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Coexpression of δ-Opioid Receptors with μ Receptors in GH_3 Cells Changes the Functional Response to μ Agonists from Inhibitory to Excitatory

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ABSTRACT

GH_3 cells show spontaneous activity characterized by bursts of action potentials and oscillations in $[Ca^{2+}]_i$. This activity is modulated by the activation of exogenously expressed opioid receptors. In GH_3 cells expressing only μ receptors (GH_3MOR cells), the μ receptor-specific ligand [D-Ala^2,*N*-Me-Phe^4,Gly^5-ol]-enkephalin (DAMGO) inhibited spontaneous Ca^{2+} signaling by the inhibition of voltage-gated Ca^{2+} channels, activation of inward-rectifying K^+ channels, and inhibition of adenylyl cyclase. In contrast, in cells expressing both μ and δ receptors (GH_3MORDOR cells), DAMGO had an excitatory effect on Ca^{2+} signaling that was mediated by phospholipase C and release of Ca^{2+} from intracellular stores. The excitatory effect of DAMGO was also inhibited by pretreatment with pertussis toxin. Despite the excitatory effect on Ca^{2+} signaling, DAMGO inhibited Ca^{2+} channels and activated inward-rectifying K^+ channels in GH_3MORDOR cells, although to a lesser extent than in GH_3MOR cells. Long-term treatment with the δ receptor-specific ligand [D-Pen^2,D-Pen^5]-enkephalin reduced the excitatory effect of DAMGO in the majority of GH_3MORDOR cells and restored the inhibitory response to DAMGO in some cells. The inhibitory effect of somatostatin on Ca^{2+} signaling was not different in GH_3MORDOR versus GH_3MOR cells. These results indicate that interaction between μ- and δ-opioid receptors causes a change in the functional response to μ ligands, possibly by the formation of a μ/δ heterodimer with distinct functional properties.

There is growing evidence that different types of opioid receptors, as well as other G protein-coupled receptors, may interact within individual cells to alter their pharmacological properties (Maggio et al., 1993; Traynor and Elliott, 1993; Quitterer and Lohse, 1999; George et al., 2000; Gomes et al., 2000, 2001; Jordan et al., 2000; Yeo et al., 2001). Interaction between μ- and δ-opioid receptor subtypes has been suggested by a variety of in vitro and in vivo evidence. In vivo evidence for μ/δ receptor interactions includes observations that treatment with δ receptor-specific antagonists reduces tolerance and dependence in response to morphine (Abdelhamid et al., 1991). Morphine dependence is also altered in δ-receptor knockout mice (Zhu et al., 1999) and in animals whose δ receptors are selectively decreased with antisense oligonucleotides (Sanchez-Blazquez et al., 1997).

At present, it is not known whether these in vivo phenomena are caused by interactions between μ- and δ-opioid receptors in individual cells, or whether different cell populations are involved. However, in vitro studies clearly show that μ- and δ-opioid receptor interaction within individual cells results in significant alterations in their pharmacology. Ligand-binding studies show that μ-specific ligands alter the binding properties of δ-specific ligands and vice versa (George et al., 2000; Gomes et al., 2000). μ-Specific agonists and antagonists also potentiate mitogen-activated protein kinase activity evoked by δ agonists in cells expressing both μ and δ receptors, and δ-specific ligands potentiate mitogen-activated protein kinase activity evoked by μ agonists. These pharmacological properties may be mediated by heterodimers of different opioid receptor subtypes (Jordan and Devi, 1999; George et al., 2000; Gomes et al., 2000). Other evidence for μ/δ receptor interaction includes observations of a pertussis toxin-resistant inhibition of adenylyl cyclase by the μ-receptor agonist DAMGO applied to cells expressing both μ and δ receptors but not μ or δ receptors alone (George et al., 2000). In other studies, the interaction of μ and δ receptors stably expressed together in GH_3 cells produced a

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ABBREVIATIONS: DAMGO, [D-Ala^2,*N*-Me-Phe^4,Gly^5-ol]-enkephalin; DPDPE, [D-Pen^2,D-Pen^5]-enkephalin; $[Ca^{2+}]_i$, intracellular Ca^{2+} concentration; K_{ir}, inwardly rectifying K^+; PLC, phospholipase C; U73122, 1-(6-[[17β-3-methoxyestra-1,3.5(10)-trien-17-yl]amino]hexyl)-^{1}H-pyrrole-2,5-dione.

synergistic inhibition of adenylyl cyclase activity by μ- and δ-receptor agonists (Martin and Prather, 2001).

Despite the strong evidence for interaction between μ and δ receptors in individual cells, functional roles for this interaction have not been clearly defined. We have used GH_3 cells, a prolactin- and growth hormone-secreting pituitary cell line, as a model for the study of opioid-receptor function. These cells express multiple functional neurotransmitter receptors and voltage-gated ion channels and have robust spontaneous activity characterized by bursts of action potentials, influx of Ca^{2+} through voltage-gated Ca^{2+} channels, and oscillations in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) (Charles et al., 1999). The spontaneous Ca^{2+} signaling of GH_3 cells provides a sensitive detection system for the effects of exogenously expressed opioid receptors (Piros et al., 2000). In this study, we used patch-clamp techniques and video imaging of $[Ca^{2+}]_i$ to show that the functional response to μ-receptor activation is altered by coexpression of δ receptors.

Materials and Methods

Cell Cultures. GH_3, GH_3MOR, and GH_3MORDOR cells were maintained in culture in Dulbecco's modified Eagle's medium/Ham's F12 media supplemented with 5% fetal bovine serum, 5% horse serum, 100 IU/ml penicillin, and 100 μg/ml streptomycin in 25-mm^2 flasks. GH_3MOR cells stably transfected with the μ-opioid receptor were selected by inclusion of 1 mg/ml geneticin (Piros et al., 1995). Inclusion of geneticin (1 mg/ml) and hygromycin (200 μg/ml) stably selected for GH_3MOR cells that were stably transfected with the δ receptor (Piros et al., 1996). Cells were passaged or transferred onto 35-mm dishes for use in electrophysiological experiments or poly(D-lysine)-coated glass coverslips for fluorescence imaging, on which they were grown for 1 to 5 days to a confluence of approximately 60 to 80% before experimentation.

Measurement of $[Ca^{2+}]$. $[Ca^{2+}]_i$ was measured in 50 to 1000 individual cells in a microscopic field, depending on the magnification, with a time resolution of 33 ms. Cells on glass coverslips were loaded with fura-2AM by incubation in 5 to 10 μM dye for 40 to 60 min. Cells were then washed and maintained in normal medium for 30 min before experimentation. Coverslips were excited with a mercury lamp through 340- or 380-nm bandpass filters, and fluorescence at 510 nm was recorded through a 20× objective with an silicon intensified tube or charge-coupled device camera to VHS videotape, optical memory disc recorder, or computer hard drive. Images were then digitized and subjected to background subtraction and shading correction, after which change in fluorescence or $[Ca^{2+}]_i$ was calculated on a pixel-by-pixel basis, as described previously, by a frame grabber and image-analysis board (Data Translation or Axon Image Lightning board) using Axon Imaging Workbench software (Axon Instruments, Inc., Union City, CA) (Charles et al., 1999).

Calcium Signaling Analysis. The frequency of Ca^{2+} oscillations was quantified for each individual cell by counting the number of Ca^{2+} peaks over a 3-min interval using the Mini Analysis Program (Synaptosoft, Decatur, GA). An excitatory response, compared with an inhibitory response, was defined as a greater than 50% increase or decrease in the frequency of Ca^{2+} oscillations for an individual cell. The percentages of cells showing excitatory or inhibitory responses in each experiment were averaged, and p values for differences in the averages were determined using a Student's t test.

Electrophysiology. The whole-cell patch-clamp (Axopatch 200A amplifier, Axon Instruments) technique was used to record currents from voltage-clamped GH_3, GH_3MOR, and GH_3MORDOR cells. Ca^{2+}-channel activity was recorded with Ba^{2+} as the charge carrier. Cells were initially bathed in a solution containing 140 mM NaCl, 2.8 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, and 10 mM HEPES, pH 7.2. After the whole-cell configuration was established, the bath solution was replaced by one composed of 125 mM NaCl, 5.4 mM CsCl, 10.8 mM $BaCl_2$, 1 mM $MgCl_2$, and 10 mM HEPES. Electrodes contained 120 mM CsCl, 10 mM EGTA, 1 mM $MgCl_2$, 3 mM Mg-ATP, and 10 mM HEPES, pH 7.2 with CsOH. The potential difference between the open electrode and the bath ground was zeroed before establishing a ≥1-Gohm resistance seal. Voltage-activated Ba^{2+} currents were recorded from cells depolarized from the holding potential of −80 to 0 mV for 80 ms at 10-s intervals. Whole-cell inwardly rectifying K^+ (K_{ir}) channel current recordings were performed using an extracellular solution containing 140 mM KCl, 4 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM HEPES, 7 mM glucose, and 5×10^{-4} mM tetrodotoxin, pH 7.2 with KOH. Recording electrodes contained a solution composed of 140 mM KCl, 10 mM EGTA, 2 mM $MgCl_2$, 10 mM HEPES, and 3 mM Mg-ATP, pH 7.2 with KOH. Opioid agonists or somatostatin were bath-applied to cells clamped at −60 mV. Currents were low-pass–filtered at 2 KHz and digitized (Digidata; Axon Instruments) at 10 KHz for storage on the hard drive of a Pentium personal computer, after which analysis was performed using pCLAMP8 software (Axon Instruments).

Electrophysiological Analysis. The significance of inhibition of Ba^{2+} currents by opioid ligands or somatostatin was determined using the Student's t test to compare the average of four current amplitudes averaged immediately before drug application with those averaged immediately before washout. Differences were considered significant when $p < 0.05$. A similar approach was used to determine whether K_{ir} current activation by opioid ligands or somatostatin was significant. In many cases, there was a steady run-down of Ba^{2+} current amplitude or a run-up of inward current amplitude during K_{ir} current recording. When this occurred, four points were used immediately before drug application and after complete drug washout to carry out a linear extrapolation. Activation of K_{ir} currents or inhibition of Ba^{2+} currents was considered significant if the average of four points immediately before drug washout was significantly different from the equivalent values from the extrapolation ($p < 0.05$). When significance was achieved, the amplitudes of Ba^{2+} current inhibition and K_{ir} current activation were calculated relative to the corresponding current amplitudes from the fit.

[^{3}H]Diprenorphine Binding Assay. Binding assays were performed as described previously (Von Zastrow et al., 1993). Approximately 200,000 cells (determined by counting in a hemocytometer) were placed in polyvinyl chloride microtiter plates (BD Biosciences, San Jose, CA) with 100 nM [^{3}H]diprenorphine (21 Ci/mmol; Amersham Biosciences Inc., Piscataway, NJ) in a total volume of 100 μl. After incubation on ice for 60 min, the mixture was harvested quickly in an M24RS harvester (Brandel Inc., Gaithersburg, MD) using GT100 GF/B glass filters and washed with ice-cold phosphate-buffered saline. After drying, the filters were counted in a Beckman LS1600 scintillation counter using CytoScint (ICN Biomedicals, Inc Costa Mesa, CA). Nonspecific binding was determined by performing radioligand binding in the presence of 10 μM diprenorphine. DPDPE-specific [^{3}H]diprenorphine binding was determined by incubation in [^{3}H]diprenorphine in the presence of 10 μM DPDPE.

Results

DAMGO Inhibits Spontaneous Ca^{2+} Oscillations in GH_3MOR Cells. GH_3 cells show spontaneous Ca^{2+} oscillations that are generated by spontaneous depolarization resulting in the influx of Ca^{2+} through voltage-gated channels (Charles et al., 1999). The pattern of Ca^{2+} signaling in individual cells is heterogeneous, as is their response to the activation of membrane receptors. In GH_3 cells expressing only the μ-opioid receptor (GH_3MOR cells), the μ-receptor agonist DAMGO inhibited spontaneous Ca^{2+} signaling (Fig. 1). DAMGO (100 nM) abolished Ca^{2+} oscillations in the majority of cells and reduced the frequency of Ca^{2+} signaling in

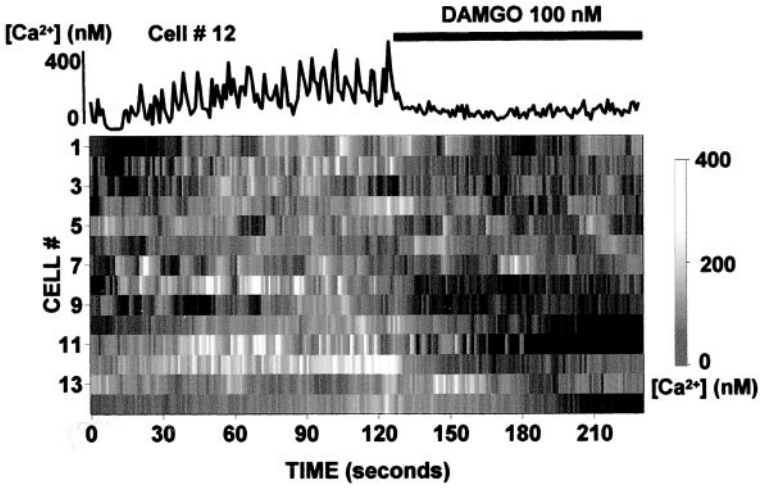

Fig. 1. DAMGO inhibits Ca^{2+} signaling in GH_3 cells expressing μ-opioid receptors. Raster plot of $[Ca^{2+}]$ versus time in a microscopic field of GH_3MOR cells. Each row represents an individual cell and $[Ca^{2+}]_i$ for each cell is indicated by grayscale (bar). The top tracing is a line trace of a representative individual cell. Individual cells show a heterogeneous pattern of spontaneous Ca^{2+} oscillations. Bath application of DAMGO inhibits Ca^{2+} oscillations in the majority of cells.

others (Figs. 1 and 4). Spontaneous Ca^{2+} signaling resumed in most cells 1 to 3 min after washout DAMGO.

DAMGO Has an Excitatory Effect on Ca^{2+} Signaling in GH_3MORDOR Cells. GH_3MORDOR cells express both δ receptors and μ receptors with a ratio of approximately 11:1 (B_{max} for [^{3}H]DPDPE binding = 3.45 pmol/mg of protein compared with B_{max} for [^{3}H]DAMGO binding = 0.3 pmol/mg of protein) (Piros et al., 1996). We reported previously that the δ-specific ligand DPDPE inhibits spontaneous Ca^{2+} signaling in cells expressing both the μ- and δ-opioid receptors (Piros et al., 2000). However, in contrast to the inhibition of Ca^{2+} signaling observed in GH_3MOR cells, DAMGO increased both baseline $[Ca^{2+}]_i$ and the frequency of Ca^{2+} oscillations in GH_3MORDOR cells (Figs. 2 and 4). This effect was concentration-dependent, with a maximal response observed at 1 μM DAMGO.

DAMGO Inhibits Ba^{2+} Currents and Activates K_{ir} Currents in GH_3MORDOR cells. We reported previously that μ receptors that are stably expressed in GH_3 cells inhibit L-type Ca^{2+} channels and adenylyl cyclase activity and activate K_{ir} channels (Piros et al., 1995, 1996, 2000). Despite its differential effect on Ca^{2+} signaling in GH_3MORDOR compared with GH_3MOR cells, DAMGO inhibited Ba^{2+} currents and activated K^+ currents recorded from both GH_3MOR and GH_3MORDOR cells. The average Ba^{2+} current inhibition in response to DAMGO was reduced in GH_3MORDOR cells compared with GH_3MOR cells, but this difference was not statistically significant. The average K_{ir} current amplitude evoked by DAMGO was reduced to a statistically significant ($p < 0.05$) degree in GH_3MORDOR versus GH_3MOR cells. DAMGO had no effect on currents recorded from untransfected GH_3 cells that lack opioid receptors but contain endogenous somatostatin receptors that coupled to Ca^{2+} and K_{ir} channels (Fig. 3, A and B). Despite the fact that there are more than 10-fold more δ than μ receptors in GH_3MORDOR cells (Piros et al., 1996), the degree of Ba^{2+} current inhibition and K_{ir} channel activation by the DAMGO (1 μM) and DPDPE (1 μM) was not significantly different (Fig. 3, C and D). Furthermore, the activation of K_{ir} currents by the two agonists was not additive, suggesting the involvement of similar signaling mechanisms (data not shown). These data indicate that although DAMGO retains some inhibitory effect via Ca^{2+} and K_{ir} channels in cells expressing both μ and δ receptors, this inhibitory effect is overcome by a simultaneous excitatory effect to result in an increase in baseline $[Ca^{2+}]_i$ and in the frequency of Ca^{2+} oscillations.

The Excitatory Effect of DAMGO on Ca^{2+} Signaling in GH_3MORDOR Cells Is Abolished by Pretreatment with Thapsigargin or U73122. To determine the mechanism of the excitatory effect of DAMGO on GH_3MORDOR

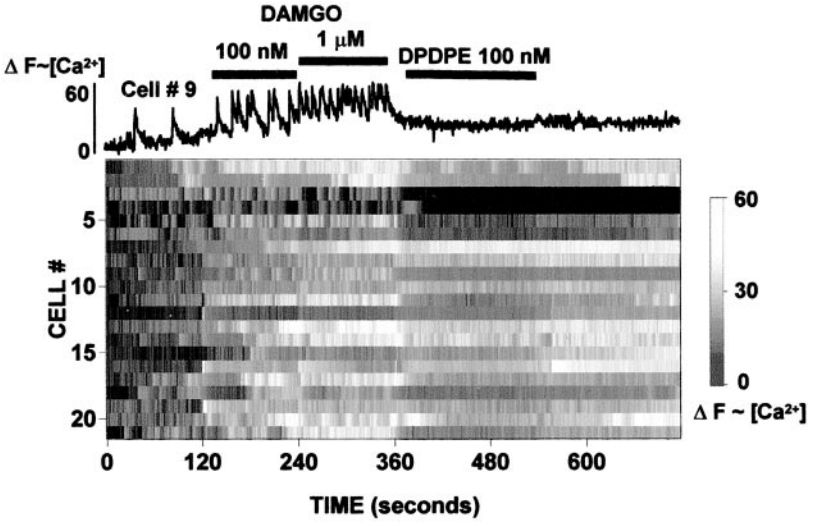

Fig. 2. DAMGO has excitatory effects on Ca^{2+} signaling in GH_3 cells expressing both μ and δ receptors. Raster plot of ΔF (~$[Ca^{2+}]_i$) versus time in a microscopic field of GH_3MORDOR cells. Each row represents an individual cell: the top trace shows a representative individual cell. DAMGO evokes a concentration-dependent increase in the frequency of Ca^{2+} oscillations in GH_3MORDOR cells, whereas DPDPE reversibly inhibits Ca^{2+} oscillations.

cells, we used the endoplasmic reticulum Ca^{2+} pump-inhibitor thapsigargin to deplete releasable intracellular Ca^{2+} stores. Pretreatment with thapsigargin (1 μM) for 5 min abolished the excitatory effect of DAMGO on Ca^{2+} signaling in the majority of GH_3MORDOR cells and revealed a small but significant inhibitory effect (Fig. 4). A similar effect was observed in cells pretreated with the phospholipase C inhibitor U73122 (1 μM; Fig. 4). These results indicate that the excitatory effect of DAMGO is mediated by the activation of phospholipase C and subsequent release of Ca^{2+} from intracellular Ca^{2+} stores. The inhibitory response to DAMGO after U73122 or thapsigargin pretreatment is consistent with unveiling of a $G_{i/o}$-mediated inhibition of Ca^{2+} signaling when the excitatory response to DAMGO is blocked.

The Excitatory Effect of DAMGO on Ca^{2+} Signaling in GH_3MORDOR Cells Is Abolished by Pretreatment with Pertussis Toxin. To investigate the role of G_i/G_o in the excitatory response to DAMGO in GH_3MORDOR cells, we treated cells for 24 h with pertussis toxin (200 ng/ml) before the application of DAMGO. A significantly reduced percentage of cells pretreated with pertussis toxin showed an excitatory response to DAMGO compared with untreated cells (Fig. 4).

Somatostatin Inhibits Ca^{2+} Signaling in a Similar Fashion in GH_3, GH_3MOR, and GH_3MORDOR Cells. GH_3 cells express somatostatin receptors, and activation of these receptors inhibits Ca^{2+} channels and adenylyl cyclase via $G_{i/o}$-coupled mechanisms (Piros et al., 1995, 1996, 2000). Somatostatin (10 nM–1 μM) significantly inhibited or abolished spontaneous Ca^{2+} oscillations in GH_3 cells. To determine whether the expression of opioid receptors individually or in combination altered the functional response to a different G protein-coupled receptor, we investigated the response to somatostatin in GH_3, GH_3MOR, and GH_3MORDOR cells. We found that the inhibitory effect of somatostatin (100 nM) was not significantly different in any of these cells (n = 4 coverslips for each cell type, data not shown). These results indicate that the Ca^{2+}-signaling response to at least one other $G_{i/o}$-coupled receptor is not significantly altered by the expression of μ- and δ-opioid receptors in GH_3 cells.

Long-Term Treatment with DPDPE Restores the Inhibitory Response of Some GH_3MORDOR Cells to DAMGO. Long-term treatment with DPDPE may reduce the numbers of δ receptors by multiple mechanisms, including increased internalization and decreased expression (Prather et al., 1994b; Jordan and Devi, 1999). We found that long-term treatment of GH_3MORDOR cells for 24 h with 1 μM DPDPE resulted in an 86 ± 6% reduction (n = 4) in DPDPE-specific [^{3}H]diprenorphine binding. Short-term treatment with DPDPE for 2 min had no significant effect on DPDPE-

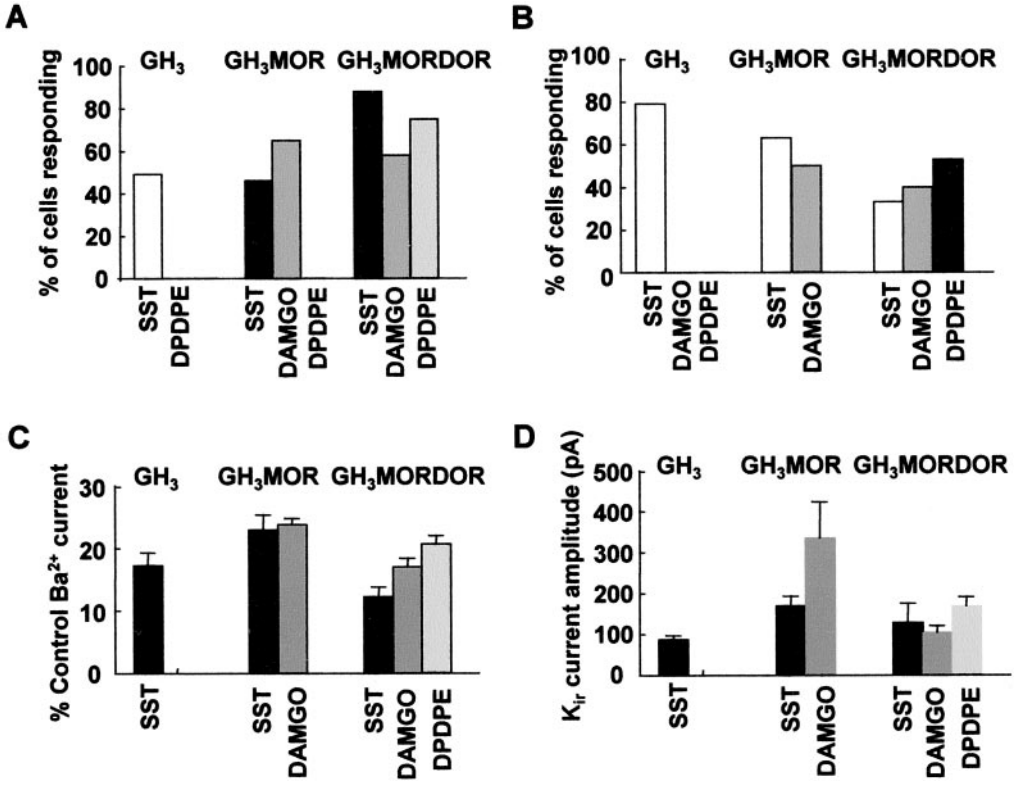

Fig. 3. Effects of somatostatin and opioids on Ca^{2+} and K_{ir} channel activity are similar in GH_3MOR and GH_3MORDOR cells. A, percentage of cells responding with a reduction in Ba^{2+} current amplitude to somatostatin (SST; 1 μM, n = 18, 14, and 7 for GH_3, GH_3MOR, and GH_3MORDOR, respectively), DAMGO (1 μM, n = 5, 49, and 24, respectively), or DPDPE (1 μM, n = 14, 5, and 24, respectively). B, percentage of cells responding with an increase in inward K^+ current to somatostatin (1 μM, n = 17, 25, and 16 for GH_3, GH_3MOR, and GH_3MORDOR, respectively), DAMGO (1 μM, n = 3, 20, and 9, respectively), or DPDPE (1 μM, n = 13, 23, and 29, respectively). C, the amplitude of Ba^{2+} current inhibition by somatostatin, DAMGO, or DPDPE is expressed as a percentage of the control Ba^{2+} current amplitude. Bars represent average data from at least four separate recordings. Error bars, ± S.E.M. There was a slight decrease in the Ba^{2+} current inhibition by DAMGO in GH_3MORDOR cells compared with GH_3MOR cells, but this decrease was not statistically significant. The difference between the effects of DAMGO and DPDPE on GH_3MORDOR cells were not statistically significant. D, the amplitude of inward K^+ currents activated by somatostatin, DAMGO, or DPDPE is expressed in picoamperes (pA). Bars represent the average data from at least four separate recordings. Error bars, ± S.E.M. There was a significant ($p < 0.05$) reduction in the activation of K_{ir} currents in GH_3MORDOR cells compared with GH_3MOR cells. The difference between the effect of DAMGO and DPDPE on GH_3MORDOR cells was not statistically significant.

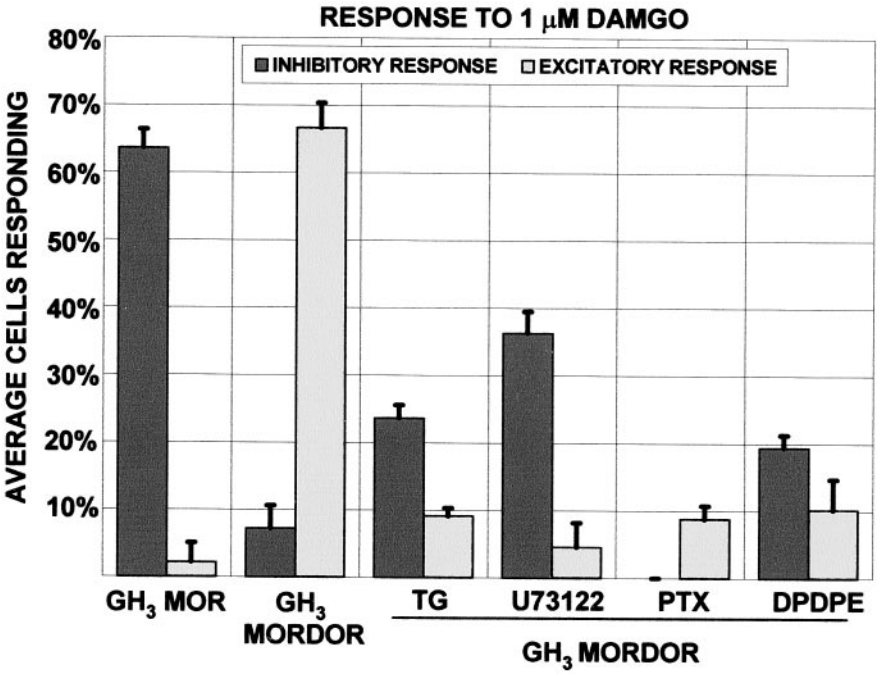

Fig. 4. Average percentage of cells showing excitatory versus inhibitory responses to DAMGO in GH_3MOR and $GH_3MORDOR$ cells. An excitatory versus inhibitory response was defined as a greater than 50% increase or decrease in the frequency of Ca^{2+} oscillations for each individual cell. The number of cells showing each type of response was averaged over all experiments for a given condition (error bars represent standard deviation). In GH_3MOR cells, DAMGO had an inhibitory response in the majority of cells ($n = 300$ cells, 10 experiments), whereas in $GH_3MORDOR$ cells, the response to DAMGO was predominantly excitatory ($n = 360$ cells, 12 experiments; $p < 10^{-8}$ for difference in either the percentage of inhibitory or excitatory response in GH_3MOR versus $GH_3MORDOR$ cells). The excitatory response to DAMGO was inhibited by pretreatment with either thapsigargin (1 μM, $n = 100$ cells, three experiments; $p < 10^{-7}$) or U73122 (1 μM, $n = 100$ cells, three experiments; $p < 0.001$), and a greater percentage of cells showed an inhibitory response ($p < 0.001$ for thapsigargin, $p < 0.005$ for U73122). The excitatory response to DAMGO was also inhibited by pretreatment for 24 h with pertussis toxin (200 ng/ml, $n = 120$ cells, four experiments; $p < 10^{-8}$). Treatment of cells for 24 h with 10 μM DPDPE also inhibited the excitatory response to DAMGO ($n = 150$ cells in five experiments; $p < 10^{-6}$) and increased the percentage of cells showing an inhibitory response ($p < 10^{-4}$).

specific [^{3}H]diprenorphine binding. We also found that after treatment with 1 μM DPDPE for 24 h, exposure to DAMGO had an excitatory effect in only a minority of cells and, in fact, either abolished spontaneous Ca^{2+} oscillations or reduced their frequency in 20 to 30% of $GH_3MORDOR$ cells (Figs. 4 and 5). In contrast, less than 5% of untreated $GH_3MORDOR$ cells showed an inhibitory response to DAMGO.

Discussion

Our results show that coexpression of δ receptors with μ receptors changes the response to DAMGO from an inhibition to a stimulation of Ca^{2+} signaling. The excitatory response to DAMGO in $GH_3MORDOR$ cells was inhibited by the inhibition of phospholipase C with U73122 or by the depletion of intracellular Ca^{2+} stores with thapsigargin, consistent with a Gq-mediated activation of PLC as the pathway for the excitatory response. The change in the response to DAMGO induced by δ-receptor expression therefore seems to involve differential coupling to different G proteins.

It is possible that the expression of multiple opioid-receptor subtypes, in combination with a limited pool of G proteins, causes the "switching" of specific receptors from one G protein to another (Prather et al., 1994a; Quitterer and Lohse, 1999; Sanchez-Blazquez et al., 2001; Yeo et al., 2001). Because $GH_3MORDOR$ cells express δ receptors and μ receptors at an 11:1 ratio (Piros et al., 1996; Prather et al., 2000), it is possible that the δ receptors occupy the majority of $G_{i/o}$ proteins, causing μ receptors to "switch" to G_q. However, evidence against this mechanism is the observation that the response to somatostatin does not seem to be altered by the level of expression of different opioid receptors, suggesting that the function of other G_i-coupled receptors is not altered by the coexpression of opioid receptors. Furthermore, the pattern of μ-receptor coupling to $G_{i/o}$ protein subtypes is not changed in $GH_3MORDOR$ cells compared with GH_3MOR

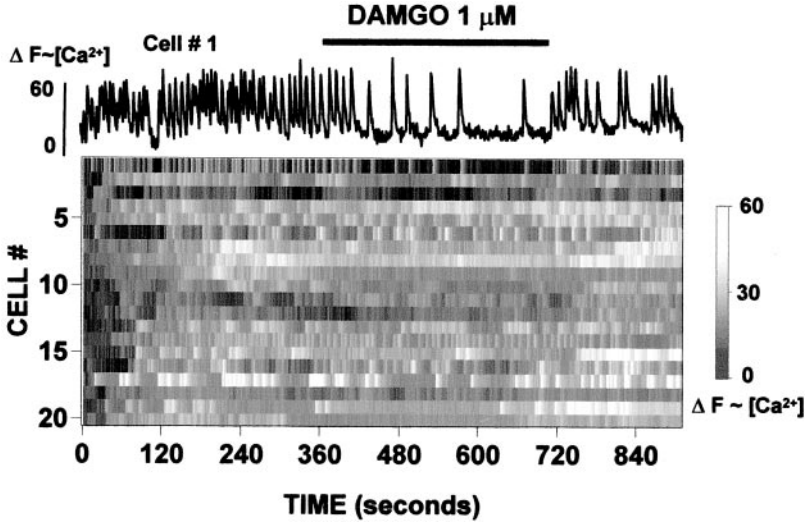

Fig. 5. Long-term treatment with DPDPE modulates the excitatory response to DAMGO. In cells treated with 10 μM DPDPE for 24 h, DAMGO does not evoke an excitatory response as in untreated $GH_3MORDOR$ cells but instead has an inhibitory effect in approximately 20% of cells (Fig. 4).

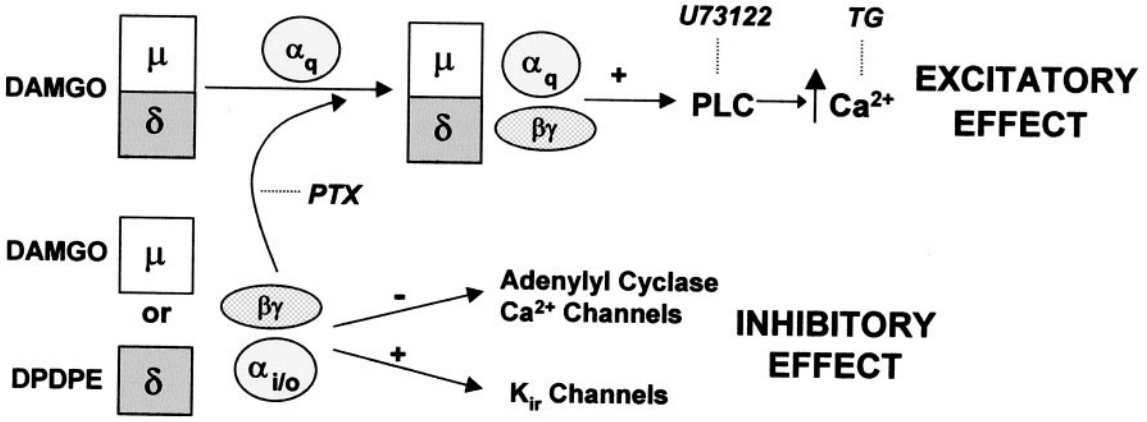

Fig. 6. Schematic diagram of proposed signaling mechanisms involved in response to DAMGO and DPDPE in GH_3 cells expressing opioid receptors. The excitatory response to DAMGO in GH_3MORDOR cells is sensitive to pertussis toxin (PTX) as well as to U73122 and thapsigargin (TG). The diagram (adapted from Quitterer and Lohse, 1999) indicates signaling mechanisms that are consistent with these results.

cells, despite the presence of δ receptors in the former (Martin and Prather, 2001). Coupling of μ receptors to Ca^{2+} channels and K_{ir} channels is also retained in the GH_3MORDOR cells.

Another potential mechanism for this phenomenon is the formation of hetero-oligomeric complexes that have unique functional properties. Opioid receptor heterodimers have been reported in cells either endogenously or exogenously expressing different types of opioid receptors (Jordan and Devi, 1999; George et al., 2000; Gomes et al., 2000). Our results could be explained by the formation of μ/δ heterodimers with functional properties distinct from those of either homomeric μ or δ receptors. In this scenario, μ receptors would exist primarily (but not exclusively) in a heterodimer configuration because of the preponderance of δ receptors in GH_3MORDOR cells. DAMGO would therefore primarily activate heterodimers that are coupled to G_q, leading to an excitatory response. DPDPE would primarily activate monomeric δ receptors or homodimers, explaining the inhibitory response. Also, DPDPE may not activate heterodimers because of structural specificity of this configuration, as suggested by Gomes et al. (2000).

The pertussis toxin sensitivity of the excitatory response to DAMGO suggests an interaction between $G_{i/o}$ and G_q (Fig. 6). Similar interactions between $G_{i/o}$- and G_q-coupled receptors within individual cells have been reported for a variety of receptor types (Quitterer and Lohse, 1999). In cases in which $G_{i/o}$-coupled receptors evoke an increase in $[Ca^{2+}]_i$, the majority of studies support a mechanism by which βγ subunits released by $G_{i/o}$ activation subsequently activate phospholipase C (Quitterer and Lohse, 1999; Yoon et al., 1999), although one study suggested that βγ subunits may act downstream from PLC (Yeo et al., 2001). Possible pathways for the effects of the βγ subunits include a direct activation of PLC (Camps et al., 1992), or an activation of PLC that requires interaction with $G\alpha_q$ (Quitterer and Lohse, 1999; Chan et al., 2000). Direct activation of PLC by βγ subunits seems less likely given that in most cells, the $G_{i/o}$-mediated release of Ca^{2+} only occurs if there is coactivation of G_q-coupled receptors. We propose that in GH_3MORDOR cells, DAMGO-evoked activation of PLC via G_q requires βγ subunits contributed by activation of $G_{i/o}$ (Fig. 6), as has been proposed for other cell types (Quitterer and Lohse, 1999; Chan et al., 2000). Simultaneous activation of both G_q and $G_{i/o}$ could be achieved by the binding of DAMGO to μ receptors in both heteromeric (μ/δ) and homomeric configurations.

Opioid receptors generally have inhibitory effects mediated by their coupling through pertussis toxin-sensitive $G_{i/o}$ proteins to adenylyl cyclase, Ca^{2+} channels, and K^+ channels (Piros et al., 1995, 2000; Williams et al., 2001). However, in some cells, opioids may have direct stimulatory effects, via either the release of Ca^{2+} from inositol phosphate-3–sensitive stores or the stimulation of Ca^{2+} entry (Jin et al., 1992; Smart et al., 1995; Smart and Lambert, 1996; Spencer et al., 1997; Yoon et al., 1999; Chen et al., 2000). In other cells, opioid-receptor activation does not evoke an increase in $[Ca^{2+}]_i$ on its own , but it does potentiate Ca^{2+} signaling stimulated by other G_q-coupled receptors (Chen et al., 2000; Yeo et al., 2001). Each of the cell types in which opioids have been reported to evoke an increase in $[Ca^{2+}]_i$ express both μ and δ receptors. This correlation suggests that μ/δ receptor interactions may be involved in an excitatory response to opioids.

An involvement of G_q and PLC in the antinociceptive effects of δ-2 agonists in mice has been reported (Sanchez-Blazquez and Garzon, 1998). This result is particularly interesting in light of the conclusion of Gomes et al. (2000), stating that δ-2 agonists activate μ/δ heterodimers. Additional evidence of a role for PLC in opioid-induced analgesia comes from PLC B3 knockout mice, in which a markedly increased sensitivity to antinociceptive effects of morphine has been observed (Xie et al., 1999). An alteration of opioid-receptor levels that leads to activation of PLC could therefore result in both short- and long-term changes in the response to opioid ligands.

Regardless of the exact mechanisms involved, the observations that opioids are capable of both inhibitory and excitatory actions on cells and that these actions depend on the level of receptor expression within cells have multiple significant implications. First, ligands or ligand combinations that differentially activate μ versus δ receptors may have different analgesic effects determined from the μ/δ receptor interactions and excitatory versus inhibitory actions. Similarly, the development of tolerance and dependence may be a function of relative μ- versus δ-receptor activation. We found that long-term exposure of GH_3MORDOR cells to DPDPE caused a decline in the excitatory response to DAMGO. One explanation for this result is that long-term exposure to the δ-re-

ceptor agonist reduces the numbers of δ receptors in the membrane, thereby reducing the excitatory μ/δ receptor interaction. Long-term treatment with opioid agonists has been reported to reduce receptor the number by multiple mechanisms, including increased internalization and decreased expression (Prather et al., 1994b; Jordan and Devi, 1999). Consistent with these previous reports, we found that long-term treatment with DPDPE resulted in a significant reduction in DPDPE-specific [^{3}H]diprenorphine binding in GH_3MORDOR cells. Long-term exposure to opioids that alter the ratio of μ- versus δ-receptor expression could therefore have dramatic effects on the function of opioid receptors, both via their response to opioid ligands and also potentially via constitutive activation of these receptors (Wang et al., 1994; Liu et al., 2001). An increased understanding of these mechanisms and the identification of individual ligands or ligand combinations that preferentially activate different signaling pathways as a result of μ/δ interaction could lead to the development of more effective pharmacological approaches to analgesia with reduced levels of tolerance and dependence.

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