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Voltage-Dependent Inhibition of Ca^{2+} Channels in GH_3 Cells by Cloned μ- and δ-Opioid Receptors

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SUMMARY

To study cloned opioid receptor binding and modulation of both adenylyl cyclase and ion channel activity, we stably expressed μ- and δ-opioid receptors in the rodent pituitary-derived GH_3 cell line. GH_3 cells express G proteins and voltage-activated Ca^{2+} channels (predominantly of the L-type). Activation of cloned rat μ-opioid receptors expressed in GH_3 cells (termed GH_3MOR cells) inhibits L-type Ca^{2+} channel activity. GH_3MOR cells, further transfected with mouse δ receptor cDNA (termed GH_3MORDOR cells), bound both [D-Ala^2,*N*-MePhe^4,Gly-ol^5]enkephalin (DAMGO) and [D-Pen^2,D-Pen^5]enkephalin (DPDPE). These opioid ligands inhibited adenylyl cyclase activity (IC_{50} = 174 and 0.53 nM, respectively). This action of DAMGO and DPDPE was attenuated selectively by μ- and δ-opioid receptor-specific antagonists. Activation of both opioid receptors also led to inhibition of Ca^{2+} channel activity, measured with Ba^{2+} as the charge carrier using the whole-cell patch-clamp technique. Both DAMGO (1 μM) and DPDPE (1 μM) reversibly inhibited Ba^{2+} currents (by 17.0 ± 1.4% and 20.7 ± 1.3%, respectively) in GH_3MORDOR cells. The inhibitory action of DPDPE was dose dependent (IC_{50} = 1.6 nM) and was attenuated by pretreatment with pertussis toxin (200 ng/ml) or by the inclusion of guanosine-5′-*O*-(2-thio)diphosphate (2 mM) in the recording electrode. Ba^{2+} current inhibitions by both DAMGO and DPDPE were completely reversed by depolarizing (to >50 mV) prepulses in GH_3MORDOR cells. In summary, cloned μ- and δ-opioid receptors expressed in GH_3 cells voltage-dependently couple through G_i/G_o proteins to L-type Ca^{2+} channels.

Since their cloning, μ-, δ-, and κ-opioid receptors have been expressed and characterized in various unexcitable cell lines lacking endogenous receptors. This approach enabled studies of receptor binding and coupling to adenylyl cyclase and phosphatidyl inositol turnover (for reviews, see Refs. 1–3). In addition to these second messenger systems, endogenous opioid receptors couple to both Ca^{2+} and K^+ channels (4, 5). Opioid-induced activation of K^+ channels and inhibition of Ca^{2+} channels leads to reduced neurotransmitter release from presynaptic terminals (5, 6). The coupling of opioid receptors to both K^+ and Ca^{2+} channels can be reconstituted when both receptors and ion channels are expressed in *Xenopus laevis* oocytes (7–10). However, the specificity with which opioid receptors couple to their effectors may be dictated by the availability of the appropriate G proteins, and it is not clear whether the G proteins expressed by oocytes are representative of those in mammalian neurons. Indeed, to establish reliable coupling between opioid receptors and cloned Ca^{2+} channels, oocytes also have to be injected with the synthetic G protein subunit $G_{\alpha o}$ (10). In an attempt to avoid these difficulties, we sought a suitable excitable mammalian cell line in which to study the coupling of cloned opioid receptors to both adenylyl cyclase and ion channels.

Clonal rat pituitary GH_3 cells (rat pituitary growth hormone- and prolactin-secreting cell line) lack endogenous opioid receptors but express other G protein-coupled receptors that functionally couple to various effector mechanisms, including ion channels. The membranes of GH_3 cells transfected with cloned rat μ-opioid receptors (termed GH_3MOR cells) bind the selective agonist DAMGO. Cloned μ-opioid receptors in GH_3MOR cells couple to both adenylyl cyclase and voltage-activated L-type Ca^{2+} channels (11). In the current study, GH_3MOR cells were also transfected with δ receptors (termed GH_3MORDOR cells). We compared the bind-

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ABBREVIATIONS: DAMGO, [D-Ala^2,*N*-MePhe^4,Gly-ol^5]enkephalin; DPDPE, [D-Pen^2,D-Pen^5]enkephalin; CTOP, D-Pen-Cys-Tyr-D-Trp-Orn-Thr-Pen-Thr-NH_2; TIPP-Ψ, H-Tyr-Tic-Ψ-[CH_2NH]Phe-Phe-OH; PTX, pertussis toxin; GDPβS, guanosine-5′-*O*-(2-thio)diphosphate; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; IBMX, 3-isobutyl-1-methylxanthine; DMEM, Dulbecco's modified Eagle's medium.

ing profiles of μ- and δ-opioid receptors and their interaction with both adenylyl cyclase and Ca^{2+} channels.

We also examined the nature of the coupling between activated opioid receptors and Ca^{2+} channels using PTX, the nonhydrolyzable analogue of GDP (GDPβS) and double-pulse voltage protocols. Inhibitions of neuronal N-type Ca^{2+} channels by receptors coupled to PTX-sensitive G proteins are largely voltage dependent. Receptor activation causes a shift in the gating of Ca^{2+} channels, making them "reluctant" to open. This change in channel kinetics does not involve a diffusible messenger (i.e., is membrane delimited) and is thought to result from a direct interaction between activated G protein subunit(s) and Ca^{2+} channels (12, 13). Such a mechanism may also couple cloned opioid receptors to Ca^{2+} channels in GH_3 cells. We found that both μ- and δ-opioid receptor activation causes a voltage-dependent, PTX-sensitive inhibition of L-type Ca^{2+} channels.

Materials and Methods

Cell culture. GH_3 cells were grown as described previously (11). Briefly, cells were grown in a DMEM-based medium supplemented with 10% (v/v) fetal bovine serum, 50 units/ml penicillin, and 50 μg/ml streptomycin. Cells were harvested once each week by detachment with 0.1% phosphate-buffered saline supplemented with 0.4% EDTA and reseeded at 20% of their original density. The incubation medium was changed every 2–3 days. All experiments were conducted with cells maintained between passages 4–15 after transfection with δ-opioid receptors.

Transfection. Previously, we transfected μ-opioid receptor cDNAs into GH_3 cells (GH_3MOR) (11). To obtain the clone used in the current study, which expressed both μ- and δ-opioid receptors, GH_3MOR cells were subsequently stably transfected by electroporation with δ-opioid receptor cDNA. The DOR-1 construct was subcloned into the pREP4 plasmid (InVitrogen, San Diego, CA) and consisted of a 1.8-kbp cDNA representing the coding region and a 700-bp 3′ noncoding region of the mouse δ-opioid receptor. Transient transfection into COS7 cells established that this construct exhibited δ-opioid receptor-specific binding (14). GH_3 cells (5×10^6 cells in 0.5 ml of phosphate-buffered saline, pH 7.4) were stably transfected by electroporation (500 μF, 250 V) in the presence of 10 μg of the expression vector. GH_3MOR cells that stably incorporated these plasmids were selected by picking 25 colonies that survived culturing in the presence of both 1 mg/ml geneticin (for μ-opioid receptors) and 200 μg/ml hygromycin-B (for δ-opioid receptors). During the selection process, the growth medium was supplemented with 30% filtered conditioned medium. Initial screening for expression of the δ-opioid receptors in GH_3MOR cells was achieved by displacing [^{3}H]diprenorphine (2 nM) binding with DPDPE (50 nM), as described below. The clone in which DPDPE produced the greatest degree of displacement of [^{3}H]diprenorphine binding was selected for further characterization and designated as the GH_3MORDOR clone.

Receptor binding. Membranes used in binding assays were prepared by homogenization of cells in 50 mM Tris·HCl, pH 7.6, with seven strokes of a glass Dounce Homogenizer (Wheaton, Philadelphia, PA). The cell homogenates were then centrifuged at 20,000 × *g* for 30 min, and the resultant pellet was homogenized twice. The final pellet was resuspended in 50 mM Tris·HCl, pH 7.6. All opioid receptor binding was performed using 250 μg of membrane protein. Binding incubation was performed in 50 mM Tris·HCl, pH 7.6, with 10 mM $MgCl_2$ at room temperature for 90 min, as described previously (15). For saturation binding studies, concentrations of 0.05–20 nM [^{3}H]DAMGO (for μ-opioid receptors) or [^{3}H]DPDPE (for δ-opioid receptors) were used with nonspecific binding determined in the presence of nonradioactive DAMGO (5 μM) or DPDPE (1 μM), respectively. In competition binding experiments, the ability of increasing concentrations (0.01 nM to 100 μM) of various opioid receptor ligands to displace the binding of [^{3}H]DAMGO (2.5 nM) or [^{3}H]DPDPE (10 nM) was assessed. The concentrations of opioid receptor ligands required to produce a 50% reduction of [^{3}H]DAMGO or [^{3}H]DPDPE binding (IC_{50}) were determined with the computer program SigmaPlot (Jandel Scientific, San Rafael, CA) and then converted to a measure of receptor affinity (K_i) using the Cheng-Prusoff equation (15).

Measurement of opioid receptor-mediated inhibition of adenylyl cyclase activity. The conversion of the [^{3}H]adenine-labeled ATP pools to cAMP was used as a measurement of the effect of opioid ligands on cAMP levels as described previously (15). Briefly, measurements were made with cells seeded onto 17-mm (24-well) plates (4×10^6 cells/plate), which became confluent when cultured for 4 days. The incubation medium was changed 24 hr before the assay. On the day of the assay, medium was removed and replaced with an incubation mixture (DMEM containing 0.09% NaCl, 500 μM IBMX, and 2 μCi/well [^{3}H]adenine) at 37° for 1 hr. At the time of the assay, plates were placed in an ice-water bath for 5 min. The incubation mixture was then removed and replaced with ice-cold assay mixture with Krebs/Ringer/HEPES buffer (110 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1.8 mM $CaCl_2$, 25 mM glucose, 55 mM sucrose, 10 mM HEPES, pH 7.4) and 500 μM IBMX, 10 μM forskolin, and the opioid ligand to be tested. The plates were then incubated at 37° for 15 min and placed back into the ice-water bath for 5 min. After termination of incubations with 50 μl of 3.3 N perchloric acid and the subsequent addition of [^{32}P]cAMP as an internal standard, radioactive cAMP was separated from other ^{3}H-labeled nucleotides by a double-column chromatographic method (16). Scintillation fluid (7 ml) was added, and samples were immediately counted in a Beckman LS2800 scintillation counter (Beckman Instruments, Palo Alto, CA).

Electrophysiology. GH_3 cells used in electrophysiological experiments were cultured as described above, except DMEM was supplemented with 10% (v/v) calf serum instead of fetal bovine serum. For current recordings, cells were plated onto 35-mm-diameter culture dishes. Ca^{2+} channel activity in GH_3MORDOR cells was recorded by the whole-cell voltage-clamp technique using a List EPC-7 patch-clamp amplifier (Campbell, CA). Immediately before recording, the culture medium was replaced by superfusion (2 ml/min) of a saline composed of 140 mM NaCl, 2.8 mM KCl, 2 mM $MgCl_2$, 1 mM $CaCl_2$, and 10 mM HEPES, pH 7.2 with NaOH. The solution in the recording electrode contained 120 mM CsCl, 10 mM EGTA, 1 mM $MgCl_2$, 3 mM Mg-ATP, and 10 mM HEPES, pH 7.2 with CsOH. In some experiments, GDPβS (2 mM) was included in the electrode solution. Once the whole-cell configuration was obtained, the extracellular saline was replaced by a solution containing 125 mM NaCl, 5.4 mM CsCl, 10.8 mM $BaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES, and 5×10^{-4} mM tetrodotoxin, pH 7.2 with NaOH (all from Sigma Chemical, St. Louis, MO). The uncompensated liquid junction potential was <1 mV.

In most experiments, Ba^{2+} currents were evoked by step depolarizations to 0 mV (100-msec duration) from a −80 mV holding potential. In experiments in which the voltage-dependence of opioid-induced current inhibition was investigated, cells were first depolarized from a holding potential of −80 mV to between −50 and +60 mV (10-mV increments, 95-msec duration); this was followed 9 msec after return to −80 mV by a 9-msec pulse to 0 mV. Capacitance and series resistance compensations were achieved using the patch-clamp amplifier. Residual artifacts and leakage currents were nulled using P/4 subtraction.

Patch electrodes were manufactured from thin-walled borosilicate glass pipettes (World Precision Instruments, New Haven, CT) using a Narishige PP-83 electrode puller (Tokyo, Japan). Whole-cell currents, monitored using the EPC-7 amplifier, were low-pass filtered with the internal filter at 1 KHz and digitized (Labmaster DMA, Axon Instruments, Burlingame, CA) at a frequency of 5 KHz onto the hard drive of an IBM personal computer. Data were acquired (Labmaster DMA interface, Axon Instruments) and analyzed using pClamp 6.0 software (Axon Instruments).

Drugs (prepared daily from frozen stock solutions) were bath applied, and all recordings were performed at room temperature (20–22°).

Curve fitting and statistics. The Scatchard analysis of ligand-binding data was performed using LIGAND software, as described previously (17). A computer-generated "best fit" of linear regression data was used to discriminate a one-site fit from a two site-fit and to provide estimates of K_d and B_{max}. Dose-response data generated by electrophysiological experiments were fitted with the logistics function:

$$I = I_{max} - E_{max}/(1 + (IC_{50}/x)^n)$$

where I is the peak Ba^{2+} current amplitude, I_{max} is maximum current, and E_{max} is the maximum DPDPE-inhibited current amplitude (all three are expressed as a percentage of control); IC_{50} is the concentration of opioid required to cause half-maximal effect; x is the DPDPE concentration; and n is the Hill coefficient. Data obtained from the voltage-dependent reversal of Ba^{2+} current inhibitions were fitted with the Boltzmann equation:

$$I = 100 + F_{max}/(1 + \exp(-(x - F_{50})/S)$$

where I is the Ba^{2+} current amplitude as a percentage of control amplitude, F_{max} is the maximum percent facilitation of the current amplitude, x is the prepulse potential, F_{50} is the prepulse potential required for half-maximal facilitation, and S is the slope factor. In these experiments, control amplitude was the amplitude of the current activated by a test pulse from −80 mV to 0 mV after a prepulse to −50 mV. In previous experiments, such currents were similar in amplitude to currents recorded from the same cells activated by stepping from −80 mV to 0 mV with no prepulse.

Electrophysiological data are presented as mean ± standard error, and binding and cyclase data are presented as mean ± standard deviation. Comparisons between data sets were made using the Student's t test.

PTX treatment. GH_3 cells were placed in culture media containing 200 ng/ml PTX 24–36 hr before electrophysiological recordings. Ba^{2+} currents were recorded from control and PTX-treated cells for comparison on the same day.

Reagents and drugs used. Somatostatin, DPDPE, DAMGO, and CTOP were obtained from Peninsula Laboratories (Belmont, CA). TIPP-Ψ was a generous gift of Dr. P. Schiller (Clinical Research Institute of Montreal, Montreal, Quebec, Canada). All tissue culture reagents, including geneticin (G418) and hygromycin-B, were purchased from GIBCO BRL (Gaithersburg, MD). [^{3}H]DAMGO (37 Ci/mmol) and [^{3}H]DPDPE (18 Ci/mmol) were provided by the National Institute on Drug Abuse (Bethesda, MD). [^{3}H]Adenine (27 Ci/mmol), [^{3}H]diprenorphine (36 Ci/mmol), and α-[^{32}P]ATP (17 Ci/mmol) were purchased from Amersham (Arlington Heights, IL). Forskolin and IBMX were obtained from List Biochemicals (Campbell, CA). PTX, GDPβS, ATP, and all other reagents were purchased from Sigma.

Results

Opioid receptor binding in GH_3MORDOR cells. Control GH_3 cells do not bind opioid ligands; however, cells stably transfected with μ-opioid receptor cDNA and selected on the basis of their resistance to geneticin, termed GH_3MOR cells, bind the μ-opioid receptor-selective ligand DAMGO (11). GH_3MOR cells do not bind δ-opioid receptor-selective ligands with high affinity. To compare the properties of μ- and δ-opioid receptors, we transfected GH_3MOR cells with δ receptor cDNA. Stable transfectants were selected on the basis of their resistance to hygromycin, and the clone from which DPDPE caused the greatest displacement of [^{3}H]diprenorphine (see Materials and Methods), termed GH_3MORDOR, was used in all subsequent experiments. Studies of saturation binding to GH_3MORDOR membranes using the selective μ-opioid receptor ligand [^{3}H]DAMGO indicated the presence of a single high affinity binding site (K_d = 0.58 ± 0.04 nM) with a receptor density (B_{max}) of 0.30 ± 0.02 pmol/mg of protein (Fig. 1). The density of μ-opioid receptors in the GH_3MORDOR clone was slightly lower than that observed previously in the parental GH_3MOR clone (0.39 pmol/mg of protein) (11). Scatchard analysis of saturation binding studies with [^{3}H]DPDPE using membranes obtained from GH_3MORDOR cells revealed a single high affinity δ-opioid binding site (K_d = 3.20 ± 0.15 nM) with a B_{max} of 3.45 ± 0.14 pmol/mg of protein (Fig. 1). To further characterize the μ- and δ-opioid receptors expressed in the GH_3MORDOR clone, the abilities of various selective opioid ligands to compete for [^{3}H]DAMGO or [^{3}H]DPDPE binding were assessed. The IC_{50} values for these competition experiments were subsequently converted to K_i values and are presented in Table 1. The μ-opioid agonist DAMGO had >600-fold higher affinity for μ-opioid receptors relative to the δ-opioid receptors expressed in the GH_3MORDOR clone, as indicated by the K_i values of 0.51 and 341 nM, respectively. Also, the μ-opioid receptor antagonist CTOP bound selectively to the expressed μ-opioid receptors with a K_i of 5.8 nM compared with >25,000 nM for the δ-opioid receptor (Table 1). The δ-opioid agonist DPDPE was found to have a 68-fold greater affinity for δ-opioid receptors relative to μ-opioid receptors in GH_3MORDOR cell membranes (Table 1). The δ-opioid receptor antagonist TIPP-Ψ also bound selectively to δ-opioid receptors as opposed to μ-opioid receptors, as indicated by K_i values of 3.4 and >40,000 nM, respectively (Table 1). In summary, the radioligand binding data demonstrate that the GH_3MORDOR cells stably express both μ- and δ-opioid receptors.

Inhibition of adenylyl cyclase activity by μ- and δ-opioid receptors. Opioid ligands have no effect on adenylyl cyclase activity in GH_3 cells. Activation of μ-opioid receptors in GH_3MOR cells inhibits cAMP accumulation (11). In

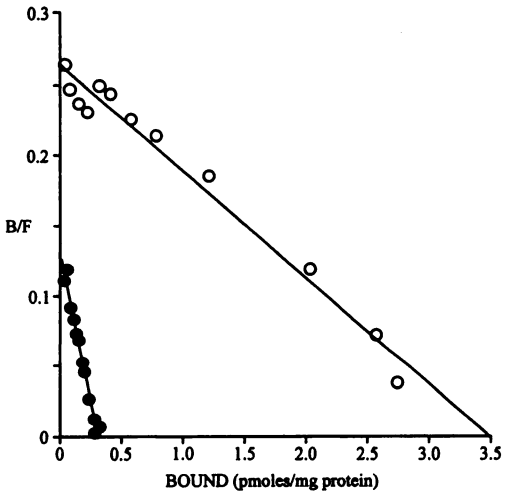

Fig. 1. Scatchard analyses of [^{3}H]DPDPE and [^{3}H]DAMGO binding to GH_3MORDOR cell membranes. Saturable [^{3}H]DPDPE (○) binding (0.05–20 nM) was best described by a one-site model. The K_d and B_{max} values were 3.2 nM and 3.45 pmol/mg of protein, respectively. [^{3}H]DAMGO binding was also saturable, and the data indicated the presence of a single μ-opioid binding site (K_d = 0.58 nM and B_{max} = 0.3 pmol/mg of protein). Each point represents mean values obtained from experiments performed in triplicate. Standard deviations were <10%.

TABLE 1

GH_3MORDOR cells exhibit μ- and δ-opioid receptor-specific binding

Affinity (K_i) values were determined from competition binding experiments using [^{3}H]DAMGO (2.5 nM) or [^{3}H]DPDPE (10 nM) with increasing concentrations of various opioid ligands (0.01 nM to 10 μM) as described in Materials and Methods. Data represent the mean ± standard deviations determined from experiments using 11 concentrations, performed in triplicate.

Opioid ligand	Opioid receptor affinity (K_i)	
	μ Receptor	δ Receptor
	nM	
μ Selective		
DAMGO	0.51 ± 0.06	341 ± 40
CTOP	5.8 ± 0.8	>25,000
δ Selective		
DPDPE	49.0 ± 0.8	0.72 ± 0.04
TIPP-Ψ	>40,000	3.4 ± 1.1

the GH_3MORDOR cell line, the μ-selective agonist DAMGO produced 74.5 ± 7.2% inhibition of adenylyl cyclase activity, with an IC_{50} value of 174 ± 77 nM (Table 2). The μ-opioid receptor antagonist CTOP (300 nM) competitively antagonized the inhibition of cAMP accumulation by DAMGO (0.01 nM to 10 μM) (Table 2), whereas the δ-agonist TIPP-Ψ (10 nM) had no effect. The δ-opioid receptor-selective agonist DPDPE also potently (IC_{50} = 0.53 ± 0.15 nM) and efficaciously reduced (by 79.1 ± 2.2%) the production of cAMP. The potency of DPDPE was decreased by the coapplication of TIPP-Ψ (10 nM) but not CTOP (300 nM) (Table 2). Taken collectively, data obtained from both opioid receptor binding and adenylyl cyclase assays suggest that GH_3MORDOR cells express functional μ- and δ-opioid receptors.

Cloned δ- and μ-opioid receptors couple to voltage-activated Ca^{2+} channels. The whole-cell configuration of the patch-clamp technique was used to investigate the actions of DAMGO and DPDPE on Ca^{2+} channels in GH_3MORDOR cells. GH_3 cells predominantly express dihydropyridine-sensitive L-type Ca^{2+} channels. Voltage-activated Ca^{2+} channels in GH_3 cells are insensitive to opioids. In GH_3MOR cells, activation of cloned μ receptors by DAMGO dose-dependently inhibits L-type Ca^{2+} channels (11). In contrast, Ca^{2+} channels are insensitive to DPDPE (1 μM) in GH_3MOR cells. Depolarization of GH_3MORDOR cells activated inward Ba^{2+} currents with a current-voltage relationship similar to that seen in GH_3 and GH_3MOR cells (five experiments; data not shown). Activation of either μ- or δ-opioid receptors in GH_3MORDOR cells inhibited Ca^{2+} channel activity. The time course of Ba^{2+} current inhibition by both DPDPE (1 μM) and DAMGO (1 μM) is illustrated in Fig. 2. Inhibitions by both of these peptides reversed on washout. Both opioid ligands were applied at 1 μM; this dose of DAMGO is required for maximal Ca^{2+} current inhibitions in GH_3MOR cells and other preparations. DAMGO (1 μM) had no effect on Ba^{2+} currents recorded from the δ-opioid receptor expressing NG108-15 cells,[1] suggesting that this agonist discriminates between μ and δ receptors. DPDPE (1 μM) had no effect on currents recorded from GH_3MOR cells (1), suggesting that the actions of this agonist are mediated specifically by δ receptors. The selectivity of action of both DAMGO (1 μM) and DPDPE (1 μM) is also verified by the inhibition of their actions on adenylyl cyclase by the selective μ and δ antagonists CTOP and TIPP-Ψ, respectively (Table 2). The inhibitory action of DPDPE on Ba^{2+} currents was dose dependent (Fig. 3) (IC_{50} = 1.6 nM, Hill slope = 0.8) in GH_3MORDOR cells. DAMGO (1 μM) caused a 17.0 ± 1.4% inhibition (five experiments). Interestingly, not all GH_3MORDOR cells responded to both DPDPE (1 μM) and DAMGO (1 μM) when applied consecutively (Table 3). Of the 24 cells in which both compounds were tested, 50% responded to both and 17% did not respond to either. Inhibitions due to DAMGO but not to DPDPE were observed in 8% of the cells, and 25% responded to DPDPE but not to DAMGO (Table 3). Taken together, these observations again suggest that the actions of DAMGO (1 μM) and DPDPE (1 μM) on Ca^{2+} channel activity occur through activation of μ and δ receptors, respectively.

The inhibition of Ba^{2+} currents by DAMGO (1 μM) and DPDPE (1 μM) was not additive. When coapplied, DAMGO and DPDPE caused a 19.0 ± 0.9% (four experiments) inhibition (data not shown). This was not significantly different from the inhibition of 17.0 ± 1.4% (five experiments) and 20.7 ± 1.3% (12 experiments) in response to either DAMGO or DPDPE alone. In conclusion, in GH_3MORDOR cells there is functional coupling between both activated μ- and δ-opioid receptors and Ca^{2+} channels.

Coupling of opioid receptors to Ca^{2+} channels is mediated by a PTX-sensitive G protein. Cloned μ-opioid receptors couple to L-type Ca^{2+} channels in GH_3MOR cells through PTX-sensitive G proteins (11). We examined the role of G proteins in the coupling of δ-opioid receptors to Ca^{2+} channels either by applying the nonhydrolyzable analog of GDP, GDPβS, within the recording electrode or by pretreating cells for 24 hr with PTX. In GH_3MORDOR cells, GDPβS (2 mM; nine experiments) significantly ($p < 0.05$) reduced DPDPE (1 μM)-evoked Ba^{2+} current inhibitions (6.3 ± 2.9%) compared with control values (20.7 ± 1.3%) (Fig. 4). Furthermore, pretreatment with PTX (200 ng/ml, 10 experiments) also attenuated DPDPE-mediated Ba^{2+} current inhibitions in GH_3MORDOR cells (3.4 ± 1.9%) (Fig. 4). These data

[1] E. T. Piros and T. G. Hales, unpublished observations.

TABLE 2

Activated μ- and δ-opioid receptors inhibit forskolin-stimulated adenylyl cyclase activity in GH_3MORDOR cells

The ability of increasing concentrations of DAMGO and DPDPE (both 0.01 nM to 10 μM) to inhibit 10 μM forskolin-stimulated intracellular [^{3}H]cAMP production in the presence and absence of the μ-selective antagonist CTOP (300 nM) or the δ-selective antagonist TIPP-Ψ (10 nM). Data are expressed as mean ± standard deviation. Numbers in parentheses represent the number of experiments performed in triplicate.

Opioid ligand		Adenylyl cyclase inhibition		
		No antagonist	+CTOP (300 nM)	+TIPP-Ψ (10 nM)
DAMGO	IC_{50} (nM)	174 ± 77 (9)	897 ± 142 (2)	130 ± 53 (4)
	INH_{max}	74.5 ± 7.2%	80.3 ± 2.6%	70.1 ± 4.0%
DPDPE	IC_{50} (nM)	0.53 ± 0.15 (5)	0.92 ± 0.32 (1)	2.43 ± 1.2 (3)
	INH_{max}	79.1 ± 2.2%	72.0 ± 3.7%	71.5 ± 3.8%

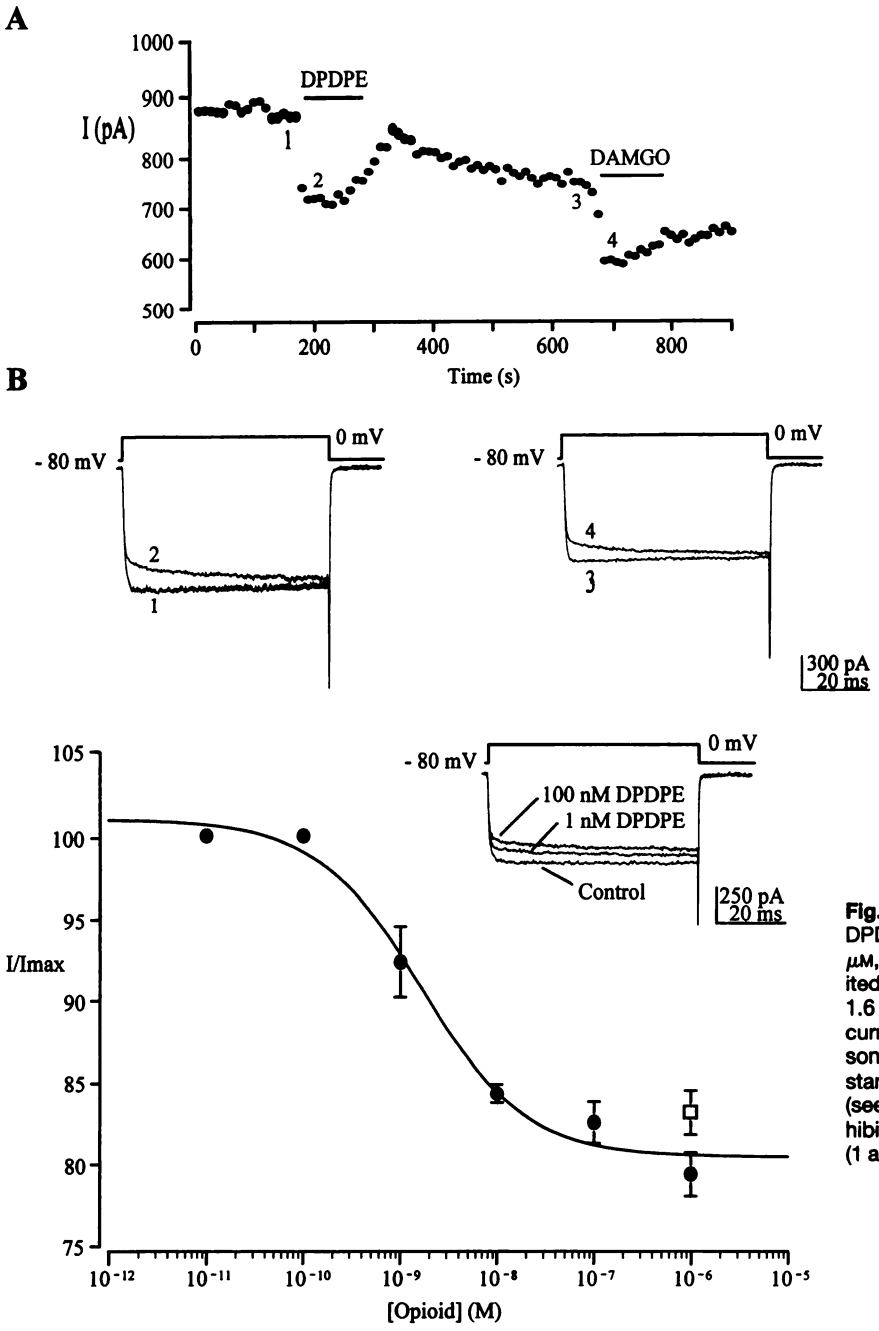

Fig. 2. Both DPDPE and DAMGO inhibit Ba^{2+} currents in $GH_3MORDOR$ cells. A, Time course of Ba^{2+} current inhibition by DPDPE (1 μM) and DAMGO (1 μM). Every 10 sec, currents were activated by 100-msec depolarizations to 0 mV from a holding potential of −80 mV. Current amplitudes were measured 5 msec after the beginning of the pulse and plotted as a function of time. B: *Left*, superimposed currents recorded in the absence (*1*) and presence (*2*) of DPDPE (1 μM). *Right*, superimposed current traces before (*3*) and during (*4*) the application of DAMGO (1 μM). *1–4*, correspond to time points indicated in A.

Fig. 3. Dose-dependent inhibition of Ba^{2+} currents by DPDPE in $GH_3MORDOR$ cells. DPDPE (●) (0.01 nM to 1 μM, five or more experiments) dose-dependently inhibited Ca^{2+} channel activity in $GH_3MORDOR$ cells (IC_{50} = 1.6 nM, Hill slope = 0.8). The mean inhibition of Ba^{2+} currents by DAMGO (□) (1 μM) is included for comparison (five experiments). Data are presented as mean ± standard error and were fitted with a logistic equation (see Materials and Methods). *Inset*, dose-dependent inhibition of Ba^{2+} currents by the δ-opioid agonist DPDPE (1 and 100 nM) recorded from a single $GH_3MORDOR$ cell.

TABLE 3

Fraction of $GH_3MORDOR$ cells exhibiting Ba^{2+} current inhibition in response to μ and δ opioids

Ba^{2+} current inhibition by DAMGO (1 μM) and DPDPE (1 μM) in $GH_3MORDOR$ cells. Drugs were applied as shown in Fig. 2. The inhibitory response is symbolized by +, and the absence of a response is denoted by −.

Opioid agonist		Cells responding/cells tested
DAMGO	DPDPE	
+	+	12/24 (50%)
+	−	2/24 (8 %)
−	+	6/24 (25%)
−	−	4/24 (17%)

indicate that like μ-opioid receptors in GH_3MOR cells, δ-opioid receptors in $GH_3MORDOR$ cells couple to voltage-gated Ca^{2+} channels through PTX-sensitive G proteins.

Cloned opioid receptors couple to Ca^{2+} channels in a voltage-dependent manner. Previous studies demonstrated that the inhibitory action of numerous G protein-coupled receptors on N-type Ca^{2+} channels is voltage dependent (12, 18). In this study, we used a double-pulse recording protocol to examine the voltage dependence of opioid receptor coupling to Ca^{2+} channels in $GH_3MORDOR$ cells. Under control conditions (in the absence of opioids), depolarizing prepulses to +60 mV caused a modest enhancement of Ba^{2+} current amplitude (see Fig. 6). Depolarizing prepulses caused a larger increase in the Ba^{2+} current amplitude in the presence of either DPDPE (1 μM) (Figs. 5 and 6) or DAMGO (1 μM) (Fig. 7). During δ-opioid receptor activation, the maximum current facilitation to 120% of control amplitude occurred after a prepulse to +40 mV. This represents a complete reversal of the inhibition of Ba^{2+} currents by DPDPE (1 μM) (Fig. 3). The Boltzmann fit (see Materials and Methods) to

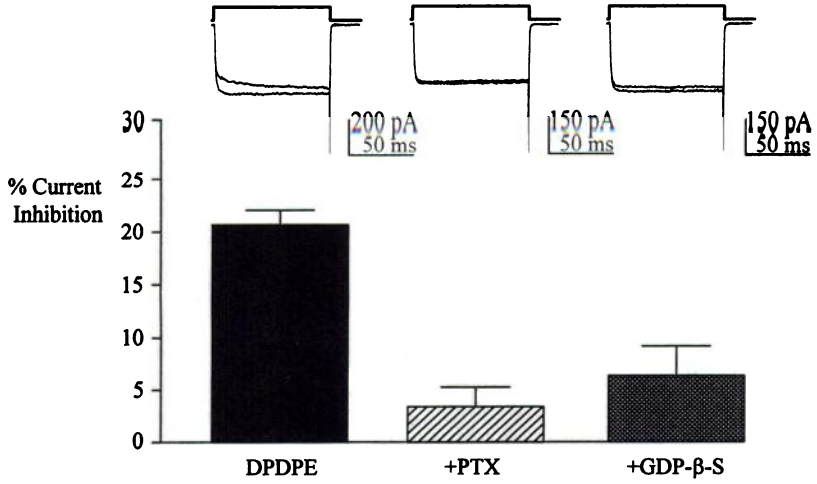

Fig. 4. Ba^{2+} current inhibition by DPDPE is attenuated by PTX or with intracellular GDPβS. Pretreatment of GH_3MORDOR cells with PTX (200 ng/ml, 10 experiments) for 24–36 hr attenuates Ba^{2+} current inhibitions by DPDPE (1 μM, 12 experiments). Inclusion of the GDP analog GDPβS (2 mM, nine experiments) in the recording electrode also reduces the ability of DPDPE (1 μM) to inhibit Ba^{2+} currents. *Histogram bars*, mean values; *vertical bars*, standard error; *inset*, superimposed Ba^{2+} current traces in the presence or absence of DPDPE (1 μM) under control conditions (*left*), after PTX treatment (*middle*), and with intracellular GDPβS (*right*). Currents, which were activated by depolarizing from −80 to 0 mV, were recorded from three separate cells.

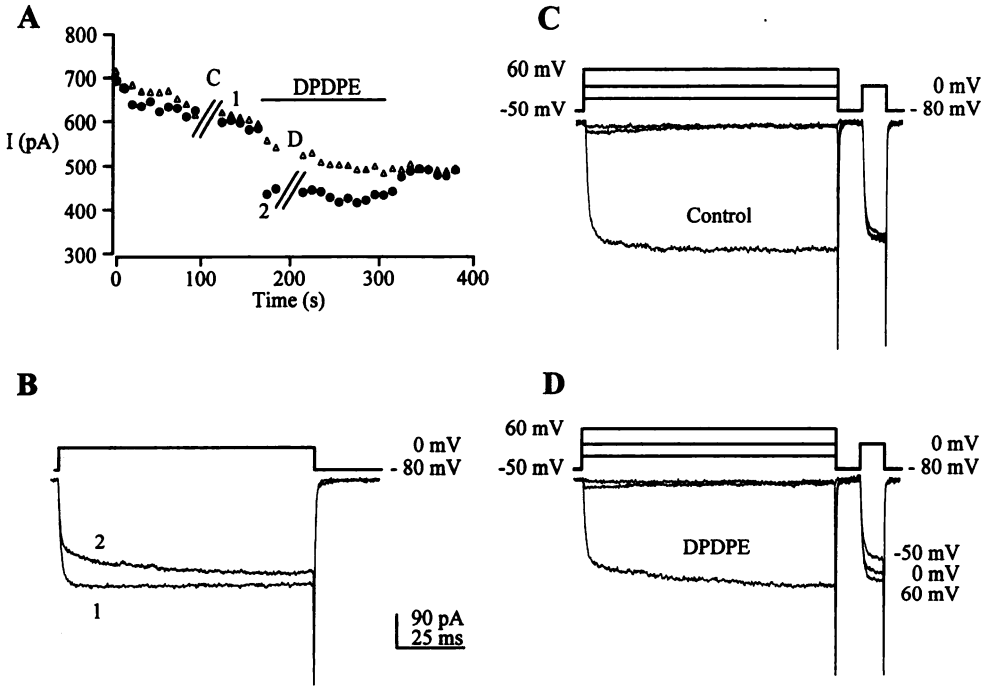

Fig. 5. Prepulse-dependent reversal of Ba^{2+} current inhibition by the δ-opioid agonist DPDPE. A, Time course of DPDPE (1 μM)-evoked inhibition of Ba^{2+} currents recorded from a GH_3MORDOR cell. Current amplitudes were measured 5 msec after the depolarization from −80 to 0 mV (●) and 5 msec before repolarization to −80 mV (△). Recordings were interrupted at time points (*C* and *D*), and a double-pulse protocol was applied (see Materials and Methods). B, Ba^{2+} currents before (*1*) and during (*2*) the application of DPDPE (1 μM). *1 and 2*, time points shown in A. C, Ba^{2+} currents activated using a double-pulse protocol before the application of DPDPE (performed at time point *C* in A). Prepulse steps to −50, 0 and +60 mV had little effect on currents activated by the subsequent step to 0 mV. D, Same protocol used in C (performed at time point *D* in A) causes a voltage-dependent reversal of the DPDPE inhibition of Ba^{2+} current amplitude.

the data points (Fig. 5) revealed a half-maximal facilitation with a depolarization to −10 mV. Depolarizing prepulses also reversed inhibitions of Ba^{2+} currents caused by DAMGO (1 μM; five experiments) and somatostatin (1 μM; three experiments) in GH_3MOR cells (data not shown). The characteristics of the voltage-dependent reversal of μ-opioid receptor-mediated L-type Ca^{2+} channel inhibition in GH_3MORDOR cells (Fig. 7) were similar to those for reversal of δ receptor inhibition. Taken together, these results demonstrate that cloned μ and δ receptors expressed in GH_3 cells couple to similar Ca^{2+} channels through PTX-sensitive G proteins.

Discussion

The three opioid receptor subtypes (μ, δ, and κ) couple to adenylyl cyclase, K^+ channels, and voltage-activated Ca^{2+} channels (19). These receptors were recently cloned, and their binding profiles and negative coupling to adenylyl cyclase have been extensively studied in nonexcitable cell lines transfected with appropriate cDNAs (2–4). In contrast, few studies have investigated recombinant opioid receptor coupling to ion channels. In the central nervous system, modulation of the activity of specific ion channels by opioids inhibits neuronal excitability. For example, the activation of μ-opioid receptors in locus ceruleus neurons, δ receptors in the submucosal plexus, and κ receptors in guinea pig trigeminal nuclei hyperpolarizes cells by opening K^+ channels (4, 5). In addition, all three receptor subtypes inhibit high voltage-activated Ca^{2+} channel activity (4, 5). The combined actions of opioids on these ion channels reduce neurotransmitter release. We chose a mammalian heterologous expression system, lacking endogenous opioid receptors, in which to study the binding characteristics of cloned μ and δ receptors and their coupling to both adenylyl cyclase and Ca^{2+} channels. In a previous report, we established that μ-opioid receptors stably expressed in GH_3 cells (termed GH_3MOR cells) bind μ-opioid ligands, and receptor activation causes inhibition of both adenylyl cyclase and L-type Ca^{2+} channels (11). For the current study, GH_3MOR cells were also stably trans-

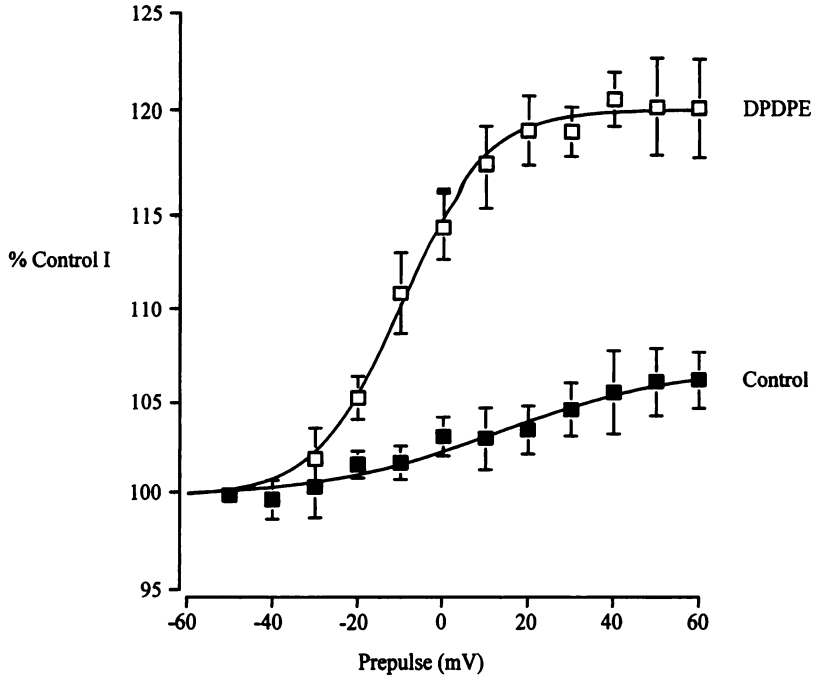

Fig. 6. Voltage dependence of the reversal of DPDPE-mediated Ba^{2+} current inhibition in $GH_3MORDOR$ cells. The double-pulse recording protocol was used to measure the current amplitude during the test pulse (to 0 mV). Prepulse potentials to between −50 and +60 mV (10-mV increments) were applied. Current (*I*) amplitudes were normalized to the value obtained after a prepulse to −50 mV (expressed as percentage of control) and plotted as a function of the prepulse potential (see Materials and Methods). *Points*, mean data from 15 cells under control conditions (■) and 5 cells in the presence of DPDPE (1 μM) (□) were fitted using the Boltzmann equation (see Materials and Methods). The Boltzmann fit indicated that currents in the presence of DPDPE were maximally facilitated (F_{max}) by 19.8%, half-maximal facilitation (F_{50}) occurred at −10 mV, and the slope factor (S) was 9.6. Under control conditions, current facilitation was characterized by the following parameters: F_{max} = 6.8%, F_{50} = +13.0 mV, and S = +20.4. *Vertical bars*, standard error.

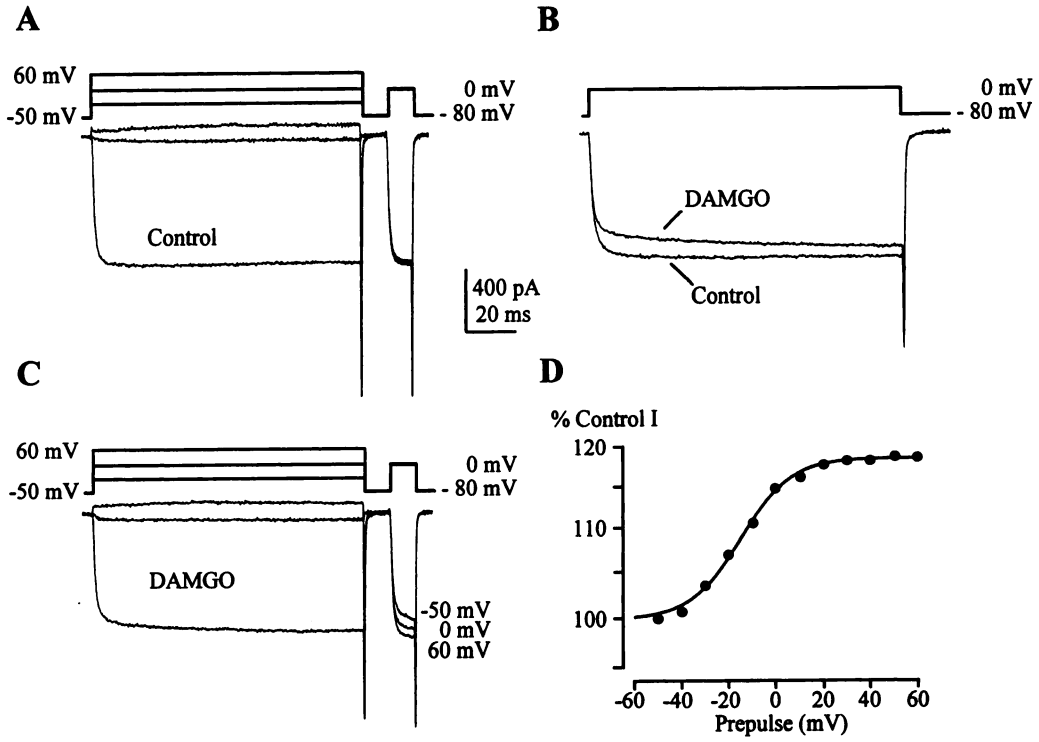

Fig. 7. Voltage-dependent reversal of DAMGO-evoked Ba^{2+} current inhibition in a $GH_3MORDOR$ cell. A, Ba^{2+} currents recorded by applying the double-pulse recording protocol (see Materials and Methods) in the absence of DAMGO (*Control*). B, Inhibitory effect of DAMGO (1 μM) on the Ca^{2+} channel activity recorded by the single-pulse protocol (from −80 to 0 mV). Same cell as in A. C, Ba^{2+} currents recorded from the same $GH_3MORDOR$ cell (as in A and B) in the presence of DAMGO (1 μM), using the double-pulse protocol. D, Current (*I*) amplitudes were normalized to the value obtained after a prepulse to −50 mV (expressed as percentage of control) and plotted as a function of the prepulse potential (see Materials and Methods). Data were obtained from the same cell (as in C) during DAMGO (1 μM) application. *Points*, fitted with the Boltzmann equation, which resulted in fit parameters (F_{max} = 17.9%, F_{50} = −14.5 mV, S = +10.7) similar to those for the reversal of DPDPE-mediated inhibition (see legend to Fig. 6).

fected with cDNA encoding the δ-opioid receptor, generating the $GH_3MORDOR$ cell line.

$GH_3MORDOR$ cells have high affinity binding sites for the μ-and δ-selective agonists DAMGO and DPDPE. Subsequent experiments investigated whether both receptors functionally couple to adenylyl cyclase. Activation of μ receptors in both GH_3MOR (11) and $GH_3MORDOR$ cells inhibits forskolin-stimulated cAMP accumulation. It is noteworthy that the potency of the DAMGO-induced inhibition of adenylyl cyclase activity in $GH_3MORDOR$ cells was less (IC_{50} = 174 nM) than

that previously observed in GH_3MOR cells (IC_{50} = 21.9 nM) (11). This difference might be explained by the fact that $GH_3MORDOR$ cells contain ~25% less μ-opioid receptors than do GH_3MOR cells. Also, high concentrations of DAMGO may activate δ receptors in $GH_3MORDOR$ cells. However, the fact that the actions of DAMGO were inhibited by the μ receptor antagonist CTOP but unchanged by the δ receptor antagonist TIPP-Ψ argues against this possibility. DPDPE had no effect on forskolin-stimulated cAMP accumulation in GH_3MOR cells but inhibited adenylyl cyclase activity in $GH_3MORDOR$ cells. Thus, $GH_3MORDOR$ cells express functional μ- and δ-opioid receptors.

It is interesting that the relationship between affinity and potency for DAMGO and DPDPE in $GH_3MORDOR$ cells is dissimilar. The potency of DAMGO as an inhibitor of adenylyl cyclase is considerably lower than the affinity of the ligand for the μ receptor in GH_3MOR (11), $GH_3MORDOR$, and μ receptor-expressing Chinese hamster ovary cells (20). The inhibition of Ca^{2+} channel activity by DAMGO also occurs with a lower potency than would be predicted by its affinity alone (11). However, comparisons of opioid binding affinities with their potencies in functional assays are made here, cautiously. The conditions used for measurements of affinities and potencies were markedly dissimilar. Most notably, binding assays were performed on membrane preparations, whereas adenylyl cyclase assays and patch-clamp experiments used whole cells. Only high affinity opioid binding sites are observed in membrane preparations in the presence of Mg^{2+} and the absence of Na^+ and GTP, agents that reduce ligand binding affinities (15, 20, 21). Membrane preparations are often used rather than whole-cell binding assays because in the latter, activated opioid receptors have a lower affinity state, making their detection more difficult (i.e., low levels of specific binding are observed) (22). The lower affinity state of the receptor occurs after its dissociation from a G protein on activation. Differing affinity states in binding and functional assays may explain why the dose-response curves for DAMGO are dextral to the binding isotherms. In contrast, the affinity of DPDPE for the δ receptor is similar to its potency in both functional assays. There are ~10-fold more δ than μ receptors expressed in $GH_3MORDOR$ cells. Because more receptors are available, less-than-complete occupancy of these receptors may be required for a full effect. If this is the case, then the dose-response curve would be expected to be to the left of the binding isotherm. Data presented here support this hypothesis. DPDPE inhibited intracellular cAMP accumulation with a higher potency than DAMGO in $GH_3MORDOR$ cells (Table 2). An alternative explanation for these observations is that DAMGO has low intrinsic activity at the μ-opioid receptor relative to the activity of DPDPE at the δ-opioid receptor. However, comparison between the intrinsic activities of these ligands is valid only when their respective activated receptors couple to an effector through the same G proteins. Although the inhibition of Ca^{2+} channel and adenylyl cyclase activities by DAMGO and DPDPE occurs through PTX-sensitive inhibitory G proteins, it is not clear whether the two receptors couple to their effectors using the same G protein subtypes. Further experiments are required to determine which types of G proteins are involved in the coupling between cloned opioid receptors and effectors in $GH_3MORDOR$ cells. Regardless of the disparity between opioid binding affinities and functional potencies, the pharmacological data from adenylyl cyclase assays demonstrate that micromolar concentrations of DAMGO and DPDPE exert selective actions on μ- and δ-opioid receptors, respectively. Similarly, electrophysiological experiments also confirm a lack of effect of DAMGO through δ receptors and of DPDPE through μ receptors on Ca^{2+} channel activity. In the μ-opioid receptor expressing GH_3MOR cells, DAMGO, but not DPDPE, caused a dose-dependent inhibition of Ba^{2+} currents (1). In the cotransfected $GH_3MORDOR$ cells, both DAMGO and DPDPE reversibly inhibited Ba^{2+} currents.

GH_3 cells express both transient T-type and dihydropyridine-sensitive L-type Ca^{2+} channels as demonstrated pharmacologically (23) and based on their differential current deactivation kinetics (24). L-type Ca^{2+} channels mediate >90% of the whole-cell Ba^{2+} current in GH_3 cells (11). Activation of μ-opioid receptors inhibits the dihydropyridine-sensitive current component in GH_3MOR cells (11). DAMGO and DPDPE inhibit a similar Ba^{2+} current component in $GH_3MORDOR$ cells. Both agonists caused a reduction in the rate of current activation. A two-pulse protocol revealed that depolarizing prepulses reverse Ba^{2+} current inhibitions by the opioids. The fact that complete recovery from opioid-induced inhibition occurs with depolarizing prepulses confirms that T-type Ca^{2+} channels are not involved in this inhibitory effect because T-type channels exhibit voltage-dependent inactivation. Therefore, taken together, our observations suggest that both recombinant μ- and δ-opioid receptors negatively couple to L-type Ca^{2+} channels in $GH_3MORDOR$ cells.

It is not known whether L-type Ca^{2+} channels are involved in the central actions of opioids; however, inhibition of dihydropyridine-sensitive Ca^{2+} channels mimics their central analgesic effects (25, 26). Coupling of δ-opioid receptors to L-type channels has been shown outside the nervous system in chromaffin cells (27) and ventricular myocytes (28). There have been relatively few studies directly investigating the actions of opioids on Ca^{2+} channels in central neurons. In acutely dissociated nucleus tractus solitarius neurons, activation of μ-opioid receptors inhibits N- and P/Q-type Ca^{2+} channels (29). Coupling of μ and δ receptors has been investigated more extensively in peripheral neurons and cell lines. In dorsal root ganglion cells, voltage-clamp recordings demonstrated that opioids inhibit voltage-gated N-type (30, 31), P/Q-type (30), and T-type (30) Ca^{2+} channels. Endogenous μ and δ receptors in neuroblastoma cell lines (SH-SY5Y and NG108-15 cells, respectively) seem to couple exclusively to N-type Ca^{2+} channels (33, 34). In addition, cloned κ- and μ-opioid receptors couple to N-type Ca^{2+} channels when expressed in pheochromocytoma (PC-12) (35) and NG108-15 (36) cell lines, respectively. It is clear that in these neuronal preparations, N-type Ca^{2+} channels are consistently modulated by opioid receptors. However, it is worth noting that the contribution of L-type channels is relatively minor in most of these cells. It is possible that inhibition of this component could be overshadowed by the more obvious attenuation of N-type channels. Perhaps the clearest approach to this problem would be to study each channel subtype in isolation. This is possible subsequent to the cloning of various Ca^{2+} channel isoforms.

Ca^{2+} channels consist of several proteins: an α_1 subunit, which provides a voltage-gated channel, and ancillary sub-

units, including a β subunit and disulfide-linked $\alpha_2\delta$ subunit (37). There are six known α_1 gene products (α_{1S} and α_{1A-E}), which have different pharmacological and biophysical properties. Adding to this complexity, there are three splice variants of α_{1C}. There are functional correlates between some of the cloned Ca^{2+} channels and their naturally expressed counterparts. When expressed in *X. laevis* oocytes with ancillary subunits, α_{1A} and α_{1B} resemble P/Q- and N-type Ca^{2+} channels, respectively. Activation of coexpressed μ-opioid receptors inhibits Ba^{2+} currents mediated by either of these cloned channels (1). Several of the α_1 subunits (α_{1S}, α_{1C}, and α_{1D}) form dihydropyridine-sensitive high-voltage-activated Ca^{2+} channels when expressed in oocytes and cell lines (37). Thus, there is considerable L-type Ca^{2+} channel heterogeneity. μ-Opioid receptors do not couple to channels formed by α_{1C} subunits expressed with ancillary α_2 and β_4 subunits in oocytes (10). However, the coupling of opioid receptors to α_{1S} and α_{1D} remains untested. The α_{1D} subunit is particularly relevant in the context of the current report because its transcript is abundant in GH_3 cells (38). This subunit is also found in other endocrine cells and in the brain, where it could participate in the central actions of opioids. It is also possible that GH_3 cells express additional dihydropyridine-sensitive α_1 subunits.

Inhibitions of L-type Ca^{2+} channels by opioids through activation of μ (11) and δ receptors are attenuated by PTX, indicating a role for G_i and/or G_o proteins. The inhibitory actions of activated G_i/G_o protein-coupled receptors on N-type Ca^{2+} channels are largely voltage dependent (12, 18). It is thought that N-type Ca^{2+} channels are "reluctant" to open as a consequence of activated G protein actions, but they can be converted into a more "willing" state by strong depolarizations (12, 39). Consistent with this model, Ca^{2+} currents exhibit slowed activation kinetics after the activation of inhibitory G proteins. Depolarizing prepulses reverse this inhibition (12, 34, 39–41). We observed that in the presence of the μ- and δ-opioid agonists DAMGO and DPDPE, Ba^{2+} currents had a slower rate of activation than in the absence of these agents. Furthermore, Ba^{2+} current inhibitions were completely reversed by depolarizing prepulses. Voltage-dependent inhibition of L-type Ca^{2+} channels by G protein-coupled receptors is not without precedence; L-type Ca^{2+} channel inhibition by D_2 receptor stimulation is also reversed by depolarizing prepulses in rat pituitary melanotropes (42).

In summary, cloned μ- and δ-opioid receptors expressed in GH_3 cells inhibit adenylyl cyclase and L-type Ca^{2+} channel activities. L-type Ca^{2+} channel inhibition by opioid receptors occurs through activation of G_i/G_o proteins and is reversed by depolarization. The similarity between the inhibition of L-type channels in this preparation and N-type channels in neuronal preparations suggests that a common mechanism exists for G protein-mediated modulation of both channel subtypes.

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