Cloned δ-Opioid Receptors in GH3 Cells Inhibit Spontaneous Ca2+ Oscillations and Prolactin Release Through KIR Channel Activation

ELEMER T. PIROS,1 ANDREW C. CHARLES,2 LEI SONG,3 CHRIS J. EVANS,4 AND TIM G. HALES3
1Department of Physiology, Cornell University, New York, New York 10021; 2Department of Neurology, University of California Los Angeles School of Medicine, Los Angeles, California 90095; 3Department of Pharmacology, The George Washington University, Washington, DC 20037; and 4Department of Psychiatry, University of California Los Angeles School of Medicine, Los Angeles, California 90095

PIROS, Elemer T., Andrew C. Charles, Lei Song, Chris J. Evans, and Tim G. Hales. Cloned δ-opioid receptors in GH3 cells inhibit spontaneous Ca2+ oscillations and prolactin release through KIR channel activation. J. Neurophysiol. 83: 2691–2698, 2000. Opioid receptors can couple to K+ and Ca2+ channels, adenylyl cyclase, and phosphatidyl inositol turnover. Any of these actions may be important in the regulation of neurotransmitter and hormone release from excitable cells. GH3 cells exhibit spontaneous oscillations of intracellular Ca2+ concentration ([Ca2+]i) and prolactin release. Activation of cloned δ-opioid receptors stably expressed in GH3 cells inhibits both spontaneous Ca2+ 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 biochemical techniques to examine the contribution of KIR 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 phosphatidyl inositol turnover elevating inositol 1,4,5-trisphosphate (IP3) levels and subsequently releasing intracellular Ca2+ (Harrison et al. 1998). In central neurons, opioid receptor activation inhibits N- and P/Q-type Ca2+ channels and adenylyl cyclase, and activates inwardly rectifying K+ (KIR) 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 Ca2+ channel activity and increases the activity of Ca2+ and voltage-activated K+ channels (KCaVA) (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 Ca2+ 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 Ca2+ concentration ([Ca2+]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 Ca2+ 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 humidified atmosphere of 5% CO2-95% air, at 37°C. GH3MOR or GH3MORDOR cells were grown under positive selection using geneticin (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 reseeded 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 HEPES, 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 HEPES (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-Pen1, 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 antiserum (PRL-S-9) and standards (PRL-100, 300, 1000, 3000, 10,000, 30,000, 100,000 units) 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/106 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
lactin concentration (all 3 expressed as percentage of control), IC\textsubscript{50} is the opioid concentration required to achieve the half-maximal effect. The concentration of opioid required to achieve the half-maximal effect, \( IC_{50} \), was calculated using the equation (see METHODS), yielding an IC\textsubscript{50} value for DPDPE of 3.8 nM.

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

where \( P \) represents the prolactin concentration, \( P_{\text{max}} \) is the maximum prolactin concentration, \( E_{\text{max}} \) is the maximum opioid inhibited prolactin concentration (all 3 expressed as percentage of control), IC\textsubscript{50} 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 genetin (G418) and hygromycin, were obtained from GIBCO/BRL. Nimbodipine (a gift from Miles Pharmaceuticals) was diluted fresh each day from a 10 mM stock solution in ethanol. \( K^+ \) channel inhibitors 4-AP, apamin, charybdotoxin, and iberiotoxin from RBI (Natick, MA) were added to the wells, followed by the addition of 50 \( \mu \)l 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. \( H_2SO_4 \) (1 N) terminated the peroxidase reaction and a microplate reader (Molecular Devices) measured absorbance at 450 nm. All samples were assayed in quadruplicate 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

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

receptors inhibits \( Ca^{2+} \) channel and adenylyl cyclase activity recorded from GH\textsubscript{3}MORDOR 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, \( \mu \)-opioid-induced presynaptic inhibition of GABA release from hippocampal neurons appears to be independent of \( K^+ \) channel activity (Capogna et al. 1993). We previously demonstrated that in GH\textsubscript{3} cells blockade of \( K_R \) channels, and \( K_{(Ca,V)} \) channels increased the frequency and amplitude of \( [Ca^{2+}]_i \) oscillations, respectively. Only the former caused enhanced prolactin release (Charles et al. 1999).

\[ \delta \text{ receptor activation inhibits spontaneous } [Ca^{2+}]_i \text{, oscillations} \]

Bath application of DPDPE (10 nM) reversibly abolished spontaneous \( Ca^{2+} \) oscillations and decreased baseline \( [Ca^{2+}]_i \) in the majority of GH\textsubscript{3}MORDOR cells (122/189 cells in 6 experiments, Fig. 2). In some cells, it reduced the frequency of \( Ca^{2+} \) 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 \( Ca^{2+} \) oscillations in untransfected GH\textsubscript{3} cells (n = 3 coverslips) or GH\textsubscript{3}MOR cells expressing \( \mu \) receptors alone (n = 3 coverslips). The inhibitory effect of DPDPE on GH\textsubscript{3}MORDOR 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 \( \mu \)M, n = 90 cells in 3 experiments, data not shown). The ability of DPDPE to inhibit \( [Ca^{2+}]_i \), oscillations was unaffected by pretreatment with 10 \( \mu \)M thapsigargin (n = 60 cells in 3 experiments, Fig. 2B), a compound that dumps \( Ca^{2+} \) from
IP$_3$-sensitive intracellular stores, indicating that the effects of DPDPE are not mediated through modulation of Ca$^{2+}$ release. [Ca$^{2+}$]$_i$ oscillations in GH$_3$ cells involve the activity of K$_1$ and L-type Ca$^{2+}$ channels (Charles et al. 1999). Regulation of either K$_1$ or L-type Ca$^{2+}$ channels by δ receptors therefore could lead to the observed changes in [Ca$^{2+}$]$_i$ 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 ± 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 (Giangiacomo et al. 1992) conductance Ca$^{2+}$-

![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 μM) 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, K(Ca,Vr), 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 = 12, respectively). The more selective μ receptor agonist DAMGO (1 μM) also activated inward currents recorded from GH3MOR and GH3MORDOR cells (data not shown).
DPDPE inhibits \([Ca^{2+}]_i\) oscillations and prolactin release by activating \(K_{IR}\) channels

We exploited the selective \(K^+\) channel blocking actions of extracellular \(Ba^{2+}\) and TEA to examine whether DPDPE induces inhibition of \([Ca^{2+}]_i\) oscillations and prolactin release through activation of \(K_{IR}\) current. The inhibition of \([Ca^{2+}]_i\) oscillations by DPDPE \((10-100\ nM)\) was attenuated by the preapplication of \(1\ mM\ Ba^{2+}\) \((n = 60\ cells\ in\ 3\ experiments,\ Fig. 6A)\) 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 \(Ba^{2+}\) \((1\ mM)\) or \(Cs^+\) \((1\ mM)\), another ion capable of inhibiting \(K_{IR}\) channel activity \((Fig. 6C)\). The increase in prolactin release in the presence of \(Ba^{2+}\) and DPDPE was not significantly different from that observed when \(Ba^{2+}\) was applied alone to GH3 cells \((Charles\ et\ al.\ 1999)\), confirming that DPDPE has no discernible effect on prolactin release when \(K_{IR}\) channels are inhibited \((Fig. 6C)\). There was also no significant difference between the change in prolactin release when \(Cs^+\) \((1\ mM)\) was applied alone to GH3 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 GH3 cells had no effect on prolactin release \((Charles\ et\ al.\ 1999)\).

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

**DISCUSSION**

We previously established excitable, prolactin-secreting anterior pituitary-derived GH3 cells stably expressing either \(\mu\) receptors alone \((GH3_MOR)\) or \(\mu\) and \(\delta\) receptors \((GH3_MORDOR)\) together \((Piros\ et\ al.\ 1995,\ 1996b)\). Here we demonstrate that opioid receptors in GH3_MORDOR cells activate inwardly rectifying \(K^+\) channels leading to a reduction in spontaneous \([Ca^{2+}]_i\) oscillations and prolactin release.
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\(^{2+}\)]\(_i\), 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\(^{2+}\)]\(_i\) 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\(^{2+}\)]\(_i\), oscillations in GH3MORMOR 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\(^{2+}\)]\(_i\) 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 GH3MORMOR cells because the DPDPE-induced reduction in [Ca\(^{2+}\)]\(_i\), 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 GH3MORMOR 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\(^+\) 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\(_R\) 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\(_R\) channels in spontaneous [Ca\(^{2+}\)]\(_i\), oscillations and prolactin release from GH3 cells (Charles et al. 1999). TEA (1 mM) selectively inhibits outward K\(^+\) current, Ca\(^{2+}\) (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\(_R\), the DPDPE-mediated inhibitions of Ca\(^{2+}\) signaling and prolactin release were attenuated by Ba\(^{2+}\) but not by TEA.

Opioid receptors are known to couple to K\(_R\) channels in a variety of neurons (Grudt and Williams 1993; North and Williams 1985; Wimpey and Chavkin 1991), an effect that can be mimicked by expressing opioid receptors with G-protein-coupled inwardly rectifying K\(^+\) channels (GIRK) in Xenopus oocytes (Chen and Yu 1994; Henry et al. 1995; Ikeda et al. 1995; Ma et al. 1995). This action appears to be mediated by activation of inhibitory G proteins liberating \(\beta\gamma\) subunits that bind directly to an amino acid motif found on GIRK channels and several other effectors regulated by G-protein-coupled receptors (Ford et al. 1998). The molecular identity of the G-protein-regulated K\(_R\) channel in GH3 cells remains unknown (Falk et al. 1995). Early reports implicate G-protein \(\alpha\) subunits in the activation of K\(_R\) channels in GH3 cells (Codina et al. 1987; Yatani et al. 1988). Whether G-protein \(\alpha\) subunits are involved in the regulation of K\(_R\) channels by recombinant opioid receptors expressed in GH3 cells remains to be examined.

In summary, taken together the results of this study indicate that on activation cloned opioid receptors stimulate K\(_R\) channels native to GH3 cells preventing depolarization and the influx of Ca\(^{2+}\) through voltage-activated Ca\(^{2+}\) channels thus reducing spontaneous Ca\(^{2+}\) oscillations and prolactin release. Our findings with this model system may be more widely applicable to other neuronal and endocrine cells at times of tonic neurotransmitter or hormone release when opioid-receptor-mediated activation of K\(_R\) channels can prevent spontaneous depolarization. We speculate that the other actions mediated by opioid receptors in GH3MOR and GH3MORDOR cells, inhibition of L-type Ca\(^{2+}\) channel activity and adenyl cyclase (Piros et al. 1995, 1996b), may play a role when hormone release is stimulated by exogenous signals causing depolarization and increased intracellular cAMP levels, respectively. Multiple effector mechanisms may enable opioid receptor agonists to inhibit either basal or evoked Ca\(^{2+}\) signaling and hormone release.

This work was supported by National Institutes of Health Grants NS-32283 and NS-02808 (A. C. Charles), DA-05010 (T. G. Hales and C. J. Evans), and DA-05627 (E. T. Piros).

Address for reprint requests: T. G. Hales, Dept. of Pharmacology, The George Washington University, 2300 Eye St, NW, Washington, DC 20037.

Received 3 December 1999; accepted in final form 7 February 2000.

REFERENCES


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


