Cloned δ-Opioid Receptors in GH3 Cells Inhibit Spontaneous Ca\(^{2+}\) Oscillations and Prolactin Release Through \(K_{IR}\) Channel Activation

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Piros, Elemer T., Andrew C. Charles, Lei Song, Chris J. Evans, and Tim G. Hales. Cloned δ-opioid receptors in GH3 cells inhibit spontaneous Ca\(^{2+}\) oscillations and prolactin release through \(K_{IR}\) channel activation. J. Neurophysiol. 83: 2691–2698, 2000. Opioid receptors can couple to K\(^{-}\) and Ca\(^{2+}\) channels, adenylyl cyclase, and phosphatidyl inositol turnover. Any of these actions may be important in the regulation of neurotransmitter and hormone release from excitatory cells. GH3 cells exhibit spontaneous oscillations of intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_{i}\)) and prolactin release. Activation of cloned δ-opioid receptors stably expressed in GH3 cells inhibits both spontaneous Ca\(^{2+}\) signaling and basal prolactin release. The objective of this study was to examine a possible role for K\(^{-}\) channels in these processes using the patch-clamp technique, fluorescence imaging, and a sensitive ELISA for prolactin. The selective δ receptor agonist \([\text{D-Pen}^2,\text{D-Pen}^2]\)enkephalin (DPDPE) inhibited [Ca\(^{2+}\)]\(_{i}\), oscillations in GH3 cells expressing both μ and δ receptors (GH3MORDOR cells) but had no effect on control GH3 cells or cells expressing μ receptors alone (GH3MOR cells). The inhibition of [Ca\(^{2+}\)]\(_{i}\), oscillations by DPDPE was unaffected by thapsigargin pretreatment, suggesting that this effect is independent of inositol 1,4,5-triphosphate-sensitive Ca\(^{2+}\) stores. DPDPE caused a concentration-dependent inhibition of prolactin release from GH3MORDOR cells with an IC\(_{50}\) of 4 nM. DPDPE increased inward K\(^{-}\) current recorded from GH3MORDOR cells but had no significant effect on K\(^{-}\) currents recorded from control GH3 cells or GH3MOR cells. The μ receptor agonist morphine also had no effect on currents recorded from control cells but activated inward K\(^{-}\) currents recorded from all three cell lines. The DPDPE-sensitive K\(^{-}\) current was inwardly rectifying and was inhibited by Ba\(^{2+}\) but not TEA. DPDPE had no effect on delayed rectifier-, Ca\(^{2+}\)-, and voltage-activated or A-type K\(^{+}\) currents, recorded from GH3MORDOR cells. Ba\(^{2+}\) attenuated the inhibition of [Ca\(^{2+}\)]\(_{i}\), and prolactin release by DPDPE, whereas TEA had no effect, consistent with an involvement of \(K_{IR}\) channels in these actions of the opioid.

INTRODUCTION

Opioids activate μ, δ, and κ receptors that couple through pertussis-toxin-sensitive G proteins to adenylyl cyclase and ion channels (for review, see Piros et al. 1996a). Opioid receptors can also increase phosphatidylinositol turnover elevating inositol 1,4,5-triphosphate (IP\(_3\)) levels and subsequently releasing intracellular Ca\(^{2+}\) (Harrison et al. 1998). In central neurons, opioid receptor activation inhibits N- and P/Q-type Ca\(^{2+}\) channels and adenylyl cyclase, and activates inwardly rectifying K\(^{+}\) (\(K_{IR}\)) channels (Childers et al. 1992; North and Williams 1985; Rhim and Miller 1994; Williams et al. 1988; Wimpey and Chavkin 1991). Any one of these actions can reduce neuronal excitability and neurotransmitter release (see Boehm and Huck 1997; Miller 1998). Opioid receptors also couple to multiple effectors in peripheral neurons and chromaffin cells (Kleppisch et al. 1992; Moises et al. 1994; Schroeder et al. 1991; Twitchell and Rane 1994). The activation of μ opioid receptors in chromaffin cells inhibits Ca\(^{2+}\) channel activity and increases the activity of Ca\(^{2+}\) and voltage-activated K\(^{+}\) channels (K\(_{Ca,VA}\)) (Twitchell and Rane 1994). This raises the question: why are so many transduction pathways required for opioid receptors to inhibit neurotransmitter and hormone release? One possibility is that the combined regulation of all of the effectors is required to effectively attenuate release. Several reports suggest that this is not the case, instead the regulation of neurotransmitter release by G-protein-coupled receptors can be explained by the involvement of specific effectors. The identity of the relevant effector varies according to the receptor and/or the preparation being studied (Boehm and Huck 1997; Capogna et al. 1993; Cherubini and North 1985; Koyama et al. 1999). An alternative possibility is that specific effectors must be regulated to inhibit release from cells receiving different stimuli. For example, the activation of K\(^{+}\) channels may be sufficient to inhibit spontaneous release, whereas the inhibition of adenylyl cyclase and Ca\(^{2+}\) channels might be necessary to prevent release evoked by specific stimuli.

In this study, we used a combination of electrophysiological, fluorescence imaging, and biochemical techniques to examine the contribution of K\(^{+}\) channels to the opioid-induced inhibition of spontaneous changes in intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_{i}\)) and release. We required a homogeneous population of cells that both expressed opioid receptors and released measurable quantities of a neurotransmitter or hormone. Although there are opioid receptor-expressing cell lines that have been studied extensively using electrophysiological and Ca\(^{2+}\) imaging techniques (Connor and Henderson 1996; Seward et al. 1991) none of these is suitable for the study of spontaneous release. We circumvented this problem by expressing cloned opioid receptors in the prolactin-secreting rat pituitary GH3 cell line (Piros et al. 1995, 1996b) and developed a sensitive enzyme-linked immunosorbent assay (ELISA) for...
measuring secreted prolactin levels (Charles et al. 1999; Piros et al. 1996a). GH3 cells have been extensively studied for several years; they have well characterized K+ and Ca2+ channels and exhibit spontaneous oscillations of intracellular Ca2+ concentration and hormone release (Barros et al. 1992; Bauer et al. 1990, 1994; Charles et al. 1999; Dubinsky and Oxford 1985; Matteson and Armstrong 1986; Oxford and Wagoner 1989). The inhibition of KIR current stimulates the frequency of [Ca2+]i oscillations and leads to an increase in prolactin release (Charles et al. 1999). Conversely, inhibition of L-type Ca2+ channel activity inhibits [Ca2+]i oscillations and prolactin secretion. We stably transfected GH3 cells with μ receptor cDNA to establish GH3 cells stably expressing rat μ receptors (GH3MOR) (Piros et al. 1995). This cell clone was subsequently stably transfected with δ receptor cDNA to establish GH3 cells expressing both rat μ and mouse δ receptors (GH3MORDOR) (Piros et al. 1996b). The activation of μ receptors expressed in either clone or δ receptors expressed by GH3MORDOR cells, leads to the inhibition of adenyl cyclase and L-type Ca2+ channel activity. Either or both of these actions may be involved in the opioid-induced reduction in prolactin release from GH3MORDOR cells.

The goal of this study was to use the whole cell recording technique to examine whether opioid receptors, stably expressed in GH3MOR and GH3MORDOR cells, couple to K+ channels. We also sought to examine the contribution of KIR channels in the δ receptor-mediated inhibition of spontaneous [Ca2+]i oscillations and prolactin release.

**METHODS**

**Cell culture**

GH3 cells, obtained from the American Type Culture Collection, Rockville, MD (CCL 82.1), were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% (vol/vol) calf serum, penicillin (100 IU/ml), and streptomycin (100 μg/ml) and incubated in a humid atmosphere of 5% CO2-95% air, at 37°C. GH3MOR or GH3MORDOR cells were grown under positive selection using genticin (G418) alone or G418 plus hygromycin B, as described previously (Piros et al. 1995, 1996b). Cells were harvested once a week by treatment with a phosphate-buffered saline containing EDTA (3 mM) and reseded at 20% of their original density, either into six-well plates for prolactin release assays, 35-mm-diameter culture dishes for electrophysiological studies, or poly-d-lysine-coated coverslips for Ca2+ imaging studies. The incubation medium was changed every 2-3 days.

**Electrophysiological recordings**

Single GH3, GH3MOR, or GH3MORDOR cells were voltage-clamped and voltage-activated K+ channel activity was recorded using a List EPC-7 patch-clamp amplifier (Cambell, CA). Cells were superfused with a solution containing (in mM) 140 NaCl, 2.8 KCl, 2 MgCl2, 1 CaCl2, 10 HEPS, 6 glucose, and 5 × 10−4 TTX (pH 7.2 with NaOH) when recording outward K+ currents. The recording electrode contained a solution composed of (in mM) 120 KCl, 1 EGTA, 1 MgCl2, 3 Mg-ATP, and 10 HEPS (pH 7.2 with KOH). Currents were activated by step-depolarizations of membrane potential from a holding potential of −80 mV for 100 ms every 10 s. Capacitance compensations were achieved using the patch-clamp amplifier. Residual artifacts and leakage currents were nulled using a P/4 subtraction.

Whole cell KIR current recordings were performed at room temperature (20–22°C) using an extracellular solution containing (in mM): KCl 140, MgCl2 4, CaCl2 1, HEPES 10, glucose 7, TTX 5 × 10−4 (pH 7.2 with KOH). Recording electrodes contained a solution comprised of (in mM): KCl 140, EGTA 10, MgCl2 2, HEPES 10, Mg-ATP 3 (pH 7.2 with KOH). Two protocols were used: Currents were evoked by a step hyperpolarization from the −40 mV holding potential to −90 mV (duration 1.5 s, frequency 0.03 Hz), no leak subtraction was employed. Alternatively, [d-Pen2, d-Pen2]enkephalin (DPDPE), morphine, or somatostatin was bath applied to GH3, GH3MOR, and GH3MORDOR cells clamped at −60 mV. In some cells there was a gradual increase in the amplitude of inward current even in the absence of agonist. To compensate for this effect, a linear extrapolation was used to compare control current amplitude to the current amplitude recorded in the presence of an agonist. The current-voltage relationship of the DPDPE activated KIR current was examined by ramping the potential from −100 to 50 mV (1-s duration). Leak subtraction was achieved by subtracting the control current recorded in response to the ramp depolarization in the absence of DPDPE.

Patch electrodes were manufactured from thin-walled borosilicate glass pipettes (World Precision Instruments, New Haven, CT) using a Flaming/Brown P-87 micropipette puller (Sutter, Novato, CA). Whole cell currents recorded using the EPC-7 amplifier were low-pass filtered with an 8-pole filter at 1 kHz and digitized (Labmaster DMA, Axon Instruments, Burlington, CA) at a frequency of 5 kHz onto the hard drive of a personal computer. Data were analyzed using pClamp software (Axon Instruments).

**Measurement of [Ca2+]i**

[Ca2+]i was measured using a fluorescence imaging system that has previously been described in detail (Charles et al. 1991). Briefly, cells grown on poly-d-lysine-coated glass coverslips were loaded with fura2 by incubation in 5 μM fura2-AM for 40 min. Cells then were washed and maintained in normal medium for 30 min before experimentation. Coverslips were excited with a mercury lamp through 340- and 380-nm band-pass filters, and fluorescence at 510 nm was recorded through a ×10 or ×20 objective with a SIT camera to an optical memory disk recorder. Images then were digitized and subjected to background subtraction and shading correction, after which [Ca2+]i was calculated on a pixel-by-pixel basis, as previously described, by a frame grabber and image analysis board (Data Translation). Dr. Michael Sanderson wrote data-acquisition and analysis software. Tracings in all figures are based on fluorescence of a 4 × 4 pixel area located within each cell body.

Experiments were carried out in Hanks’ balanced salt solution with 10 mM HEPES buffer, pH 7.4 (HBSS/HEPES) at 20–22°C. Agents were applied in HBSS/HEPES by perfusion of the recording chamber the base of which was a coverslip supporting the cells.

**ELISA for measuring prolactin**

The competitive ELISA makes use of an antibody raised in rabbit against rat prolactin. Both antisera (PRL-S-9) and standards (PRL-RP-3) were provided by the National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK). GH3 cells (0.5–0.7 million cells per well) were seeded into six-well tissue-culture plates 48 h before experimentation. Release experiments were conducted at 37°C in a humidified atmosphere of 5% CO2-95% air. Before each experiment, cells were washed gently with media (DMEM with 20 mM HEPES and 0.1% BSA, pH 7.4 with NaOH). After washing, aliquots of media (1 ml) were added to each well for 0.5 h at a time so that release could be monitored before, during, and after exposure to drugs. Prolactin (ng/ml/105 cells) released in 0.5 h in the presence of drugs was expressed as a percentage of release from the same cells during 0.5 h under control conditions. After incubation with the cells, each media aliquot was centrifuged at 1000 g at 4°C to pellet cells the supernatant was stored at −20°C or assayed directly to determine the prolactin concentration. Each well of a 96-well Nunc-ImmuNo Maxisorp Plate...
(Life Sciences, Denver, CO) was coated with prolactin by incubation of 100 μl of 0.1 M NaHCO₃, pH 9.5, containing 1 ng prolactin for 20–24 h at 4°C. Before the assay, prolactin-coated plates were washed with assay buffer (AB) containing 0.5 M NaCl, 20 mM NaH₂PO₄, 0.05% Tween20, 0.5% BSA, pH to 7.4, and then incubated with AB for 0.5 h at room temperature to remove prolactin bound weakly to the plate. After further washing with AB, undiluted samples (100 μl) or prolactin standards (0.02–40 ng) dissolved in 100 μl media were added to the wells, followed by the addition of 50 μl prolactin antibody at a dilution of 1:40,000. After incubation for 2 h at room temperature, bound antibody was detected using peroxidase-conjugated anti-rabbit antibody (Vector, Burlingame, CA) with tetramethylbenzidine (GIBCO/BRL, Gaithersburg, MD) as substrate. H₂SO₄ (1 N) terminated the peroxidase reaction and a microplate reader (Molecular Devices) measured absorbance at 450 nm. All samples were assayed in triplicate from three separate determinations.

Data analysis

Data are expressed as means ± SE. Statistical significance was established using Student’s t-test. Comparisons between multiple data sets were made using ANOVA followed by the post hoc application of Student’s t-test. Data points in the graph of DPDPE concentration versus prolactin release were fitted using the logistics equation

\[ P = P_{max} - E_{max}[1 + (IC_{50}/x)^n] \]

where \( P \) represents the prolactin concentration, \( P_{max} \) is the maximum prolactin concentration, \( E_{max} \) is the maximum opioid inhibited prolactin concentration (all 3 expressed as percentage of control), IC₅₀ is the concentration of opioid required to achieve the half-maximal effect, \( x \) is the opioid concentration, and \( n \) is the slope.

Drugs and reagents

Pertussis toxin (Sigma, MO) was added to the culture media at 200 ng/ml and cells were incubated for 24 h before performing the prolactin assay. Parallel control experiments were performed on the same batch of cells. All tissue culture reagents, including geneticin (G418) and hygromycin, were obtained from GIBCO/BRL. Nimodipine (a gift from Miles Pharmaceuticals) was diluted fresh each day from a 10 mM stock solution in ethanol. K⁺ channel inhibitors 4-AP, apamin, charybdoxin, and iberiotoxin from RBI (Natick, MA) were put into solution on the day of the experiment. [d-Pen², d-Pen⁵]enkephalin (DPDPE), [d-Ala²,N-MePhe⁴,Gly-ol⁵]enkephalin (DAMGO), and somatostatin were obtained from Peninsula Laboratories (Belmont, CA). Morphine sulfate was a gift from NIDA Drug Supply Program. All other reagents were obtained from Sigma Chemical (St. Louis, MO). Agonists were diluted from frozen stocks on the day of experimentation.

RESULTS

Opioid receptor activation inhibits spontaneous prolactin release

Previously we have determined that prolactin release from GH₃MOR and GH₃MORDOR cells but not GH₃ cells can be modulated by opioid ligands (Piros et al. 1996a). In this study, we examined the cellular mechanisms by which δ-opioid receptors inhibit prolactin release from GH₃MORDOR cells. The δ receptor selective agonist DPDPE (0.1 nM to 1 μM), inhibited prolactin release from GH₃MORDOR cells with an IC₅₀ of 3.8 nM (Fig. 1). The inhibition of prolactin release by DPDPE (100 nM) was attenuated by pertussis toxin pretreatment (200 ng/ml, for 24 h), demonstrating the involvement of inhibitory (Gi and/or Go) G proteins (Fig. 1).

We previously have observed that the activation of μ or δ receptors inhibits Ca²⁺ channel and adenyl cyclase activity recorded from GH₃MORDOR cells. Both of these pertussis-toxin-sensitive effects could be involved in the opioid-induced inhibition of prolactin release (Piros et al. 1996b). K⁺ channel activation is thought to be important for the inhibitory actions of opioids on neurotransmitter release from peripheral neurons (Cherubini and North 1985). However, μ-opioid-induced presynaptic inhibitory action of GABA release from hippocampal neurons appears to be independent of K⁺ channel activity (Capogna et al. 1993). We previously demonstrated that in GH₃ cells blockade of Kᵣ channels, and Kᵥ(Ca,V) channels increased the frequency and amplitude of [Ca²⁺] oscillations, respectively. Only the former caused enhanced prolactin release (Charles et al. 1999).

δ receptor activation inhibits spontaneous [Ca²⁺], oscillations

Bath application of DPDPE (10 nM) reversibly abolished spontaneous Ca²⁺ oscillations and decreased baseline [Ca²⁺], in the majority of GH₃MORDOR cells (122/189 cells in 6 experiments, Fig. 2). In some cells, it reduced the frequency of Ca²⁺ oscillations without abolishing them altogether (42/189 cells), whereas in a smaller percentage of cells (25/189), it had no effect. DPDPE (10–100 nM) had no effect on spontaneous Ca²⁺ oscillations in untransfected GH₃ cells (n = 3 coverslips) or GH₃MOR cells expressing μ receptors alone (n = 3 coverslips). The inhibitory effect of DPDPE on GH₃MORDOR cells was blocked by pretreatment of cells with pertussis toxin (200 ng/ml, for 24 h, n = 100 cells in 3 experiments, data not shown) as well as by the opioid receptor antagonist naloxone (1 μM, n = 90 cells in 3 experiments, data not shown). The ability of DPDPE to inhibit [Ca²⁺], oscillations was unaffected by pretreatment with 10 μM thapsigargin (n = 60 cells in 3 experiments, Fig. 2B), a compound that dumps Ca²⁺ from
IP$_3$-sensitive intracellular stores, indicating that the effects of DPDPE are not mediated through modulation of Ca$^{2+}$ release. Ca$^{2+}$ oscillations in GH$_3$ cells involve the activity of K$^+$ and L-type Ca$^{2+}$ channels (Charles et al. 1999). Regulation of either K$^+$ or L-type Ca$^{2+}$ channels by $\delta$ receptors therefore could lead to the observed changes in [Ca$^{2+}$], and subsequent reduction in prolactin release. We have shown previously that Ba$^{2+}$ and TEA inhibit K$^+$ currents activated in GH$_3$ cells in response to depolarizing pulses from $-80$ to $20$ mV. Bath application of TEA preferentially inhibited a sustained K$^+$ current component that is likely to be mediated by delayed rectifying K$^+$ channels (Charles et al. 1999). By contrast, 5 mM 4-aminopyridine (4-AP) reduced a fast activating current leaving a slowly rising component (Fig. 3A) reminiscent of current through the delayed rectifier (Rudy 1988). By subtracting the current in the presence of 4-AP from the control recording, the amplitude of the A current component recorded from GH$_3$ cells was observed. The 4-AP-sensitive current constituted 56 $\pm$ 4% ($n = 4$) of the peak K$^+$ current activated by depolarizing from $-80$ to $20$ mV (Fig. 3B). The inhibition of transient K$^+$ current induced by 4-AP was measured by comparing the mean current amplitudes averaged between 5 and 10 ms after depolarizing to $20$ mV in the presence and absence of the drug. Apamin (100 nM) and iberiotoxin (100 nM), inhibitors of small (Blatz and Magelby 1986) and large (Giacciomano et al. 1992) conductance Ca$^{2+}$ channels, had no effect on outward K$^+$ currents recorded from GH$_3$ MORDOR cells depolarized from $-80$ to $20$ mV ($n = 10$).

![Fig. 3. Outward K$^+$ currents recorded from GH$_3$ MORDOR cells are insensitive to DPDPE. A: 4-aminopyridine (4-AP; 5 mM) inhibits a transient outward K$^+$ current component activated by depolarizing GH$_3$ MORDOR cells from $-80$ to $20$ mV. Top: superimposed currents recorded in the absence and presence of 4-AP (5 mM). Bottom: subtraction of the current recorded in the presence of the drug from the control current reveals the transient 4-AP-sensitive A current. Traces are averages of 2 currents recorded from the same cell in the presence and absence of 4-AP. B: DPDPE (1 $\mu$M) had no effect on outward K$^+$ currents recorded from GH$_3$ MORDOR cells depolarized from $-80$ to $20$ mV ($n = 10$).](http://jn.physiology.org/content/110/11/2694/F3)

**Regulation of K$^+$ channel activity in GH$_3$MOR and GH$_3$MORDOR cells by opioids**

Depolarization of GH$_3$MORDOR cells from $-80$ to $20$ mV activates outward voltage-activated currents (Fig. 3). Such currents were mediated by the outward flow of K$^+$, and they were abolished when CsCl replaced KCl in the electrode solution and were inhibited by K$^+$ channel antagonists (e.g., Fig. 3A). Under similar recording conditions, several studies have provided evidence for the presence of K$^+$ channels that mediate delayed rectifier-, Ca$^{2+}$-, and voltage-activated- and A current in GH$_3$ cells (Dubinsky and Oxford 1985; Oxford and Wagener 1989; Ritchie 1987; Simasko 1991). We have demonstrated previously that Ba$^{2+}$ and TEA inhibit K$^+$ currents activated in GH$_3$ cells in response to depolarizing pulses from $-80$ to $20$ mV. Bath application of TEA preferentially inhibited a sustained K$^+$ current component that is likely to be mediated by delayed rectifying K$^+$ channels (Charles et al. 1999). By contrast, 5 mM 4-aminopyridine (4-AP) reduced a fast activating current leaving a slowly rising component (Fig. 3A) reminiscent of current through the delayed rectifier (Rudy 1988). By subtracting the current in the presence of 4-AP from the control recording, the amplitude of the A current component recorded from GH$_3$ cells was observed. The 4-AP-sensitive current constituted 56 $\pm$ 4% ($n = 4$) of the peak K$^+$ current activated by depolarizing from $-80$ to $20$ mV (Fig. 3B). The inhibition of transient K$^+$ current induced by 4-AP was measured by comparing the mean current amplitudes averaged between 5 and 10 ms after depolarizing to $20$ mV in the presence and absence of the drug. Apamin (100 nM) and iberiotoxin (100 nM), inhibitors of small (Blatz and Magelby 1986) and large (Giacciomano et al. 1992) conductance Ca$^{2+}$ channels, had no effect on outward K$^+$ currents recorded from GH$_3$ MORDOR cells depolarized from $-80$ to $20$ mV ($n = 10$).
activated K+ channels, caused significant parallel inhibitions of the outward K+ current amplitude of 2.7 ± 0.6 and 21 ± 6% (n = 4). The effects of these agents were averaged over 10 ms at the end of each depolarizing step and were determined to be significant using the paired t-test when compared with the current in the absence of drug application (P < 0.05). The effects of the K+ channel inhibitors reversed during washout.

Taken together with our previous observations (Charles et al. 1999), these data demonstrate that outward currents recorded from GH3 cells are mediated by several classes of K+ channels. DPDPE (1 μM) had no discernible effect on outward K+ currents (n = 10), suggesting that activation of δ receptors does not affect K+ channels mediating delayed rectifier, Kir or A current recorded from GH3MORDOR cells (Fig. 3B).

An inwardly rectifying K+ current component can also be recorded from GH3 cells under appropriate conditions (Bauer et al. 1990; Charles et al. 1999). Hyperpolarizing GH3 cells from -40 to -90 mV with equal K+ concentrations on either side of the cell membrane reveals inward currents. Such currents are inhibited by Cs+ (1 mM) and Ba2+ (1 mM) but are insensitive to 1 mM TEA (Charles et al. 1999). The bath application of DPDPE (1 μM) increased the amplitude of currents (by 31 ± 4%, n = 6) observed by hyperpolarizing GH3MORDOR cells from -40 to -90 mV, suggesting that opioid receptors couple to Kir channels (Fig. 4A). This suggestion is supported by the demonstration that currents recorded in the presence of DPDPE are inhibited by bath application of 1 mM Ba2+ (Fig. 4B). To test whether the DPDPE-induced current was truly inwardly rectifying, we examined the relationship between current amplitude and holding potential using voltage ramps from -100 to 50 mV (Fig. 4C). Currents generated by the voltage ramp under control conditions were subtracted from those observed in the presence of DPDPE. In an attempt to restrict the actions of the opioid to K+ channel activation, we performed experiments in the presence of the dihydropyridine nimodipine (100 μM) at a sufficient concentration to abolish Ca2+ channel activity in GH3 cells (Piros et al. 1995). Subtraction of ramp currents recorded in the presence of nimodipine alone from those recorded with both nimodipine and DPDPE (100 nM) revealed inwardly rectifying currents (n = 5) that reversed in sign at the K+ equilibrium potential of 0 mV (Fig. 4C).

To confirm that DPDPE was selectively activating δ receptors in GH3MORDOR cells, we tested the sensitivity of control GH3 cells and GH3MOR cells expressing μ receptors alone. Cells were held at -60 mV while DPDPE, somatostatin (an agonist of somatostatin receptors native to GH3 cells), or morphine (a μ receptor agonist) were bath applied (Fig. 5). Clearly discernible inward currents (87 ± 11 pA, n = 9) recorded from control GH3 cells developed in the presence of somatostatin (1 μM). No increase in the amplitudes of inward currents were observed on application of morphine (1 μM) or DPDPE (100 nM) to control GH3 cells (n = 5 and 8, respectively). By contrast, DPDPE (100 nM) activated robust currents (168 ± 23 pA, n = 16) recorded from GH3MORDOR cells (Fig. 5) but had no significant effect on currents recorded from GH3MOR cells (n = 11, Fig. 5). Both somatostatin (1 μM) and morphine (1 μM) activated currents recorded from GH3MOR cells (169 ± 25 pA, n = 13 and 197 ± 46 pA, n =
DPDPE inhibits $[\text{Ca}^{2+}]_{\text{i}}$ oscillations and prolactin release by activating $K_{\text{IR}}$ channels

We exploited the selective $K^+$ channel blocking actions of extracellular $\text{Ba}^{2+}$ and TEA to examine whether DPDPE induces inhibition of $[\text{Ca}^{2+}]_{\text{i}}$ oscillations and prolactin release through activation of $K_{\text{IR}}$ current. The inhibition of $[\text{Ca}^{2+}]_{\text{i}}$ oscillations by DPDPE (10–100 nM) was attenuated by the preapplication of 1 mM $\text{Ba}^{2+}$ ($n = 60$ cells in 3 experiments, Fig. 6A) but was unaffected by 1 mM TEA ($n = 75$ cells in 3 experiments, Fig. 6B). Likewise, the DPDPE (1 $\mu$M)-evoked inhibition of prolactin release was inhibited by coapplication with $\text{Ba}^{2+}$ (1 mM) or $\text{Cs}^{+}$ (1 mM), another ion capable of inhibiting $K_{\text{IR}}$ channel activity (Fig. 6C). The increase in prolactin release in the presence of $\text{Ba}^{2+}$ and DPDPE was not significantly different from that observed when $\text{Ba}^{2+}$ was applied alone to GH$_3$ cells (Charles et al. 1999), confirming that DPDPE has no discernible effect on prolactin release when $K_{\text{IR}}$ channels are inhibited (Fig. 6C). There was also no significant difference between the change in prolactin release when $\text{Cs}^{+}$ (1 mM) was applied alone to GH$_3$ cells (Charles et al. 1999) or coapplied with DPDPE (1 $\mu$M; Fig. 6C). TEA had no significant effect on the inhibition of prolactin release by DPDPE. DPDPE (1 $\mu$M) inhibited prolactin levels by 29 $\pm$ 7% ($n = 4$) and 39 $\pm$ 4% ($n = 11$) in the presence and absence of TEA (1 mM). TEA (1 mM) applied alone to GH$_3$ cells had no effect on prolactin release (Charles et al. 1999).

Taken together, these data suggest that activation of $K_{\text{IR}}$ channels by DPDPE reduces spontaneous $[\text{Ca}^{2+}]_{\text{i}}$ oscillations and prolactin release.

**DISCUSSION**

We previously established excitable, prolactin-secreting anterior pituitary-derived GH$_3$ cell lines stably expressing either $\mu$ receptors alone (GH$_3$MOR cells) or $\mu$ and $\delta$ receptors (GH$_3$MORDOR cells) together (Piros et al. 1995, 1996b). Here we demonstrate that opioid receptors in GH$_3$MORDOR cells activate inwardly rectifying $K^+$ channels leading to a reduction in spontaneous $[\text{Ca}^{2+}]_{\text{i}}$ oscillations and prolactin release.

![Figure 5](http://jn.physiology.org/)

**FIG. 5.** DPDPE activates inward rectifier $K^+$ channels through selective activation of $\delta$ receptors in GH$_3$MORDOR cells. Bar graph of the average amplitude of inward currents activated by DPDPE (100 nM), morphine (1 $\mu$M), and somatostatin (1 $\mu$M) when applied to GH$_3$ cells expressing $\mu$ receptors alone (GH$_3$MOR) and GH$_3$MORDOR cells. Data were recorded from $\geq 6$ cells. Error bars represent $\pm$ SE.

![Figure 6](http://jn.physiology.org/)

**FIG. 6.** Blockade of $K_{\text{IR}}$ channels prevents the DPDPE-induced reduction of both $[\text{Ca}^{2+}]_{\text{i}}$, and prolactin release. $A$: $\text{Ba}^{2+}$ (1 mM) caused an increase in the frequency of $[\text{Ca}^{2+}]_{\text{i}}$ oscillations and blocked the inhibitory action of DPDPE (10 nM). $B$: TEA (1 mM) caused an increase in the amplitude of $[\text{Ca}^{2+}]_{\text{i}}$ oscillations. DPDPE (10 nM) abolished $[\text{Ca}^{2+}]_{\text{i}}$ oscillations in the presence of TEA. Bottom: raster plots of $[\text{Ca}^{2+}]_{\text{i}}$, vs. time in a field of 20 different GH$_3$MORDOR cells. Each row represents an individual cell and $[\text{Ca}^{2+}]_{\text{i}}$ is represented as changes in gray-scale as shown on the calibration bar on the right. Top: line tracings of $[\text{Ca}^{2+}]_{\text{i}}$, in an exemplar individual cell. Cells in the field exhibited heterogeneous patterns of spontaneous $[\text{Ca}^{2+}]_{\text{i}}$ oscillations. $C$: bar graph illustrating the regulation of prolactin (PRL) release by DPDPE (1 $\mu$M) applied alone or in the presence of $\text{Ba}^{2+}$ (1 mM), $\text{Cs}^{+}$ (1 mM), or TEA (1 mM). The $K_{\text{IR}}$ channel inhibitors prevented the inhibition of prolactin release by DPDPE. By contrast, DPDPE was able to reduce prolactin release in the presence of TEA. Error bars are $\pm$ SE. Each experiment was performed $\geq 4$ times. Data from Charles et al. (1999) are shown here for the purpose of comparison (†).
GH3 cells were chosen as the system for expression of cloned opioid receptors as they contain a variety of well-characterized G proteins and several effectors regulated by native somatostatin and muscarinic receptors (Hescheler and Schultz 1993). Unlike unexcitable cell lines more commonly used to study cloned opioid receptors, GH3 cells also express a range of voltage-activated ion channels as well as adenyl cyclase. Furthermore these cells exhibit spontaneous oscillations of [Ca\(^{2+}\)] and prolactin release. Therefore opioid receptor properties, from ligand binding to regulation of hormone release, can be studied using this single clonal cell system (Piros et al. 1996a).

The activation of either \( \mu \) or \( \delta \) receptors expressed in GH3 cells leads to the inhibition of the activities of adenyl cyclase and dihydropyridine-sensitive Ca\(^{2+}\) channels (Piros et al. 1995, 1996b). Reductions in the intracellular concentration of cAMP and Ca\(^{2+}\) entry may contribute to the opioid-induced reduction in prolactin release. Indeed the inhibition of Ca\(^{2+}\) channel activity by nimodipine caused both a reduction in spontaneous [Ca\(^{2+}\)], oscillations and prolactin release, suggesting that the entry of Ca\(^{2+}\) through L-type channels plays an important part in these processes in GH3 cells (Charles et al. 1999).

In the present study DPDPE caused a marked reduction in spontaneous [Ca\(^{2+}\)], oscillations in GH3MORDOR cells but not GH3MOR or control GH3 cells, demonstrating that this effect required \( \delta \) receptor activation. We examined the possibility that \( \delta \) receptors could be regulating [Ca\(^{2+}\)], through modulation of IP\(_3\)-sensitive intracellular Ca\(^{2+}\) stores, a phenomenon that has been observed in NG108–15 cells (Jin et al. 1994). This pathway does not appear to be important in the actions of \( \delta \) receptors in GH3MORDOR cells because the DPDPE-induced reduction in [Ca\(^{2+}\)], oscillations persisted after application of thapsigargin. The application of thapsigargin had little effect on spontaneous Ca\(^{2+}\) oscillations, suggesting that IP\(_3\)-sensitive intracellular Ca\(^{2+}\) stores play only a minor role in the control of this process in GH3 cells (Charles et al. 1999).

The activation of \( \delta \) receptors increased the amplitude of a Ba\(^{2+}\)-sensitive inward rectifying K\(^+\) current recorded from GH3MORDOR cells. Consistent with the idea that this effect is mediated specifically by \( \delta \) receptors, DPDPE did not activate currents in GH3 or GH3MOR cells. GH3 cells express endogenous somatostatin receptors that are known to couple to K\(_{IR}\) channels. Somatostatin activated inward currents in all three cell lines, whereas the \( \mu \) receptor agonists morphine and DAMGO activated resolvable currents only in GH3MOR and GH3MORDOR cells. This action of the opioids appears to be specific to K\(_{IR}\) channels as DPDPE did not regulate outward K\(^+\) currents mediated by TEA-, 4-AP-, apamin-, charybdotoxin-, or iberiotoxin-sensitive channels. TEA, Ba\(^{2+}\), and Cs\(^{+}\) have been used previously to examine the roles of K\(_{Ca\(V\)}\) and K\(_{IR}\) channels in spontaneous [Ca\(^{2+}\)], oscillations and prolactin release from GH3 cells (Charles et al. 1999). TEA (1 mM) selectively inhibits outward K\(^{+}\) current, Cs\(^{+}\) (1 mM) selectively inhibits inward currents, whereas Ba\(^{2+}\) (1 mM) inhibits both K\(^{+}\) current components. The application of Ba\(^{2+}\) but not TEA increased the frequency of Ca\(^{2+}\) oscillations, an action that is accompanied by an increase in prolactin release (Charles et al. 1999). In the present study, we demonstrated that, consistent with an effect primarily involving K\(_{IR}\), the DPDPE-mediated inhibitions of Ca\(^{2+}\) signaling and prolactin release were attenuated by Ba\(^{2+}\) but not by TEA.

Charles, A. C., Piros, E. T., Evans, C. J., and Hales, T. G. L-type Ca"\textsuperscript{2+}\" channels and K"\textsuperscript{+}\ channels specifically modulate the frequency and amplitude of spontaneous Ca"\textsuperscript{2+}\ oscillations and have distinct roles in prolactin release in GH\textsubscript{3} cells. *J. Biol. Chem.* 274: 7508–7515, 1999.


