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Voltage-Dependent κ -Opioid modulation of Action Potential Waveform- Elicited Calcium Currents in Neurohypophysial Terminals

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Abstract

Release of neurotransmitter is activated by the influx of calcium. Inhibition of Ca^{2+} channels results in less calcium influx into the terminal and presumably a reduction in transmitter release. In the neurohypophysis (NH), 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). In this series of experiments we test whether the κ - and μ -opioid inhibition of Ca^{2+} currents in NH terminals is voltage-dependent. Voltage-dependent relief of G-protein inhibition of VGCC can be 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 κ - and μ -receptors in neurohypophysial terminals. 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 action potential 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 neuropeptide release in other CNS terminals, as well.

Keywords

action potentials; opioids; neurohypophysis; KOR; MOR

INTRODUCTION

The hypothalamic neurohypophysial system (HNS) releases both oxytocin (OT) and vasopressin (AVP) into a capillary bed for systemic delivery. Action potentials (AP) from magnocellular neurons to the neurohypophysial terminals elicit secretion of both neuropeptides by triggering the opening of voltage-gated calcium channels (VGCC) leading to the 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 & Nordmann, 1974; Bicknell, 1988; Berrino et al., 1989; Lee et al., 1992; Salzberg et al., 2000). Isolated neurohypophysial (NH) terminals show

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inhibition of VGCC by either μ -opioid receptor (MOR) agonists (Ortiz-Miranda et al., 2003; Ortiz-Miranda et al., 2005) or κ -opioid receptor (KOR) agonists (Rusin et al., 1997), and inhibition of subsequent release of both oxytocin and vasopressin (Sumner et al., 1990; Kato et al., 1992; Russell et al., 1993; Lemos et al., 2002). However, the signaling mechanisms and modulatory importance of κ - and μ -opioid receptor activation at these pre-synaptic terminals and subsequent VGCC inhibition is still not well understood.

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., 1995). However, modulation via κ - and μ -opioids is neither identical nor static and displays plasticity in response to changes in physiological status. Co-release of dynorphin, an endogenous κ -opioid receptor agonist, with vasopressin from magnocellular neuron cell bodies and dendrites has been shown to inhibit in an activity-dependent manner firing of vasopressinergic neurons (Bourque et al., 1998; Brown & Bourque, 2004; Brown et al., 2004; Roper et al., 2004; Brown et al., 2006; Sabatier & Leng, 2007). κ -opioid receptors have also been found in isolated terminals of the neurohypophysis (Hamon & Jouquey, 1990). 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 & Leng, 2000). Therefore, baseline release of AVP with its concurrent co-release of dynorphin, would represent a tonic inhibitory regulation mechanism for both OT and AVP release.

Both the μ - and κ -opioid receptors are G-protein coupled receptors that 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 & Moises, 1998; Connor & Christie, 1999; Chen et al., 2000). A membrane-delimited pathway is via a G-protein activation leading to $G_{\beta\gamma}$ 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 & Zamponi, 2006). Therefore, relief of G-protein membrane-delimited inhibition can be achieved 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 & Fox, 2002; McDavid & Currie, 2006). The ability of action-potential-like waveforms 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 examined the voltage- and frequency-dependent inhibition of calcium currents by both the κ - and μ -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 200–250g were sedated using CO₂ and immediately decapitated following the ethical guidelines laid down at U. Mass. Med. School (protocol A-1031). The neurohypophysis was isolated as

previously described (Lemos & Nordmann, 1986; Knott *et al.*, 2005). 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 (See Above). 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 μ m in diameter). The pipette solution consisted of (in mM): 145 Cs-gluconate, 15 CsCl, 2 MgCl₂, 2 NaCl, 7 Glucose, 10 HEPES (pH 7.3), at 295 mOsm. Amphotericin B at a concentration of 30 μ M (Sigma-Aldrich, St. Louis, MO) was added as a perforating agent. The bath solution consisted of (mM): 145 NaCl, 5 KCl, 1 MgCl₂, 10 HEPES, 10 Glucose, 1.2 CaCl₂, 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 M Ω . Pipettes were made of thin borosilicate glass (Drummond Scientific Co., Broomall, PA, USA). After perforation the terminals were voltage-clamped at –80 mV. Whole-cell capacitance and series resistance were compensated (nulled) before each test (run). 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, least square method, with largest error for individual τ 's at $\pm 5.35\%$) 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 to peak was measured from the beginning of the waveform to the maximum inward currents (in conditions where the sodium current was blocked). The application of multiple protocols on the same samples was made in a random order. This was necessary in order to avoid possible pre-treatment bias for those procedures that required different stimulus protocols.

Stimulus-induced (0.3 to 0.4 ms duration, 5–10 nA) action potentials (APs) were recorded from isolated neurohypophysial terminals using the perforated-patch method and the fast current-clamp mode of an EPC9 amplifier (HEKA Instruments Inc., NY, NY) 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. All data is presented as percent of control peak current, unless otherwise stated.

Statistical comparisons

All paired data were analyzed by t-test, after passing equal variance and normality tests. All values are the mean $\pm$ SEM, n being the number of terminals, and differences were considered significant at $p < 0.05$.

RESULTS

G-protein mediated inhibition by κ -opioid receptor

Ca^{2+} channel inhibition in isolated HNS terminals by κ -opioid receptor agonists have been previously shown using the “classic” whole-cell patch configuration (Rusin et al., 1997) and is now shown using the perforated-patch clamp mode (Fig. 1A). Ca^{2+} current inhibition by the μ -opioid receptor agonist DAMGO can be observed only using the perforated-patch configuration (Fig. 1B), as previously published (Ortiz-Miranda et al., 2003; Ortiz-Miranda et al., 2005). The perforated-patch configuration of the whole-cell patch-clamp method is typically utilized to prevent intracellular dialysis of diffusible components. This strongly suggests a diffusible second messenger is necessary for MOR inhibition of VGCC, and KOR mediated inhibition is potentially via a membrane delimited G-protein pathway.

In order to link κ - and μ -opioid receptor inhibition of calcium currents to the activation of a G-protein coupled receptor, we tested DAMGO (100 nM) and U50488 (100 nM) effects by pre-treating the terminals with 10 μM *N*-ethylmaleimide (NEM) for 5 min (Fig. 1). NEM is a sulfhydryl alkylating reagent, characterized as a blocker of specific G-protein ADP-ribosylation, which results in termination of downstream opioid receptor signaling initiated by agonist binding. Studies show that treating membranes with NEM abolishes signaling downstream of G-protein activation by the κ -opioid receptor agonist U50488 and μ -opioid receptor agonist DAMGO specifically (Allgaier et al., 1989; Ueda et al., 1990; Ofri & Simon, 1992; Ueda et al., 1996). Functional studies of opioid receptor cysteine residues show that agonist binding to the receptor itself is not likely to be affected within the 5-10 μM concentration range of NEM (Ueda et al., 1996; Ehrlich et al., 1998). 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). In our preparation of NH terminals, 10 μM concentrations of NEM were sufficient to block all κ - and μ -opioid inhibition of calcium currents (Fig. 1C). Inhibition of calcium currents was 58.8 ± 2.2 % from control and recovered to 99.9 ± 4.8 % in the presence of NEM during U50488 treatment. Similarly, DAMGO inhibited the calcium currents to 50.1 ± 5.6 % of control and showed a recovery from inhibition in the presence of NEM to 97.0 ± 5.1 %. This demonstrates that these responses are via a G-protein mediated pathway, potentially pertussis toxin-sensitive. Pertussis toxin itself could not be used given the necessity for long incubation periods that are incompatible with the lifetime of our isolated HNS terminal 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-types (Wang et al., 1997; Wang et al., 1999). The time of

activation kinetics (i.e., time of e-fold change in currents, τ) was measured in the presence of either κ - or μ -opioid agonists and ratioed 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 difference is expressed as a percentage of control (i.e., 0 % difference = no change, negative % difference = faster time constant, and positive % difference = slower time constant). Results show a 75 ± 16.6 % difference for U50488 and only a 1.7 ± 4.0 % 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 receptor agonists

Given the persistence of κ -opioid inhibition to intraterminal dialysis we hypothesized that κ -, but 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 a 30 ms duration, U50488 inhibition was completely blocked (Fig. 2A). DAMGO inhibition, however, was unaffected (Fig. 2B), nor was the shape of the current consistently altered. The repetitive application of a square DPP usually resulted in unstable recordings, especially when a square test pulse was used. Thus, duplicates of runs (i.e., repetitions) were limited as much as possible while still maximizing the number of samples for statistical analysis. The depolarizing pre-pulse had no facilitating effect on control calcium currents without opioid treatment or during NEM treatment (data not shown).

MOR and KOR 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., 1995; Leng et al., 1994; Whitnall et al., 1983). 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 these more physiologically relevant stimuli (See Methods). For tests made with individual APs, samples are shown representing the initial (unbroadened) and final (broadest) action potentials within a burst: from peak to half amplitude on the falling phase within a range of 1 to 4 ms (Fig. 3A, upper traces). Peak calcium currents varied with longer duration APW (Fig. 3A, lower traces). The APW calcium currents were isolated using 100 nM 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 $\text{NiCl}_2/\text{CdCl}_2$ (Fig. 3A, lower traces). The degree of inhibition with either κ - or μ -opioid receptor agonists did not significantly change with APW duration (Fig. 3B). Both DAMGO (Fig. 4A) and U50488 (Fig. 4B) inhibit APW-elicited calcium currents (APW duration was 4.5 ms from peak to half amplitude on the falling phase) to a similar extent as those elicited by rectangular pulses. Inhibition by U50488 of APW currents (71.2 ± 5.0 %) was similar to that of rectangular pulse-elicited currents (72.8 ± 12.3 %). Inhibition by DAMGO of APW currents (77.1 ± 8.1 %) was also similar to that of rectangular pulse-elicited currents (74.6 ± 8.9 %).

Voltage-dependent G-protein modulation of Ca^{2+} 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 of U50488 and DAMGO inhibition on APW elicited calcium currents. The criteria for selecting the broadest APW was based on using an

APW that would most likely be at the end of a train of APs (Gainer et al., 1986; Leng & Shibuki, 1987; Jackson et al., 1991; Branchaw et al., 1998; Muschol & Salzberg, 2000; Marrero & Lemos, 2005). Presumably, this APW would be affected by a 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; Brown et al., 2006).

KOR inhibition of APW-elicited calcium currents is voltage-dependent

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 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 (total change of +180 mV) and a duration of 30 ms (Fig. 5). Ca^{2+} current inhibition by U50488 ($60.5 \pm 1.7\%$) disappeared after DPP ($95.2 \pm 2.5\%$) whereas Ca^{2+} current inhibition by DAMGO ($64.5 \pm 2.1\%$) did not significantly ($p = 0.06$) change (to $59.7 \pm 2.4\%$) after DPP ($n = 11$ for each treatment with 2 repetitions per n) (Fig. 5). This small 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 (see Fig. 6). With a DPP pre-pulse interval of 2 ms and duration of 25 ms, DPP amplitude was varied from -70 mV (where -80 mV is no pre-pulse) to +130 mV (in steps of 20 mV). Maximum relief of U50488 inhibition was achieved between +90 to +130 mV. DAMGO inhibition was at no point relieved and exhibits a downward trend indicative of voltage-dependent inactivation (Fig. 6D). Using a fixed step amplitude of +100 mV (total change of +180 mV) and a 2 ms pre-pulse interval, DPP duration was increased from 1 ms to a maximal duration of 104 ms (in steps of 13 ms). No relief of U50488 inhibition occurred with a 1 ms duration DPP. However, following a 13 to 27 ms duration DPP, inhibition by U50488 decreased and remained attenuated thereafter. In contrast, DAMGO inhibition was not attenuated for any of these DPP durations. Pre-pulses to +100 mV (30 ms duration) 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 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 (see Fig. 3A). 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 μM application of NEM, $96.1 \pm 3\%$ and $106 \pm 10.3\%$, respectively (data not shown). In contrast, control and NEM treated terminals showed no facilitation of APW calcium currents using identical DPP protocols (data not shown).

Frequency-dependent relief of KOR inhibition

Protocols of varying frequency were designed using 1 ms duration rectangular pre-pulses (Fig. 7A-C) which, although similar to *in vivo* AP duration, were ineffective in relieving any inhibition by DAMGO (Fig. 7E). Currents decreased by DAMGO were further decreased progressively as DPP frequency increased (Fig. 7E). 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. 7D,E). At 50 and 100 Hz the currents are also significantly disinhibited (as compared to 0 Hz) but not back to control levels. 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. 7D, APW-elicited total calcium currents in the presence of U50488 show a change in activation kinetics (time-to-peak). The time-to-peak of the APW-elicited calcium current was measured in the presence of either κ - or μ -opioid agonists and ratioed 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 difference was then expressed as a percentage from control (*i.e.*, 0 % difference = no change, negative % difference = decrease in time to peak, and positive % difference = increase in time-to-peak). Results show a 8.8 ± 1.0 % difference for U50488, and a -3 ± 2.4 % difference for DAMGO. The change in activation kinetics of the APW-elicited current in the presence of U50488 was smaller than those measured for rectangular pulse elicited currents (see above). However, as noted by others (Park & Dunlap, 1998), APW-elicited currents have changing and comparatively brief activation phases which make kinetics more difficult to quantify and compare.

KOR inhibition relieved by a train of action potentials

The effect of APWs on U50488 κ -opioid inhibition of voltage-gated Ca^{2+} channels was investigated using a train of APs typical of those generated by NH terminals (Fig. 8A). The Long APW studied is present at the end of the train (#35). Burst-elicited sample calcium currents under control (gray traces) and opioid (black traces) conditions are presented in Figure 8B&C during U50488 and DAMGO treatment, respectively. The U50488 inhibition of APW-elicited currents was significantly ($* p < 0.05$) relieved within the burst (Fig. 8B). DAMGO inhibition was not relieved regardless of APW duration or AP position within the burst (Fig. 8C). The first statistically significant relief of U50488 inhibition was observed at APW #12 at an average estimated frequency of 100 Hz. All subsequent APW-elicited currents during the burst showed increasing recovery from this initial inhibition.

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 release is exceptionally receptive to μ -receptor inhibition (Russell et al., 1995; 2003; Ortiz-Miranda et al., 2005; Lemos et al., 2002) whereas both oxytocin (OT) and arginine-vasopressin (AVP) release have been shown to be similarly sensitive to κ -receptor mediated inhibition (Zhao et al., 1988; van de Heijning et al., 1991). In isolated terminals the inhibitory constraints of both μ - and κ -opioids are mirrored in the systemic output of both OT and AVP 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. 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 at the terminals. 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 the HNS

Peak release efficiency for both oxytocin and vasopressin is achieved via the specific bursting modes of activity of magnocellular neurons (Bicknell & Leng, 1981; Poulain & Wakerly, 1982). Oxytocin cells are known to fire within the 50-100 Hz range for periods of 0.5-2.5 sec. (Wang & Hatton, 2005) for optimum neuropeptide secretion (Nordmann & 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. 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). 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 & Dreifuss, 1972).

The exact reason why specific frequency stimulations optimize neuropeptide secretion is still under intense study (Lemos & Wang, 2000). 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 intraterminal calcium, have been proposed to explain the frequency dependence of both AVP and OT release (Leng & Shibuki, 1987; Muschol & Salzberg, 2000). However, a yet unexplained residual frequency-dependent facilitation of action potential-induced rise in the intracellular Ca^{2+} concentration, unrelated to action potential broadening, remain to be explained (Jackson et al., 1991). Furthermore, biophysical properties of the VGCC present in isolated terminals cannot account for the observed frequency-dependent facilitation (Wang et al., 1997b; Wang et al., 1999; Lemos & Wang, 2000).

As with learning and memory, appropriate timing of electrical signals is important in conveying an accurate physiological response (D'Angelo & Rossi, 1998; Matsumoto & Okada, 2002; Debanne et al., 2003; Thivierge et al., 2007; Caporale & 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. We propose that voltage-dependent relief of tonic κ -opioid receptor mediated inhibition of VGCC may be such a mechanism and could help explain the importance of specific frequency-dependent bursting patterns in increasing the efficacy of neuropeptide release.

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

Neurohypophysial terminals co-package and thus co-release dynorphin, the endogenous κ -opioid receptor agonist, with AVP and OT neuropeptides (Leng et al., 1994; Whitnall et al., 1983). The presence of functional κ -receptors has been determined for both OT and AVP neurohypophysial nerve terminals (Zhao et al., 1988). Here we propose that neuropeptide release is potentially tonically inhibited by endogenous κ -opioid modulation acting on both OT and AVP release in the neurohypophysis. This tonic κ -opioid inhibition could be a result of basal opioid secretion, and is only relieved by appropriate electrical stimuli from MNC neurons. Therefore, temporal integration of the appropriate electrical signals from MNC neurons can result in high frequency bursting relieving VGCC from tonic voltage-dependent κ -opioid inhibition, thus optimizing neuropeptide release (Dyball et al., 1988; Brody et al., 1997). Furthermore, during the initial high frequency phase of the action potential burst, NH

terminals could potentially prime release by mobilizing readily releasable pools of neurosecretory granules (Nowycky et al., 1998). Optimum release would thus 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., 1988; Nowycky et al., 1998; Marrero & Lemos, 2005).

Role of voltage-dependent inactivation of I_{Ca}

Our results show, that like rectangular DPPs of varying frequencies (Fig. 7), a train of APWs is also capable of relieving U50488 effects (Fig. 8). APW pulses, having comparatively slower activation kinetics than rectangular pulses, could make them capable of unmasking all of the facilitation while avoiding the competing accumulation of voltage-induced Ca^{2+} current inactivation. Thus, although less prominent when using AP waveforms, 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 & 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., 1991). We simulated burst broadening in our burst pattern and found that dis-inhibition of κ -receptor activation progressively occurs reaching a maximum during the latter part of the burst during the broadest APWs (Fig. 8). This is consistent with studies done by Brody and colleagues (Brody et al., 1997) that show the extent of G-protein dis-inhibition of P/Q-type calcium channels in HEK cells increases linearly with the duration of the action potential waveform.

Currently we have no evidence that the duration of the APWs within a burst would differentially affect relief of voltage-dependent κ -mediated inhibition in the HNS terminals. Although there are no studies differentiating between AVP and OT frequency-dependent spike broadening it is possible to speculate that since AVP bursts are longer in duration, the APs at the end of an AVP burst would be inherently broader (see Fig. 8A). Therefore, eliciting an AVP-like burst could circumvent any voltage-dependent inhibition such as that mediated by the κ -opioid receptor. This could help explain why AVP secretion is not always affected by κ -opioid inhibition or potentiated in the presence of κ -receptor antagonists during stimulated synchronized release (Bicknell et al., 1985; Bondy et al., 1988; Falke et al., 1988). Furthermore, this would suggest that under physiological conditions most neuropeptide secretion from NH terminals would be facilitated as a burst progresses, concurrent with relief of inhibition, and not at the very beginning as current dogma dictates (Cazalis et al., 1985, 1987; Bicknell 1988). For example, during physiological conditions of dehydration, the asynchronous activation of AVP neurons would result in tonic κ -opioid inhibition due to release of dynorphin from both AVP and OT neurons (Leng et al., 1994). This inhibition would restrain secretion at the start of the burst, but be increasingly relieved

during the progression of the burst. Furthermore, this relief of inhibition would theoretically favor AVP release with its potentially broader APs, enabling secretion to be maintained through an AVP bursting pattern despite tonic κ -inhibition. Although further investigation is required to determine whether a specific duration 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 with a functionally relevant range of AP durations.

CONCLUSIONS

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 HNS 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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Abbreviations

APW	action potential waveforms
AVP	vasopressin
HNS	hypothalamo-neurohypophysial system
NH	neurohypophysis
NHT	neurohypophysial terminals
NMG	n-methyl glucamine
OT	oxytocin
VGCC	voltage-gated calcium channels
NEM	n-Ethyl Maleimide
U50488	trans-3,4-dichloro-N-methyl-N-[2-(1-pyrrolidinyl)-cyclo-hexyl] benzeneacetamide
DAMGO	Tyr-D-Ala-Gly-MePhe-Gly-Ol
TTX	tetrodotoxin
DPP	depolarizing pre-pulse

PTX	pertussis toxin
MNC	magnocellular
KOR	κ -opioid receptor
MOR	μ -opioid receptor
CNS	Central Nervous System

Figure 1. *N*-ethylmaleimide blocks κ - and μ -opioid receptor mediated inhibition of calcium currents

A. Calcium currents elicited by step from -80 mV to 0 mV of single HNS terminal under control (black), 100 nM U50488 (dashed gray) and with 10 μ M NEM (gray) conditions using the perforated-patch configuration of the whole-cell patch-clamp technique. **B.** Calcium currents elicited using the same protocol on another single HNS terminal under control (black), 100 nM DAMGO (dashed gray) and with 10 μ M NEM (gray) conditions using the perforated-patch configuration of the whole-cell patch-clamp technique. Both A and B are sample averages of 2 repetitions. **C.** Bar Graph summarizing average effects of opioids on Ca^{2+} currents ($n = 5$ for U50488, $n = 5$ for DAMGO: with 2 repetitions per n for both treatments). Asterisks (*) represent statistically significant differences ($p < 0.05$).

Figure 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 (dashed black) and with a DPP in the presence of 100 nM U50488 (gray) conditions. **B.** Calcium currents under control conditions (black), with 100 nM DAMGO (dashed gray) and with a DPP in the presence of 100 nM DAMGO (gray) conditions. **C.** Bar graph quantifying differences 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 ($n = 4$ for U50488, $n = 4$ for DAMGO: with no repetitions). Asterisk (*) indicates significant difference ($p < 0.05$).

Figure 3. Calcium currents from action potential waveforms obtained from NH terminal action potentials

A. Voltage waveform action potential duration is measured from the peak to the half height of the falling phase. Shown are the narrowest recorded APW (left) with a duration of 1 ms and the broadest with a duration of 4.5 ms (right). Inward currents, remaining after application of TTX (to block inward sodium currents), are blocked by the application of 200 μ M $\text{Cd}^{2+}/\text{Ni}^{2+}$, indicating that they are calcium currents. The remaining currents, after $\text{Cd}^{2+}/\text{Ni}^{2+}$ block, were 24.47 ± 4.25 % for the narrowest and 6.46 ± 1.78 % for the broadest (% of respective calcium current $\pm$ % SE, $n = 8$). B. Peak Current normalized to maximum of APW-elicited calcium currents without treatment plotted as a function of action potential duration (dashed plot) and the percent peak control Ca^{2+} current with either κ - or μ -opioid agonist plotted as a function of APW duration (solid plots, black and gray, respectively). Data points represent means of $n = 11$ for U50488, $n = 11$ for DAMGO: with 2 repetitions included for each n . There was no significant difference between any of the opioid-reduced APW currents when compared to their respective narrower (1 ms) opioid-reduced response.

Figure 4. κ - and μ -opioid receptor activation similarly inhibits APW- and rectangular pulse-elicited calcium currents

A. & *B.* Calcium currents elicited by a broadened APW (upper trace) inhibited by either 100 nM U50488 (gray) or 100 nM DAMGO (dashed black) followed by their respective washes (dashed gray). Changes in the activation kinetics following wash were not consistent. *C.* & *D.* Same designation scheme for calcium currents elicited by rectangular pulses. All currents with κ -opioid inhibition belong to the same HNS terminal and all currents with μ -opioid inhibition presented are recorded from a different individual HNS terminal. For averages given in text, data was chosen randomly with a total of $n = 8$ for each opioid, and no repetitions.

Figure 5. APW-elicited calcium currents are also relieved by DPP of κ - but not μ -opioid inhibition

A, Sample APW-elicited calcium currents in two different terminals with control (black), in the presence of their respective opioid agonist, 100 nM κ -opioid receptor agonist U50488 (gray), or μ -opioid receptor agonist DAMGO (dashed black), and after a depolarizing pre-pulse (dashed gray). Sample traces are an average of two repetitions. **B**, Bar graph quantifying the effects of a DPP on κ - and μ -opioid inhibition of APW-elicited calcium currents ($n = 11$ for U50488, $n = 11$ for DAMGO: with 2 repetitions for each n). Asterisks (*) represent statistically significant difference ($p < 0.05$).

Figure 6. Optimizing DPP for APW-evoked calcium currents

Opioid induced inhibition of APW-evoked currents plotted as a function of pre-pulse Voltage (A. & D.), Duration (B. & E.), and Interpulse Interval (C. & F.). Voltage commands are shown in top panels. Fixed prepulse parameters (unless otherwise stated) were as follows: 2 ms initial interpulse interval, amplitude to +100 mV, 30 ms duration. (D., E. & F.) Long waveform stimulation data points in all panels represent the means of measurements for n = 4 for both U50488 and DAMGO treated terminals. No repetitions were included. Black lines are treated with 100 nM U50488 and gray lines are treated with 100 nM DAMGO. Asterisks (*) represent values with statistical difference (p > 0.05) from initial inhibition.

Figure 7. 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; black), U50488 after 0 Hz DPP (black short dashes), U50488 after 10 Hz DPP (black long dashes), U50488 after 50 Hz (gray) and U50488 after 100 Hz (gray dashes) DPP protocol. E. The effect of pre-pulse frequency on opioid inhibition of APW elicited currents vs. DPP frequency (Hz = 0 is no pre-pulses) for 100 nM U50488 (black), and 100 nM DAMGO (gray) with $n = 6$ for both treatments (no repetitions per n). Asterisks (*) represent statistically significant increase ($p < 0.05$) from control for U50488 treatment.

Figure 8. κ -opioid inhibition of APW calcium currents is relieved within a simulated burst

A. The waveform used, applied as a depolarizing stimulus from -70 to +70 mV, contained a total of 35 action potentials in progressive order of broadening. **B.** APW-elicited calcium currents using the simulated AP burst under control conditions (gray) and in the presence of 100 nM U50488 (black). Below it is a bar graph quantifying changes of all APW-elicited Ca^{2+} currents throughout the simulated burst during 100 nM U50488 treatments. Dark gray bars in **A** show statistically significant difference ($p < 0.05$) from initial inhibition shown for AP #1. Asterisk (*) indicates onset of significant difference, shown here only as a reference for comparison of significant differences with APW's at end of burst. **C.** APW-elicited calcium currents using the same protocol under control (gray) or in the presence of 100 nM DAMGO (black). Below is the corresponding bar graph quantifying changes during 100 nM DAMGO treatments of all APW-elicited currents within burst. All current traces are an average of two repetitions and all bar graphs are for $n = 11$ with 2 repetitions per n . Controls, for respective opioid treatments, were taken independently. Vertical calibration bar = 100 pA and horizontal calibration bar = 40 ms.