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Ca^{2+} Channel and Adenylyl Cyclase Modulation by Cloned μ-Opioid Receptors in GH_3 Cells

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SUMMARY

Members of the three classes of opioid receptors (μ, δ, and κ) have been cloned and characterized in unexcitable cell lines using biochemical techniques. However, an important function of these cloned receptors, their coupling to voltage-activated Ca^{2+} channels, remains untested. We stably transfected cloned rat μ-opioid receptor cDNAs into clonal pituitary GH_3 cells. GH_3 cells expressing μ-opioid receptors (GH_3MOR cells) bound the receptor-specific ligands [D-Ala^2,Me-Phe^4,Gly-ol^5]-enkephalin (DAMGO) and morphine with high affinity (K_i = 1.0 and 7.2 nM, respectively), and these ligands also potently inhibited adenylyl cyclase activity (IC_{50} = 21.9 and 55.2 nM, respectively). Functional coupling of μ-opioid receptors to voltage-activated Ca^{2+} channels was compared with that of endogenous somatostatin (SRIF) receptors in GH_3MOR cells, using the patch-clamp technique, with Ba^{2+} as the charge carrier. DAMGO (1 μM) and SRIF (1 μM) inhibited Ba^{2+} currents by 23.8 ± 1.0% and 22.9 ± 2.5%, respectively. DAMGO (0.1 nM to 10 μM) dose-dependently inhibited Ba^{2+} currents, with an IC_{50} of 105 nM. The μ-opioid receptor agonist morphine (1 μM) inhibited currents by 13.5 ± 1.1% and the δ-opioid receptor-selective ligand [D-Pen^{2,5}]-enkephalin (1 μM) caused only 3.5 ± 2.1% inhibition. The inhibitory actions of DAMGO, morphine, and [D-Pen^{2,5}]-enkephalin were reversed by naloxone. Ba^{2+} current inhibitions by DAMGO and SRIF were attenuated by pertussis toxin pretreatment. Nimodipine reduced the amplitude of Ba^{2+} current inhibition by DAMGO, suggesting that μ-opioid receptors couple to L-type Ca^{2+} channels in GH_3MOR cells.

The μ-, δ-, and κ-opioid receptors are coupled to various effector systems, such as adenylyl cyclase, Ca^{2+} and K^+ channels, and PI turnover, via different G proteins (for recent reviews, see Refs. 1 and 2). Recently, members of the three opioid receptor subtypes have been cloned (3–6). The availability of cDNA clones has facilitated research on the functional expression of the receptors in either unexcitable mammalian cell lines or *Xenopus laevis* oocytes. These studies have demonstrated that all three opioid receptor subtypes are coupled to adenylyl cyclase (3–6). Furthermore, it was found that both μ-opioid receptors (7) and δ-opioid receptors (8) are coupled to the recently cloned G protein-activated K^+ channel, when expressed in oocytes. Simultaneous coupling of the μ-opioid receptor to adenylyl cyclase and PI turnover was also demonstrated by transfection of the receptors into Chinese hamster ovary cells (9). However, coupling of the cloned opioid receptors to Ca^{2+} channels in an environment lacking endogenous opioid receptors remains to be evaluated.

In an attempt to investigate coupling of cloned μ-opioid receptors to voltage-activated Ca^{2+} channels, we stably expressed these receptors in the pituitary growth hormone- and prolactin-secreting cell line GH_3 (yielding GH_3MOR cells). This pituitary cell line has voltage-activated Ca^{2+} channels coupled to endogenous SRIF receptors by inhibitory G proteins (10) and lacks endogenous functional opioid receptors (11). Our biochemical and electrophysiological studies demonstrate that the cloned μ-opioid receptors couple to both adenylyl cyclase and voltage-activated Ca^{2+} channels in GH_3MOR cells.

Materials and Methods

Cell cultures. GH_3 cells (CCL 82.1), obtained from the American Type Culture Collection (Rockville, MD), were maintained in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal calf serum, 0.05 IU/ml penicillin, and 50 μg/ml streptomycin and were incubated in a humidified atmosphere of 5% CO_2/95% O_2, at 37°. Cells were harvested once each week by treatment with phos-

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ABBREVIATIONS: PI, phosphatidylinositol; SRIF, somatostatin; DAMGO, [D-Ala^2,Me-Phe^4,Gly-ol^5]-enkephalin; DPDPE, [D-Pen^{2,5}]-enkephalin; PTX, pertussis toxin; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

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phate-buffered saline containing EDTA (1 mM) and were reseeded at 20% of their original density, either into 75-cm^2 tissue culture flasks (for binding assays) or into 35-mm-diameter culture dishes (Costar) (for use in electrophysiological studies). The incubation medium was changed every 2–3 days.

Transfection, selection, and screening of GH_3MOR cells. GH_3 cells (5×10^6 in 0.5 ml of phosphate-buffered saline, pH 7.4) were transfected by electroporation (1000 μF, 150 V) in the presence of 10 μg of the expression vector pRC/CMV. The μ-opioid receptor cDNA (MOR-1) plasmid contained the entire coding region for the rat μ-opioid receptor along with 200 base pairs of 5′ and 30 base pairs of 3′ noncoding regions. Previous studies demonstrated μ-opioid receptor binding in COS-7 cells in which this construct was transiently expressed (5). Colonies were selected with Geneticin (1 mg/ml) starting 72 hr after electroporation. Expression of μ-opioid receptors was confirmed by screening of the selected colonies for the ability of the μ-opioid receptor-selective antagonist naloxone (1 μM) to compete with binding of the nonselective opioid receptor antagonist [^{3}H]diprenorphine (2 nM) in whole cells, as described previously (12).

Opioid 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). The cell homogenates were then centrifuged at 20,000 × g for 1 hr and the resultant pellet was resuspended in 50 mM Tris·HCl. All opioid receptor binding was performed using 250 μg of membrane protein in 50 mM Tris·HCl, pH 7.6, with 10 mM $MgCl_2$, at room temperature for 90 min, as described previously (13). For saturation binding studies, [^{3}H]diprenorphine concentrations of 0.01–10 nM were used, with nonspecific binding being determined in the presence of 1 μM levels of the opioid receptor antagonist naloxone. Data obtained were subjected to Scatchard analysis, and estimates of affinity (K_d) and receptor density (B_{max}) were obtained (see Curve fitting and statistics). In competition binding experiments the ability of increasing concentrations (0.01 nM to 10 μM) of various opioid receptor ligands to displace the binding of [^{3}H]diprenorphine (2 nM) was assessed. The concentrations of opioid receptor ligands required to produce a 50% reduction in [^{3}H]diprenorphine binding (IC_{50}) were determined and then converted to a measure of receptor affinity (K_i) using the Cheng-Prusoff equation (14).

Adenylyl cyclase assays. The ability of various opioid receptor agonists to inhibit forskolin (10 μM)-stimulated cAMP production was assessed as described previously (13). Briefly, measurements were made with cells seeded into 17-mm (24-well) plates and cultured to confluency for 4 days. Medium was changed 24 hr before the assay. [^{3}H]Adenine was then used to label intracellular ATP pools, and radioactive cAMP, generated in the presence of forskolin with increasing concentrations of various opioid receptor agonists, was separated from other nucleotides using Dowex and alumina column chromatography, as described by White and Karr (15).

Electrophysiological recordings. Single cells were voltage-clamped, and voltage-activated Ca^{2+} channel activity was recorded from whole GH_3 and GH_3MOR cells using a List EPC-7 patch-clamp amplifier. Before recording, culture dishes containing cells were superfused (flow rate, 2 ml/min) with a solution containing 140 mM NaCl, 2.8 mM KCl, 2 mM $MgCl_2$, 1 mM $CaCl_2$, and 10 mM HEPES (adjusted to pH 7.2 with NaOH). After a high resistance seal was established and access to the interior of the cell was obtained, the external solution 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 1 μM tetrodotoxin (adjusted to pH 7.2 with NaOH). In most experiments performed with a holding potential of −40 mV, the external $BaCl_2$ concentration was increased to 30 mM by equimolar substitution for NaCl. The recording electrode contained 120 mM CsCl, 10 mM EGTA, 1 mM $MgCl_2$, 3 mM Mg-ATP, and 10 mM HEPES (adjusted to pH 7.2 with CsOH) (all from Sigma). Ba^{2+} was used as the charge carrier to maximize currents through Ca^{2+} channels and to inhibit some K^+ channels. Use of intracellular CsCl and extracellular tetrodotoxin abolished currents through K^+ and Na^+ channels, respectively.

Ba^{2+} currents were usually activated by step depolarizations of membrane potential from a holding potential of −80 mV for 100 msec. In experiments investigating the voltage dependence of nimodipine inhibition of Ba^{2+} currents, cells were also held at −40 mV. Capacitance and series resistance compensations were achieved using the patch-clamp amplifier. Residual artifacts and leakage currents were nulled using a $P/4$ subtraction.

Patch electrodes were manufactured from thin-walled, borosilicate glass pipettes (World Precision Instruments) using a Narishige (PP-83) electrode puller. Whole-cell currents, monitored using the EPC-7 amplifier, were low-pass filtered with an eight-pole Bessel filter (Frequency Devices) at 1 kHz, digitized (Labmaster DMA interface; Axon Instruments) at a frequency of 5 kHz, and stored on an IBM PC hard disk. 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 (16). A computer-generated "best fit" of linear regression data was used to discriminate a one-site from a two-site fit and to provide K_d and B_{max} estimates. Dose-response curves were fitted with the logistic function $R = R_{max} - E_{max}/[1 + (E_{50}/x)^n]$. For fitting of ligand binding data, R represents bound [^{3}H]diprenorphine as a percentage of control, R_{max} is maximal [^{3}H]diprenorphine binding (percentage of control), E_{max} is the maximal inhibition of binding by DAMGO, E_{50} is the concentration of DAMGO that causes half-maximal inhibition of binding, n is the slope factor, and x is the DAMGO concentration. For adenylyl cyclase data, R represents forskolin-stimulated cAMP accumulation as a percentage of control, R_{max} is the maximal forskolin-stimulated cAMP accumulation (percentage of control), E_{max} is the maximal inhibition by DAMGO, E_{50} is the IC_{50}, n is the Hill coefficient, and x is the DAMGO concentration. For electrophysiological data, R represents the peak Ba^{2+} current amplitude as a percentage of control, R_{max} is maximal current (percentage of control), E_{max} is the maximal inhibition by DAMGO, E_{50} is the IC_{50}, n is the Hill coefficient, and x is the DAMGO concentration. All data are presented as mean ± standard error, and comparisons were made using the Student t test.

PTX treatment. GH_3 cells were placed in culture medium containing 100–200 ng/ml PTX (Sigma) for 12–36 hr before electrophysiological recordings. Ba^{2+} currents were recorded from control and PTX-treated cells on the same day, for comparison.

Drugs used. Nimodipine was a kind gift from Dr. Andrew Charles, University of California, Los Angeles, and Bay K 8644 was purchased from Calbiochem (La Jolla, CA). ω-Conotoxin GVIA was purchased from Research Biochemicals (Natick, MA), SRIF, DPDPE, and DAMGO were obtained from Peninsula Laboratories (San Carlos, CA), and morphine sulfate was obtained from Sigma Chemical Co. (St. Louis, MO). Naloxone was provided by the National Institute on Drug Abuse. All tissue culture reagents, including Geneticin (G418), were purchased from GIBCO-BRL (Gaithersburg, 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). [^{32}P]cAMP was prepared by enzymatic conversion of [α-^{32}P]ATP using NG108–15 membranes, as described (13). Forskolin was obtained from List Biologicals (Campbell, CA).

Results

Opioid receptor binding in GH_3MOR cells. GH_3 cells were transfected with pRC/CMV plasmids containing the MOR-1 construct, and 24 clones were obtained after selection with Geneticin (1 mg/ml). Subsequent screening to confirm

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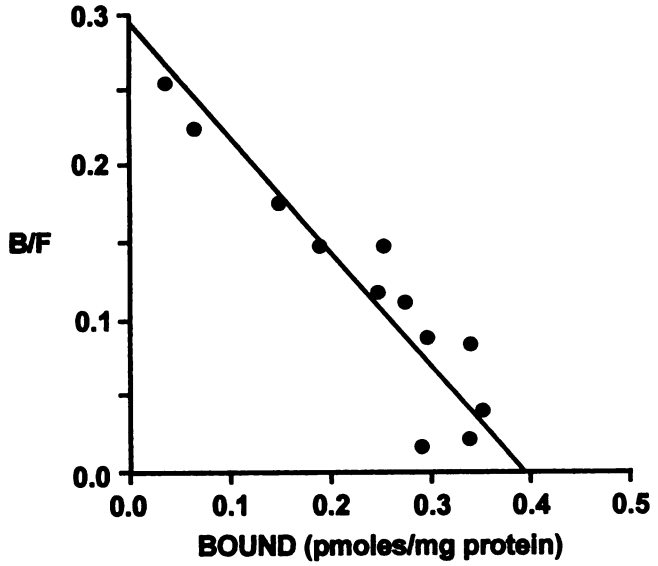

Fig. 1. Scatchard analysis of [^{3}H]diprenorphine binding to GH_3MOR cell membranes. [^{3}H]Diprenorphine binding (0.01–10 nM) was saturable and the data were best fitted with a one-site model (see Materials and Methods). The K_d and B_{max} values were 0.33 nM and 0.39 pmol/mg of protein, respectively. Each point represents the mean of triplicate determinations (standard errors were <10%).

μ-opioid receptor expression in all clones was accomplished by measuring the ability of the μ-opioid receptor antagonist naloxone (1 μM) to displace [^{3}H]diprenorphine (2 nM) binding in whole cells. Seven of these clones exhibited various degrees of μ-opioid receptor antagonist-displaceable [^{3}H]diprenorphine binding. The clone that expressed the highest level of binding upon initial screening was selected for all subsequent studies and was designated GH_3MOR. Scatchard analysis of [^{3}H]diprenorphine saturation binding in GH_3MOR membranes revealed the presence of a single high affinity binding site (K_d = 0.33 ± 0.02 nM), with a receptor density (B_{max}) of 0.39 ± 0.02 pmol/mg of protein (Fig. 1). Further investigation in competition binding experiments using [^{3}H]diprenorphine (2 nM) revealed that the μ-opioid receptor ligands DAMGO (Fig. 2A) and morphine also showed high affinity for the expressed receptor (K_i values of 1.0 ± 0.6 and 7.2 ± 1.6 nM, respectively) (Table 1). The concentration of [^{3}H]diprenorphine used in these competition binding experiments was saturating (approximately 6 times the K_d of the drug); this relatively high concentration restricts the maximal observed displacement by DAMGO (Fig. 2A). In contrast to DAMGO and morphine, the selective δ-opioid receptor ligand DPDPE bound to GH_3MOR membranes with a K_i of 310 ± 105 nM (Table 1). Untransfected GH_3 cells did not exhibit detectable [^{3}H]diprenorphine binding (11). The affinities of the μ-opioid receptor ligands presented in this study are similar to those reported for mammalian brain membranes (17) and in a previous investigation in which this μ-opioid receptor construct was expressed in Chinese hamster ovary cells.[1]

Opioid receptor-mediated inhibition of adenylyl cyclase activity in GH_3MOR cells. The inhibition of adenylyl cyclase activity by various opioid receptor agonists was investigated in GH_3MOR cells. The maximal inhibition of forskolin (10 μM)-stimulated cAMP production by DAMGO was

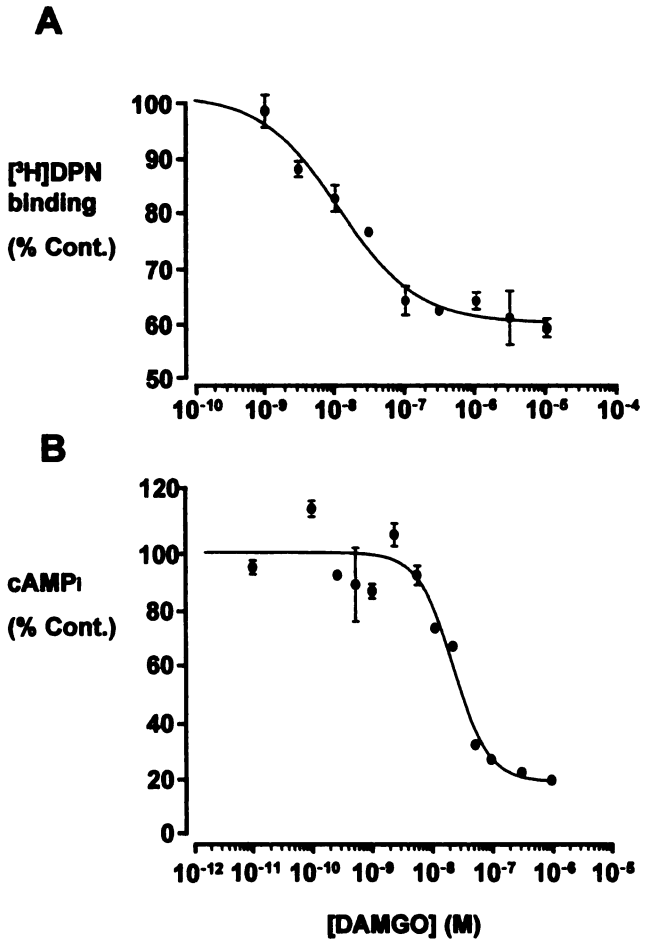

Fig. 2. Opioid receptor binding and inhibition of adenylyl cyclase activity in GH_3MOR cells. A, [^{3}H]Diprenorphine (*[^{3}H]DPN*) binding in the presence of DAMGO. GH_3MOR membranes were incubated at room temperature for 90 min with [^{3}H]diprenorphine (2 nM), in the presence of increasing concentrations (logarithmic scale) of the μ-opioid receptor ligand DAMGO (1 nM to 10 μM). B, Inhibition of forskolin (10 μM)-stimulated adenylyl cyclase activity. The ability of increasing concentrations of the μ-opioid receptor agonist DAMGO (0.01 nM to 1 μM) to inhibit [^{3}H]cAMP accumulation was assessed in GH_3MOR cells as described in Materials and Methods. Data points represent the mean values from three experiments. *Vertical bars*, standard errors. Curves were fitted to data points using a logistic equation (IC_{50} = 21.9 nM, Hill coefficient = 1.4) (see Materials and Methods).

TABLE 1

GH_3MOR cells exhibit μ-opioid receptor specific binding and activated receptors inhibit forskolin-stimulated adenylyl cyclase activity

Opioid receptor binding and measurement of forskolin-stimulated [^{3}H]cAMP production were performed as described in Materials and Methods. Data represent the mean ± standard error obtained from three experiments.

Agonist	Binding, K_i	Adenylyl cyclase inhibition	
		IC_{50}	INH_{max}[a]
	nM	nM	%
DAMGO	1.0 ± 0.6	21.9 ± 4.1	77.3 ± 3.7
Morphine	7.2 ± 1.6	55.2 ± 8.5	57.0 ± 2.9
DPDPE	310 ± 105	>10,000	58.0 ± 3.5

[a] INH_{max}, maximal agonist-induced inhibition.

[1] S. Chakrabarti, P. L. Prather, L. Yu, P. Y. Law, and H. H. Loh. Expression of the μ-opioid receptor in CHO cells: ability of μ-opioid ligands to promote [^{35}S]-α-azidoanilido-GTP labeling of multiple G protein α-subunits. Submitted for publication.

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77.3 ± 3.7% (Fig. 2B; Table 1). For comparison, morphine and the δ-opioid receptor-selective agonist DPDPE caused only 57.0 ± 2.9 and 58.0 ± 3.5% inhibition, respectively (Table 1). In addition, the amount of DAMGO required to produce 50% inhibition of adenylyl cyclase activity (IC_{50}) was only 21.9 ± 4.1 nM, whereas morphine required 55.2 ± 8.5 nM (Table 1). Although the selective δ-opioid receptor agonist DPDPE (100 μM) was able to produce 58.0 ± 3.5% inhibition of cAMP accumulation, the IC_{50} value for this ligand was >10 μM (Table 1). Inhibitions of cAMP accumulation by DAMGO (1 μM), morphine (1 μM), and DPDPE (10 μM) were blocked by naloxone (10 μM). Taken collectively, data obtained from both opioid receptor binding and adenylyl cyclase assays provide evidence that GH_3MOR cells express functional μ-opioid receptors.

Voltage-activated Ba^{2+} currents in GH_3 cells. Inward Ba^{2+} currents were activated by depolarizing cells from a holding potential of −80 mV to voltages between −60 and 50 mV (Fig. 3, A and B). The threshold of activation was approximately −50 mV and currents peaked when GH_3 cells were depolarized to 0 mV. Ba^{2+} currents activated by depolarization to voltages between −50 and 0 mV appeared to be sustained, whereas at more positive voltages currents became more transient in appearance (Fig. 3, A and B). To illustrate the Ba^{2+} current-voltage relationship in GH_3 cells, normalized peak current amplitudes (I/I_{max}) from 15 cells were averaged and plotted against voltage (Fig. 3C). A characteristic U-shaped current-voltage relationship was observed.

Various pharmacological agents and biophysical parameters have been used to characterize Ca^{2+} channels in GH_3 and other cells (18–24). For example, the dihydropyridines are commonly used to identify L-type Ca^{2+} channels (19, 21, 23, 24). We investigated the sensitivity of Ba^{2+} currents to nimodipine (1 nM to 100 μM), at holding potentials of −80 and −40 mV. Currents activated by stepping from −80 to 0 mV were dose-dependently inhibited by nimodipine, with an IC_{50} of 606 nM (Fig. 4, A and B). The time course of the inhibitory action of nimodipine (10 and 100 μM) and representative traces are illustrated in Fig. 4, A and B.

Currents activated by depolarization from −40 to 0 mV with 10.8 mM Ba^{2+} in the recording chamber declined after several minutes and only partially recovered when the holding potential was changed to −80 mV (n = 8) (data not shown). This rapid current run-down was hindered by increasing the extracellular Ba^{2+} concentration to 30 mM. Under these conditions the charge screening caused by the higher concentration of external divalent cations shifted the current-voltage relationship so that peak currents were activated by stepping to 10 mV.

Consistent with previous reports of voltage-dependent inhibition of L-type Ca^{2+} channels by dihydropyridines (19, 25, 26), nimodipine was more potent as a Ba^{2+} current inhibitor when GH_3 cells were held at −40 mV than when cells were clamped at −80 mV (Fig. 4C). When cells were held at −40 mV, nimodipine inhibited Ba^{2+} currents with an IC_{50} of 98 nM. Both the peak and sustained phases of the Ba^{2+} currents were inhibited by nimodipine; however, inhibition of the sustained component was greater, leaving a current with a more transient appearance (Fig. 4). Having demonstrated that nimodipine inhibits Ba^{2+} currents in a voltage-dependent manner in GH_3 cells, in subsequent experiments we used a holding potential of −80 mV to minimize current run-down (unless otherwise stated).

The dihydropyridine Bay K 8644 enhances current through L-type Ca^{2+} channels (27). Bay K 8644 (500 nM) potentiated

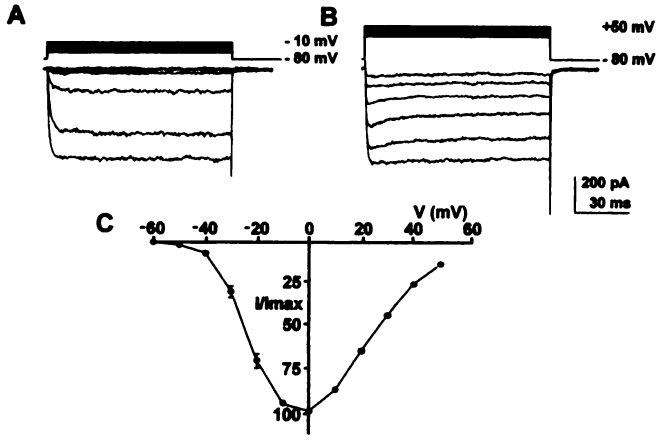

Fig. 3. Whole-cell Ba^{2+} currents in GH_3 cells. A, Ba^{2+} currents through voltage-activated Ca^{2+} channels, recorded using the whole-cell voltage-clamp technique. Currents were evoked by depolarizing voltage steps from a holding potential of −80 mV to voltages between −60 and −10 mV (10-mV increments), for 100 msec. B, Ba^{2+} currents activated by steps from −80 mV to voltages between 0 and 50 mV, recorded from the same cell as in A. C, Relationship between peak Ba^{2+} current amplitude and voltage. Current amplitudes were calculated as a percentage of the peak current recorded from individual cells (I/I_{max}). Each data point represents mean data from 15 cells. *Vertical bars*, standard error (when larger than the symbol).

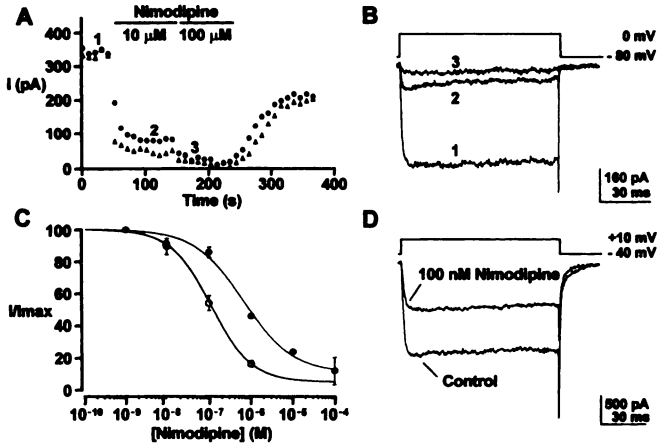

Fig. 4. Voltage-dependent inhibition of Ba^{2+} currents by nimodipine in GH_3 cells. A, Graph of peak (●) and sustained (△) Ba^{2+} current amplitude (holding potential, −80 mV) before, during, and after nimodipine (10 and 100 μM) application (sampled every 10 sec). B, Individual Ba^{2+} currents before (*1*) and in the presence of 10 μM (*2*) and 100 μM (*3*) nimodipine, sampled at the time points indicated in A. C, Graph illustrating the dose-response relationship for Ba^{2+} current inhibition by nimodipine with holding potentials of −80 and −40 mV. Currents were recorded using the voltage protocols in B (●) and in D (○). Currents measured in the presence of the drug were expressed as percentage of control currents (I/I_{max}). Data points are averages of at least four determinations. *Vertical bars*, standard errors (when larger than the symbols). Curves were fitted to data points using a logistic equation (IC_{50} = 606 nM, Hill coefficient = 0.7, and IC_{50} = 98 nM, Hill coefficient = 0.8, for holding potentials of −80 and −40 mV, respectively) (see Materials and Methods). D, Representative current traces illustrating the action of nimodipine (100 nM) on a whole-cell Ba^{2+} current activated by depolarization from −40 to +10 mV.

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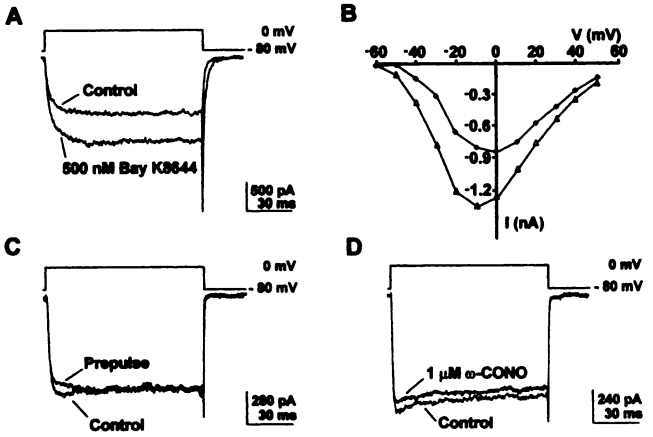

Fig. 5. Ca^{2+} channel subtypes in GH_3 cells. A, Superimposed Ba^{2+} currents in the absence and presence of Bay K 8644 (500 nM). The dihydropyridine increased the amplitude of the current and decreased the rate of deactivation (seen as a prolongation of the tail current). The drug effect was reversible after a prolonged wash. B, Current-voltage relationship of Ba^{2+} currents recorded from a cell before (♦) and during (△) the application of Bay K 8644 (500 nM). C, An example of a control Ba^{2+} current and a current recorded from the same cell after a prepulse to −20 mV for 200 msec. In this cell the prepulse inhibited a small transient Ba^{2+} current component. D, A control Ba^{2+} current and a current recorded from the same cell in the presence of ω-conotoxin (ω-CONO) (1 μM). In A, C, and D, currents were activated by depolarization of cells from a holding potential of −80 mV to 0 mV.

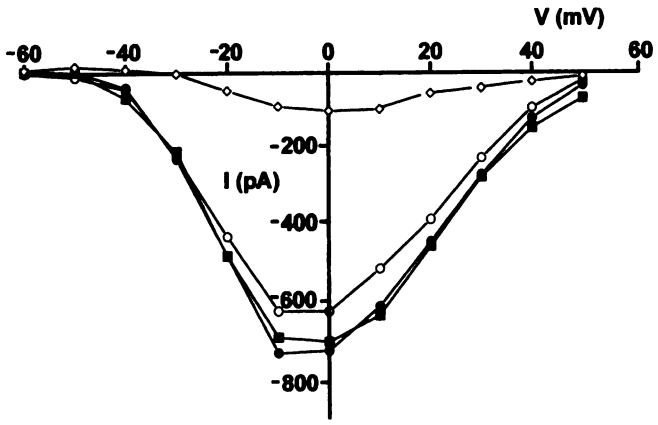

Fig. 6. Current-voltage relationship of the Ba^{2+} currents inhibited by ω-conotoxin in GH_3 cells. Ba^{2+} currents were evoked by depolarization of cells to 0 mV from the holding potential of −80 mV before (●) and during (○) the application of ω-conotoxin (1 μM). The current-voltage relationship for the ω-conotoxin-sensitive current component, calculated by subtracting currents obtained during ω-conotoxin (1 μM) application (○) from control currents (●), is also shown (◇). The current-voltage relationship of Ba^{2+} currents recorded after prepulses to −20 mV (duration, 200 msec) was plotted for comparison (■). This protocol was adopted to assess the amplitude of the T-type current component in this cell.

Ba^{2+} currents recorded from GH_3 cells by 39.3 ± 4.0% (n = 6) and caused a characteristic slowing of current deactivation (Fig. 5A). The current-voltage relationship was shifted so that the threshold of activation occurred at −50 mV and peak currents were activated by voltage steps to −10 mV (Fig. 5B). Taken together, the dramatic block of Ba^{2+} currents by nimodipine (1 μM) at −40 mV (Fig. 4) and their facilitation by Bay K 8644 (Fig. 5) suggest that GH_3 cells express predominantly L-type Ca^{2+} channels.

T-type Ca^{2+} channels in various preparations inactivate rapidly at depolarizing voltages (18, 20, 21). Depolarizing prepulses (to −20 mV for 200 msec) sufficient to inactivate T-type channels in NG108–15 cells[2] and in other preparations (20, 22) caused a small and inconsistent inhibition of the peak (2.7 ± 2.2%, n = 6) but not sustained Ba^{2+} current (Figs. 5C and 6). Thus, under these conditions the contribution of T-type channels to Ba^{2+} currents recorded from GH_3 cells appears minimal.

ω-Conotoxin has been used in previous studies to irreversibly block N-type Ca^{2+} channels (22–24). In contrast, in GH_3 cells ω-conotoxin (1 μM) reversibly inhibited Ba^{2+} currents by 8.8 ± 1.1% (n = 10) (Figs. 5 and 6). The effect of 1 μM ω-conotoxin appeared to be maximal, because when it was applied at 3 μM the drug produced no greater inhibition (data not shown). Fig. 6 shows the current-voltage relationship for the ω-conotoxin (1 μM)-sensitive Ba^{2+} current. The ω-conotoxin-sensitive Ba^{2+} current component was activated at approximately −20 mV and peaked between 0 and 10 mV.

The fact that the Ba^{2+} current inhibition by ω-conotoxin was reversible suggests that the inhibition may not be due to block of N-type Ca^{2+} channels in GH_3 cells. ω-Conotoxin has been reported to reversibly inhibit L-type Ca^{2+} channels (23). We investigated whether ω-conotoxin reversibly inhibits L-type Ca^{2+} channels in GH_3 cells, by comparing its inhibition of Ba^{2+} currents in the presence and absence of Bay K 8644. As stated above, Bay K 8644 (500 nM) enhanced Ba^{2+} currents by 39.3 ± 4.0% (n = 6) (Fig. 5A). In the presence of the dihydropyridine, ω-conotoxin inhibited currents by 17.7 ± 2.9% (n = 6), which is a significantly larger inhibition (p < 0.005) than seen in the absence of Bay K 8644. The increased Ba^{2+} current inhibition by the toxin in the presence of Bay K 8644 suggests that this effect is caused by an interaction with L-type Ca^{2+} channels.

Coupling of μ-opioid receptors to Ca^{2+} channels in GH_3MOR cells via PTX-sensitive G proteins. Whereas SRIF (1 μM) inhibited whole-cell Ba^{2+} currents recorded from GH_3 cells (49% of cells responded) by 17.2 ± 2.1% (n = 11), DAMGO (0.1–10 μM), a selective μ-opioid receptor agonist, was without effect (n = 11) (Fig. 7). In contrast to Ba^{2+} currents recorded from untransfected cells, those recorded from GH_3MOR cells (65% of cells responded) were inhibited by DAMGO (1 μM) (Fig. 8). The time course of inhibition by DAMGO (1 μM) and SRIF (1 μM) and superimposed Ba^{2+} currents in the presence and absence of drugs appeared similar (Fig. 8). The inhibition of GH_3MOR Ba^{2+} currents by DAMGO (0.1 nM to 10 μM) was dose dependent, with an IC_{50} of 105 nM (see Fig. 10). Consistent with DAMGO (100 nM) activating μ-opioid receptors in these cells, the inhibitory effect of the drug was reversed by its coapplication with naloxone (1 μM) (n = 5) (Fig. 9C). The μ-opioid receptor ligand morphine (1 μM) also inhibited Ba^{2+} currents in GH_3MOR cells (Fig. 9), although to a lesser degree (13.5 ± 1.1%, n = 9) than did the same concentration of DAMGO (Fig. 10). The effect of the δ-opioid receptor-selective ligand DPDPE (1 μM) on Ba^{2+} currents is illustrated in Fig. 9B. In this instance, no inhibition by DPDPE (1 μM) was observed. However, in some cells DPDPE (1 μM) inhibited Ba^{2+} currents to a small degree (maximal inhibition, 6.6%), with the average inhibition being only 3.5 ± 2.1% (n = 9) (Fig. 10). Consistent with DPDPE (1 μM) and morphine (1 μM) activat-

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

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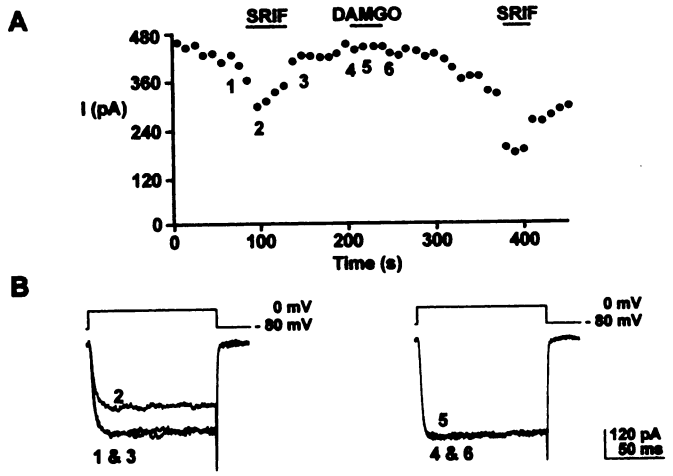

Fig. 7. Evidence that GH_3 cells express SRIF receptors but lack functional μ-opioid receptors. A, Time course of the effects of DAMGO (1 μM) and SRIF (1 μM) on Ba^{2+} currents in nontransfected GH_3 cells. Currents were activated by depolarization of the cell from −80 to 0 mV every 10 sec. B, *Left*, individual Ba^{2+} currents before (*1*), during (*2*), and after (*3*) SRIF (1 μM) application; *right*, currents before (*4*), during (*5*), and after (*6*) DAMGO (1 μM) application. *Numbers*, time points illustrated in A.

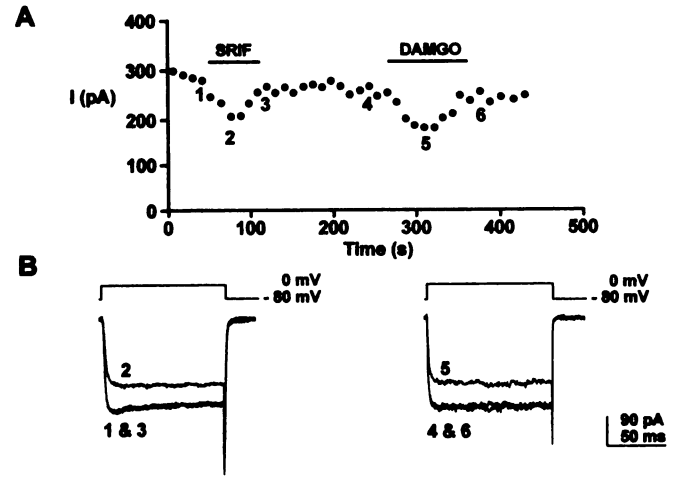

Fig. 8. Ca^{2+} channel inhibition by SRIF and DAMGO in GH_3MOR cells. A, Time course of Ba^{2+} current inhibition by DAMGO (1 μM) and SRIF (1 μM) in GH_3 cells stably transfected with the rat μ-opioid receptor. B, *Left*, individual Ba^{2+} currents before (*1*), during (*2*), and after (*3*) the application of SRIF (1 μM); *right*, currents before (*4*), during (*5*), and after (*6*) the application of DAMGO (1 μM). *Numbers*, time points illustrated in A.

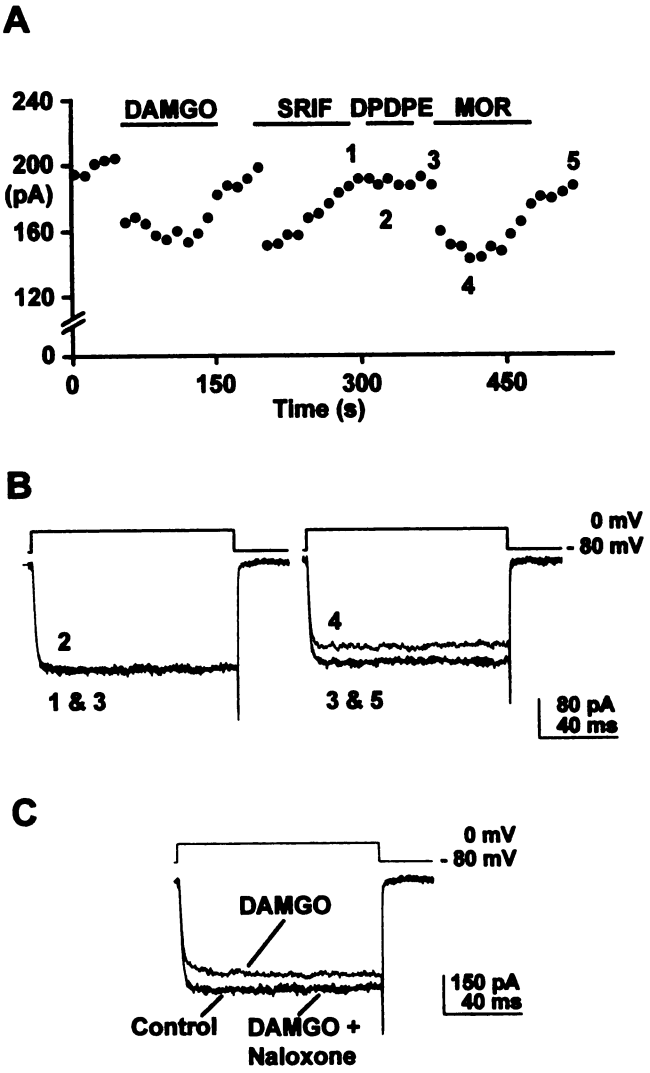

Fig. 9. μ-Opioid receptor pharmacology in GH_3MOR cells. A, Time course of the modulatory actions of DAMGO (1 μM), SRIF (1 μM), DPDPE (1 μM), and morphine (*MOR*) (1 μM) on Ba^{2+} currents in a GH_3 cell expressing the μ-opioid receptor. SRIF, DAMGO, and morphine inhibited Ba^{2+} currents in this GH_3MOR cell, but the δ-opioid receptor-selective agonist DPDPE had no effect. B, *Left*, Ba^{2+} currents before (*1*), during (*2*), and after (*3*) the application of DPDPE (1 μM); *right*, currents before (*3*), during (*4*), and after (*5*) the application of morphine (1 μM). *Numbers*, time points in A. C, Reversal of DAMGO (100 nM) inhibition of Ba^{2+} currents by the coapplication of DAMGO (100 nM) with naloxone (1 μM). Data shown in B and C were recorded from different GH_3 MOR cells.

ing μ-opioid receptors in GH_3MOR cells, their inhibitions of Ba^{2+} currents were reversed by naloxone (1 μM) (n = 3 in both cases).

Both the Ba^{2+} current inhibition by SRIF (1 μM) and that by DAMGO (1 μM) were sensitive to PTX pretreatment in GH_3MOR cells (Fig. 11). In untreated cells Ba^{2+} currents were inhibited by 23.8 ± 1.0% (n = 6) and 22.9 ± 2.5% (n = 6) by DAMGO (1 μM) and SRIF (1 μM), respectively. In parallel experiments, exposure to PTX at 100 ng/ml and 200 ng/ml reduced Ba^{2+} current inhibition by DAMGO (1 μM) to 10.7 ± 1.1% (n = 3) and 0.6 ± 0.6% (n = 6), respectively. Likewise, Ba^{2+} current inhibition by SRIF (1 μM) was reduced to 11.9 ± 1.7% (n = 3) by pretreatment with 100 ng/ml PTX and to 3.4 ± 1.2% (n = 6) by pretreatment with 200 ng/ml PTX (Fig. 11). PTX (200 ng/ml)-pretreated GH_3MOR cells remained sensitive to ω-conotoxin (1 μM) (n = 3) (data not shown).

DAMGO inhibition of L-type Ca^{2+} channels in GH_3MOR cells. Nimodipine (10 μM) inhibited Ba^{2+} currents activated by test pulses from −80 to 0 mV by 75.7 ± 1.0% (n = 11) (Figs. 4 and 12). The remaining nimodipine (10 μM)-insensitive current was further inhibited by coapplication of the dihydropyridine with either DAMGO (1 μM), SRIF (1 μM), or ω-conotoxin (1 μM) in GH_3MOR cells (Fig. 12). The amplitude of DAMGO (1 μM) inhibition of Ba^{2+} currents was significantly ($p < 0.001$) decreased in the presence of nimodipine (10 μM) (Fig. 13). This suggests that L-type channel inhibition causes a reduction in the Ca^{2+} channels available for inhibition by DAMGO and therefore μ-opioid receptors

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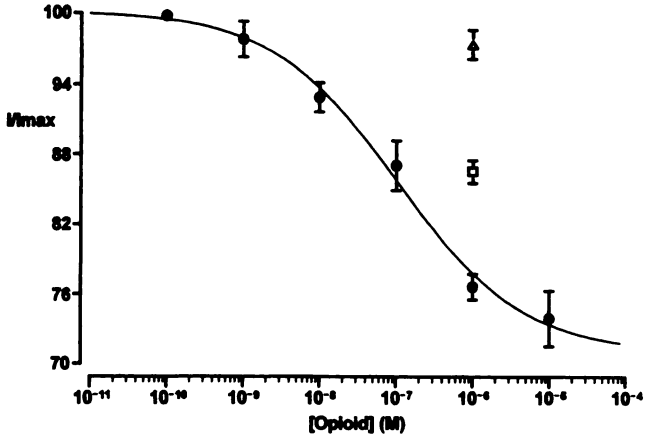

Fig. 10. Dose-dependent inhibition of Ba^{2+} currents by DAMGO in GH_3MOR cells. A graph of Ba^{2+} current amplitude (normalized to control amplitude, I/I_{max}) plotted against opioid receptor ligand concentration (logarithmic scale) is shown. DAMGO (●) (0.1 nM to 10 μM, n = 4) inhibited Ba^{2+} currents in a dose-dependent manner (IC_{50} = 105 nM, Hill coefficient = 0.5). Data points were fitted with a logistic equation (see Materials and Methods). Morphine (□) (1 μM, n = 9) and DPDPE (△) (1 μM, n = 9) also inhibited Ba^{2+} currents in GH_3MOR cells. Data points represent mean values obtained from at least four cells. *Vertical bars*, standard error.

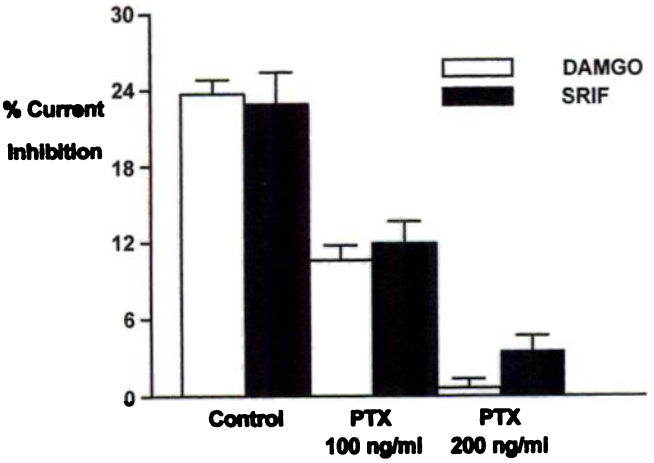

Fig. 11. PTX sensitivity of Ba^{2+} current inhibition by DAMGO and SRIF in GH_3MOR cells. The inhibitory actions of DAMGO (1 μM, n = 6) and SRIF (1 μM, n = 6) on Ca^{2+} channel activity were inhibited by PTX pretreatment (100 and 200 ng/ml, n = 3 and n = 6, respectively). Control and PTX experiments were performed in parallel cultures on the same day. Values are means; *vertical bars*, standard errors.

couple to L-type channels in GH_3MOR cells. Because it was possible that a high concentration of nimodipine could inhibit other Ca^{2+} channel types in these cells, DAMGO was applied in the presence and absence of nimodipine (100 nM) in cells clamped at −40 mV. The lower concentration of the dihydropyridine caused a 47.2 ± 4.5% (n = 4) inhibition of Ba^{2+} currents at this potential (Fig. 4). Consistent with the observation with 10 μM nimodipine at −80 mV, 100 nM nimodipine at −40 mV caused a significant ($p < 0.05$) reduction in the amplitude of the DAMGO inhibition of Ba^{2+} currents (Fig. 13).

Discussion

Using biochemical and electrophysiological techniques, we have demonstrated that activation of cloned rat μ-opioid

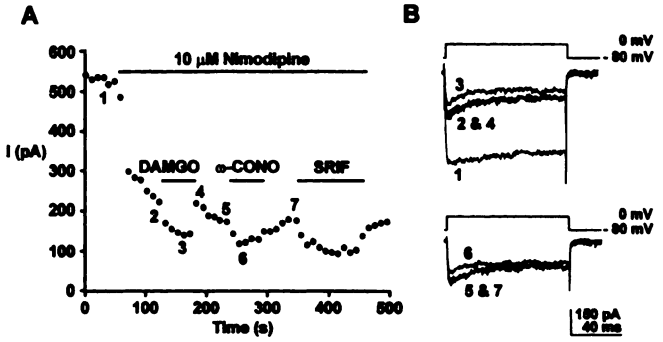

Fig. 12. DAMGO, SRIF, and ω-conotoxin inhibition of Ba^{2+} currents in GH_3MOR cells in the presence of nimodipine. A, Time course of Ba^{2+} current inhibition by DAMGO (1 μM), ω-conotoxin (*ω-CONO*) (1 μM), and SRIF (1 μM) in the presence of nimodipine (10 μM). B, *Top*, Ba^{2+} currents under control conditions (*1*) and in the presence of nimodipine (10 μM) before (*2*), during (*3*), and after (*4*) the application of DAMGO (1 μM); *bottom*, individual currents in the presence of nimodipine (10 μM) before (*5*), during (*6*), and after (*7*) the application of ω-conotoxin (1 μM). *Numbers*, time points in A.

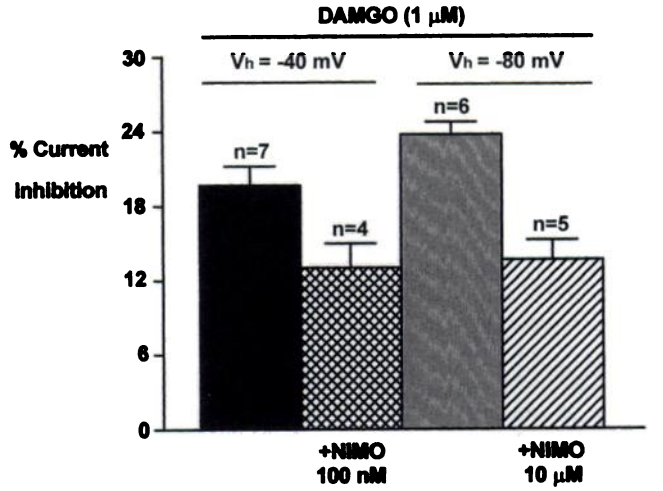

Fig. 13. Inhibition of L-type Ca^{2+} channels by DAMGO in GH_3MOR cells. The inhibitory action of DAMGO (1 μM) in the absence and presence of nimodipine (*NIMO*) (100 nM and 10 μM) is shown. The effects of nimodipine at 100 nM and 10 μM were observed with holding potentials of −40 and −80 mV, respectively. Ba^{2+} current inhibition by DAMGO was expressed as a percentage of the control current (in the absence of any drug). Measurements were obtained from different cells (*numbers above each bar*). Data are presented as mean ± standard error.

receptors, stably expressed in GH_3 cells (GH_3MOR cells), can modulate both adenylyl cyclase and Ca^{2+} channel activity. A large number of G protein-coupled receptors modulate Ca^{2+} conductances via stimulatory or inhibitory G proteins (28). Although the number of cloned G protein-coupled receptors is increasing, coupling between these cloned receptors and Ca^{2+} channels has been tested in only a few cases (29–31). Stable expression of the μ-opioid receptor clone in GH_3 cells provides a system in which to assess the coupling of the receptor to native Ca^{2+} channels. The interaction between endogenous opioid receptors and ion channels has been well characterized in cultured neurons and cell lines (32); however, some of these cells express multiple types of opioid receptors (33). In contrast, GH_3MOR cells are advantageous for studying the mechanism of action of a single cloned opioid receptor subtype.

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In addition to demonstrating that the μ-opioid receptor-specific ligand DAMGO inhibited Ba^{2+} currents in GH_3MOR cells, we also sought to determine which Ca^{2+} channel subtypes were involved. Previous studies, conducted in dorsal root ganglion neurons and SH-SY5Y cells, concluded that activated μ-opioid receptors inhibit N-type Ca^{2+} channels (34–37), and one group reported an additional inhibition of T-type Ca^{2+} channels (34). Although tail current analysis has revealed T-type Ca^{2+} channel activity in GH_3 cells (18), under our recording conditions the low voltage-activated component contributed <3% of the total Ba^{2+} current. This observation suggests that T-type channel inhibition is not responsible for the relatively large effects of DAMGO and SRIF in GH_3MOR cells. In addition, DAMGO inhibited Ba^{2+} currents when cells were held at −40 mV, a potential at which T-type channels inactivate (18, 19, 21).

In agreement with previous studies of Ca^{2+} channel pharmacology in GH_3 cells (19, 20), Ba^{2+} currents were inhibited by nimodipine and enhanced by Bay K 8644. The inhibitory dihydropyridine was more potent when cells were clamped at a more depolarized potential (−40 mV); under such conditions most of the current was blocked by low concentrations of nimodipine. These observations are consistent with GH_3 cells expressing predominantly L-type Ca^{2+} channels.

Previous reports suggested that Ba^{2+} currents recorded from GH_3 cells are sensitive to the N-type Ca^{2+} channel inhibitor ω-conotoxin (20). We also demonstrated that Ba^{2+} currents were reversibly inhibited by ω-conotoxin, both in the absence and in the presence of 10 μM nimodipine. Although reversible inhibition of a component of N-type Ca^{2+} channels has been seen in PC-12 cells and sympathetic neurons (24), the effects of ω-conotoxin on this channel subtype are thought to be predominantly irreversible (22–24). In addition to N-type Ca^{2+} channels, ω-conotoxin reversibly inhibits native L-type channels in chick sensory neurons (23) and recombinant L-type channels expressed in *Xenopus laevis* oocytes (38). In GH_3 cells, Ba^{2+} current inhibition by ω-conotoxin was enhanced by its coapplication with Bay K 8644, suggesting that facilitation of L-type Ca^{2+} channels increases the efficacy of the toxin. Adding to the case against N-type Ca^{2+} channels in GH_3 cells, the cell line has previously been shown to lack transcript for this channel subtype (39). Hence, the pharmacological and molecular biological data suggest that GH_3 cells express predominantly L-type Ca^{2+} channels.

We investigated whether cloned μ-opioid receptors couple to L-type channels in GH_3MOR cells, by coapplying DAMGO with nimodipine. Reduction of the Ba^{2+} current inhibition by DAMGO in the presence of the antagonist suggests that DAMGO inhibits L-type Ca^{2+} channels in GH_3MOR cells. These experiments demonstrate that, in addition to N- and T-type Ca^{2+} channels (34–37), μ-opioid receptors can also couple to L-type Ca^{2+} channels. It is possible that in the previous studies modest coupling of native μ-opioid receptors to L-type channels was overshadowed by more robust coupling to N- and T-type channels. Alternatively, different G proteins may be required to mediate the inhibitory actions of μ-opioid receptors on L-type Ca^{2+} channels, and these may not be present in the other systems studied (34–37). It is also possible that GH_3 cells express L-type Ca^{2+} channels with different properties, compared with their neuronal counterparts.

Our observation of PTX blockade of Ba^{2+} current inhibition by DAMGO and SRIF implies that either G_i or G_o subtypes of G proteins are involved in these inhibitory responses. In dorsal root ganglion neurons, Moises *et al.* (36) have demonstrated that μ-opioid receptors inhibit currents through Ca^{2+} channels via G_o. Furthermore, SRIF receptors have been shown to couple to Ca^{2+} channels via G_{oB} in GH_3 cells (40). These data suggest that the coupling of μ-opioid receptors to Ca^{2+} channels is mediated via G_o in GH_3MOR cells.

From the experiments reported here it is clear that the cloned rat μ-opioid receptor can couple to both adenylyl cyclase and Ca^{2+} channels. Taken together with the observations of Johnson *et al.* (9) and Chen and Yu (7), the data imply that these receptors can modulate adenylyl cyclase, PI turnover, G protein-activated K^+ channels, and voltage-activated Ca^{2+} channels. Interactions of μ-opioid receptors with single or multiple effector systems may depend on the availability of selective G protein complexes.

The potencies of DAMGO in our ligand binding, adenylyl cyclase, and electrophysiological assays were markedly different. Of course, the conditions used for these assays were not consistent; for example, binding assays were performed on membrane preparations, whereas whole cells were used in adenylyl cyclase and patch-clamp experiments. Such disparities between the potencies of G protein-coupled receptor agonists in these assays have been observed previously (1, 35). It is possible that this discrepancy could be due in part to an involvement of different G protein subtypes in coupling to the various effector systems.

In summary, this is the first report of functional coupling between cloned μ-opioid receptors and voltage-activated Ca^{2+} channels. This significant advance provides a system for studying the contribution of ion channels and second messenger systems to the control of vesicular release by μ-opioid receptors.

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