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**μ-Opioid Inhibition of Ca\(^{2+}\) Currents and Secretion in Isolated Terminals of the Neurohypophysis Occurs via Ryanodine-Sensitive Ca\(^{2+}\) Stores**

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**μ-Opioid agonists have no effect on calcium currents (I\(_{Ca}\)) in neurohypophysial terminals when recorded using the classic whole-cell patch-clamp configuration. However, μ-opioid receptor (MOR)-mediated inhibition of I\(_{Ca}\) is reliably demonstrated using the perforated-patch configuration. This suggests that the MOR-signaling pathway is sensitive to intraterminal dialysis and is therefore mediated by a readily diffusible second messenger. Using the perforated patch-clamp technique and ratio-calcium-imaging methods, we describe a diffusible second messenger pathway stimulated by the MOR that inhibits voltage-gated calcium channels in isolated terminals from the rat neurohypophysis (NH). Our results show a rise in basal intracellular calcium ([Ca\(^{2+}\)]\(_i\)) in response to application of [d-Ala\(^2\)-N-Me-Phe\(^4\),Gly\(^5\)-ol]-Enkephalin (DAMGO), a MOR agonist, that is blocked by d-Phe-Cys-Tyr-d-Trp-Orn-Thr-Pen-Thr-NH\(_2\) (CTOP), a MOR antagonist. Buffering DAMGO-induced changes in [Ca\(^{2+}\)]\(_i\) with BAPTA-AM completely blocked the inhibition of both I\(_{Ca}\) and high-K\(^+\)-induced rises in [Ca\(^{2+}\)]\(_i\), due to MOR activation, but had no effect on κ-opioid receptor (KOR)-mediated inhibition. Given the presence of ryanodine-sensitive stores in isolated terminals, we tested 8-bromo-cyclic adenosine diphosphate ribose (8Br-cADPr), a competitive inhibitor of cyclic ADP-ribose (cADPr) signaling that partially relieves DAMGO inhibition of I\(_{Ca}\), and completely relieves MOR-mediated inhibition of high-K\(^+\)-induced and DAMGO-induced rises in [Ca\(^{2+}\)]\(_i\). Furthermore, antagonist concentrations of ryanodine completely blocked MOR-induced increases in [Ca\(^{2+}\)]\(_i\) and inhibition of I\(_{Ca}\) and high-K\(^+\)-induced rises in [Ca\(^{2+}\)]\(_i\), while not affecting KOR-mediated inhibition. Antagonist concentrations of ryanodine also blocked MOR-mediated inhibition of electrically-evoked increases in capacitance. These results strongly suggest that a key diffusible second messenger mediating the MOR-signaling pathway in NH terminals is [Ca\(^{2+}\)]\(_i\), released by cADPr from ryanodine-sensitive stores.

**Key words:** exocytosis; I\(_{Ca}\); MOR modulation; NH terminals; RyR

Introduction

The μ-opioid receptor (MOR)-mediated inhibition of the hypothalamic neurohypophysial system (HNS) becomes increasingly evident during pregnancy, but is interrupted before parturition, allowing for strong excitation of oxytocin cells that facilitates birth (Russell et al., 1995; Russell et al., 2003). Corelease of dynorphin-A, an endogenous κ-opioid receptor (KOR) agonist, with vasopressin from dendrites facilitates activity-dependent modulation of vasopressinergic neurons (Brown and Bourque, 2004; Brown et al., 2004; Roper et al., 2004; Brown et al., 2006; Sabatier and Leng, 2007). Isolated HNS terminals demonstrate inhibition of release in the presence of either MOR or KOR agonists for both oxytocin and vasopressin (Sumner et al., 1990; Kato et al., 1992; Russell et al., 1993); I\(_{Ca}\) is similarly inhibited (Rusin et al., 1997; Ortiz-Miranda et al., 2003; Ortiz-Miranda et al., 2005). The signaling mechanism and modulatory importance of MOR and KOR activation at these presynaptic terminals and subsequent I\(_{Ca}\) inhibition, however, is not well understood. Given the level of endogenous opioid modulation in the HNS, this has considerable physiological relevance.

MOR and KOR are G-protein-coupled receptors that could potentially mediate inhibitory effects of opioids/opiates on I\(_{Ca}\) through either a membrane-delimited or diffusible second messenger pathway (Wilding et al., 1995; Soldo and Moises, 1998; Kaneko et al., 1998; Connor and Christie, 1999; Chen et al., 2000). The MOR-signaling pathway seems to contrast sharply with that documented for the KOR in the same isolated neurohypophysis (NH) terminals (Velázquez-Marrero et al., 2010),

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MOR agonists did not inhibit $I_{Ca}$ when recorded using the whole-cell patch-clamp configuration, in contrast to KOR-mediated inhibition (Rusin et al., 1997). However, MOR-mediated inhibition of $I_{Ca}$ is demonstrated using the perforated-patch configuration of the patch-clamp method (Ortiz-Miranda et al., 2005). This strongly suggests that the MOR-signaling pathway at the NH terminals is sensitive to intraterminal dialysis and is therefore mediated by a readily diffusible second messenger. Furthermore, unlike the KOR-mediated inhibition of $I_{Ca}$, MOR inhibition is not relieved by a strong depolarizing prepulse (Velázquez-Marrero et al., 2010).

Rytophosphate-sensitive calcium stores have been shown to be targets of G-protein opioid activation (Tai et al., 1992; Hauser et al., 1996) Samways and Henderson, 2006). In isolated HNS terminals, we have characterized rythropine- and voltage-sensitive $[Ca^{2+}]_r$, release events, known as syntillias (De Crescenzo et al., 2004). Furthermore, the cyclic ADP-ribose (cADPr) signaling pathway initiates a signaling cascade leading to activation of the ryanodine receptor in vitro and subsequent release of $[Ca^{2+}]_r$ 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 oxytocin (OT) release from isolated HNS terminals (Higashida et al., 2007; Jin et al., 2007). We propose that, in HNS terminals, activation of the MOR triggers a rise in intraterminal basal calcium released from ryanodine-sensitive stores via activation of the cADPr signaling cascade. This rise in $[Ca^{2+}]_r$ could lead to calcium-dependent inhibition of $I_{Ca}$ and subsequent inhibition of depolarization-induced neuropeptide release.

### Materials and Methods

#### Isolation of nerve endings

Male Sprague Dawley rats (Taconic Farms) weighing 200–250 g were sedated using CO2 and immediately decapitated. The pituitary gland was isolated as described previously (Lemos et al., 1986; Knott et al., 2005). Briefly, after removal of the anterior and intermediate lobes, the posterior pituitary was homogenized in 270 μl of buffer at 37°C containing the following (in mM): 270 sucrose, 0.004 EGTA, and 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 consisting of modified normal Locke’s (NL) containing the following (in mM): 145 NaCl, 2.5 KCl, 10 HEPES, 1.0 glucose, 0.8 CaCl2, and 0.4 MgCl2, pH 7.4, 298–302 mOsmol/L.

**Electrophysiological experiments.** The NH was isolated and homogenized as described previously (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 the following (in mM): 145 Cs-glucosinate, 15 CsCl, 2 MgCl2, 2 NaCl, 7 glucose, and 10 HEPES, pH 7.3, 295 mOsm. Amphotericin B at a concentration of 30 μg (Sigma) was added as a perforating agent. The bath solution consisted of the following (in mM): 145 NaCl, 5 KCl, 1 MgCl2, 10 HEPES, 10 glucose, 1.2 CaCl2, pH 7.5, in NL. 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 MΩ. Pipettes were made of thin borosilicate glass (Drummond Scientific). After perforation, the terminals were voltage clamped at −80 mV. Depolarization was applied every 30 s to 0 mV for 250–300 ms. The preparation was either continually perfused via a gravity-driven perfusion system or was left in a static, nonperfused bath (as noted). Agonists and antagonists were either applied through the gravity-driven perfusion system or was left in the static bath. All experiments were performed at room temperature (25°C). Data were acquired, stored, and analyzed using a PC and 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., measuring any small capacitance change). In this particular case, the parameters were tuned using a sine wave of 1000 Hz at ±25 mV (approximately the holding potential), with the program reporting a

#### Inactivation time constants ($\tau$) were obtained from single exponential fits (Igor; Waveformics or PClamp6) on inward $I_{Ca}$ for 5 ms after peak current and 3 ms before the end of stimulus.

**Calcium imaging.** Freshly dissociated nerve terminals (Nordmann et al., 1987) were prepared from adult Sprague Dawley CD rats weighing 25.5 μm Fura-2 AM for 45 min at 37°C and thoroughly washed with NL solution containing the following (in mM): 145 NaCl, 5 KCl, 10 HEPES, 10 glucose, 1.0 MgCl2, and 2.2 CaCl2, pH 7.4. Ca2+ free bath solution contained the following (in mM): 145 NaCl, 5 KCl, 10 HEPES, 10 glucose, 0.0002 EGTA, and 1 MgCl2, pH 7.4, and a calculated free $[Ca^{2+}]_r$ of zero. Cytosolic $[Ca^{2+}]_r$ was determined with ratiometric indicator Fura-2 AM-loaded terminals. Calibration of Fura-2 was done both in vitro using the Invitrogen Calcium Calibration Buffer Kit (catalog #F-6774) and in situ using predetermined $[Ca^{2+}]_r$ buffered solutions on terminals loaded with Fura-2 AM and using the Br-A23187 divalent cation ionophore. The $K_D$ was calculated from a plot generated by scanning the ratio of the emission at the two corresponding wavelengths against $[Ca^{2+}]_r$ across the zero to saturating $[Ca^{2+}]_r$ concentrations. The results were plotted as a double log of the calcium response resulted in a straight line, with the x-intercept being equal to the log of the apparent $K_D$, indicator, which was determined under in situ conditions to be −200 nm across the physiologically relevant concentration range of 0–35 μM $[Ca^{2+}]_r$. The calculations were performed as described previously (Grynkiewicz et al., 1985). Resting values for global cytosolic $[Ca^{2+}]_r$ in the presence and absence of extracellular Ca2+ 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. In all cases, changes in $[Ca^{2+}]_r$ determined by measuring total calcium.

**Fluorescence images using Fura-2 AM as a calcium indicator were viewed with a Nikon Diaphot TMD microscope using a Zeiss Plan-NEOFLUAR 100× oil-immersion lens and fitted with a Photometrics SenSys CCD camera. The camera was interfaced to the inverted microscope.**
Furthermore, pretreatment with 100 nM DAMGO (a MOR agonist) or puffed with control 0 mM [Ca\(^{2+}\)]\(_o\) NL (Fig. 1C). Application of the MOR antagonist CP-99,994 (Fig. 1D) blocked the DAMGO-induced rise in basal [Ca\(^{2+}\)]. Changes in basal intracellular calcium concentration were 26.9 ± 0.8 nM by 100 nM DAMGO in 2.2 mM [Ca\(^{2+}\)]\(_o\) NL and (28.7 ± 5.1 nM) in 0 mM [Ca\(^{2+}\)]\(_o\) with 100 nM DAMGO. Differences in [Ca\(^{2+}\)]\(_i\) were –3.3 ± 5.2 nM due to 100 nM U50488, control was –2.4 ± 3.4 nM, and 100 nM DAMGO in the presence of CTOP was –1.8 ± 1.0 nM (all in 0 mM [Ca\(^{2+}\)]\(_o\)). Control applications (Fig. 1C) caused an artifactual drop in [Ca\(^{2+}\)]\(_i\), which may be an inherent factor in all treatment applications and could therefore lead to an underestimation of the DAMGO-induced rise in [Ca\(^{2+}\)]. Control baseline [Ca\(^{2+}\)]\(_i\), averaged 71.6 ± 15.7 nM.

**Figure 1.** MOR but not KOR agonists trigger a rise in basal [Ca\(^{2+}\)], preceding MOR-mediated inhibition of high-K\(^+\)-induced rise in [Ca\(^{2+}\)]. **A.** Images of a Fura2-AM-loaded single terminal – 10 s apart challenged with high-K\(^+\) (HiK\(^+\)) in 2.2 mM [Ca\(^{2+}\)]\(_o\) for 5 s with and without 100 nM DAMGO preexposure. **B.** Plot of changes in [Ca\(^{2+}\)]\(_i\) over time of a different single isolated HNS terminal loaded with Fura-2 AM; high K\(^+\) (as labeled) exposures for 5 s and DAMGO (as labeled) pretreatment for 10 s followed by high K\(^+\) in the presence of 100 nM DAMGO (as labeled). Double forward slashes in x-axis represent 30 s breaks in image acquisition to allow recovery from high K\(^+\) treatment. **C.** [Ca\(^{2+}\)]\(_i\) change from baseline in response to 100 nM U50488, 100 nM DAMGO, and control treatment containing modified NL without Ca\(^{2+}\) (as labeled); n = 5 terminals. **D.** Rise in [Ca\(^{2+}\)]\(_i\) due to 100 nM DAMGO application is blocked in the presence of 100 nM BAPTA-AM (as labeled); n = 4 terminals. Asterisks (*) represent statistical differences (p = 0.002).

MOR- but not KOR-mediated inhibition is blocked by intraterminal calcium buffering

To determine whether the rise in [Ca\(^{2+}\)]\(_i\) due to MOR agonist application is essential for subsequent inhibition of I\(_{Ca}\), we partially buffered [Ca\(^{2+}\)], using BAPTA-AM and monitored the effects on both I\(_{Ca}\) and high-K\(^+\)-induced rises in [Ca\(^{2+}\)]. Empirically determined incubation periods of 5–10 min using low concentrations (10 μM) of BAPTA-AM were followed by a brief wash. Using this procedure, BAPTA-AM buffered DAMGO-induced rises in intraterminal calcium from 22.7 ± 2.4 nM down to 0.2 ± 0.5 nM (Fig. 2A). However, this concentration of BAPTA-AM did not block total high-K\(^+\)-induced rise in [Ca\(^{2+}\)]\(_i\), (Fig. 2B, C). Effects on both high-K\(^+\)-induced rises in [Ca\(^{2+}\)]\(_i\) and I\(_{Ca}\) were measured for both MOR- and KOR-mediated inhibitions using the same concentration of BAPTA-AM for identical incubation periods. All measurements were made as either changes in basal [Ca\(^{2+}\)]\(_i\), or as a percentage of control. Control measurements were under identical conditions without opioid/opiate treatments. High-K\(^+\)-induced rises in [Ca\(^{2+}\)]\(_i\) in the presence of 100 nM DAMGO were 54.1 ± 3.4% of control responses. High K\(^+\) in the presence of 100 nM U50488 was also inhibited to 70.9 ± 2.8% of controls (Fig. 2C). However, after incubation with 10 μM BAPTA-AM, terminals exposed to 100 nM DAMGO showed no significant inhibition (109.9 ± 12.8%) of the high-K\(^+\)-induced rises in [Ca\(^{2+}\)]\(_i\) (p = 0.05), as opposed to without BAPTA-AM treatment (p = 0.002). In contrast, inhibition (78.5 ± 2.8%) of the high K\(^+\) response by 100 nM U50488 remained the same with versus without BAPTA-AM treatment (p = 0.3). Baseline [Ca\(^{2+}\)]\(_i\), before high K\(^+\) was not statistically different (p = 0.16) with (77.9 ± 12.8 nM) versus without (86.5 ± 27.5 nM) 5 min preincubation by 10 μM BAPTA-AM. Total [Ca\(^{2+}\)]\(_i\), was not significantly changed (see legend to Fig. 2). Furthermore, nM capacitance averaged for every 30 points (24 μs sampling rate). The current was filtered at a bandwidth of 5000 Hz.

**Results**

**MOR but not KOR agonist elicits a rise in basal intraterminal [Ca\(^{2+}\)]**

To determine whether intraterminal Ca\(^{2+}\) ([Ca\(^{2+}\)]\(_i\)) is part of a diffusible second messenger pathway mediating opioid receptor signaling in isolated HNS terminals, we monitored it in response to both MOR and KOR activation. Fura-2 AM ratio calcium imaging of isolated HNS terminals showed a significant inhibition of high-K\(^+\)-induced rises in [Ca\(^{2+}\)]\(_i\), when treated with either MOR agonists (Fig. 1A,B) or KOR agonists (Fig. 2C). Furthermore, pretreatment with 100 nM DAMGO (a MOR agonist) alone at either 2.2 (Fig. 1A,B) or 0 nM (Fig. 1C,D) external
BAPTA is needed to affect high K$^+$ peaks in \([\text{Ca}^{2+}]_i\). Therefore, changes in responses to opioids cannot be attributed to initial differences in baseline \([\text{Ca}^{2+}]_i\).

Effects on calcium currents

Buffering \([\text{Ca}^{2+}]_i\) also blocked inhibition of \(I_{\text{Ca}}\) by MOR but not KOR agonists. Terminals treated with 100 nM DAMGO were 82.2 ± 4.0% of control currents and were 88.7 ± 2.3% with 100 nM U50488 (Fig. 3). After preincubation with 10 \(\mu\text{M}\) BAPTA-AM, 100 nM DAMGO elicited no inhibition, with \(I_{\text{Ca}}\) being 98.9 ± 1.2% of control. Exposing BAPTA-AM-treated terminals to 100 nM U50488 again showed no change in KOR-mediated inhibition, with \(I_{\text{Ca}}\) remaining at 78.8 ± 4.9% of controls. Statistical analysis determined no significant difference (\(p = 0.09\)) between terminals treated with 100 nM DAMGO, with versus without BAPTA-AM preincubation.

\(c\text{ADPr}\) pathway

Because activation of the MOR-induced rise in \([\text{Ca}^{2+}]_i\) appears to be essential in mediating MOR inhibition, we investigated whether cADPr was part of this receptor’s intraterminal signaling pathway. We examined the effects of the membrane-permeant cADPr antagonist 8-bromo-cyclic adenosine diphosphate ribose (8Br-cADPr) on MOR-mediated inhibition of both high-K$^+$-induced rises in \([\text{Ca}^{2+}]_i\) (Fig. 4A, B) and \(I_{\text{Ca}}\) (Fig. 4C, D). Control high-K$^+$-induced changes in \([\text{Ca}^{2+}]_i\) were 457.1 ± 13.5 nM, but in the presence of 100 nM DAMGO high-K$^+$-induced changes in \([\text{Ca}^{2+}]_i\) decreased to 392.7 ± 19.6 nM (\(p = 0.04\)). After incubation with 100 nM 8Br-cADPr, terminals exposed to 100 nM
DAMGO-induced rise in $[\text{Ca}^{2+}]_i$ (Fig. 5A), suggesting that the cADPr pathway is involved in MOR signaling.

**MOR effects are blocked by ryanodine receptor antagonist**

We next addressed whether the release of $[\text{Ca}^{2+}]_i$, due to MOR activation was from ryanodine-sensitive intraterminal stores. The pharmacology of the ryanodine receptor indicates that 10–100 µM concentrations of ryanodine can block ryanodine channel activity (Coronado, Morrise et al., 1994; Ehrlich, Kaftan et al., 1994). Therefore, we tested the effects of 100 µM ryanodine on DAMGO-induced rises in basal $[\text{Ca}^{2+}]_i$ (Fig. 5A) and its inhibition of high-$K^+$-induced rises in $[\text{Ca}^{2+}]_i$ (Fig. 5B,C), $I_{\text{Ca}}$ (Figs. 6, 7), and electrically induced capacitance changes (Fig. 7).

DAMGO-induced changes in basal $[\text{Ca}^{2+}]_i$ were 21.3 ± 3.2 nM, but after preincubation with 100 µM ryanodine (Fig. 5A), these were significantly ($p = 0.0002$) reduced to −0.1 ± 0.8 nM. Likewise, DAMGO-induced changes in basal $[\text{Ca}^{2+}]_i$ were blocked by 100 nM 8Br-cADPr (Fig. 5A).

Control high-$K^+$-induced changes in $[\text{Ca}^{2+}]_i$ were 219.9 ± 21.4 nM. In the presence of 100 nM DAMGO, high-$K^+$-induced changes in $[\text{Ca}^{2+}]_i$ were 112.7 ± 8 nM, which represents 51% of the control response (Fig. 5B,C). 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 controls without DAMGO (209 ± 13.4 nM) in the presence of 100 µM ryanodine. There was no significant difference ($p = 0.11$) 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. However, there was a statistically significant difference ($p = 0.01$) in high-$K^+$-induced changes in $[\text{Ca}^{2+}]_i$, between terminals treated with versus without 100 nM DAMGO, which were not preincubated with 100 µM ryanodine. Control baseline $[\text{Ca}^{2+}]_i$ was 62.2 ± 11.3 nM and 76.1 ± 11.5 nM after preincubation with 100 µM ryanodine ($n = 3$). There was no statistical difference between baseline $[\text{Ca}^{2+}]_i$ with versus without 100 µM ryanodine incubation ($p = 0.06$).

Inhibition of $I_{\text{Ca}}$ by 100 nM DAMGO was measured as percentage of controls. Calcium currents treated with 100 nM DAMGO (Fig. 6A) were inhibited to 76.3 ± 1.8% of control currents (Fig. 7C). During incubation with 100 µM ryanodine (Fig. 6B), $I_{\text{Ca}}$ treated with 100 nM DAMGO showed no significant inhibition, being 94.6 ± 2.5% of controls ($n = 6$). There was no statistically significant difference ($p = 0.10$) between terminals in the presence of 100 µM ryanodine with and without 100 nM DAMGO.

Inactivation of $I_{\text{Ca}}$ via $\text{Ca}^{2+}$-dependent inactivation could be reflected in the time constants of inactivation of the total recorded currents. We therefore measured the time constants of...
inactivation of the $I_{Ca}$ (see Materials and Methods) under control (no opioid or ryanodine), 100 nm DAMGO, 100 µM ryanodine, or 100 µM ryanodine with 100 nm DAMGO conditions (Fig. 7D). The results were plotted as percentage of controls. The average inactivation time constant tau ($\tau$) for the control currents (±SE) was 137.0 ± 25.6 s. Calcium currents remaining in the presence of DAMGO showed slower inactivation kinetics that were 133 ± 2.6% of control. Currents with 100 nm DAMGO treatment had a $\tau = 182.14 ± 8.7$ ms, which is different from control values ($p = 0.001$). Most interestingly, terminals treated with 100 nm DAMGO and 100 µM ryanodine had a $\tau = 131.67 ± 12.8$ ms. In the presence of 100 µM ryanodine, these $\tau$’s were 105.4 ± 3.9% ($p = 0.77$), and, in the presence of 100 µM ryanodine and 100 nm DAMGO, they were 96.1 ± 3.7% ($p = 0.22$).

Effects on capacitance
Reduction of depolarization-induced neuropeptide release has been shown to be due to MOR-mediated inhibition of $I_{Ca}$ (Ortiz-Miranda et al., 2010, 2005). If MOR-mediated inhibition of $I_{Ca}$ can be blocked by 100 µM ryanodine, will it also block the MOR-mediated reduction in neuropeptide release? To address this question, we monitored changes in exocytosis/endocytosis reflected as capacitance changes in individual HNS terminals (see Materials and Methods). Changes in capacitance in perforated-patch-clamped isolated terminals in response to rectangular pulse depolarizations are inhibited by 100 nm DAMGO (Fig. 7A). This inhibition is almost completely reversed by 100 nm CTOP (data not shown), indicating that it is mediated by activation of the MOR. In the presence of 100 nm DAMGO, capacitance changes were 43.8 ± 8.2% of controls (Fig. 7A, B). When incubated in 100 µM ryanodine, capacitance changes were 102.9 ± 3.5% of controls. Application of 100 nm DAMGO in the presence of 100 µM ryanodine resulted in 98.2 ± 2.7% of control capacitance changes and was statistically no different from controls ($p = 0.99$) or capacitance changes in the presence of 100 µM ryanodine alone ($p = 0.16$). Time constants (two exponential fits) for each capacitance change were not significantly different between the treatments, allowing us to conclude that these effects were not on...
of high-K⁺-induced rises in [Ca²⁺]i can be blocked by antagonist concentrations of ryanodine and reduced by inhibition of the cADPr pathway. Changes in the inactivation kinetics of I_{Ca} in the presence of MOR agonists suggests that release of intraterminal Ca²⁺ could be responsible for Ca²⁺-dependent inactivation of I_{Ca}. However, because stores cannot be depleted and ryanodine receptor (RyR) KO animals are not viable (De Crescenzo et al., 2012), it is not possible to prove this. Nevertheless, showing that BAPTA, ryanodine and 8Br-cADPR are all capable of blocking DAMGO’s effects is strong evidence in support of the role of intraterminal Ca²⁺ stores in mediating MOR inhibition.

Furthermore, MOR-mediated inhibition of capacitance in individual terminals can also be blocked by antagonist concentrations of ryanodine. These data support the hypothesis (Fig. 8) that MOR activation leads to the release of Ca²⁺ from ryanodine-sensitive stores via activation of the cADPr pathway, possibly leading to Ca²⁺-dependent inactivation of I_{Ca} and subsequent inhibition of depolarization-secretion coupling (DSC).

cADPr pathway and ryanodine-sensitive stores in HNS terminals
Oxytocin secretion is most sensitive to MOR-mediated inhibition (Wright and Clarke, 1984; Bicknell et al., 1985; Zhao et al., 1988; Leng et al., 1992; Russell et al., 1995; Ortiz-Miranda et al., 2003). Vasopressin release, although responsive to MOR-mediated inhibition, is less sensitive to MOR agonists (Ortiz-Miranda et al., 2003). Interestingly, in HNS terminals, blocking cADPr signaling was shown to attenuate high-K⁺-induced rises in [Ca²⁺]i, but only inhibited OT release from isolated terminals (Higashida et al., 2007; Jin et al., 2007). This strongly suggests that the cADPr pathway is both present in OT terminals and linked to neuroepitode release and thus may be responsible for OT-terminal-heightened sensitivity to MOR-mediated inhibition (Fig. 8).

CD38 is a catalyst for the formation of cADPr and nicotinic acid adenine dinucleotide phosphate by ADP-ribosyl cyclase from NAD⁺ and NAD phosphate (Lee, 1998). Both are known to release Ca²⁺ from intracellular ryanodine-sensitive pools as part of a second messenger-signaling pathway. In studies monitoring depolarization-induced OT secretion in murine NH terminals (Jin et al., 2007), CD38⁻/⁻ mice showed severe and selective impairment of OT release. Currently, this contribution of the cADPr pathway to depolarization-induced responses appears to be in conflict with our results showing the cADPr pathway mediating I_{Ca} inhibition triggered by activation of the MOR (Fig. 8). Although it is clear that the

endocytosis alone. Furthermore, we have shown previously (Ortiz-Miranda et al., 2003; Ortiz-Miranda et al., 2005) that AVP/OT release induced by high K⁺ is inhibited by DAMGO, indicating that the net effect is inhibition of exocytosis. Therefore, antagonist concentrations of ryanodine are able to block inhibition of exocytosis by MOR activation. Nevertheless, effects on endocytosis are also possible (see Discussion).

Discussion
The present study demonstrates that, in isolated HNS terminals, activation of the MOR elicits release of intraterminal Ca²⁺ from ryanodine-sensitive stores. MOR-mediated inhibition of I_{Ca} and
cADPr pathway in HNS terminals can modulate the depolarization-induced response, our results suggest that it may do so differentially depending on the compartments/circumstances in which the pathway is activated. For example, in our model (Fig. 8), MOR activation of the cADPr pathway releases Ca$^{2+}$ from RyRs and, via PK/PP, leads to inactivation of nearby, closed VGCCs. Upon depolarization, these VGCCs would not be available for activation, leading to less neuropeptide release. Direct activation of a specific pool of intraterminal calcium stores during depolarization-induced responses, however, via the cADPr pathway could directly target specific release sites, resulting in potentiation of the response. We have previously demonstrated Ca$^{2+}$ sparks or “syntillas” in murine HNS terminals that are both ryanodine and voltage sensitive (De Crescenzo et al., 2004, 2006). Syntillas result from the activation of both type-1 and type-2 RyRs. Presumably, the type-1 RyR confers the voltage sensitivity via direct interaction with dihydropyridine receptors (De Crescenzo et al., 2006), as documented in skeletal muscle (Schneider and Chandler, 1973; Inui et al., 1987). The cADPr pathway may assist in amplifying this direct effect in HNS terminals during DSC. Inhibition of voltage-dependent release of calcium from ryanodine-sensitive stores may account for the inhibition of both the depolarization-induced rise in [Ca$^{2+}$i], and oxytoxin release observed in the presence of 200 μM ryanodine, as described previously (Jin et al., 2007). However, Jin et al. (2007) showed no effects on AVP release, which would be consistent with our lack of neuropeptide release. This could theoretically have the same effects as those seen for MOR activation, leading to subsequent calcium-dependent inactivation of I$_{Ca}$ and attenuated neuropeptide release. Therefore, the presence of 8Br-cADPr at higher concentrations would, by itself, saturate the calcium-induced inhibitory response in the HNS rat preparation.

Furthermore, IP$_3$ receptor effects have been observed in NH terminals (Cazalis et al., 1987) and, because they often work in concert with RyRs, there could also be such a role for IP$_3$ receptors.

**Opioid Intracellular Mechanisms**

![Figure 8. Model for opioid modulation of calcium currents. Activation of the MOR initiates cADPr signaling, presumably via G$_{o}$ activation of ADP-ribosyl cyclase/CD38 complex catalyzing the conversion of NAD$^+$ into cADPr. Cyclic-cADPr subsequently leads to activation of RyRs and neuropeptide release. Direct activation of the cADPr-pathway via the MOR could amplify this direct effect in HNS terminals during DSC.](image)

**MOR effects on calcium currents and subsequent release**

In cardiac tissue, changes in [Ca$^{2+}$i] can lead to inactivation of VGCCs (Morad, 1995). Calcium-dependent inactivation of VGCCs in HNS terminals has been demonstrated previously (Branchaw et al., 1997; Wang et al., 1999). Similarly, μ-opioid inhibition of I$_{Ca}$ results in a decreased rate of inactivation of the remaining I$_{Ca}$ during a depolarizing stimulation pulse (Fig. 7D). Presumably, the fraction of I$_{Ca}$ remaining after calcium-dependent inactivation represents currents shielded from the effects of intraterminal Ca$^{2+}$. It is also possible that a Ca$^{2+}$-dependent process (e.g., protein kinase or phosphatase; Ortiz-Miranda et al., 2010) is involved (see model legend in Fig. 8). Although the mechanism underlying this phenomenon is unknown, VGCCs in terminals are unique in other ways. The N-type Ca$^{2+}$ channels in the NH (Lemos and Nowycky, 1989; Wang et al., 1993) have lower single-channel conductance and more rapid inactivation kinetics than those observed for conventional somatic channels (Bean, 1989; Wang et al., 1993). In our model, release of calcium from ryanodine-sensitive stores may lead to calcium-dependent inactivation of VGCCs and a reduced I$_{Ca}$. The remaining I$_{Ca}$ has slower inactivation kinetics, possibly indicating that they are only exhibiting Ca$^{2+}$-independent inactivation (Fig. 7D). This reduction in I$_{Ca}$ would lead to decreased exocytosis (Fig. 7A) of neuropeptides from the terminals.

We cannot say unequivocally that exocytosis is the sole contributor to the effects of MOR modulation of I$_{Ca}$ on release dynamics that we report here. It is possible that our observations include effects on endocytosis or on exo-endocytosis (e.g., increases in “kiss and run”). However, we believe the exocytotic
component is a major contributor to the modulation we observe. Among other evidence, the most compelling to suggest this is the fact that when we blocked endocytosis using Ba^{2+} instead of Ca^{2+} (data not shown), DAMGO still inhibited the elicited capacitance changes.

Conclusions
Diffusible second messenger amplification of MOR signaling via cADPr-mediated release of Ca^{2+} from a ryanodine-sensitive stores seems well suited for regulating OT release during the relatively long period of gestation. It is during this time that endogenous MOR-mediated inhibition of release accumulates OT, but is then interrupted during parturition and subsequent lactation (Russell et al., 1989; Douglas et al., 1995; Russell et al., 1995; Ortiz-Miranda et al., 2003). Interestingly, opioid-receptor-induced Ca^{2+} mobilization has also been observed in both murine 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 circumstances. Given the emerging role of ryanodine-sensitive stores and the cADPr signaling pathway in the CNS, our results represent an important contribution to our understanding of their physiological role in presynaptic terminals during DSC.

References
Medline


