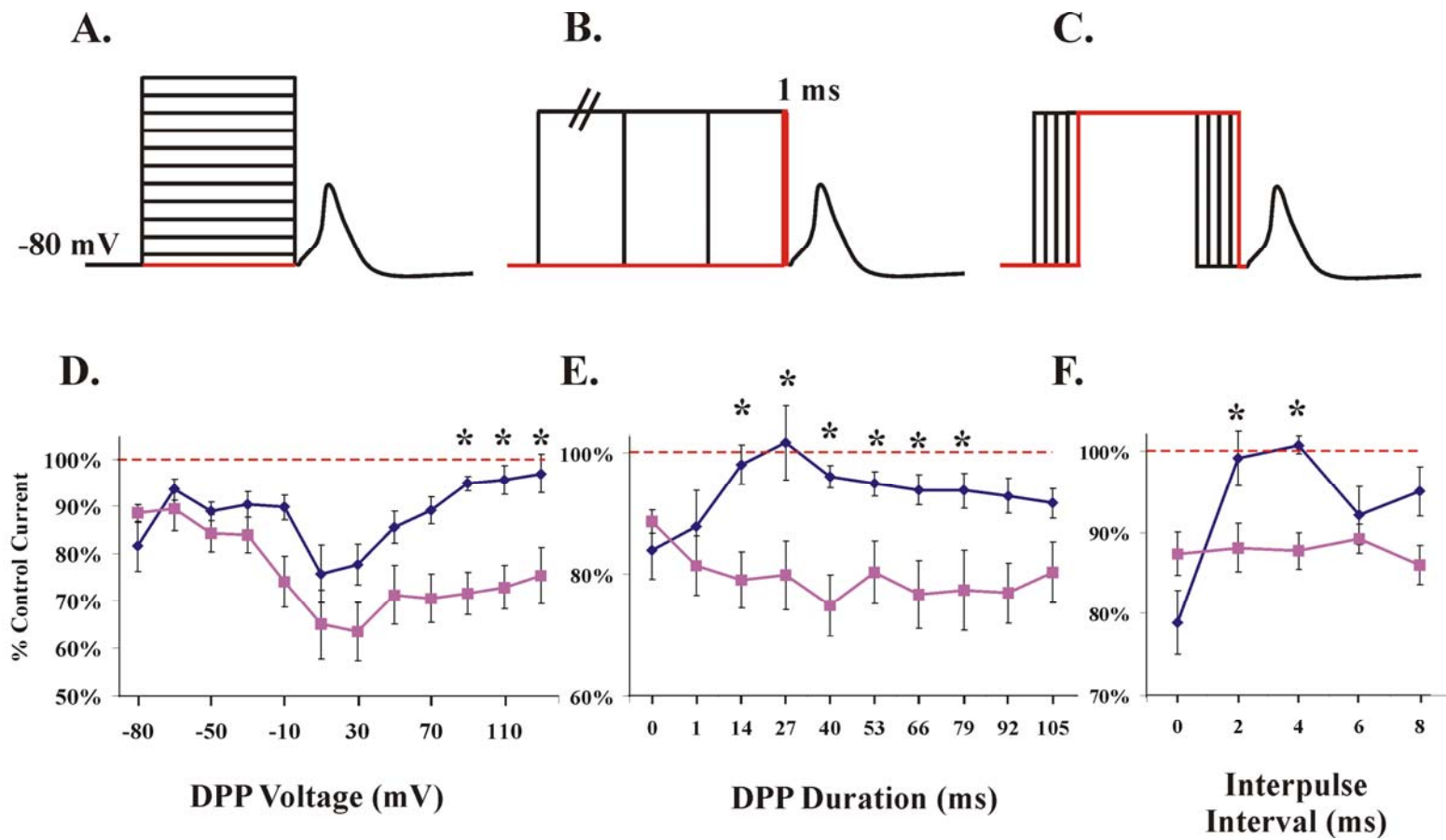


Figure 3.7 Optimizing DPP for APW calcium currents. Opioid induced inhibition of APW-evoked currents plotted as a function of pre-pulse (A.&D.) AMPLITUDE, (B.&E.) DURATION, and (C.&F.) INTERPULSE INTERVAL. Voltage commands are shown in top panel. Fixed prepulse parameters (unless otherwise stated) were as follows: 2 ms initial interpulse interval, amplitude to +100 mV (delta of 180 mV), 30 ms duration, Long waveform stimulation data points in all panels represent the means of measurements for 4-5 terminals with their respective SEM. Asterisks (*) represent values which are both statistically different from Ca²⁺ currents with opioid and no DPP, p<0.03 and at the same time are not statistically different from control currents (without opioid or DPP) p>0.05.

Figure 3.8

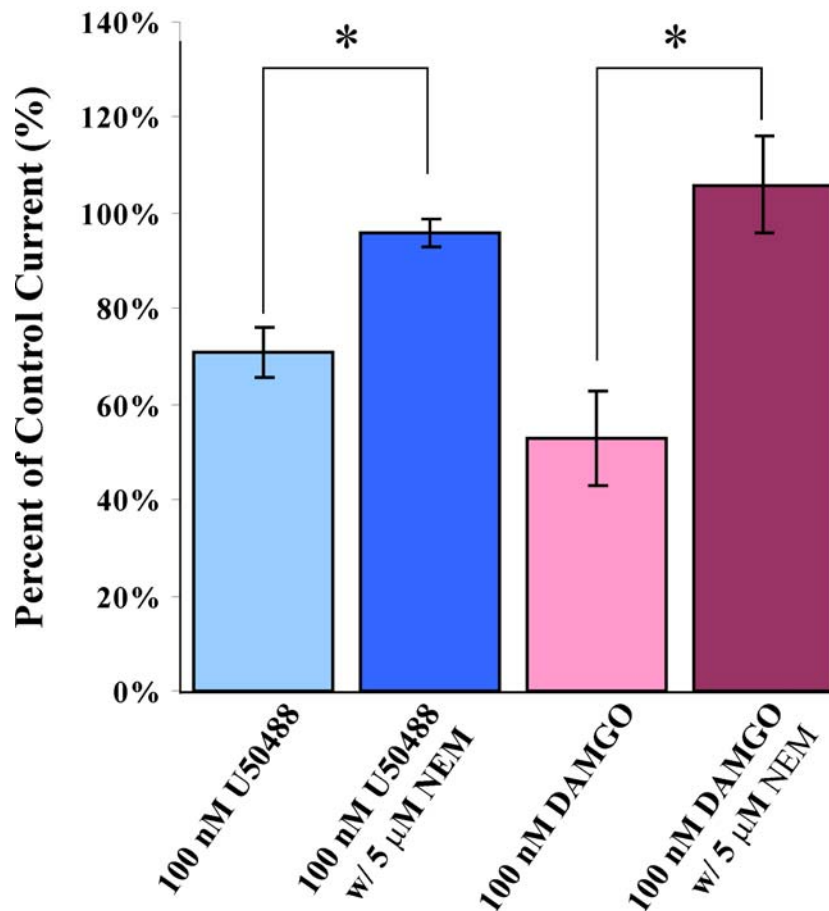


Figure 3.8 NEM relieves both κ - and μ -opioid inhibition of action potential waveform elicited Ca^{2+} -currents. Inhibition of the APW-elicited calcium current with 100 nM U50488 or 100 nM DAMGO was not statistically different ($p>0.09$) nor are both treatments with 5 μ M NEM significantly different ($p>0.19$) from each other or from control peak currents ($n=3$ for each). Asterisks (*) represent statistically significant differences ($p<0.02$).

Figure 3.9

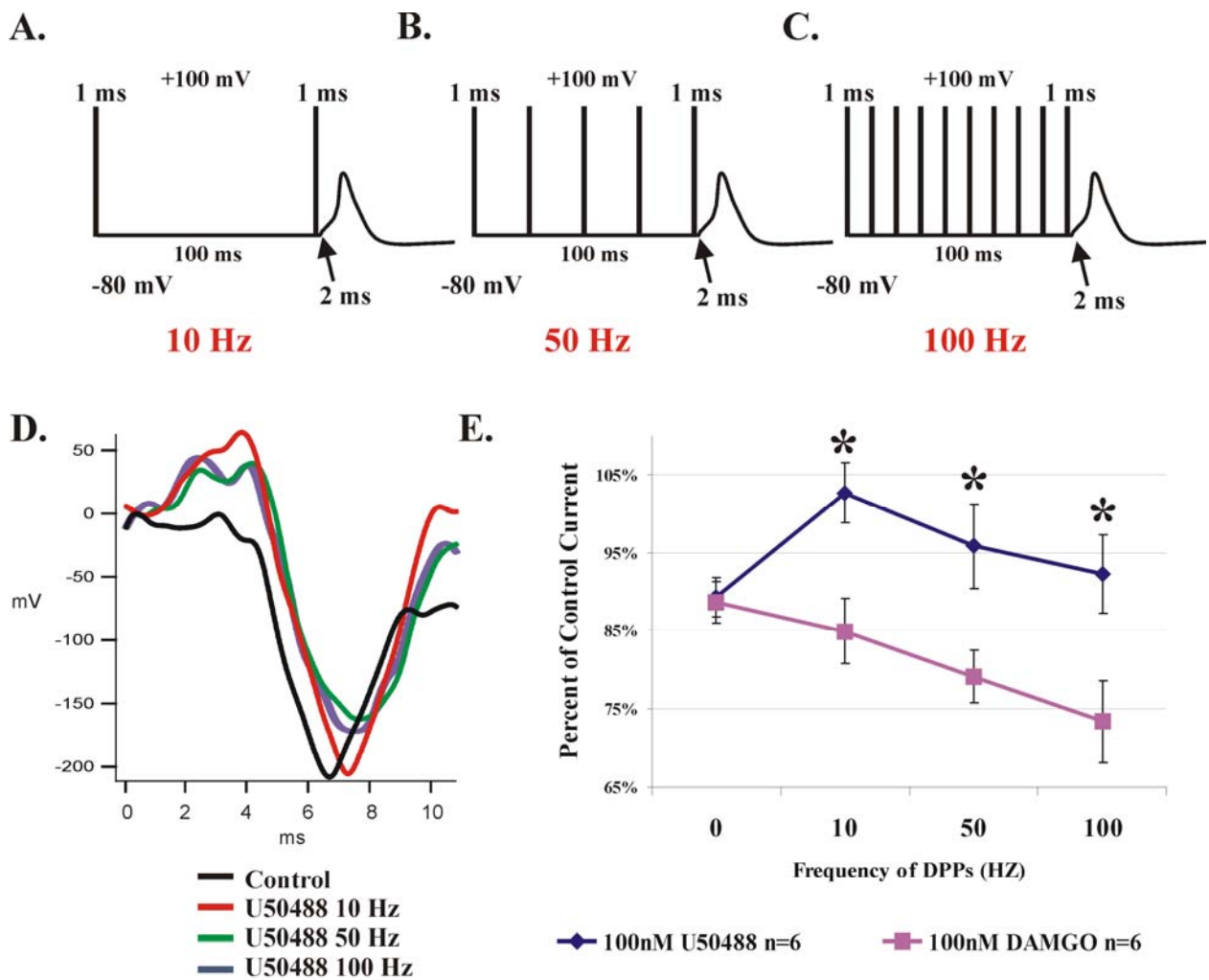


Figure 3.9 Frequency-dependent relief of κ -opioid inhibition. Voltage command protocols of varying frequency using 1 ms duration pre-pulse, stepped to +100 mV and a 2 ms interval between the last pulse and the Long APW: *A.* 10 Hz, *B.* 50 Hz, and *C.* 100 Hz. *D.* Sample Long AWP elicited calcium currents under control (no opioid, no DPP), U50488 after 10 Hz DPP (red), U50488 after 50 Hz (green) and U50488 after 100 Hz (purple) DPP protocol. *E.* Percent of control current without treatment vs. average of APW elicited current with 100 nM U50488 (blue), and 100 nM DAMGO (pink). Asterisks (*) represent statistically significant $p \leq 0.05$ differences between U50488 treatment and DAMGO.

Figure 3.10

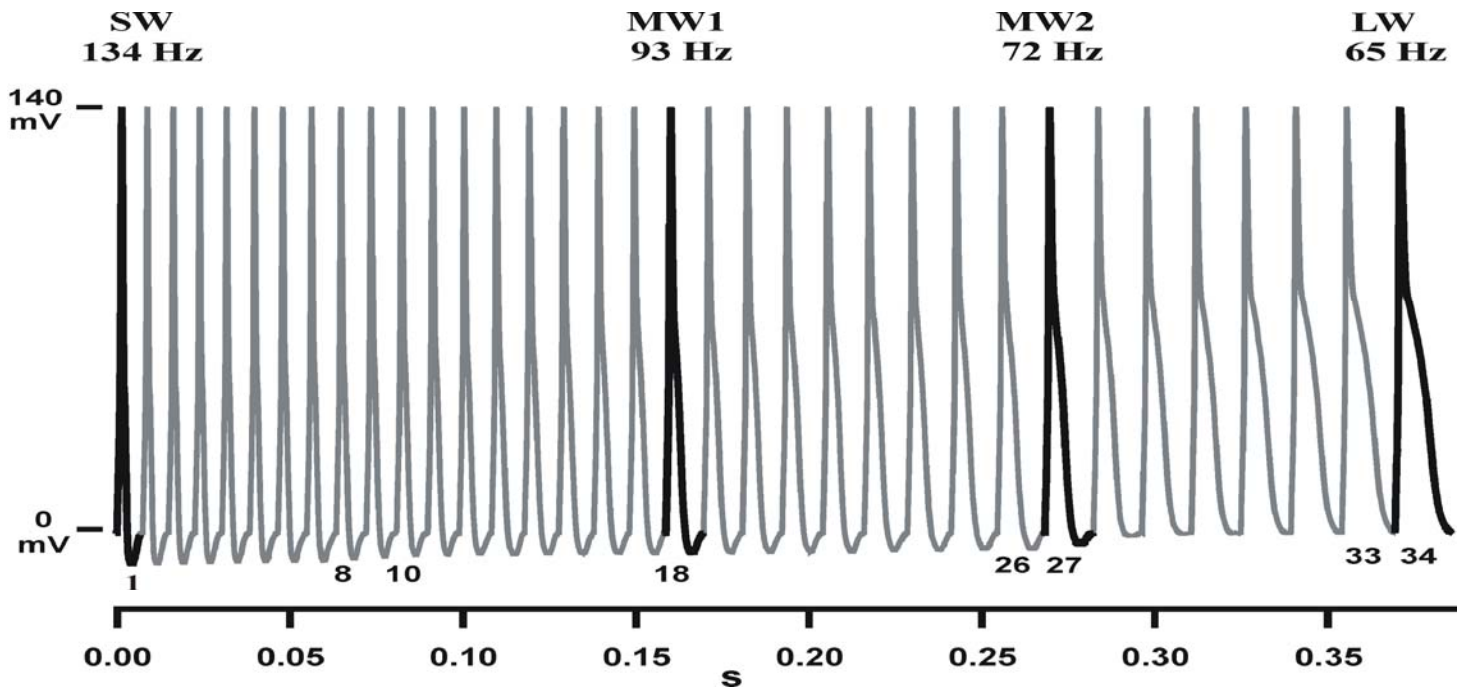


Figure 3.10 Waveform used for simulating action potential burst (gray). The waveform was applied as a relative stimulus (left voltage scale, i.e., depolarizing). Darkened segments within the burst waveform indicate action potential equivalents to the ones used individually (single action potentials) as designated by the top labels (See Fig. 3), with the frequencies that would correspond to their position within the burst (Hz, below top labels). The numbers below some of the action potentials (between time scale and burst) represent action potentials of a specific duration and illustrate their placement within the simulated burst.

Figure 3.11

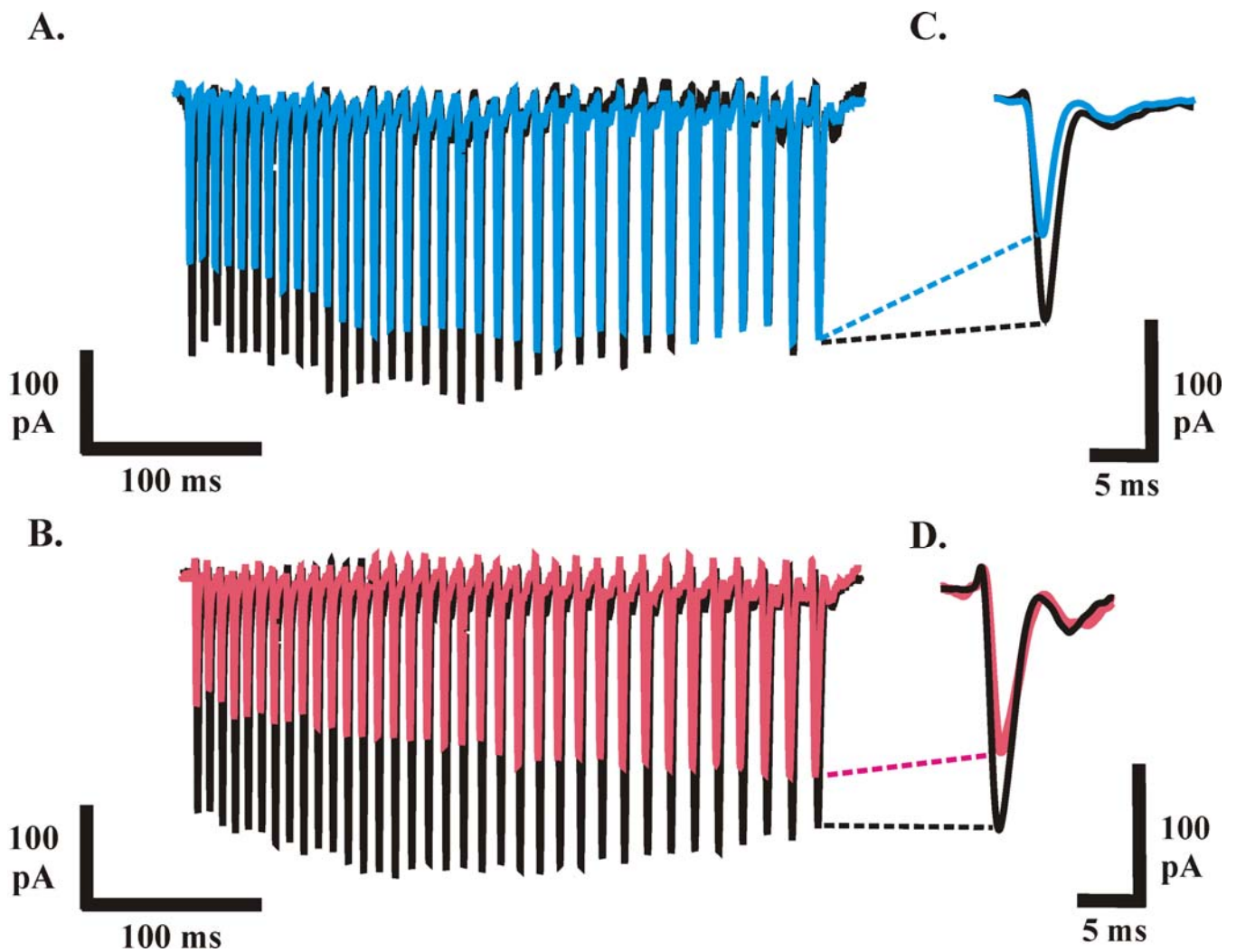


Figure 3.11 κ -opioid inhibition of APW calcium currents is relieved within a simulated burst. *A.* Calcium currents elicited by a simulated AP burst under control conditions (black line) and in the presence of 100 nM U50488. *B.* Calcium currents using the same protocol as in *A.* under control (black) or in the presence of 100 nM DAMGO (red). *C.&D.* Calcium currents elicited with a single long APW (LW2) stimulation identical to the one at the end of the burst under their respective control conditions (black) or in the presence of 100 nM U50488-blue (*C*) or 100 nM DAMGO-pink (*D*). Dotted lines are for comparison between same opioid modulation of APW-elicited Ca^{2+} -current within (*A&B*) and outside (*C&D*) of the burst.

Figure 3.12

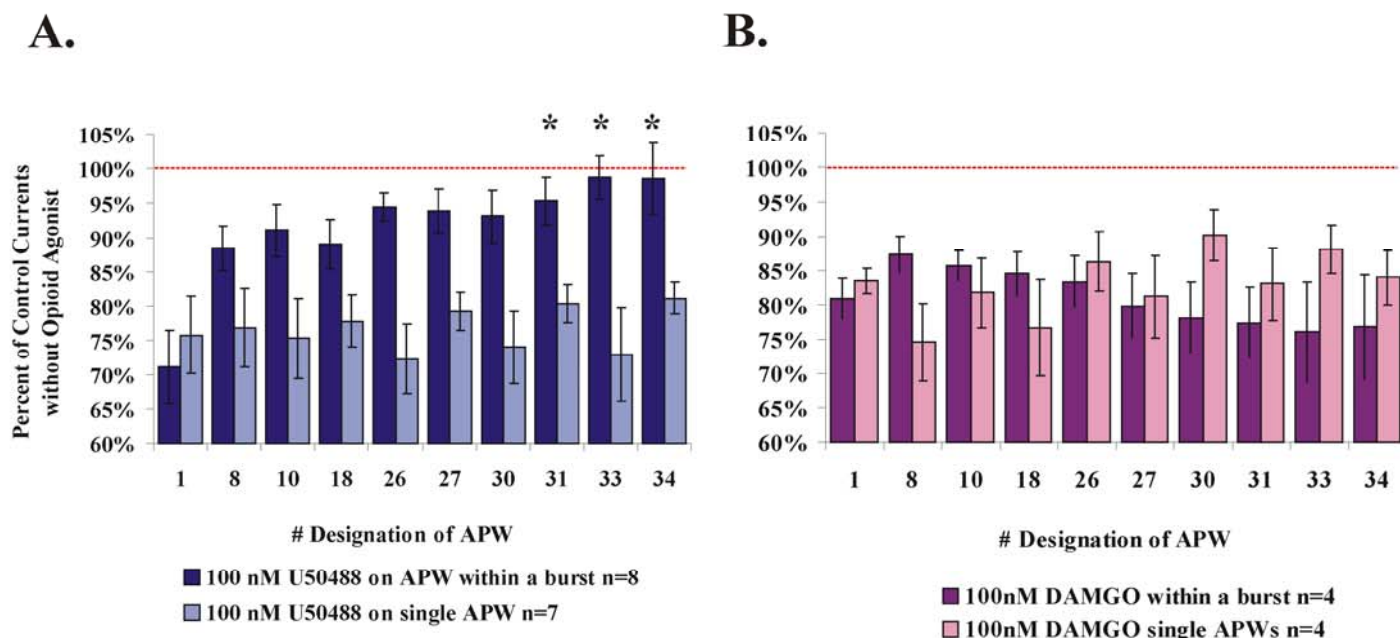


Figure 3.12 AP-elicited Currents are relieved of κ -inhibition within a Burst. **A.** Bar

Graph showing percent of control (currents without κ -opioid agonists elicited with identical APW duration as experimental with opioid agonist) during 100 nM U50488 application on APW-elicited currents of differing durations within the simulated burst (dark blue) compared to identical APWs individually elicited Ca^{2+} currents (light blue).

B. Bar Graph showing percent of control (currents without μ -opioid agonists elicited with identical APW duration as experimental with opioid agonist) during 100 nM DAMGO application on APW-elicited Ca^{2+} currents of differing duration within the simulated burst (dark pink) compared to identical APW Ca^{2+} currents elicited individually (light pink). Asterisks (*) represent no statistically significant $p > 0.5$ difference between treatment (U50488) and control (without opioid) conditions.

Chapter IV

μ -Opioid signaling via ryanodine-sensitive Ca^{2+} stores in isolated terminals of the Neurohypophysis

μ -Opioid signaling via ryanodine-sensitive Ca^{2+} stores in isolated terminals of the Neurohypophysis

Cristina Velázquez-Marrero, Sonia Ortíz-Miranda, Héctor G. Marrero, and José R. Lemos

Abstract

μ -opioid agonists do not inhibit calcium currents when recorded using the classic whole-cell patch-clamp configuration (Rusin et al., 1997b). However, μ -receptor mediated inhibition of calcium currents is consistently demonstrated using the perforated-patch configuration (Ortiz-Miranda et al., 2003; Ortiz-Miranda et al., 2005). This strongly suggests that the μ -opioid receptor-signaling pathway at the terminals is sensitive to intraterminal dialysis and therefore mediated by a readily diffusible second messenger. Using perforated patch-clamp technique and ratio calcium imaging methods, we describe a diffusible second-messenger pathway stimulated by the μ -opioid receptor that inhibits voltage-gated calcium channels (VGCC) in isolated terminals from the Neurohypophysis. Our results show a rise in basal $[\text{Ca}^{2+}]_i$ in response to application of 100 nM DAMGO, a μ -opioid receptor agonist, which is blocked by 100 nM CTOP, a μ -opioid receptor antagonist. Buffering DAMGO-induced changes in $[\text{Ca}^{2+}]_i$ with Bapta-AM completely blocked the inhibition of both calcium currents and High K^+ -induced rises in $[\text{Ca}^{2+}]_i$ due to μ -opioid receptor activation. Bapta-AM had no effect on κ -opioid receptor mediated inhibition of either calcium currents or High K^+ -induced rises in $[\text{Ca}^{2+}]_i$. Cyclic ADP-ribose has been characterized as the endogenous ryanodine receptor agonist (Jin et al., 2007). Given the presence of ryanodine-sensitive stores in isolated HNS terminals (De

Crescenzo et al., 2004b), we tested 8Br-cADP-ribose, a competitive inhibitor of cyclic ADP-ribose signaling. 8Br-cADP-ribose partially relieves DAMGO inhibition of calcium currents and completely relieves μ -opioid inhibition of High K^+ -induced rises in $[Ca^{2+}]_i$. Furthermore, antagonist concentrations of ryanodine completely blocked μ -opioid inhibition of calcium currents and High K^+ -induced rises in $[Ca^{2+}]_i$ while not affecting κ -opioid mediated inhibition. 100 μ M ryanodine also blocked μ -opioid inhibition of electrically evoked changes in capacitance. These results strongly suggest that a key diffusible second messenger mediating the μ -opioid receptor-signaling pathway at the terminals is intraterminal calcium released by cADPr from ryanodine-sensitive stores in the isolated HNS terminals.

Introduction

The μ -opioid inhibition of the magnocellular neurosecretory system becomes increasingly evident during pregnancy, and is interrupted prior to parturition allowing for strong excitation of oxytocin cells and thus facilitating birth (Russell et al., 1995b; Russell et al., 2003). Co-release of dynorphin, an endogenous κ -opioid agonist, with vasopressin from dendrites facilitates activity-dependent modulation of vasopresinergic neurons (Brown and Bourque, 2004; Brown et al., 2004; Roper et al., 2004; Brown et al., 2006; Sabatier and Leng, 2007). Isolated hypothalamic neurohypophysial (HNS) terminals also demonstrate inhibition of release in the presence of either μ - or κ -agonists for both oxytocin and vasopressin (Sumner et al., 1990; Kato et al., 1992; Russell et al., 1993), VGCC are similarly inhibited (Rusin et al., 1997a; Ortiz-Miranda et al., 2003; Ortiz-Miranda et al., 2005). The signaling mechanism and modulatory importance of μ - and κ -receptor activation at these pre-synaptic terminals and subsequent VGCC inhibition is still not well understood however it will likely prove to be physiologically relevant given the importance of endogenous opioid modulation at the level of the cell body.

Both the μ - and κ -opioid receptors are G-protein coupled receptors, which can potentially mediate inhibitory effects of opiates on VGCC through either a membrane-delimited or diffusible second-messenger pathway (Wilding et al., 1995; Kaneko et al., 1998; Soldo and Moises, 1998; Connor and Christie, 1999; Chen et al., 2000). The μ -opioid receptor signaling pathway seems to contrast sharply with that documented for the κ -opioid receptor in the same isolated terminals. The first indication of differences in

signaling mechanism emerged when it was shown that μ -opioid agonists did not inhibit calcium currents when recorded using the classic whole-cell patch-clamp configuration (Rusin et al., 1997b). However, μ -receptor mediated inhibition of calcium currents is consistently demonstrated using the perforated-patch configuration of the patch-clamp method (Ortiz-Miranda et al., 2005). This strongly suggests that the μ -opioid receptor-signaling pathway at the terminals is sensitive to intraterminal dialysis and therefore mediated by a readily diffusible second-messenger. Furthermore, unlike the κ -opioid inhibition of VGCC, μ -opioid inhibition is not relieved with a strong depolarizing pre-pulse (See Chapter III).

Several diffusible second-messenger pathways can be potential candidates mediating μ -opioid inhibition of VGCC in the HNS. Mobilization of internal calcium in response to activation of opioid receptors has been well documented in both neuronal and non-neuronal systems (for review; Samways and Henderson, 2006). Ryanodine-sensitive calcium stores have been shown to be targets of G-protein opioid activation. For example, activation of the μ -opioid receptor in isolated mouse astrocytes elicited a release of $[Ca^{2+}]_i$ blocked by the ryanodine-sensitive antagonist, dantrolene (Hauser et al., 1996). In isolated rat ventricular myocytes the rise in $[Ca^{2+}]_i$ due to opioid receptor activation was blocked when ryanodine-sensitive stores were depleted with ryanodine pretreatment (Tai et al., 1992). In isolated HNS terminals we have characterized small ryanodine- and voltage-sensitive calcium release events, known as syntillas (De Crescenzo et al., 2004b). The cyclic ADP-ribose (cADPr) signaling pathway initiates a signaling cascade leading to activation of the ryanodine receptor *in-vivo* and subsequent release of intracellular calcium from ryanodine-sensitive stores (Galione, 1994;

Sitsapesan et al., 1995; Morita et al., 2002). Interestingly, recent studies have shown the cADPr signaling pathway has a significant role in neuropeptide release from isolated HNS terminals (Higashida et al., 2007; Jin et al., 2007). We propose that in HNS terminals, activation of the μ -opioid receptor triggers a rise in intraterminal basal calcium, released from ryanodine-sensitive stores possibly via activation of the cADPr signaling cascade. The rise in $[Ca^{2+}]_i$ would lead to calcium-dependent inhibition of VGCC and subsequent inhibition of depolarization-induced neuropeptide release.

Materials and Methods

Isolation of nerve endings:

Male Sprague-Dawley rats (Taconic Farms, Germantown, NY) weighing 200–250 g were sedated using CO₂ and immediately decapitated. The pituitary gland was isolated as previously described (Lemos et al., 1986; Knott et al., 2005). Briefly, following removal of the anterior and intermediate lobes, the posterior pituitary was homogenized in 270 μ l of buffer at 37°C containing (in mM): 270 sucrose, 0.004 EGTA, 10 HEPES-Tris, buffered at pH 7.25; 298–302 mOsmol/L. The solution containing the homogenate was plated on a 35 mm petri dish and carefully washed in Low-calcium Locke's solution which consists of modified Normal Locke's (mM): 145 NaCl, 2.5 KCl, 10 HEPES, 1.2 glucose, 0.8 CaCl₂, 0.4 MgCl₂, pH 7.4; 298–302 mOsmol/L.

Electrophysiological experiments:

The neurohypophysis was isolated and homogenized as previously described (Brethes et al., 1987; Brown et al., 2006). Current recordings were obtained using the perforated-patch configuration on isolated HNS terminals. Using an inverted microscope the terminals were identified visually by their characteristic appearance, spherical shape, lack of nuclei, and size (5–10 μm in diameter). The pipette solution consisted of (in mM): 145 Cs-gluconate, 15 CsCl, 2 MgCl_2 , 2 NaCl, 7 Glucose, 10 HEPES (pH 7.3), at 295 mOsm. Amphotericin B at a concentration of 30 μM (SIGMA) was added as a perforating agent. The bath solution consisted of (mM): 145 NaCl, 5 KCl, 1 MgCl_2 , 10 HEPES, 10 Glucose, 1.2 CaCl_2 , pH 7.5 Normal Locke's. In all experiments TTX (100 nM) was added to the bath to block sodium influx via voltage-gated sodium channels. The pipette resistance was 5-8 $\text{M}\Omega$. Pipettes were made of thin borosilicate glass (Drummond Scientific Co., Broomall, PA, USA). After perforation the terminals were voltage-clamped at -80 mV. Depolarization was applied every 30 seconds to 0 mV for 250-300 ms. The preparation was either continually perfused, via a gravity driven perfusion, or left in a static non-perfused bath (as noted). Agonists and antagonists were either applied through the gravity driven perfusion system, or added to the static bath. All experiments were performed at room temperature (25°C). Data was acquired, stored and analyzed using a Pentium I computer (Gateway) and pClamp 7 (Axon Instruments, Foster City, CA). Currents were corrected online using an inverted P/4 protocol. All time constants (τ 's) were obtained using a single exponential curve fit (Igor, Wavemetrics) on inward calcium currents. For the decay time (inactivation) the fit was made between 5 ms

after peak (to avoid multiple exponentials) and 3 ms before the end of stimulus (to avoid transient artifacts).

Calcium imaging

Freshly dissociated nerve terminals (Nordmann et al., 1987) prepared from adult Sprague-Dawley CD rats were incubated with 2.5 μM Fura-2 AM for 45 min. at 37°C and thoroughly washed with Normal Locke's solution. Normal Locke's contained (mM): 145 NaCl, 5 KCl, 10 Hepes, 10 Glucose, 1 MgCl_2 and 2.2 CaCl_2 , pH 7.4. Ca^{2+} free bath solution contained (mM): 145 NaCl, 5 KCl, 10 Hepes, 10 Glucose, 0.0002 EGTA, 1 MgCl_2 , pH 7.4, and gave a calculated free $[\text{Ca}^{2+}]$ of zero. Cytosolic $[\text{Ca}^{2+}]$ was determined with ratiometric indicator fura-2 AM loaded terminals and calibrated utilizing an *in-vitro* calibration kit (Invitrogen, Carlsbad CA). This was performed according to the method of (Grynkiewicz et al., 1985) with an assumed Ca^{2+} -fura 2 KD of 200 nM, as previously described (Becker et al., 1989). Resting values for global cytosolic $[\text{Ca}^{2+}]$ in the presence and absence of extracellular Ca^{2+} were 73.3 ± 6.9 nM ($n = 12$) and 46.2 ± 7.5 nM ($n = 8$), respectively, and these values demonstrated a statistically significant difference ($p < 0.05$). In all cases, data are reported as mean \pm SEM; n being the number of terminals. Statistical analyses of differences were made with paired t-tests, with $p < 0.05$ considered significant.

Fluorescence images using Fura-2 AM as a calcium indicator were viewed with a Nikon Diaphot TMD microscope, using a Zeiss Plan- NEOFLUAR 100X oil immersion lens, and fitted with a Photometrics SenSys CCD camera. The camera was interfaced to the inverted microscope adapted with a Chroma 71000A Fura2 filter cube. The terminals

were excited using a Xenon arc lamp within a Lambda DG4 high-speed filter changer (Sutter Instruments Incorporated, Novato, CA) with the appropriate filters (340 and 380 nm wavelengths). Intraterminal emission of Fura-2 Ca^{2+} indicator was gathered at 510 nm wavelength. Fluorescent images were acquired and processed with Axon Imaging Workbench 2.1 software (Axon Instruments, Foster City, CA).

Capacitance Measurements

Freshly dissociated terminals (Nordmann et al., 1987) from adult adult Sprague-Dawley CD rats were plated in Normal Locke's solution with 1.2 mM CaCl_2 . Tight seal "whole terminal" recordings were obtained using the perforated-patch configuration described above. The pipettes resistance ranged from 5-8 M Ω . Perforation of the terminals' membrane was obtained by adding 30 μM amphotericin B (SIGMA) to the pipette solution containing (mM): 145 Cs-gluconate, 15 CsCl, 5 NaCl, 2 MgCl_2 , 7 Glucose, 10 HEPES pH 7.3. The bath solution contained (mM): 145 NaCl, 5 KCl, 1 MgCl_2 , 10 HEPES, 10 Glucose, 1.2 CaCl_2 or 0.2 EGTA, pH 7.5. Capacitance measurements were obtained using the piecewise-linear method (Knott et al., 2007). The changes in capacitance induced by a depolarizing pulse (750 ms duration) were measured 1 second after cessation of stimulus, in order to avoid interference of stimulus "end-tail" effects. These stimulus-induced capacitance changes were measured for isolated terminals (perforated-patch) using the piece-wise method (Neher and Marty, 1982; Lindau and Neher, 1988; Gillis, 1995). Briefly, the method consists in applying a sinusoidal voltage of low amplitude to the sample (in order to avoid voltage-elicited channel currents) and obtaining the phase shift of the resultant sinusoidal current.

Changes in this phase shift (“locked-in”) are used in a formula (computer software that emulates a lock-in amplifier) for the determination of the capacitance changes (i.e., the capacitance that would cause such change of phase shift). The method is sensitive to very small changes in capacitance and, in practice, large baseline capacitance and resistance (series) transients must be compensated (i.e., null) before measuring any small capacitance change. In this particular case the parameters used were a sine wave of 1000 Hz at ± 25 mV (about holding potential), with the program reporting a capacitance averaged for every 30 points (24 μ s sampling rate). The current was filtered at a bandwidth of 5000 Hz.

Statistical comparisons.

In all cases, data are reported as mean \pm SEM; n being the number of terminals. Statistical analysis of difference was made with paired t-test, with $p < 0.05$ considered significant.

Results

μ - but not κ -opioid agonist elicits a rise in basal intraterminal $[Ca^{2+}]_i$

To determine if intraterminal Ca^{2+} was part of a diffusible second-messenger pathway mediating opioid receptor signaling in isolated HNS terminals, we monitored intraterminal calcium in response to μ - and κ -opioid receptor activation. Fura-2 AM ratio calcium imaging of isolated HNS terminals has shown a significant inhibition of High K^+ -induced rise in $[Ca^{2+}]_i$ when treated with either μ - or κ -agonists (Velázquez-Marrero

et al., in preparation; See Chapter III) (Fig.4.1). Furthermore, pretreatment with 100 nM DAMGO (μ -opioid agonist) alone, in 2.2 or 0 mM $[Ca^{2+}]_o$, elicited a rise in basal $[Ca^{2+}]_i$ (Figs. 4.1 & 4.2). This did not occur when terminals were pretreated with 100 nM U50488 (κ -opioid agonist), or puffed with control 0 mM $[Ca^{2+}]_o$ Normal Lockes' (NL) (Fig. 4.2A). Application of 100 nM DAMGO in the presence of the μ -opioid receptor antagonist CTOP (Fig. 4.2B) blocked the DAMGO-induced rise in basal $[Ca^{2+}]_i$. Fura-2 AM calcium measurements were expressed as changes in basal intraterminal calcium concentration; 100 nM DAMGO in 2.2 mM $[Ca^{2+}]_o$ NL was 26.9 ± 0.8 , similar to basal changes in 0 mM $[Ca^{2+}]_o$ with 100 nM DAMGO (28.7 ± 5.1). Changes in $[Ca^{2+}]_i$ due to 100 nM U50488 were -3.3 ± 5.2 , control was -2.4 ± 3.4 , and 100 nM DAMGO in the presence of CTOP = -1.8 ± 1.0 , all in 0 mM $[Ca^{2+}]_o$. These applications caused an artifactual dip in $[Ca^{2+}]_i$ which may be inherent factor in all treatment applications and could lead to an underestimation of the DAMGO-induced rise in $[Ca^{2+}]_i$. Baseline $[Ca^{2+}]_i$ averaged 71.6 ± 15.7 (n=4).

μ - but not κ -opioid inhibition is blocked by intraterminal calcium buffering

In order to determine if the rise in $[Ca^{2+}]_i$ due to μ -opioid agonist application is essential for subsequent inhibition of VGCC, we partially buffered $[Ca^{2+}]_i$ using Bapta-AM and monitored the effects on both calcium currents and High K^+ -induced rise in $[Ca^{2+}]_i$. Empirically determined incubation periods of 5-10 min. using low concentrations (10 μ M) Bapta-AM were followed by a brief wash. Using this procedure Bapta-AM buffered DAMGO-induced rises in intraterminal calcium from a 22.7 ± 2.4 nM change in $[Ca^{2+}]_i$ to 0.2 ± 0.5 nM (Fig. 4.3), but without blocking High K^+ -induced rise in $[Ca^{2+}]_i$

(Fig.4.4). Effects on both High K^+ -induced rise in $[Ca^{2+}]_i$ and calcium currents were measured for both μ - and κ -opioid mediated inhibition using the same concentration of Bapta-AM for identical incubation periods. All measurements were made as either changes in basal $[Ca^{2+}]_i$ or percent of control, specifically, control measurements were under identical conditions without opioid treatments. Inhibition of High K^+ -induced rise in $[Ca^{2+}]_i$ due to 100 nM DAMGO represented $54.1 \pm 3.4\%$ of control K^+ -induced rise in $[Ca^{2+}]_i$ and $70.9 \pm 2.8\%$ in the presence of 100 nM U50488 (Fig.4.4B). After incubation of 10 μ M Bapta-AM, terminals exposed to 100 nM DAMGO now showed no inhibition ($109.9 \pm 12.8\%$) of the High K^+ -induced rise in $[Ca^{2+}]_i$, while exposing Bapta-AM treated terminals to 100 nM U50488 showed no change in κ -opioid mediated inhibition ($78.5 \pm 2.8\%$). T-test analysis determined no statistically significant difference ($p \geq 0.3$) between terminals treated with 100 nM U50488, with vs. without Bapta-AM pre-incubation. In contrast, there was a statistically significant difference ($p \leq 0.0005$) between terminals treated with 100 nM DAMGO, with vs. without Bapta-AM pre-incubation (Fig.4.4B). Baseline $[Ca^{2+}]_i$ without Bapta-AM pre-incubation was 86.5 ± 27.5 nM and after pre-incubation with Bapta-AM it was 77.9 ± 12.8 nM. There was no statistical difference ($p \geq 0.16$) between baseline $[Ca^{2+}]_i$ with vs. without 5 min. pre-incubation with 10 μ M Bapta-AM.

Buffering $[Ca^{2+}]_i$ also blocked Inhibition of calcium currents by μ - but not κ -opioid agonists. Calcium currents treated with 100 nM DAMGO represented $82.2 \pm 4.0\%$ of control current and $88.7 \pm 2.3\%$ in the presence of 100 nM U50488 (Fig. 4.5). After pre-incubation with 10 μ M Bapta-AM, 100 nM DAMGO treatment now elicited no inhibition, with calcium currents $98.9 \pm 1.2\%$ of control. Exposing Bapta-AM treated

terminals to 100 nM U50488, again showed no change in κ -mediated inhibition, with calcium currents at $78.8 \pm 4.9\%$ of control. T-test analysis determined no statistically significant difference ($p \geq 0.09$) between terminals treated with 100 nM U50488, with vs. without Bapta-AM pre-incubation (Fig.4.5C). There was a statistically significant difference ($p \leq 0.001$) between terminals treated with 100 nM DAMGO, with vs. without Bapta-AM pre-incubation.

8Br-cADP-ribose also blocks μ -opioid inhibition of High K^+ -induced rise in $[Ca^{2+}]_i$ and partially relieves μ -opioid inhibition of calcium currents.

Since the μ -opioid induced rise in $[Ca^{2+}]_i$ is essential in mediating μ -opioid inhibition, we then wanted to know if the cADPr pathway was part of the receptor intraterminal signaling. We therefore, examined the effects of the membrane-permeant cADPR antagonist, 8Br-cADP-ribose, on μ -opioid inhibition of both High K^+ -induced rises in $[Ca^{2+}]_i$ (Fig. 4.6) and calcium currents (Fig. 4.7). Control High K^+ -induced changes in $[Ca^{2+}]_i$ were 457.1 ± 13.5 nM. In the presence of 100 nM DAMGO High K^+ -induced change in $[Ca^{2+}]_i$ decreased to 392.7 ± 19.6 nM. After incubation with 100 nM 8Br-cADP-ribose, terminals exposed to 100 nM DAMGO now showed a change in $[Ca^{2+}]_i$ of 425.9 ± 11.3 nM in response to High K^+ , similar to those in 100 nM 8Br-cADP-ribose without DAMGO treatment 427.2 ± 8.9 nM. T-test analysis determined no statistically significant difference ($p \geq 0.43$) in High K^+ -induced changes in basal $[Ca^{2+}]_i$ between terminals treated with 100 nM 8Br-cADP-ribose, with vs. without 100 nM DAMGO treatment (Fig.4.6). However, there was a statistically significant difference ($p \leq 0.0004$) in High K^+ -induced changes in basal $[Ca^{2+}]_i$ between terminals treated with

vs. without 100 nM DAMGO, which were not pre-incubated with 100 nM 8Br-cADP-ribose. Interestingly, baseline $[Ca^{2+}]_i$ without 100 nM 8Br-cADP-ribose pre-incubation was 79.2 ± 4.0 nM and after pre-incubation with 100 nM 8Br-cADP-ribose was 68.9 ± 2.8 nM; n=8, and these baselines were statistically different ($p \leq 0.02$).

Inhibition of calcium currents due to 100 nM DAMGO was measured as percent of control (Fig. 4.7). As stated above, controls were measurements under identical conditions without opioid treatments. Calcium currents treated with 100 nM DAMGO represented $76 \pm 3\%$ of control current (Fig. 4.7). After pre-incubation with 100 nM 8Br-cADP-ribose, 100 nM DAMGO treatments now showed a partial relief of inhibition, with calcium currents at $90 \pm 2\%$ of control. T-test analysis determined there was a statistically significant difference between terminals treated with 100 nM DAMGO, with or without 100 nM 8Br-cADP-ribose pre-incubation ($p \leq 0.02$).

μ -opioid effects are blocked by 100 μ M Ryanodine

We next addressed whether the release of $[Ca^{2+}]_i$ due to μ -opioid receptor activation was from ryanodine-sensitive intraterminal stores. The pharmacology of the ryanodine receptor indicates that 10-100 μ M concentrations of ryanodine can block ryanodine channel activity (Coronado et al., 1994; Ehrlich et al., 1994). Therefore, we tested the effects of 100 μ M ryanodine on DAMGO inhibition of High K^+ -induced rise in $[Ca^{2+}]_i$ (Fig. 4.8), calcium currents (Fig. 4.9-4.11), and electrically-induced capacitance changes (Fig. 4.10 & 4.11). Control High K^+ -induced change in basal $[Ca^{2+}]_i$ was 219.9 ± 21.4 nM. In the presence of 100 nM DAMGO High K^+ -induced change in $[Ca^{2+}]_i$ was 112.7 ± 8 nM which represents 51% of the control response (Fig. 4.8). After incubation

with 100 μM ryanodine, terminals exposed to 100 nM DAMGO now showed a change in $[\text{Ca}^{2+}]_i$ of 236.8 ± 8.2 nM in response to High K^+ , similar to control without DAMGO (209 ± 13.4 nM) in the presence of 100 μM ryanodine. T-test analysis determined no statistically significant difference ($p \geq 0.11$) in High K^+ -induced changes in basal $[\text{Ca}^{2+}]_i$ between control terminals in 100 μM ryanodine and those in 100 μM ryanodine treated with 100 nM DAMGO. There was a statistically significant difference ($p \leq 0.01$) in High K^+ -induced changes in $[\text{Ca}^{2+}]_i$ between terminals treated with vs. without 100 nM DAMGO, which were not pre-incubated with 100 μM ryanodine. Control baseline $[\text{Ca}^{2+}]_i$ was 62.2 ± 11.3 nM and 76.1 ± 11.5 nM after pre-incubation with 100 μM ryanodine; ($n=3$). There was no statistical difference between baseline $[\text{Ca}^{2+}]_i$ with vs. without 100 μM ryanodine incubation ($p \geq 0.05$).

Inhibition of calcium currents due to 100 nM DAMGO (Fig. 4.9) was measured as percent of control (Fig. 4.11). Calcium currents treated with 100 nM DAMGO were inhibited to $76.3 \pm 1.8\%$ of control current (Fig. 4.11). During incubation with 100 μM ryanodine, calcium currents treated with 100 nM DAMGO showed no significant inhibition at $94.6 \pm 2.5\%$ of control; ($n=6$). T-test analysis determined there was no statistically significant difference ($p \geq 0.10$) between terminals in the presence of 100 μM ryanodine with and without 100 nM DAMGO. Inactivation of Ca^{2+} currents via Ca^{2+} -dependent inactivation could be reflected in the time constants of inactivation of the total recorded currents. We therefore tested the time constants of inactivation of the Ca^{2+} currents under control (no opioid or ryanodine), 100 nM DAMGO, 100 μM ryanodine, and 100 μM ryanodine with 100 nM DAMGO conditions (Fig. 4.11). The results were plotted as percent control (no opioid or ryanodine). Calcium currents in the presence of

DAMGO showed slowed inactivation kinetics resulting in $133 \pm 2.6\%$ of control. These were statistically different from control values with $p \leq 0.001$. In the presence of $100 \mu\text{M}$ ryanodine they were $105.4 \pm 3.9\%$, (statistically not different from control $p \geq 0.77$) and in the presence of $100 \mu\text{M}$ ryanodine and 100 nM DAMGO $96.1 \pm 3.7\%$ (also statistically not different from control $p \geq 0.22$; $n=3$).

Reduction of depolarization-induced neuropeptide release is presumably due to μ -opioid receptor inhibition of VGCC. If μ -opioid inhibition of VGCC can be blocked by $100 \mu\text{M}$ ryanodine can we also block the μ -opioid reduction in neuropeptide release? To address this question we monitored changes in neuropeptide release reflected as capacitance changes in individual terminals (See Methods). Changes in capacitance in perforated-patched isolated HNS terminals in response to rectangular pulse depolarizations are inhibited by 100 nM DAMGO (Fig. 4.10). The inhibition is almost completely reversed by 100 nM CTOP (data not shown) indicating it is mediated by activation of the μ -opioid receptor. In the presence of 100 nM DAMGO capacitance changes were $43.8 \pm 8.2\%$ of control without DAMGO (Fig. 4.10 & 4.11). When incubated in $100 \mu\text{M}$ ryanodine, capacitance changes returned to $102.9 \pm 3.5\%$ of control. Application of 100 nM DAMGO in the presence of $100 \mu\text{M}$ ryanodine resulted in $98.2 \pm 2.7\%$ of control capacitance change and was statistically no different than control ($p \geq 0.99$) or capacitance changes in the presence of $100 \mu\text{M}$ ryanodine alone ($p \geq 0.16$). T-test analysis determined there was a statistically significant difference between terminals with vs. without 100 nM DAMGO ($p \leq 0.001$) that were not treated with $100 \mu\text{M}$ ryanodine.

Discussion

In the present study we have found that in isolated HNS terminals activation of the μ -opioid receptor elicits release of intraterminal Ca^{2+} from ryanodine-sensitive stores. Results show that μ -opioid inhibition of calcium currents and High K^+ -induced rise in $[\text{Ca}^{2+}]_i$ can be blocked in the presence of antagonist concentrations of ryanodine and reduced by competitive inhibition of the cADPr pathway. Changes in the inactivation kinetics of calcium currents in the presence of μ -opioid agonists suggests that release of intraterminal Ca^{2+} can be responsible for Ca^{2+} -dependent inactivation of VGCC. Furthermore, μ -opioid inhibition of neuropeptide release from individual terminals can also be blocked in the presence of antagonist concentrations of ryanodine. The evidence supports the hypothesis that μ -opioid receptor activation leads to release of Ca^{2+} from ryanodine-sensitive stores possibly via activation of the cADPr pathway leading to Ca^{2+} -dependent inactivation of VGCC and subsequent inhibition of DSC.

μ -opioid effects on the magnocellular neurosecretory system

Endogenous opioids play an important part in modulation of neuropeptide secretion from magnocellular neurons in the neurohypophysis (Clarke et al., 1979; Bicknell and Leng, 1981; Clarke et al., 1981; Bicknell et al., 1985b; Nordmann et al., 1986a; Bondy et al., 1988). Isolated HNS terminals show a reduction in depolarization-induced release of both OT and AVP in response to both μ - (Zhao et al., 1988b; Leng et al., 1992; Russell et al., 1995a; Ortiz-Miranda et al., 2003; Ortiz-Miranda et al., 2005) and κ -opioid (Bicknell et al., 1988; Bondy et al., 1988; Zhao et al., 1988a; Rusin et al., 1997b) receptor activation.

cADPr pathway and ryanodine-sensitive stores in HNS terminals

Oxytocin secretion is most sensitive to μ -opioid inhibition (Wright and Clarke, 1984; Bicknell et al., 1985a; Zhao et al., 1988b; Leng et al., 1992; Russell et al., 1995a; Ortiz-Miranda et al., 2005). AVP release, while responsive to μ -opioid inhibition, is less sensitive to lower concentrations of agonists (Ortiz-Miranda et al., 2003). Interestingly, in HNS terminals, blocking cADPr signaling was shown to attenuate High K^+ induced rise in $[Ca^{2+}]_i$ and oxytocin release from isolated terminals (Higashida et al., 2007; Jin et al., 2007). This strongly suggests that the cADPr pathway is present in OT terminals where μ -opioid inhibition is most sensitive. However, the contribution of the cADPr pathway to the depolarization-induced response seems to contradict the current results showing inhibition by μ -opioid agonist mediated, at least partially, via the cADPr pathway.

There are some important considerations, which must be addressed in order to interpret our current results. First, the concentration of 8-br cADPr in our study is 100 nM as compared to 100 μ M used in previous studies (Jin et al., 2007). Preliminary calcium imaging results done in our laboratory on rat isolated HNS terminals, shows a significant rise in basal $[Ca^{2+}]_i$ in the presence of 100-300 μ M concentrations of 8-Br cADPr. This would theoretically have the same effects as those seen for μ -opioid agonist activation, leading to subsequent calcium-dependent inactivation of VGCC and attenuated neuropeptide release. Second, in murine HNS terminals “syntillas”, are both ryanodine- and voltage-sensitive (De Crescenzo et al., 2004b; De Crescenzo et al., 2006). Syntillas result from the activation of both ryanodine-receptor (RyR) type-1 and type-2 receptors. Presumably, the type-1 RyR confers the voltage-sensitivity via direct interaction with VGCC (De Crescenzo et al., 2006), as documented in skeletal muscle

(Schneider and Chandler, 1973; Inui et al., 1987). Inhibition of voltage-dependent release of calcium from ryanodine-sensitive stores may account for the inhibition of depolarization-induced rise in $[Ca^{2+}]_i$ and oxytocin release observed in the presence of 100 μ M ryanodine as previously published (Jin et al., 2007). However, unlike murine hypothalamic nerve terminals, chromaffin cells do not display syntilla activation by depolarization of the plasma membrane, nor do they have type 1 RyRs (ZhuGe et al., 2006). Furthermore, it has yet to be determined if ryanodine-sensitive stores in rat HNS terminals are voltage-sensitive.

μ -opioid effects on the inactivation kinetics of calcium currents

Calcium-dependent inactivation of VGCC is present in HNS terminals (Branchaw et al., 1997; Wang et al., 1999b). By saturating the calcium-dependent inactivation, μ -opioid inhibition of VGCC results in less measurable inactivation of calcium currents after a stimulation pulse. In our current model, release of calcium from ryanodine-sensitive stores leads to calcium-dependent inactivation of VGCC. Therefore, the lack of the Ca^{2+} -dependent component of inactivation during depolarization results in the slower rate of the remaining fraction of inactivation observed in the presence of μ -opioid agonist (Fig. 4.11C).

Conclusion

Amplification of μ -opioid receptor activation via cADPr-mediated release of Ca^{2+} from a ryanodine-sensitive signaling cascade seems well suited for regulating OT release in a voltage-independent manner, during the relatively long period of gestation. It is during this time that endogenous μ -opioid inhibition of OT release accumulates to be interrupted during parturition and subsequent lactation (Russell et al., 1989; Douglas et

al., 1995b; Russell et al., 1995a; Ortiz-Miranda et al., 2003). While inhibition of VGCC may only be one possible role for ryanodine-sensitive stores in HNS terminals, it is consistent with its effects on VGCC in cardiac (Schneider and Chandler, 1973; Inui et al., 1987) and skeletal muscle (Fabiato, 1985; Carl et al., 1995; Sun et al., 1995). Opioid-receptor induced Ca^{2+} mobilization has also been observed in both mouse astrocytes (Hauser et al., 1996) and isolated rat ventricular myocytes (Tai et al., 1992). Intraterminal calcium release and its associated Ca^{2+} microdomains likely have a wide range of possible targets and subsequent effects on depolarization-secretion coupling (Berridge, 2006; Oheim et al., 2006). Therefore, ryanodine-sensitive stores in HNS terminals may prove to be bimodal regulators of release depending on the physiological context. Given the emerging role of ryanodine-sensitive stores and the cADPr-signaling pathway in the CNS, our results may prove important in understanding their physiological role in presynaptic structures during depolarization-secretion coupling.

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Figure 4.1

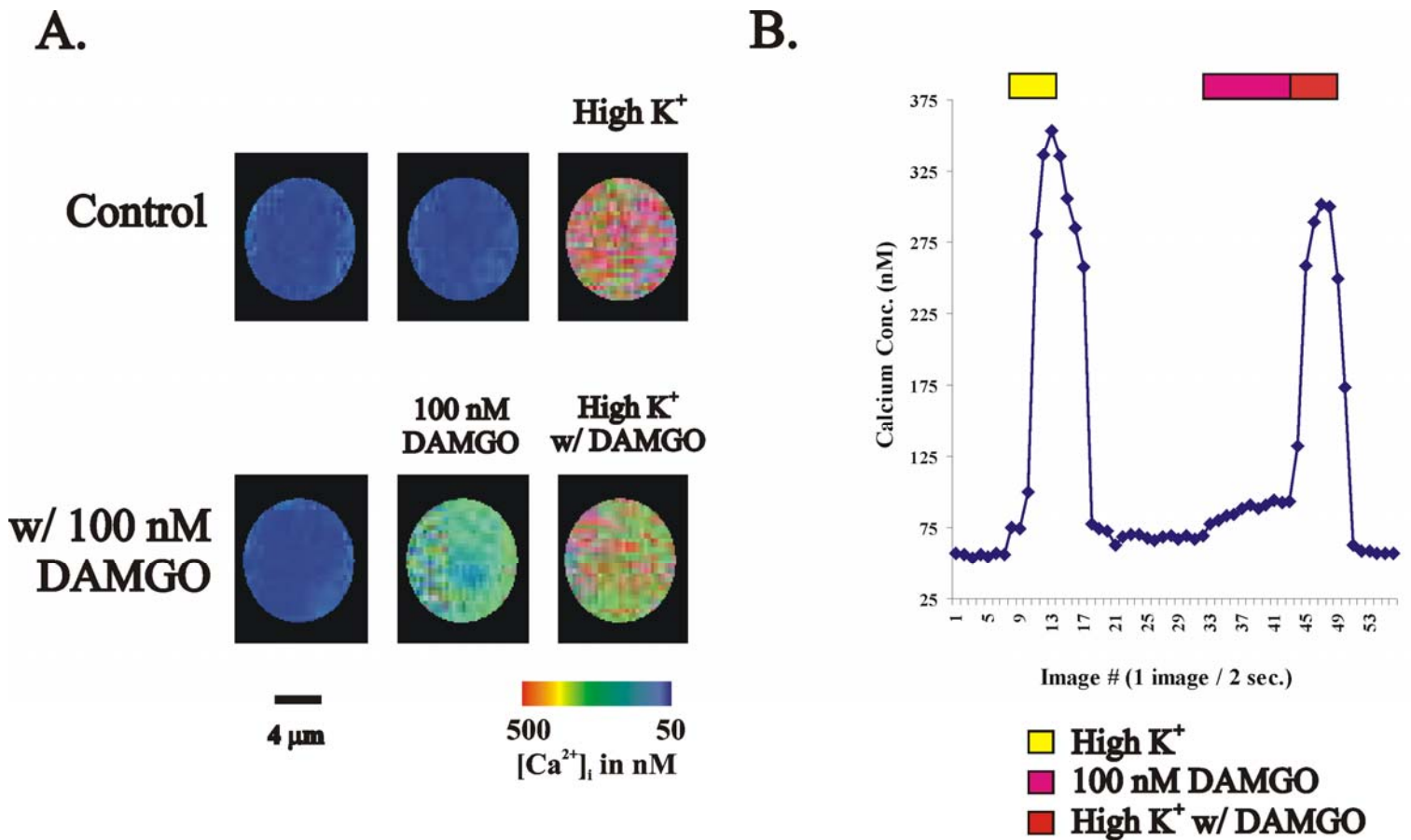


Figure 4.1 μ -opioid agonist triggers a rise in basal $[Ca^{2+}]_i$ preceding μ -opioid inhibition of High K^+ -induced rise in $[Ca^{2+}]_i$. **A.** Images of Fura-2 AM loaded single terminal approx. ten seconds apart challenged with High K^+ in 2.2 mM $[Ca^{2+}]_o$ for 5 sec. with and without 100 nM DAMGO. **B.** Plot of changes in $[Ca^{2+}]_i$ over time of a different single isolated HNS terminal loaded with Fura 2 AM. High K^+ (yellow-bar) exposures for 5 sec. and DAMGO (dark pink-bar) pretreatment for 10 sec. followed by High K^+ in the presence of 100 nM DAMGO (red-bar).

Figure 4.2

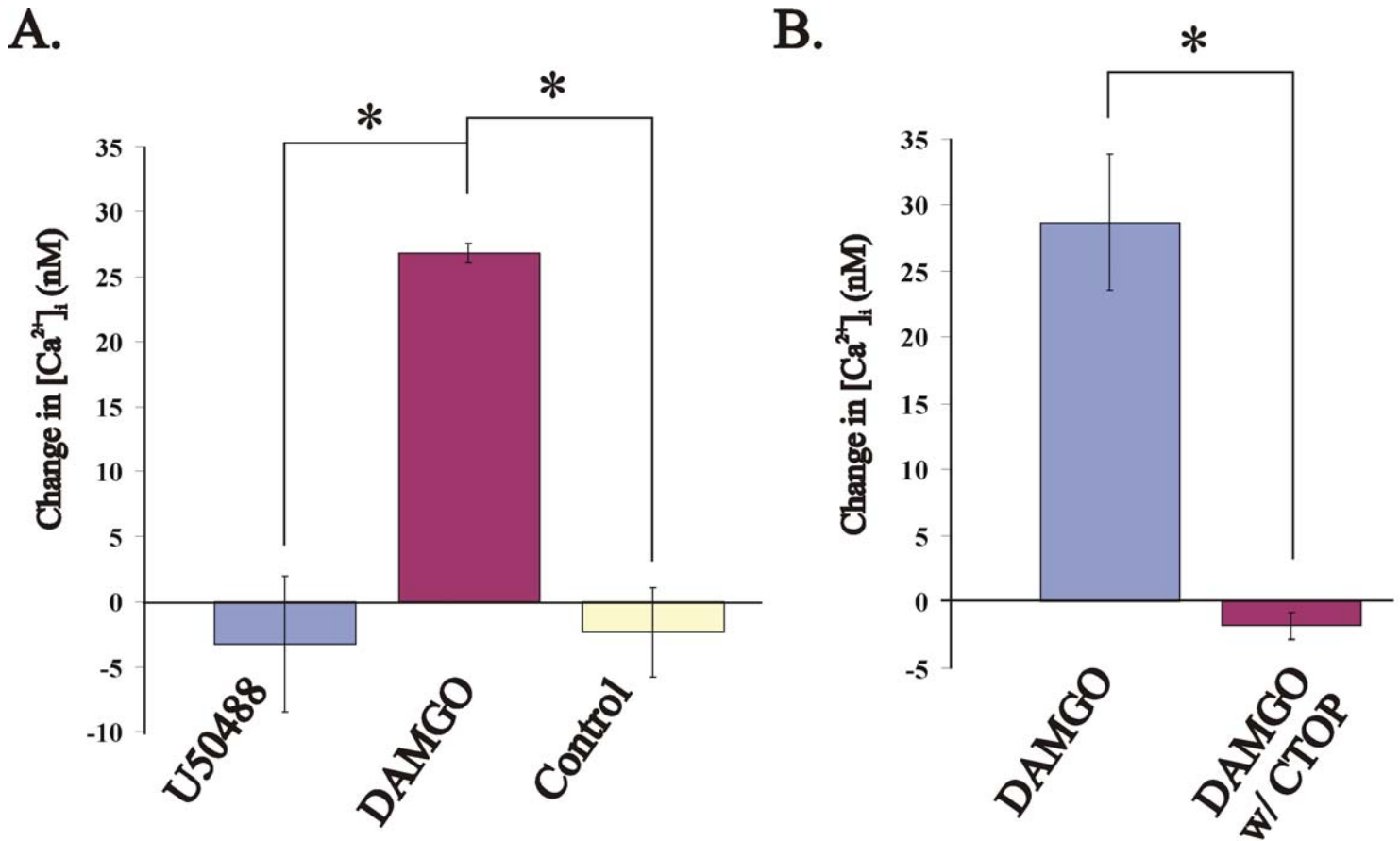


Figure 4.2 Only μ -opioid agonist triggers release of calcium from intraterminal stores. **A.** Change in [Ca²⁺]_i of isolated HNS terminals in 0 mM [Ca²⁺]_o in response to 100 nM U50488 (blue), 100 nM DAMGO (dark pink), and control treatment containing modified Normal Locke's without Ca²⁺ (cream); n=5. **B.** Rise in [Ca²⁺]_i due to 100 nM DAMGO application (blue) is blocked in the presence of 100 nM CTOP (dark pink); n=4. Asterisks (*) represent statistical differences (p<0.002).

Figure 4.3

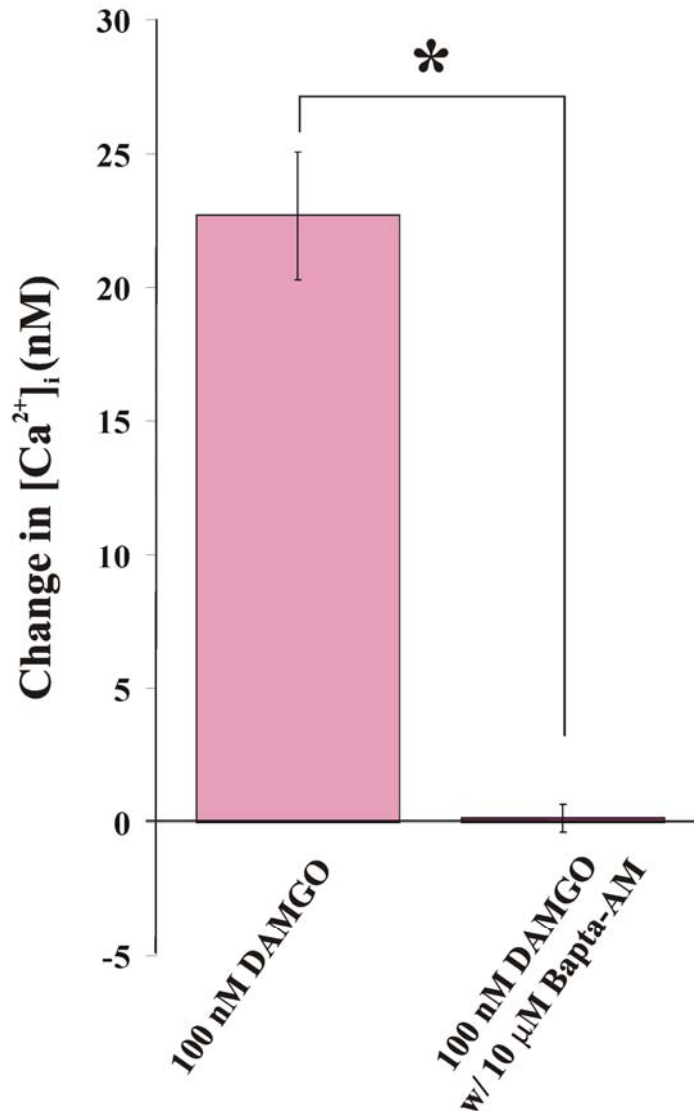


Figure 4.3 Rise in basal [Ca²⁺]_i due to μ-opioid agonist is blocked when pre-incubated with Bapta-AM. Bar graph of percent increase of [Ca²⁺]_i increase above baseline in the presence of 100 nM DAMGO with and without, a 5 min. pre-incubation with 10 μM Bapta-AM, in 2.2 mM [Ca²⁺]_o. Pre-incubation with Bapta-AM completely blocks the rise in basal [Ca²⁺]_i due to μ-opioid agonist. Asterisk (*) represents statistical difference (p<0.004) between 100 nM DAMGO and 100 nM DAMGO pre-treated with 10 μM Bapta-AM (n=3).

Figure 4.4

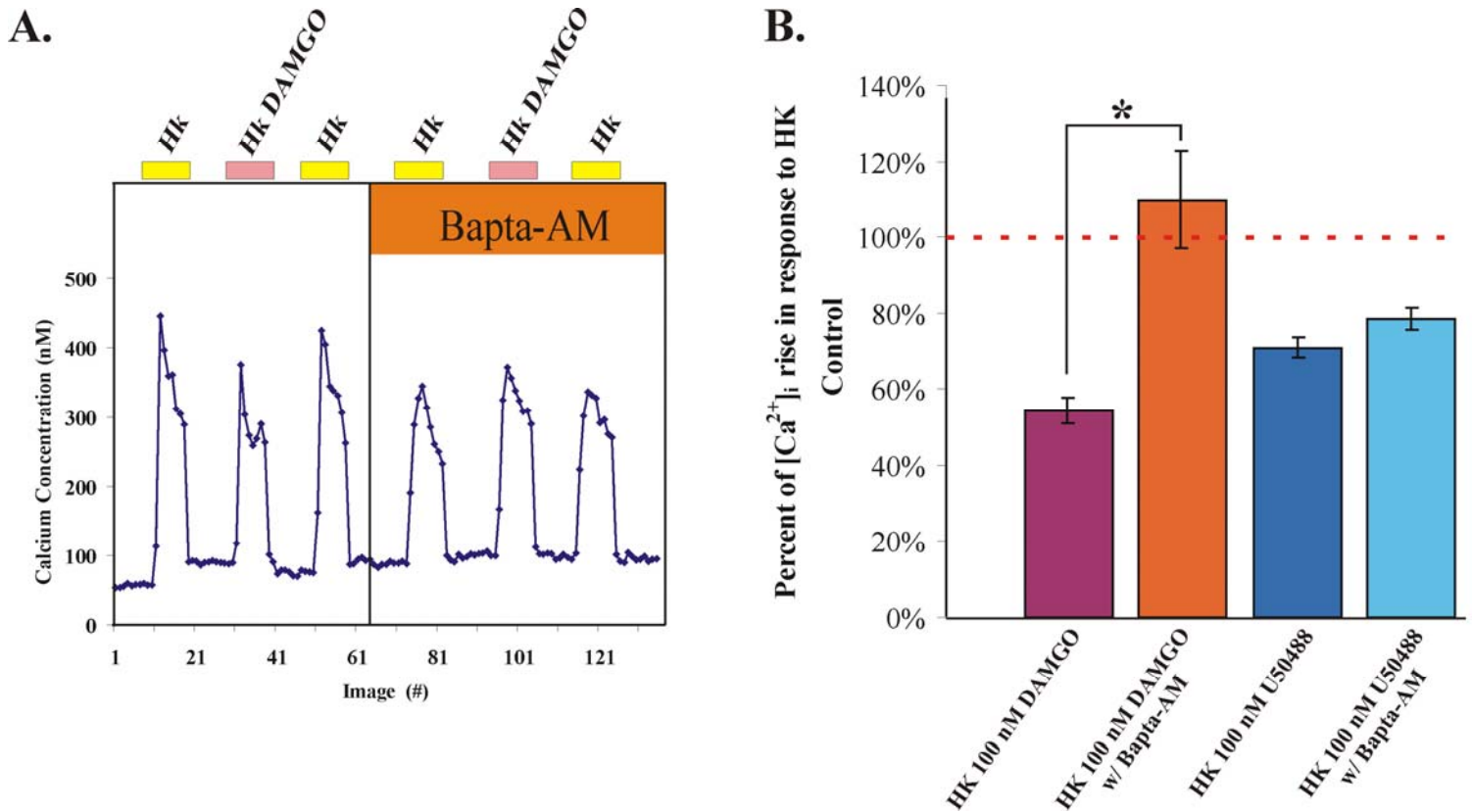


Figure 4.4 Bapta-AM relieves μ - but not κ -opioid mediated inhibition of High K^+ -induced rise in $[Ca^{2+}]_i$. **A.** Plot of changes in $[Ca^{2+}]_i$ of a single HNS terminal loaded with Fura-2 AM, with and without a brief 10 μ M Bapta-AM pre-incubation, treated with High K^+ , and High K^+ with 100 nM DAMGO in Normal Locke's solution. **B.** Bar graph quantifying changes in $[Ca^{2+}]_i$ in response to High K^+ , and High K^+ with 100 nM DAMGO or 100 nM U50488 with and without Bapta-AM pre-incubation (n=4). Asterisk (*) represents statistically significant differences (p<0.001).

Figure 4.5

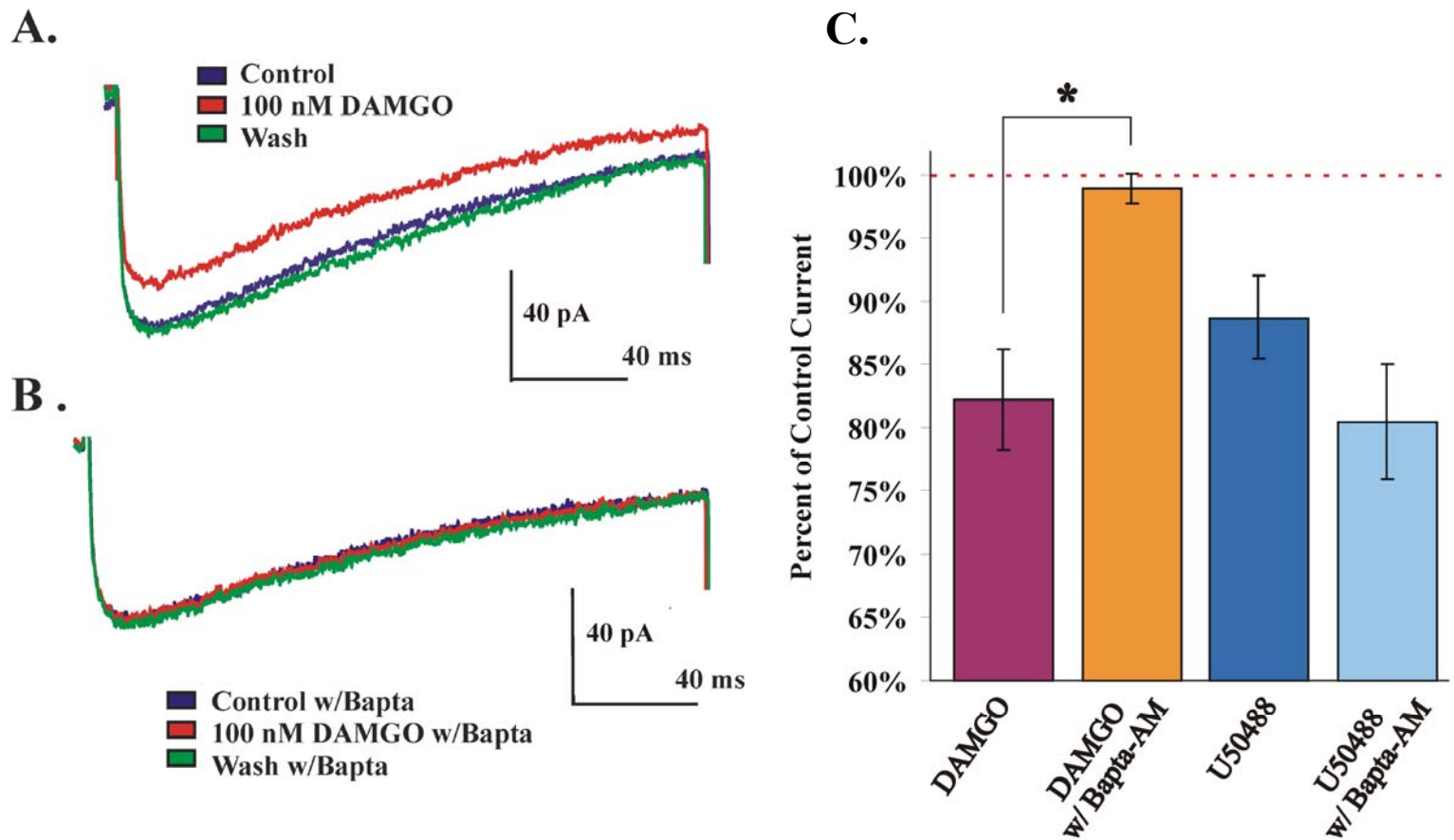


Figure 4.5 Bapta-AM pre-incubation relieves μ - but not κ -opioid inhibition of calcium currents. *A.* Sample calcium currents of isolated HNS terminal; control currents (blue), 100 nM DAMGO (red), and wash (green). *B.* The same isolated HNS terminal with the control pre-incubated in Bapta-AM (blue) trace compared to trace pre-incubated in Bapta-AM with 100 nM DAMGO (red), and wash (green). *C.* Bar graph quantifying peak barium currents (n=4) as percent of control current without opioid. Asterisk (*) represents statistically significant differences (p<0.001).

Figure 4.6

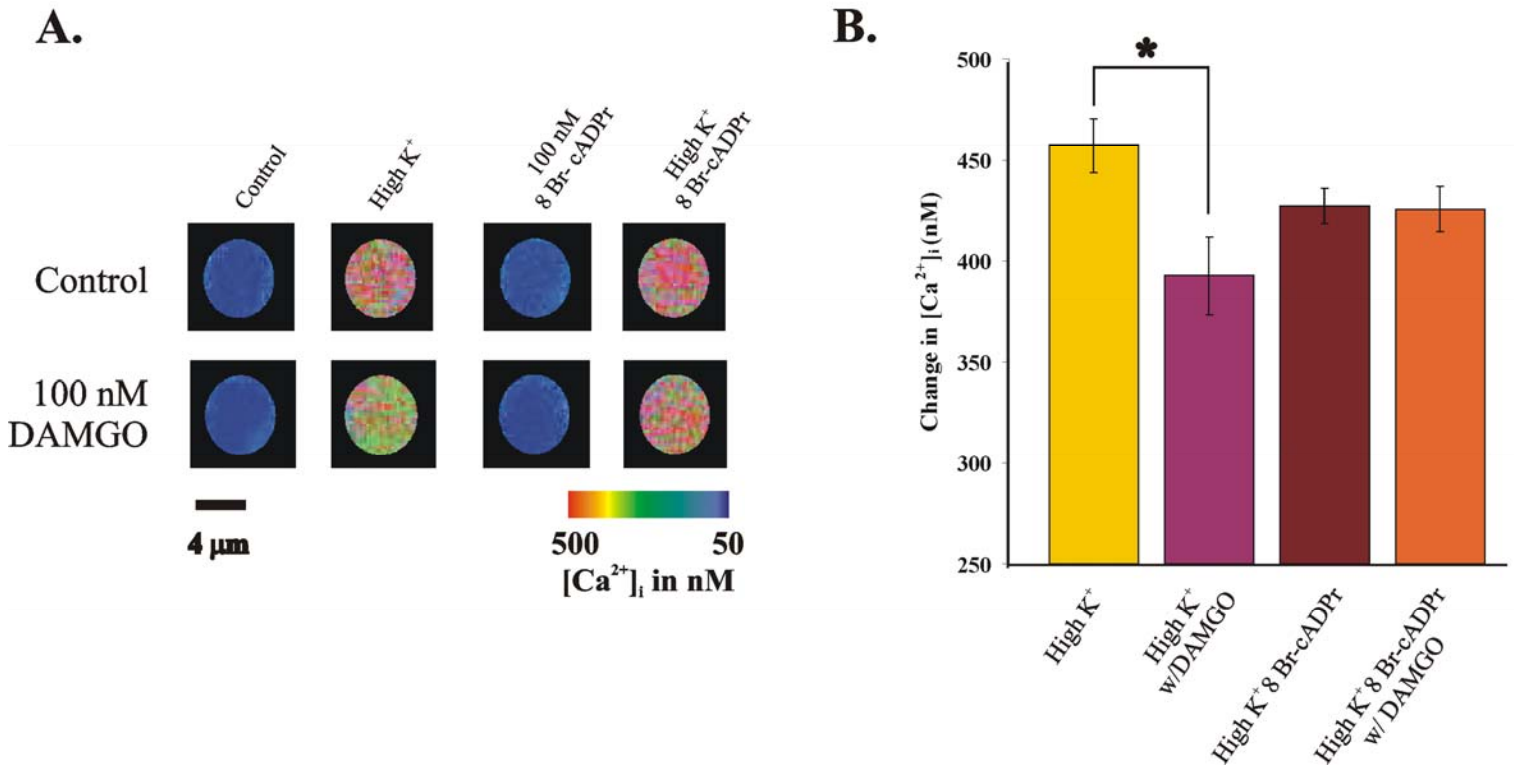


Figure 4.6 cADPr antagonist relieves μ -opioid mediated inhibition of High K^+ -induced rise in $[Ca^{2+}]_i$. **A.** Images of Fura-2 AM loaded single HNS terminal approx. ten seconds apart challenged with High K^+ in 2.2 mM $[Ca^{2+}]_o$ for 10 sec. with and without 100 nM DAMGO and with and without pretreatment with 8 Br-cADPr. **B.** Bar graph of change in $[Ca^{2+}]_i$ in response to High K^+ challenge with and without 100 nM DAMGO and with and without incubation with 100 nM 8Br-cADPr (n=8 each). Asterisk (*) represents statistically significant differences ($p \leq 0.0003$).

Figure 4.7

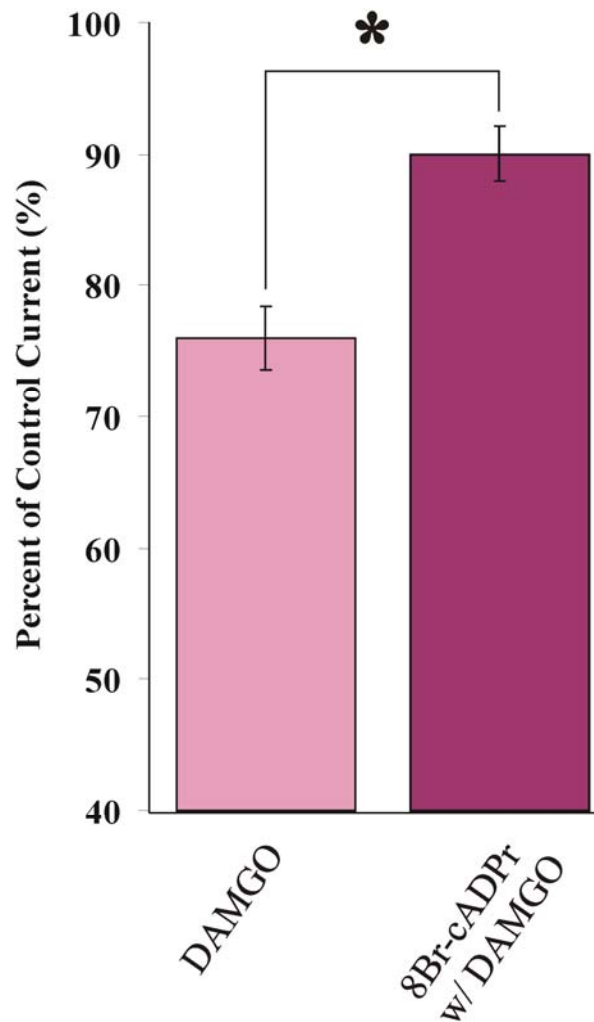


Figure 4.7 cADPr antagonist partially relieves μ -opioid inhibition of calcium currents. Bar graph quantifying the peak barium currents (n=5), as percent of control current without DAMGO. Calcium currents with 100 nM DAMGO (pink-bar) and with 100 nM DAMGO pre-incubated with 100 nM 8Br-cADPr (dark pink-bar). Asterisk (*) represents statistically significant differences (p<0.02).

Figure 4.8

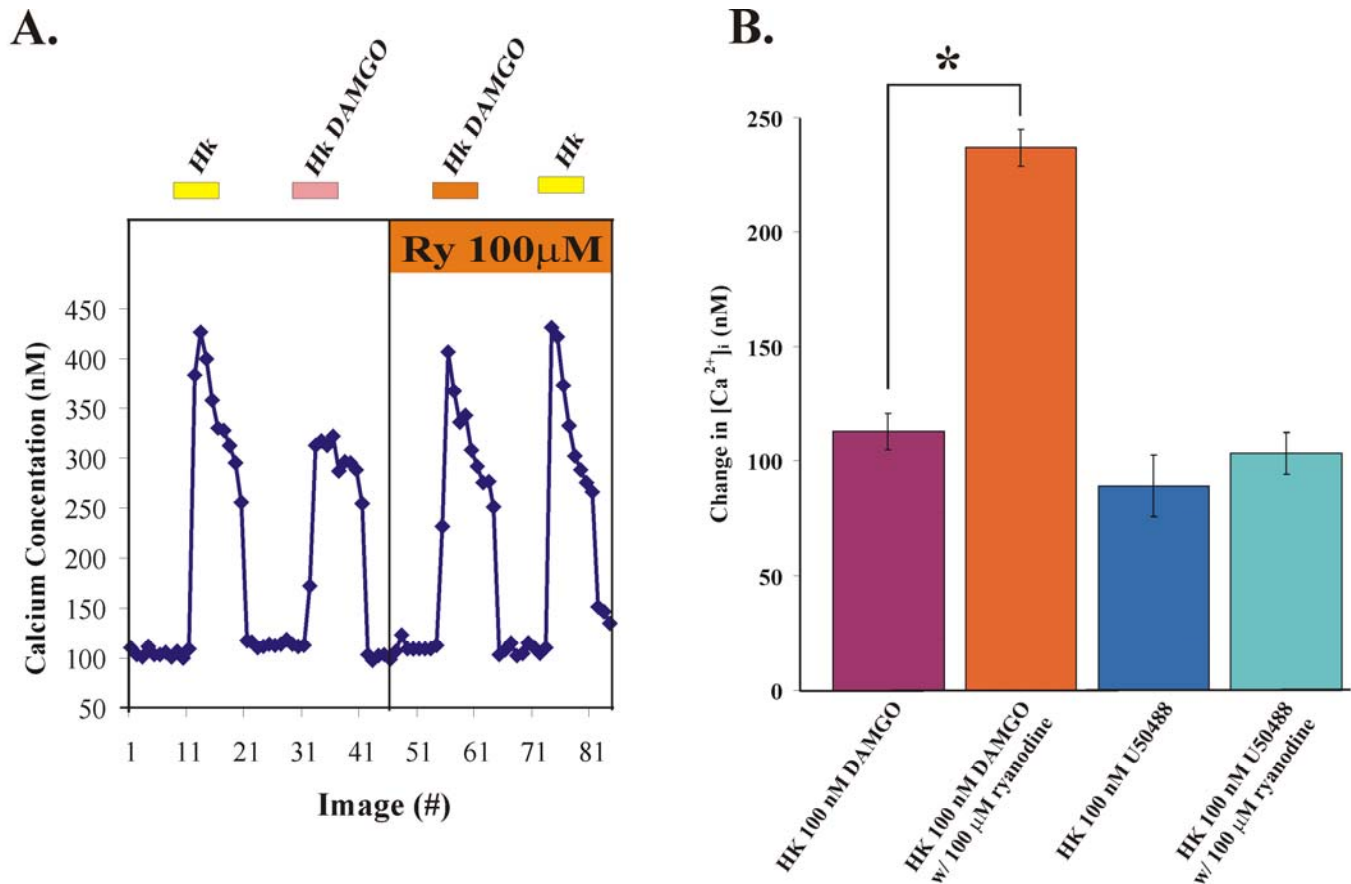


Figure 4.8 Ryanodine antagonist relieves μ - but not κ -opioid mediated inhibition of High K^+ -induced rise in $[Ca^{2+}]_i$. **A.** Trace of changes in $[Ca^{2+}]_i$ of a single HNS terminal with and without 100 μ M ryanodine incubation treated with High K^+ , and High K^+ with 100 nM DAMGO. **B.** Bar graph quantifying changes in $[Ca^{2+}]_i$ in response to High K^+ , and High K^+ with 100 nM DAMGO or 100 nM U50488, with and without 100 μ M ryanodine (n=7). Asterisk (*) represents statistically significant differences (p<0.001).

Figure 4.9

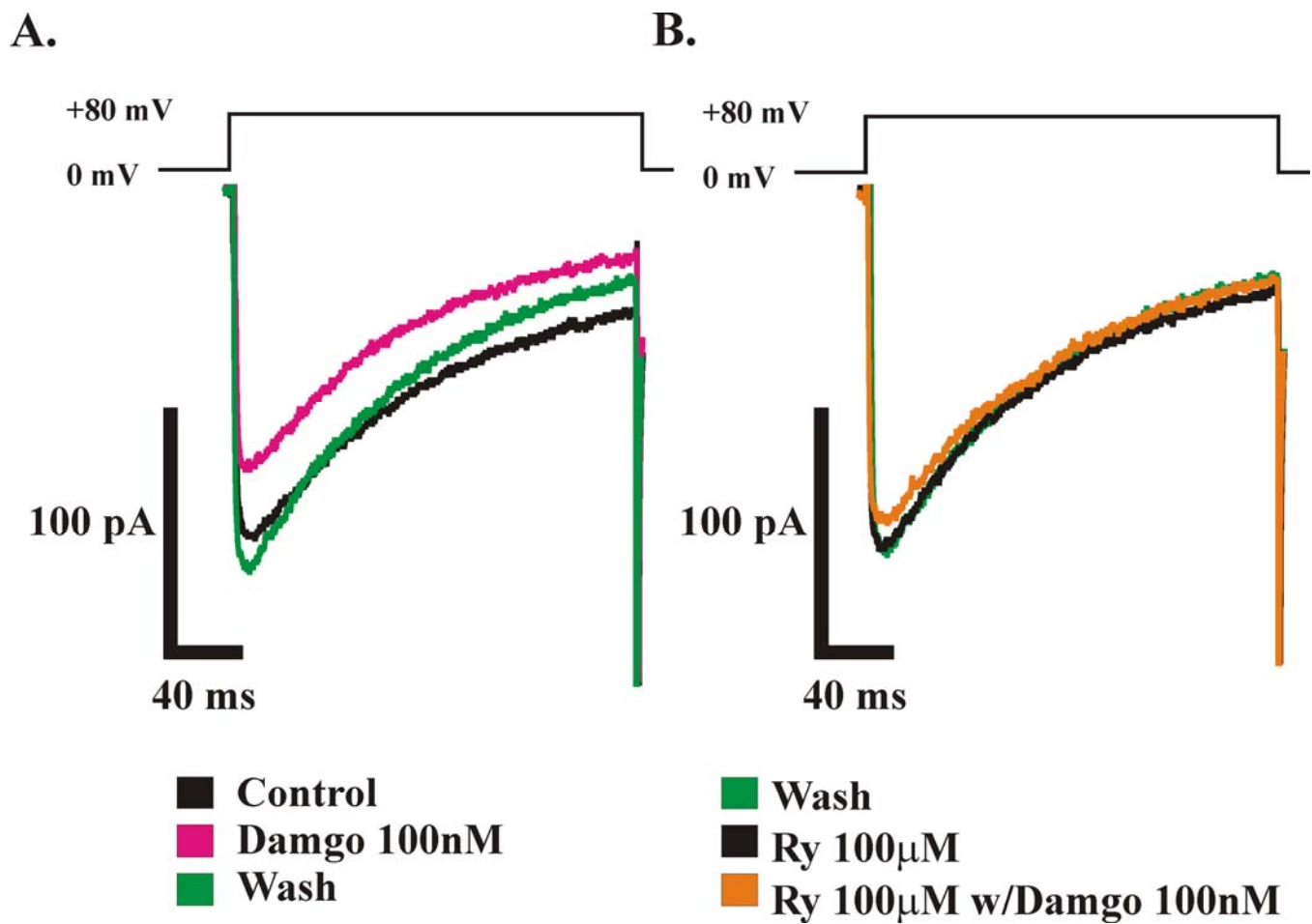


Figure 4.9. Ryanodine antagonist relieves μ -opioid inhibition of calcium currents. *A.* Sample calcium currents of isolated HNS terminal; control currents (black), 100 nM DAMGO (pink), and wash (green). *B.* The same isolated HNS terminal with the wash (green) trace compared to a current trace in 100 μ M ryanodine (black), and 100 μ M ryanodine with 100 nM DAMGO (yellow).

Figure 4.10

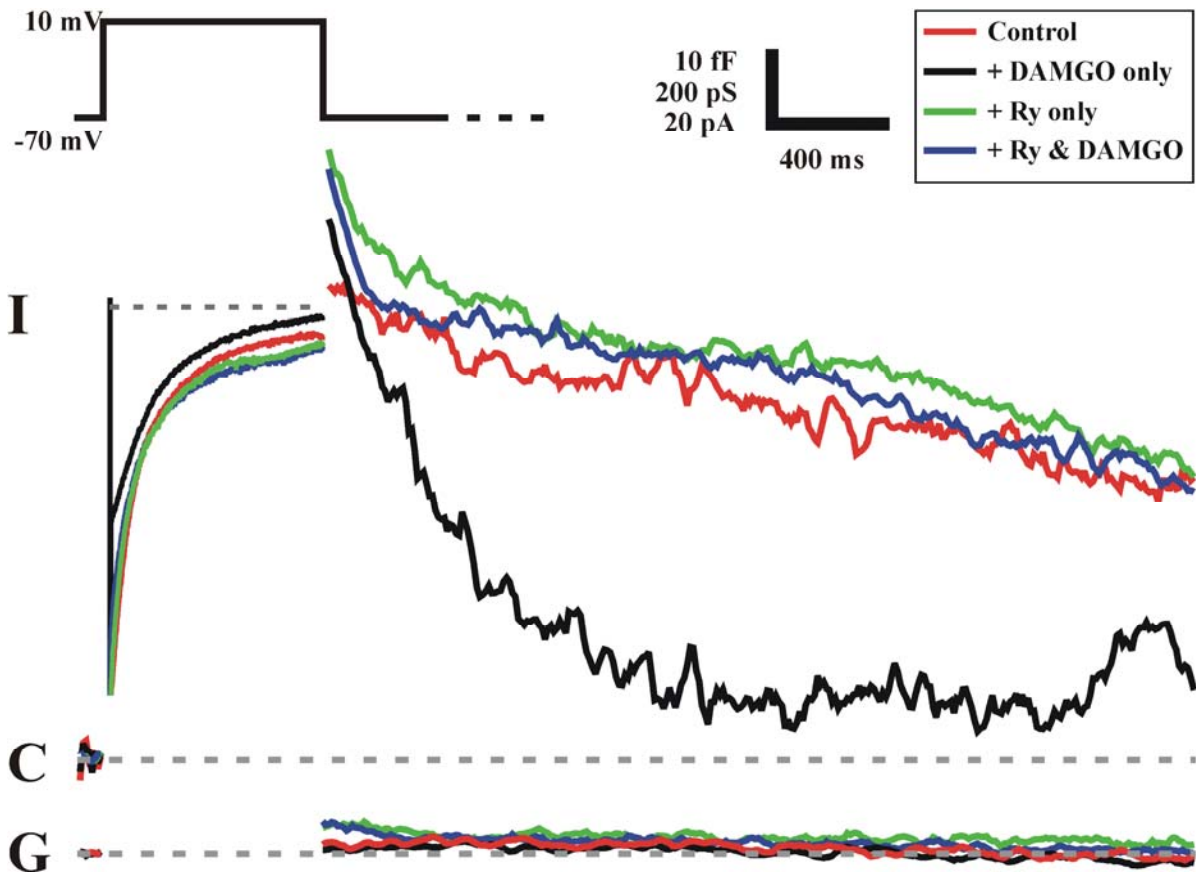


Figure 4.10 Ryanodine antagonist relieves μ -opioid inhibition of depolarization-induced exocytosis. Capacitance measurements of single terminal in the absence (red) and presence (black) of 100 nM DAMGO, in the presence of 100 μ M ryanodine (green), and of 100 μ M ryanodine and 100 nM DAMGO (blue) using a square pulse of 80 mV for a duration of 750 ms. Inset shows the currents obtained from the actual stimulus (same color indicators). The generalized conductance changes (“G”) are shown for reference. Dashed gray lines represent the zero-change baselines. Peak capacitance measurements in the presence of DAMGO were statistically significantly different from all other treatments ($p < 0.002$; $n = 5$).

Figure 4.11

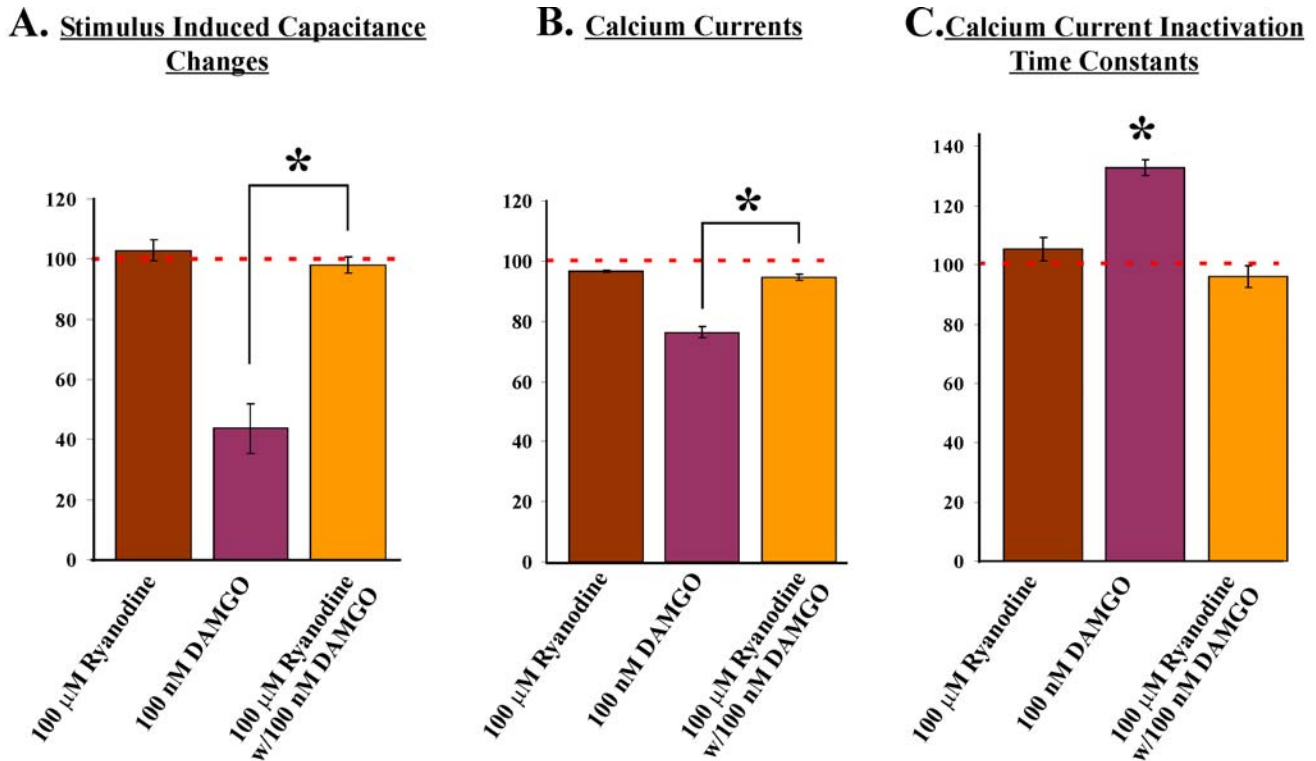


Figure 4.11 Summary of ryanodine antagonist effects on μ -opioid inhibition. A.

Bar graph quantifying changes in stimulus-induced capacitance (n=3) with 100 μ M ryanodine (dark red), 100 nM DAMGO (pink), and 100 μ M ryanodine with 100 nM DAMGO (orange), plotted as percent of control without treatment. **B.** Bar graph quantifying peak calcium currents (n=5) as percent of control peak current without treatment (same color indicators). **C.** Bar graph quantifying the inactivation time constants of calcium currents (n=5) as percent of control inactivation time constant without treatment (same color indicators). Asterisks (*) represent statistically significant differences (p<0.009).

Chapter V

General Discussion

For the past twenty years the Hypothalamic-Neurohypophysial System (HNS) has advanced our knowledge of the basic principles underlying depolarization-secretion coupling (DSC). The work presented in this thesis aims to expand our current understanding of the underlying mechanisms modulating DSC and their physiological implications. In this thesis I present research into both voltage-dependent and -independent mechanisms mediating modulation of DSC. Within the category of voltage-dependent effects on DSC, I show ground-breaking research unlinking depolarization-induced neuropeptide release from calcium influx through voltage-gated calcium channels (VGCC) in presynaptic structures. Furthermore, I show that changes in voltage can relieve G-protein mediated inhibition of VGCC through κ -opioid receptor activation that has important implications on activity-dependent modulation *in vivo*. Voltage-independent interactions mediating modulation of DSC include VGCC inhibition via μ -opioid receptor activation. This voltage-independent signaling pathway involves release of calcium from ryanodine-sensitive intraterminal stores. It is the first time, to my knowledge, that a physiological role has been determined for these recently characterized, ryanodine-sensitive calcium stores in isolated HNS terminals.

The historical cornerstone of DSC was established from early research done on the neuromuscular junction (Katz and Miledi, 1965b, a; Miledi, 1973; Smith and Augustine, 1988). It was here where the calcium hypothesis gained its foothold and from where it has only recently been challenged. The calcium hypothesis states that electrical stimulation opens VGCC, triggering calcium influx from the extracellular space into the cytoplasm. This initiates key steps in vesicular exocytosis and release of vesicular content. The main point of the calcium hypothesis is that calcium is the key component

for initiation and termination of neurotransmitter release. While most current scientific research is based on this premise, recent evidence has introduced the possibility of neurotransmitter release being both calcium- and voltage-dependent (Parnas and Parnas, 1986; Hochner et al., 1989; Parnas et al., 1991). Proponents of the Ca^{2+} -voltage hypothesis state that not only is Ca^{2+} required, but membrane potential as such also plays a pivotal role in promoting depolarization-induced release (Parnas and Parnas, 2002). A depolarizing stimulus relieves a tonic inhibition of the exocytotic machinery by an autoinhibitory receptor. This hypothesis emerged mainly to explain the differences in the time course of release in the presence of $[\text{Ca}^{2+}]_o$ where release is quickly terminated after depolarization despite residual high $[\text{Ca}^{2+}]_i$ (Hochner 1989, Barrett 1979, Matzner 1988, Parnas 1989, Datyner, 1980, Andreu 1989). Interestingly the kinetics of extracellular calcium-independent release in HNS terminals are reminiscent of the latter part of release in the presence of extracellular calcium suggesting a biphasic response to depolarization (Brethes et al., 1987; Muschol and Salzberg, 2000). First an extracellular calcium-dependent response, which leads to a peak of release is followed by a rapid decay. Second, a new baseline release in the continued presence of High K^+ is established, which is independent of extracellular calcium. This second phase of sustained release best mimics intraterminal calcium dynamics in the presence of a sustained depolarization (Lindau et al., 1992; Stuenkel and Nordmann, 1993a). The existence of voltage- and ryanodine-sensitive calcium stores in isolated HNS terminals seemed like a potential link between the calcium requirement for exocytosis and voltage during High K^+ -induced release in 0 mM $[\text{Ca}^{2+}]_o$. Therefore, unlike the Ca^{2+} -voltage hypothesis, I attempted to explain persistent depolarization-induced changes in both capacitance and neuropeptide

release in the absence of calcium influx through VGCC coincident with changes in $[Ca^{2+}]_i$.

While my experiments showed that extracellular calcium is not necessary for depolarization-induced neuropeptide release, it was clear that ryanodine- and voltage-sensitive internal calcium stores were not involved in eliciting such release, either. However, intraterminal calcium appears to play a role, but it is unclear what, if any, calcium stores are involved. Previously I introduced the possibility of calcium being released from IP_3 stores known to elicit release in isolated terminals (See Chapter III – discussion; Fig. 5.2). While this may help explain the intraterminal calcium-dependent portion of the depolarization-induced release of neuropeptide in 0 mM $[Ca^{2+}]_o$ from HNS terminals, a calcium-independent component remains. Interestingly, there is another step in the evolution of the calcium hypothesis known as the calcium-independent yet voltage-dependent release proposed by Zhang and Zhou (2002). Their research shows persistent depolarization-induced changes in capacitance in the absence of extracellular or intracellular calcium from DRG neuronal somata. These results indicate the existence of Ca^{2+} -independent but voltage-dependent vesicular secretion (CIVDS) which may be present in HNS terminals.

The molecular mechanism underlying CIVDS is unknown. Zhang and Zhou (2002), speculate about the existence of an intrinsic plasma membrane protein capable of sensing the changes in transmembrane potential and responding with a conformational change that facilitates exocytosis of the docked vesicles in a Ca^{2+} -independent manner. In fact, in pancreatic beta-cells voltage-driven conformational changes that engage the ion/EEEE interface of the L-type Ca^{2+} channel are relayed to the exocytotic machinery

prior to ion influx, allowing for a fast and tightly regulated process of release independent of Ca^{2+} influx or release of Ca^{2+} from intracellular stores (Wiser et al., 1999; Brown et al., 2000b; Trus et al., 2007). Therefore, given the CIVD component of depolarization-induced ECIR in HNS terminals, future experiments will explore the connection between VGCCs and proteins directly linked to the exocytotic machinery.

Alternatively, depolarization has been shown to cause changes in membrane tonicity (Kim et al., 2007) similar to those observed during osmotic challenges in which the cell or terminal swells or shrinks to accommodate changes in water flow due to variations in the osmotic gradient (Ishikawa et al., 1982; Cazalis et al., 1987c; Back et al., 2000; Hussy et al., 2001). The Neurohypophysis is exquisitely tuned to such changes as would be predicted from vasopressin's critical role in systemic fluid homeostasis. In HNS terminals hypo-osmotic challenges induce release of both vasopressin and oxytocin (Bacova et al., 2006). Interestingly, the kinetics of the CIVDS are not unlike calcium-independent hypo-osmotic-induced release. Therefore, we should not underestimate the possibility that changes in membrane tonicity or sodium influx due to either higher concentrations of extraterminal potassium, such as those seen during High K^+ stimulation, or electrical stimulation, observed during capacitance and calcium current recordings, may converge on sensitive osmotic regulatory mechanisms in the HNS underlying release.

Membrane tonicity may not be an important factor during calcium-dependent neuropeptide release, given that permeablized terminals still show transient vasopressin secretory responses to manipulations in $[\text{Ca}^{2+}]_o$ (Stuenkel and Nordmann, 1993a). However, also in permeablized terminals, addition of Na^+ in the absence of Ca^{2+} gives

rise to increases in vasopressin secretion (Stuenkel and Nordmann, 1993b). Therefore, influx of sodium due to depolarization may contribute to neuropeptide release in the absence of extracellular calcium. However, it is yet to be determined if intact terminals may have mechanically-sensitive triggers for neuropeptide release responding to changes in membrane tonicity due to depolarization in the absence of $[Ca^{2+}]_o$. Interestingly, there is a clear physiological equivalent to the High K^+ in 0 mM $[Ca^{2+}]_o$ conditions under which I have tested these isolated terminals. During a physiological burst, action potentials are translated into very high frequency stimulations due to the accumulation of extraterminal potassium in the interstitial space, while at the same time calcium is being depleted (Marrero and Lemos, 2005). Therefore, extraterminal calcium-independent yet voltage-dependent release of neuropeptide may serve to extend release, past the biophysical limits of VGCC, under the continued demands of high levels of physiologically relevant electrical activity.

If ryanodine- and voltage-sensitive calcium stores in HNS terminals are not involved in extracellular Ca^{2+} -independent depolarization-induced release, the question persists; do they serve a role during DSC? Amperometric studies have demonstrated no correlation between syntillas and quantal release. However, agonist concentrations of ryanodine show significant increases in the rate of spontaneous amperometric events, and inhibitory concentrations of ryanodine cause the opposite effect (McNally et. al, 2007). AVP and OT release from isolated terminals significantly increase in response to ryanodine receptor agonists (Velazquez-Marrero et. al., in preparation; See Chapter II), in both the absence and presence of $[Ca^{2+}]_o$. These data suggest that spontaneous syntillas, may release Ca^{2+} into cytosolic microdomains not associated with docked, primed

vesicles (Berridge, 2006; Oheim et al., 2006; ZhuGe et al., 2006). However, lack of coincident release of neuropeptide with syntilla events does not preclude the possibility that release of calcium from intraterminal stores may indirectly modulate DSC by increasing the probability of exocytosis from a larger readily-releasable pool of granules (Fig. 5.2). In HNS terminals, intraterminal calcium stores can prime vesicles for activity-dependent neuropeptide release (Ludwig et al., 2002; Ludwig and Leng, 2006). Furthermore, application of either antagonist concentrations of ryanodine, or the competitive ryanodine receptor antagonist, 8-Br cADPR, significantly inhibit both depolarization-induced release of OT and its coincident rise in $[Ca^{2+}]_i$ (Jin et al., 2007). I have carried out experiments measuring AVP release in response to 50 mM High K^+ -induced release, which show a reproducible potentiation of the High K^+ -induced release in the presence of 20 mM Caffeine in 2.2 mM $[Ca^{2+}]_o$. This potentiation does not occur in the absence of $[Ca^{2+}]_o$, suggesting that calcium influx through VGCC during DSC is a necessary prerequisite for “syntilla” modulation of DSC. The evidence therefore suggests that CICR likely plays an important role in the amplification of the DSC response as it does in other systems (Usachev and Thayer, 1997; Kang and Holz, 2003).

As previously mentioned, recent studies targeting oxytocin release in isolated terminals of the Neurohypophysis using CD38 knockout mice, clearly shows inhibition of depolarization-induced OT release in CD38(-) mice, and in wild-type mice when treated with antagonists of the ryanodine receptor or the cADPr pathway (Higashida et al., 2007; Jin et al., 2007). I found similar results by calcium imaging with High K^+ -induced $[Ca^{2+}]_i$ rise. In seeming contradiction, release of intraterminal Ca^{2+} from ryanodine-sensitive stores during application of the μ -receptor agonist leads to inhibition of VGCC and of

subsequent High K^+ -induced neuropeptide release (Velazquez-Marrero et.al, in preparation; See Chapter IV). However, studies show that micro-domains of calcium can affect specific targets via select signaling pathways. Graded activation of the ryanodine cADPr pathway can encompass a range of physiological responses in combination with activation via depolarization. Treating isolated terminals with 8-Br cADPR, a competitive antagonist of the ryanodine receptor, at a concentration of 100 μ M significantly decreases both OT release and the High K^+ -induced rise in $[Ca^{2+}]_i$ (Jin et al., 2007). However, a concentration of 100 nM does not affect either, yet effectively targets μ -opioid inhibition (Velazquez-Marrero et.al, in preparation; See Chapter IV). The concentration dependent effect of 8-Br cADPr on DSC suggests changes in cADPr and subsequent baseline $[Ca^{2+}]_i$ in terminals has a biphasic effect on DSC (see Fig. 5.2). A similar phenomenon was observed in *Xenopus* oocyte nerve growth cones which respond to a known chemoattractant guidance cue with opposite turning behavior, depending on the level of cytosolic cAMP and $[Ca^{2+}]_i$ (Ming et al., 1997; Hopker et al., 1999). Furthermore, brief periods of electrical stimulation of cultured *Xenopus* spinal neurons resulted in a marked alteration in the chemoattractive turning responses of the growth cone, suggesting depolarization may also modulate cAMP concentrations and/or $[Ca^{2+}]_i$ (Ming et al., 2001). Therefore, given that HNS ryanodine-sensitive calcium stores are likely Ca^{2+} - and voltage-sensitive, specific holding potentials and baseline $[Ca^{2+}]_i$ concentrations should be taken into consideration when interpreting their effects on both basal and stimulus-evoked release.

Our hypothesis states that release of calcium from ryanodine-sensitive stores leads to VGCC inhibition presumably via calcium-dependent inactivation (Fig. 5.1). The μ -

opioid receptor seems to preferentially inhibit OT release by its strong inhibition of the R-type calcium channel (Fig. 1.6), where 79% of the isolated R-type currents was blocked by DAMGO (Ortiz-Miranda et al., 2005). However, it inhibits L-type by only 15% and, N-type by 17% in OT terminals (Ortiz-Miranda et al., 2006). Research on calcium-dependent inactivation of the R-type calcium channel indicates there are hallmark Ca^{2+} -dependent calmodulin interacting domains in the cytoplasmic loops of the channel indicating the possibility for calcium-dependent modulation of the channel (Pate et al., 2000; Liang et al., 2003). Experiments measuring inactivation rate constants of total channel currents in isolated HNS terminals showed slower inactivation with Ba^{2+} as compared with Ca^{2+} as the charge carrier for total channel currents (Wang et al., 1999b), indicating VGCC of HNS terminals do inactivate in a calcium-dependent manner. However, the isolated R-type current showed no difference in inactivation with either Ba^{2+} or Ca^{2+} (Wang et al., 1999b). This seemed to suggest there is no calcium-dependent inactivation of the R-type calcium channel in HNS terminals. Nevertheless, it is important to note that Ba^{2+} can bind to the inactivation site of certain VGCC channels, giving rise to Ba^{2+} -dependent inactivation (Ferreira et al., 1997) and thus occluding any differences in inactivation during Ba^{2+} substitution. Future experiments should include intraterminal calcium buffering with Bapta to best address the possibility of calcium-dependent inactivation.

While the possibility for calcium-dependent inactivation of the R-type channel in the HNS may be debatable, other VGCC targeted by μ -opioid inhibition show significant calcium-dependent inactivation (Branchaw et al., 1997; Wang et al., 1999b). This may indicate that either the R-type channel is sensitive to calcium-dependent modulation (still

present during Ba^{2+} substitution as Ba^{2+} -dependent inactivation) or AVP terminals, which lack R-type VGCCs, are being preferentially selected. However, significant differences in the rates of inactivation of calcium currents during μ -opioid inhibition support the involvement of a calcium-dependent process mediating inhibition. As stated in Chapter IV, inactivation rates decrease in μ -opioid-treated calcium currents. While these results are somewhat unexpected, they are consistent with previous research characterizing calcium-dependent VGCC inactivation in HNS isolated terminals. It is interesting to note that, unlike other systems, in isolated HNS terminals the time constants for calcium current inactivation are faster with Ba^{2+} substitution and intracellular Bapta when compared with Ca^{2+} (Branchaw et al., 1997). This is the opposite of what would be expected for calcium-dependent inactivation of VGCC. To explain these results, Branchaw et al. (1997), proposed a simple two-state VGCC model, where open and inactivated states are governed by a forward rate when the channel moves from open to inactive and a reverse rate when the channel moves from inactive to open. Ca^{2+} is hypothesized to inhibit both the forward and reverse rates, with a greater effect on the reverse rate.

In this thesis, I show that inhibition of VGCC via μ -opioid agonist reduces the rate of inactivation, as expected for Ca^{2+} -dependent inhibition where calcium's net effect is increasing the rate at which inactive channels transition into an open state. While this would predict facilitation of calcium influx through the channel it does not take into consideration other hallmarks, not yet tested during μ -opioid inhibition, of calcium-dependent inactivation of VGCC in HNS such as Ca^{2+} -dependent inhibition of recovery from inactivation, and the hallmark U-shape of a steady-state inactivation curve

(Branchaw et al., 1997). A simpler explanation for the slower inactivation rates in μ -opioid treated calcium currents takes into account that calcium-dependent inactivation can be saturated during release of calcium from ryanodine-sensitive stores. Therefore, rate constants during stimulated release would lack the calcium-dependent component of inactivation typically elicited via calcium influx and thus seem slower than control currents.

From the research and results presented in this thesis a working hypothesis emerges. Action potentials reach the Neurohypophysis initiating important electrical and biochemical events that at different points overlap to ensure optimum efficiency of neuropeptide release. Once membrane potential at the terminals reaches threshold, initiation of APs triggers VGCC opening which allow extracellular calcium influx. These events trigger release of both AVP, with co-release of Dynorphin A, and OT, with co-release of Met-Enkephalin (see Fig. 1.6). This initiates autocrine and paracrine activation of both the κ - and μ -opioid receptors and their respective G-protein signaling pathways. The κ -opioid signaling pathway catalyzes the association of the $G_{\beta\gamma}$ subunits of the G-protein coupled receptor with cytoplasmic components of VGCC triggering slower channel opening and consequently calcium influx (Fig. 5.1). Upon high frequency stimulation from the soma, activity-dependent relief of κ -opioid inhibition occurs in nerve terminals. Met-Enkephalin co-released from HNS terminals triggers activation of the μ -opioid receptor which initiates a slower response, at lower concentrations inhibiting OT release first. With eventual μ -opioid agonist accumulation, higher concentrations also target AVP release. G-protein activation initiates a diffusible second-messenger cascade, which is both slower than a membrane-delimited pathway and voltage-independent,

triggering activation of ADP-ribosyl cyclase/CD38, which synthesizes cADPr from beta-NAD⁺ (Higashida et al., 2007)(Fig. 5.1 & 5.2). Cyclic ADPr then either activates or modulates ryanodine receptors in large dense core vesicles. This triggers subsequent release of calcium from ryanodine-sensitive stores followed by calcium-dependent inactivation of VGCC and possibly calcium-dependent activation of BK channels. Termination of this signaling pathway may occur by negative feedback triggered by high cADPr concentrations, μ -opioid receptor desensitization, and/or μ -opioid receptor internalization.

In summary, with increasing frequency of electrical activity (i.e., bursts) from the soma, κ -opioid receptor inhibition in the terminals is attenuated and μ -opioid agonists, co-released with neuropeptide, accumulate in the terminal interstitial space prior to diffusion into the adjacent capillary bed or degradation. Subsequent changes in ionic concentrations in the terminal interstitial space may then trigger extracellular calcium-independent yet voltage-dependent release of neuropeptide towards the latter phase of the burst. During inter-burst intervals, both μ - and κ -opioid agonist accumulation either diffuses away or are broken-down in the interstitial space surrounding the terminals. The rate of opioid dispersal and/or degradation may dictate whether subsequent stimulation will elicit optimum neuropeptide release. While autoinhibitory effects of co-secreted κ -opioid agonists have been observed in the somatodendritic region (Brown et al., 2007), in HNS terminals I have now demonstrated the capacity for physiologically relevant activity-dependent relief. This represents an interesting adaptation serving the unique function of the terminals in the magnocellular neurosecretory system. In contrast, the slower, voltage-insensitive mechanism mediating μ -opioid inhibition in HNS terminals is

best suited for its primary role during gestation. It is during this stage that μ -opioid inhibition of OT release progressively increases throughout pregnancy, allowing for OT accumulation and OT receptor sensitization. This inhibition is interrupted during parturition and later during lactation where OT release from the Neurohypophysis plays a critical role in milk letdown. Therefore, integration of both voltage-dependent and – independent modulatory inputs at the HNS terminals serves to fine-tune the response to physiologically relevant input and facilitate optimum depolarization-secretion coupling.

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Figure 5.1

Opioid Intracellular Mechanisms

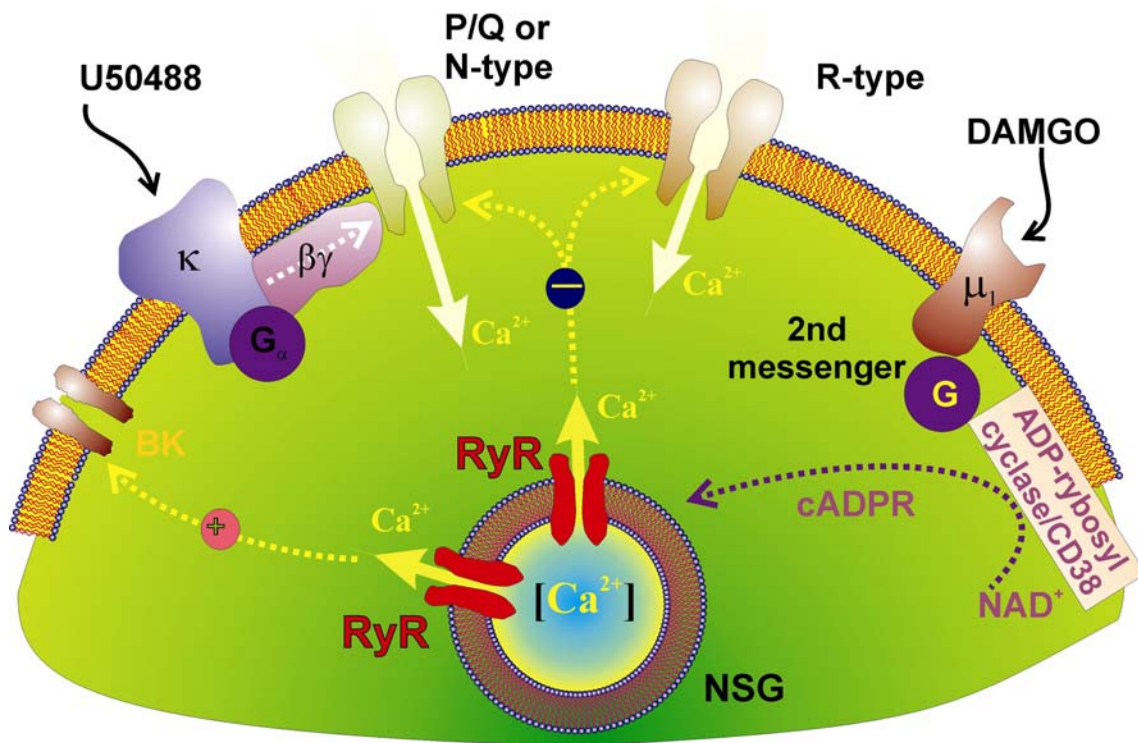


Figure 5.1 Model for Opioid modulation of VGCC. Activation of the κ -opioid receptor initiates $G\alpha$ -protein dissociation from $G_{\beta\gamma}$ subunits. $G_{\beta\gamma}$ mediates membrane-delimited voltage-dependent inhibition of VGCC via direct interaction with the channel. Activation of the μ -opioid receptor initiates cADPR signaling, presumable via activation of ADP-ribosyl cyclase/CD38 complex catalyzing the conversion of NAD^+ into cADPr. Cyclic ADPr subsequently leads to activation/modulation of ryanodine receptors on neurosecretory granules (NSG) in the terminals. Release of Ca^{2+} from ryanodine-sensitive stores subsequently results in Ca^{2+} -dependent inactivation of voltage-gated calcium channels. Figure modified from Lemos, 2002.

Figure 5.2

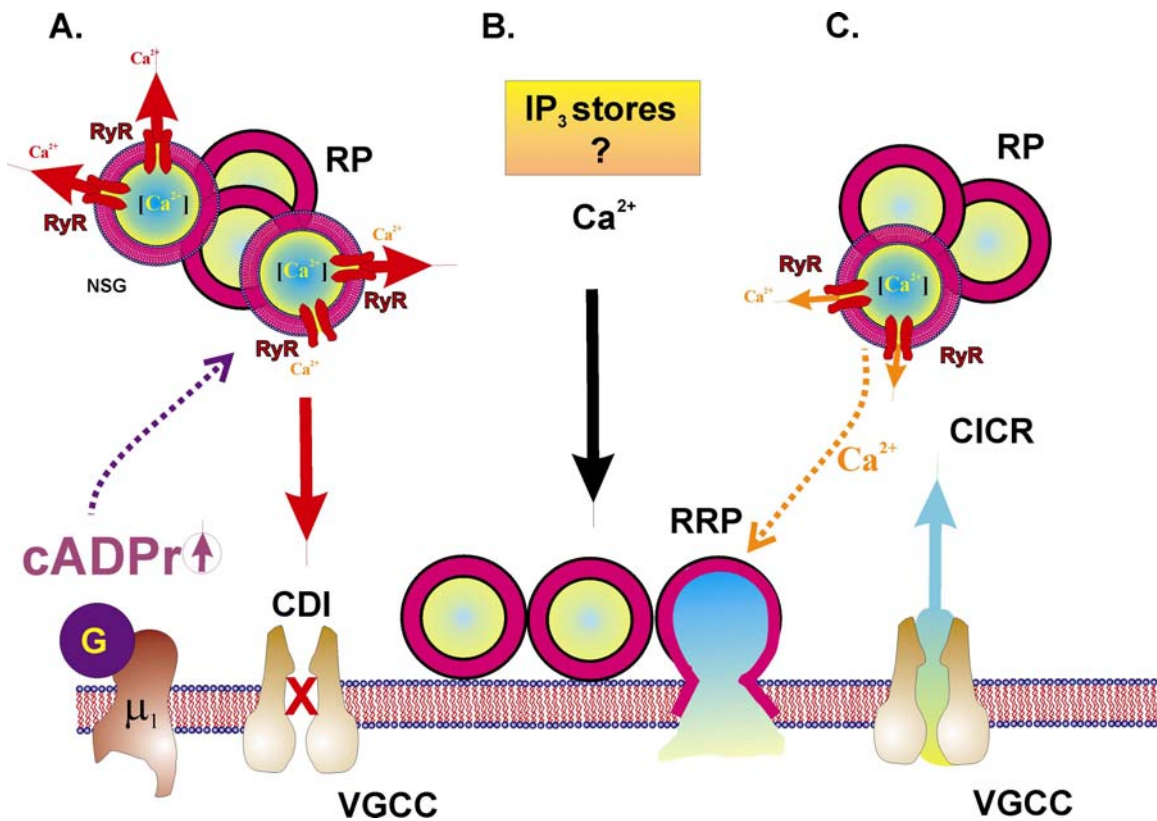


Figure 5.2 Intraterminal Ca^{2+} regulation of neuropeptide release in HNS terminals. *A.* μ -opioid agonist binds to its receptor initiating a diffusible second-messenger cascade resulting in cADPr-induced release of Ca^{2+} from ryanodine-sensitive stores. Calcium release leads to calcium-induced inactivation (CDI) of voltage-gated calcium channels (VGCC). Inactivation of VGCC inhibits Ca^{2+} influx and subsequent depolarization-induced release. *B.* In the absence of Ca^{2+} influx through VGCC, depolarization mobilizes intraterminal Ca^{2+} from an unknown source, possibly IP_3 stores, eliciting neuropeptide release in a Ca^{2+} -independent manner. *C.* During depolarization, VGCC allows influx of Ca^{2+} from the extracellular space resulting in activation of ryanodine-sensitive stores via calcium-induced calcium release (CICR) and subsequent mobilization of granules from the releasable pool (RP) to the readily releasable pool (RRP) amplifying the DSC response. Figure inspired by Leng et al.(2002).

