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Induction of δ Opioid Receptor Function by Up-Regulation of Membrane Receptors in Mouse Primary Afferent Neurons

Wendy Walwyn, Nigel T. Maidment, Matthew Sanders, Christopher J. Evans, Brigitte L. Kieffer, and Tim G. Hales

Department of Psychiatry and Biobehavioral Sciences, Center for Health Sciences, University of California, Los Angeles, California (W.W., N.T.M., C.J.E.); Psychology Department, University of California, Los Angeles, Los Angeles, California (M.S.); Institute of Genetics and Molecular and Cellular Biology, Centre National de la Recherche Scientifique/ Institut National de la Sante et de la Recherche Medicale/Université Louis Pasteur, Illkirch, France (B.L.K.); and Departments of Pharmacology & Physiology and Anesthesiology & Critical Care Medicine, the George Washington University, Washington, DC (T.G.H.)

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ABSTRACT

It is not clear whether primary afferent neurons express functional cell-surface δ opioid receptors. We examined δ receptor coupling to Ca^{2+} channels in mouse dorsal root ganglion neurons under basal conditions and after δ receptor up-regulation. [D-Ala^2,Phe^4,Gly^5-ol]-enkephalin (DAMGO), [D-Ala^2,D-Leu^5]-enkephalin (DADLE), *trans*-(±)-3,4-dichloro-*N*-methyl-*N*-(2-[1-pyrrolidinyl]cyclohexyl) benzene-acetamide methanesulfonate (U-50,488H; 1 μM), and baclofen (50 μM) inhibited Ca^{2+} currents, whereas the δ-selective ligands [D-Pen^2,Pen^5]-enkephalin (DPDPE) and deltorphin II (1 μM) did not. The effect of DADLE (1 μM) was blocked by the μ-antagonist D-Phe-Cys-Tyr-D-Trp-Arg-Thr-Pen-Thr-NH_2 (CTAP; 300 nM) but not by the δ-antagonist Tyr-1,2,3,4-tetrahydroisoquinoline-Phe-Phe-OH (300 nM), implicating μ receptors. Despite a lack of functional δ receptors, flow cytometry revealed cell-surface δ receptors. We used this approach to identify conditions that up-regulate δ receptors, including μ receptor gene deletion in dorsal root ganglion neurons of μ−/− mice and 18-h incubation of μ+/+ neurons with CTAP followed by brief (10-min) DPDPE exposure. Under these conditions, the expression of cell-surface δ receptors was up-regulated to 149 ± 9 and 139 ± 5%, respectively; furthermore, DPDPE and deltorphin II (1 μM) inhibited Ca^{2+} currents in both cases. Viral replacement of μ receptors in μ−/− neurons reduced δ receptor expression to μ+/+ levels, restored the inhibition of Ca^{2+} currents by DAMGO, and abolished δ receptor coupling. Our observations suggest that δ receptor-Ca^{2+} channel coupling in primary afferent fibers may have little functional significance under basal conditions in which μ receptors predominate. However, up-regulation of cell-surface δ receptors induces their coupling to Ca^{2+} channels. Pharmacological approaches that increase functional δ receptor expression may reveal a novel target for analgesic therapy.

Three genes encoding μ, δ, and κ opioid receptors are expressed throughout mammalian pain pathways. All three receptors couple to adenylyl cyclase, inwardly rectifying K^+ channels, and high-threshold voltage-activated Ca^{2+} channels (Williams et al., 2001). Opioid ligands that activate μ, δ, or κ receptors are antinociceptive (Kolesnikov et al., 1996). Morphine induces analgesia by activating μ receptors, a phenomenon that is blocked by μ receptor gene deletion in μ−/− mice. However, analgesia by δ ligands is more complex. Deletion of δ receptors attenuates DPDPE- and deltorphin II-induced spinal analgesia, although these agonists remain fully analgesic through supraspinal mechanisms (Zhu et al., 1999). When administered by intracerebroventricular injection, δ agonists are analgesic in δ−/− mice during tail immersion and inactive in this respect in μ−/− mice. However, in the hotplate test, δ ligands remain analgesic in μ−/− mice, and deltorphin II prolongs jump latencies in double μ/κ−/− mice. Therefore, μ receptors seem to mediate much of the analgesic response of δ ligands, but DPDPE and deltorphin II can also induce analgesia through the activation of δ recep-

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ABBREVIATIONS: DPDPE, [D-Pen^2,Pen^5]-enkephalin; DAMGO, [D-Ala^2,Phe^4,Gly^5-ol]-enkephalin; DADLE, [D-Ala^2,D-Leu^5]-enkephalin; U-50,488H, *trans*-(±)-3,4-dichloro-*N*-methyl-*N*-(2-[1-pyrrolidinyl]cyclohexyl) benzene-acetamide methanesulfonate; TIPP, Tyr-1,2,3,4-tetrahydroisoquinoline-Phe-Phe-OH; CTAP, D-Phe-Cys-Tyr-D-Trp-Arg-Thr-Pen-Thr-NH_2; qPCR, quantitative polymerase chain reaction; DELT II, deltorphin II; PBS, phosphate-buffered saline; GFP, green fluorescent protein; CT, count threshold; PerCP, peridinin-α chlorophyll protein; RFI, relative fluorescence intensity; SP, Substance P; CGRP, Calcitonin-Gene Related Peptide; ANOVA, analysis of variance; FSC-H, forward scatter; SSC-H, side scatter; IB4, isolectin B4; OR, opiate receptor; FL, fluorescent channel; R1, region 1.

tors in μ−/− and μ/κ−/− mice (Scherrer et al., 2004). It is unclear whether this represents a compensatory increase in δ receptor participation in analgesia caused by the absence of μ receptors (Qiu et al., 2000).

The δ receptor participates in morphine tolerance; reduction or abolition of δ receptor expression through antisense treatment and gene deletion, respectively, attenuates the development of morphine tolerance and dependence (Sanchez-Blazquez et al., 1997; Zhu et al., 1999). Similar findings in mice lacking a functional preproenkephalin gene suggest that tolerance to morphine requires the activation of δ receptors by endogenous opioids (Nitsche et al., 2002).

Opioid receptors can interact with each other either through convergence of their signaling pathways or through direct physical association (Jordan and Devi, 1999). Opioid receptors either exist as homomers or form heteromers containing at least two receptor subtypes. Recombinant heteromeric receptors have altered pharmacology and internalization properties compared with their homomeric counterparts (Jordan and Devi, 1999; George et al., 2000). The functional relevance of opioid receptor oligomerization in neurons has not been established. However, there may be a role for heterodimerization in the actions of δ-agonists in enhancing morphine analgesia (He and Lee, 1998; Gomes et al., 2004).

All three opioid receptor subtypes colocalize, albeit in different combinations, at different development stages in primary afferent fibers (Fields et al., 1980). Under resting conditions, most δ receptors are located in large dense core vesicles (Zhang et al., 1998; Bao et al., 2003) within the cytoplasm (Wang and Pickel, 2001), and their availability for rapid signaling remains uncertain (Bao et al., 2003; Pradhan and Clarke, 2005). Various stimuli, including δ agonists (Bao et al., 2003), chronic inflammatory pain, forced swimming, and prolonged morphine exposure, increase trafficking of δ receptors to neuronal plasma membranes and increase the analgesic efficacy of δ agonists (Cahill et al., 2001, 2003; Commons, 2003).

A lack of functional δ receptors on nociceptive primary afferent neurons may contribute to the limited efficacy of δ agonists in analgesia. Selective μ and κ agonists inhibit Ca^{2+}-channel activity recorded from rat dorsal root ganglion neurons in culture. However, functional coupling of δ receptors to Ca^{2+} channels in dorsal root ganglion neurons is controversial. Several studies suggest a lack of coupling between δ receptors and Ca^{2+} channels in dorsal root ganglion neurons (Schroeder et al., 1991; Moises et al., 1994; Liu et al., 1995). A single report describes the inhibition of Ca^{2+}-channel activity by the δ-2 agonist DADLE but not the δ-1 agonist DPDPE (Acosta and Lopez, 1999). However, DADLE has a relatively low selectivity for the δ receptor, and much of its analgesia is probably mediated through μ receptor activation (Chaillet et al., 1984).

In this study, we examined the coupling of μ, δ, and κ receptors to Ca^{2+} channels in cultured mouse dorsal root ganglion neurons. The μ agonist DAMGO inhibits Ca^{2+}-channel activity in neurons of μ+/+ but not μ−/− mice (Walwyn et al., 2004). Our data demonstrate that DADLE inhibits Ca^{2+}-channel activity in μ+/+ dorsal root ganglion neurons through the activation of μ receptors. More selective δ agonists, DPDPE and deltorphin II, have no effect on μ+/+ neurons; however, in μ−/− neurons, in which the surface expression of δ receptors is up-regulated, δ receptor activation inhibits Ca^{2+}-channel activity. Pharmacological up-regulation of δ receptor expression also initiates inhibitory coupling between δ receptors and Ca^{2+} channels in μ+/+ dorsal root ganglion neurons.

Materials and Methods

Dorsal Root Ganglion Neuron Cultures. Dorsal root ganglia were harvested from early postnatal mice (postnatal days 0–2), which contained one (μ+/−), both (μ+/+), or neither (μ−/−) μ receptor alleles in the C57BL/6 background (Matthes et al., 1996). This line has been fully backcrossed to the C57BL/6 background, and the pups used were littermates or were within two generations of μ+/− matings. For control experiments examining the specificity of the anti-δ receptor antibody, dorsal root ganglion neurons were harvested from δ receptor −/− mice (Filliol et al., 2000). The dorsal root ganglion neurons were enzymatically and physically dissociated, and the neurons were plated in modified formats for electrophysiology, flow cytometry, and quantitative polymerase chain reaction (qPCR) experiments. For electrophysiology experiments, dissociated cells were plated at a density of 1×10^5 cells/cm^2 onto glass coverslips coated with poly-L-ornithine (Sigma-Aldrich, St. Louis, MO) and laminin (BD Biosciences Discovery Labware, Bedford, MA), forming a well in 35-mm Petri dishes (MatTek, Ashland, MA). The cell suspension was dropped into the well and was allowed to settle in 100 μl of dorsal root ganglion media (Neurobasal-A B27, 0.5 mM GlutaMAX, 12 U/ml PSF) before adding an additional 2 ml of media containing 5-fluoro-2-deoxyuridine to inhibit non-neuronal growth (5-fluoro-2′-deoxyuridine, 20 mg/ml; Sigma-Aldrich) and nerve growth factor (10 μg/ml; Roche Diagnostics, Indianapolis, IN) 2 h later. A similar approach was used for flow cytometry and quantitative qPCR experiments, but because many more cells were required, the wells were formed by a 4-cm^2 hole cut in the bottom of a 100-mm dish under which a poly-L-ornithine- and laminin-coated coverslip was glued. This formed a 16-cm^2 well, into which 2×10^6 cells from μ−/− or μ+/+ mice were plated in 1 ml of media and allowed to settle before adding an additional 9 ml of dorsal root ganglion media/5-fluoro-2′-deoxyuridine/nerve growth factor 2 h later. After 2 to 3 days in vitro at 37°C and 5% CO_2, the cells were harvested on ice in phosphate-buffered saline containing 2 mM EDTA (PBS/EDTA).

Viral Expression of Recombinant μ Receptors in Cultured Dorsal Root Ganglion Neurons of μ−/− Mice. After 1 day in vitro, most of the media was removed, leaving sufficient media to cover the cells. An adenovirus expressing both GFP and the μ receptor (Ad-μ receptor; Walwyn et al., 2004) was applied at a multiplicity of infection between 1 and 5 infectious units/cell. After 1 h of adsorption, the removed media were returned to the cells, which were incubated at 37°C and 5% CO_2 for 48 h before use. The Ad-μ receptor virus expresses a Flag-tagged μ receptor under the control of a cytomegaloviral promoter and has been shown to return μ receptor expression and function to dorsal root ganglion neurons from μ−/− mice (Walwyn et al., 2004).

qPCR. Cultured dorsal root ganglion neurons from μ −/− or μ+/+ C57BL/6 mice were harvested in PBS/EDTA, spun (300*g*) for 5 min at 4°C, and lysed. RNA was isolated (RNAqueous; Ambion, Austin, TX) and reverse-transcribed (Superscript II; Invitrogen, Carlsbad, CA), including trace amounts of [α-^{32}P]dCTP. The yield of cDNA was determined, and concentration was adjusted to 5 ng/μl. A primer and probe set was designed to the mouse δ receptor mRNA (GenBank accession no. NM_013622); forward (5′-3′), GGGACACTGTGACCAAGAT; probe, FAM-GGTGTTTGGCTTCCTGAA-TAMRA; reverse, CAGTAGCATGAGGCCATAGC. A second primer and probe set to the mouse synaptophysin mRNA (GenBank accession no. NM_009305) was included: forward (5′-3′), GACTTCAGGACTCAACACCTC; probe, FAM-GGTGTTTGGCTTCCTGAA-TAMRA; reverse, ATAGGTTGCCAACCCAGA. The cycle number at which the gene-specific fluorescence increased higher than the preset thresh-

old, the count threshold (CT) was used to determine the expression of the δ receptor and synaptophysin (Chen et al., 2001) over a 100-fold dilution of the template (0.1–10 ng). All samples were collected in duplicate, and each experiment was repeated three times. The CT at the y-intercept for the δ receptor were determined and normalized to the synaptophysin CT at this intercept to control for any variance in starting template content. The data were then analyzed by multivariate linear regression analysis.

Flow Cytometry. Flow cytometry was used to analyze δ receptor cell-surface expression of cultured μ−/−, μ+/+, and AD-μ receptor-transduced μ−/− dorsal root ganglion neurons. Control experiments examining the specificity of the anti-δ receptor antibody flow cytometry were performed using cultured dorsal root ganglion neurons from δ−/− mice (Filliol et al., 2000). After 3 days in vitro, cultured dorsal root ganglion neurons were harvested in ice-cold PBS/EDTA and spun at 300*g* for 5 min at 4°C. The cells were washed in ice-cold PBS containing 2% fetal bovine serum and 0.1% sodium azide (PBS/FBS/NaN_3) and incubated in an amino-terminal (3–17) anti-δ receptor antibody (Chemicon International, Temecula, CA) for 30 min at 4°C (1:100 dilution in PBS/FBS/NaN_3). After a further wash and incubation for 30 min in the secondary antibody (biotinylated anti-rabbit IgG, 1:200; Vector Laboratories, Burlingame, CA), the antibody was visualized by 30-min incubation in streptavidin-peridinin-α chlorophyll protein (PerCP, 1:1000; BD Biosciences). After a final wash, 5,000 to 14,000 cells per sample were analyzed on a FACScalibur flow cytometer using CellQuest 3.0.1 for acquisition (BD Imunocytochemistry Systems, Mountain View, CA,) and FCS Express version 2.29 for analysis (De Novo Software, Thornhill, ON, Canada).

Each sample within each experiment was acquired using the same parameters of size (forward scatter, FSC-H), granularity (side scatter, SSC-H) and fluorescence in the first fluorescent channel for GFP (FL1-H) and the third fluorescent channel for PerCP (FL3-H). For each experiment, the neuronal population of an unlabeled sample was first defined as region 1 (R1) by size and granularity (Fig. 3A) (Walwyn et al., 2004). Gating on this population the PerCP-δ receptor fluorescence of positively labeled samples was acquired in the third fluorescent channel (FL3-H, Fig. 3B). Nonspecific fluorescence from an "isotype only" or no primary antibody control sample was also acquired. This was subtracted (M1), and the mean PerCP-δ receptor relative fluorescence intensity (RFI) was obtained (M2, Fig. 3D). Within each experiment, the mean RFI values of all samples were normalized to the RFI of the untreated μ+/+ or μ−/− sample, where appropriate. Where μ receptor expression was returned to the μ−/− background after Ad-μ receptor treatment, the sample was both neuron- and GFP-gated to obtain δ receptor fluorescence of neurons expressing GFP and, by extension, the μ receptor. Each experiment was repeated a minimum of three times, and the data were analyzed by the two-tailed Student's *t* test for paired or unpaired samples.

Flow cytometry was also used to identify pharmacological approaches that up-regulate δ receptor expression. After 2 days in vitro, dishes of cultured dorsal root ganglion neurons were either treated with opioid receptor antagonists, as described in the text, or left untreated (controls) and then incubated for 18 h at 37°C and 5% CO_2. Cells were harvested in ice-cold PBS/EDTA and then processed to label δ receptors present on the cell membrane and analyzed as described above.

Characterization of μ−/− and μ+/+ Dorsal Root Ganglion Cultures by Flow Cytometry. Matched cultures from μ−/− and μ+/+ C57BL/6 were cultured, harvested, and spun as described above. They were fixed in 2% paraformaldehyde for 5 min at 4°C, washed in PBS/FBS/NaN_3, and incubated in one of two of the following: anti-rabbit TrkA (Chemicon), Griffinia Simplicifolia Isolection B_4 (IB4, Vector Laboratories), anti-Substance P (SP), anti-calcitonin-gene related peptide (CGRP; Chemicon), anti-somatostatin, and anti-Neuropeptide Y (Diasorin, Stillwater, MN). After 30 min at room temperature, the samples were washed and incubated in the following secondary antibodies: Fluorescein isothiocyanate-conjugated anti-rabbit, anti-rabbit SP-Biotin IgG, and anti-rat SP-Biotin IgG (Jackson ImmunoResearch Laboratories Inc., West Grove, PA) for 30 min at room temperature, followed by PerCP and/or Alexa-fluor-Allophycanin-750 conjugated streptavidin (Invitrogen). After a final wash and resuspension in PBS/FBS/NaN_3, the samples were acquired on a FACSCaliburX (BD Immunocytochemistry). Similar to the methodology described above, the neuronal population was initially defined by size and granularity (FSC-H and SSC-H) and selected as R1. The number of cells in R1 labeled with fluorescein isothiocyanate (FL1-H), PerCP (FL3-H), or Alexa-fluor-Allophycanin-750 (FL5-H) was then quantified and expressed as a percentage of the total number of cells within R1.

Patch-Clamp Recordings. The whole-cell patch-clamp technique was used to record voltage-activated Ca^{2+}-channel activity from cultured dorsal root ganglion neurons (Axopatch 200A amplifier; Molecular Devices, Sunnyvale, CA). Culture media were replaced by an external solution that contained 130 mM TEA-Cl, 10 mM $CaCl_2$, 5 mM HEPES, 25 mM D-glucose, and 2.5×10^{-4} mM tetrodotoxin at pH 7.2. Recording electrodes contained 105 mM CsCl, 40 mM HEPES, 5 mM D-glucose, 2.5 mM $MgCl_2$, 10 mM EGTA, 2 mM Mg-ATP, and 0.5 mM GTP, pH 7.2. The potential difference between the open electrode and the bath ground was zeroed before establishing a ≥1-GΩ resistance seal. No compensation was made for the cancellation of liquid junction potential. Ca^{2+} currents were activated by depolarizing neurons from −80 to 10 mV for 100 ms at 10-s intervals. Currents were low-pass-filtered at 2 kHz and digitized (Digidata; Molecular Devices) at 10 kHz for storage on the hard drive of a Pentium PC. Leak currents were nulled using the P/4 subtraction method. Dorsal root ganglion neurons were continuously superfused (5 ml/min) with external solution in the chamber formed by the coverslip insert at the bottom of the 35-mm Petri dish. Opioid agonists and antagonists were diluted into external solution on the day of the experiment and applied through the perfusion system. Experiments were performed at room temperature (22–24°C). Mean Ca^{2+}-current amplitudes were measured (pCLAMP 9.0; Molecular Devices) between 5 and 10 ms after initiating the depolarizing step. Mean current amplitudes were then plotted against time. Recordings that exhibited marked rundown were discarded. Stable recordings were fitted by a linear function to compare, by extrapolation, control current amplitude with the current amplitude recorded in the presence of opioid agonists and antagonists. Data are expressed as mean ± S.E.M. and were compared using ANOVA with a post hoc Tukey's test.

Results

δ Receptors Do Not Couple to Ca^{2+} Channels under Basal Conditions in Mouse Dorsal Root Ganglion Neurons. The μ, δ, and κ agonists DAMGO (1 μM), DADLE (1 μM), and U-50,488H (1 μM) inhibited voltage-activated Ca^{2+} currents recorded, using the whole-cell patch-clamp configuration, from cultured neonatal dorsal root ganglion neurons of μ+/+ mice (Fig. 1). Ca^{2+} currents were inhibited by 41 ± 5% (n = 13; 100% responded), 30 ± 3% (n = 12; 100% responded), and 13 ± 6% (n = 8; 63% responded), respectively. When nonresponders were omitted from the analysis, the inhibition by U-50,488H grew to 19 ± 8% (n = 5). The $GABA_B$ receptor agonist baclofen (50 μM) also inhibited Ca^{2+} currents by 22 ± 8% (n = 10; 60% responded). When nonresponders were omitted from the analysis, the inhibition by baclofen was 37 ± 9% (n = 10). The inhibition of Ca^{2+} currents by DADLE observed here is consistent with a previous report of functional coupling between δ receptors and Ca^{2+} channels in rat dorsal root ganglion neurons (Acosta and Lopez, 1999). However, there are several reports of an

absence of coupling between δ receptors and Ca^{2+} channels (Schroeder et al., 1991; Moises et al., 1994; Liu et al., 1995). We tested the ability of more selective δ-agonists DPDPE (1 μM, $n = 14$) and deltorphin II (1 μM, $n = 9$) to couple to Ca^{2+} channels in dorsal root ganglion neurons. Both agonists failed to inhibit Ca^{2+} currents in all cells tested (Fig. 2), raising the possibility that the inhibitory effects of DADLE are mediated through μ receptor activation.

In keeping with the idea that DADLE is activating μ receptors, the IC_{50} value for DADLE as an inhibitor of Ca^{2+}-channel activity was >100 nM, indicating a potency much lower than would be expected for a selective effect mediated by the δ receptor (Fig. 2A). We further investigated the identity of the receptor mediating the inhibition of Ca^{2+}-current activity by DADLE by using μ- and δ-selective antagonists (Fig. 2, B and C). The application of the δ-selective antagonist TIPP (300 nM) had no effect on the amplitude of the inhibition mediated by DADLE (1 μM; Fig. 2B). In contrast, the μ-selective antagonist CTAP (300 nM) abolished inhibition by DADLE (1 μM; Fig. 2C).

Surface Expression of δ Receptors Is Up-Regulated in Dorsal Root Ganglion Neurons of μ−/− Mice. δ-Agonist induced analgesia is modest compared with that of μ agonists and may involve μ receptor activation (Zhu et al., 1999; Scherrer et al., 2004). However, deletion of the gene encoding the μ receptor in μ−/− mice reveals an antinociceptive contribution of δ receptors that is independent of the μ receptor (Matthes et al., 1998; Qiu et al., 2000). We examined the possibility that the expression of δ receptors is up-regulated in dorsal root ganglion neurons of μ−/− mice. We harvested neurons from μ+/+ and μ−/− mice after 2 days in culture and used flow cytometry to look for differences in the levels of mature membrane δ receptor protein (Fig. 3). Figure 3 illustrates how these samples were analyzed. The neuronal population was first defined by size (FSC-H) and granularity (SSC-H) and is shown as region 1 (R1; Fig. 3A). Selecting only these cells, we obtained the fluorescence of the PerCP-labeled δ receptor from the third fluorescent channel (FL3-H) as shown in Fig. 3B, where one pixel = one cell. This scatter plot shows a single population with a range of δ receptor fluorescence concentrated around 10^2 fluorescent units for this sample. We confirmed the specificity of the anti-δ receptor antibody by applying it to cultures of dorsal root ganglion neurons from δ −/− mice (Filliol et al., 2000). There was a low level of background staining under immunocytochemical conditions, and in the flow cytometry scatter graph, most cells fell well below the 10^2 fluorescent units observed for μ+/+ neurons (Fig. 3C). Figure 3D is a histogram of the FL3-H fluorescence (from Fig. 3B) and shows typical nonspecific background fluorescence (M1) and the mean δ receptor fluorescence of the positively labeled cells (M2).

Using this approach, we found that a similar proportion of dorsal root ganglion neurons from μ+/+ (93 ± 2%, $n = 5$) and μ−/− (94 ± 1%, $n = 5$) mice expressed δ receptors. However, comparison of δ receptor density on the cell membrane revealed an up-regulation in fluorescence intensity in neurons from μ−/− mice to 149 ± 9% of the levels observed in dorsal root ganglion neurons from μ+/+ mice, indicating an increase in cell-surface δ receptor number (Fig. 3E). We examined further whether the cell-surface expression of δ receptors was influenced by μ receptor expression by reintroducing μ receptors into cultured dorsal root ganglion neurons from μ−/− mice using the adenoviral vector containing μ receptor cDNA (Ad-μ receptor). Returning expression of the μ receptor to the μ−/− background decreased the level of δ receptors present on the cell membrane to 93 ± 12% of control μ+/+ (Fig. 3E).

Lack of Compensatory Changes in δ Receptor mRNA Expression in μ−/− Dorsal Root Ganglion Neurons. An up-regulation of mature δ receptor protein present on the cell surface of dorsal root ganglion neurons from μ−/− mice compared with those from μ+/+ mice could be caused by a compensatory adaptation occurring at the level of the δ receptor transcript. However, qPCR analysis, with gene-specific probes for synaptophysin and the δ receptor (see *Materials and Methods*), showed no difference in δ receptor mRNA levels over a range of starting μ+/+ and μ−/− templates (5.0–0.1 ng), as evidenced by a lack of difference in the intercepts and slopes ($p = 0.694$ and 0.888, respectively) of the linear regression equations used to fit data from dorsal root ganglion neurons of μ−/− and μ+/+ mice (data not shown). These data suggest that alterations in cell-surface δ receptor expression seen in flow cytometry experiments

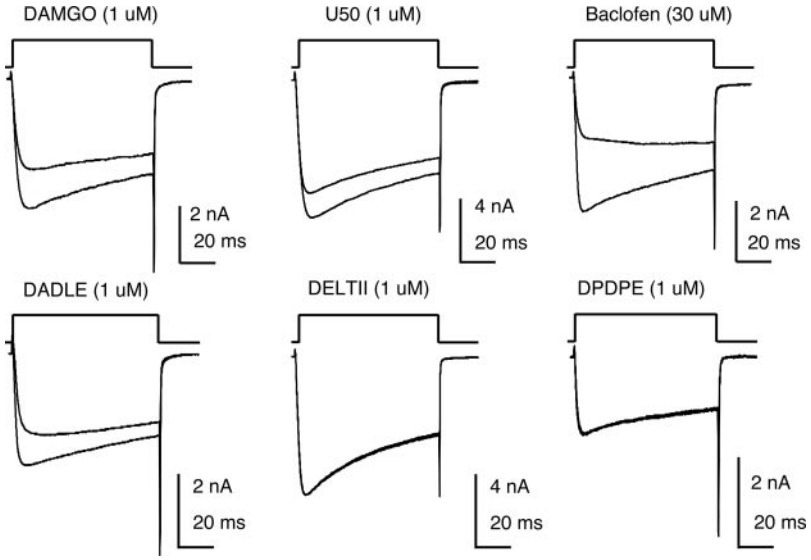

Fig. 1. Inhibitory coupling of opioid and $GABA_B$ receptors to Ca^{2+} channels in culture murine dorsal root ganglion neurons. Representative superimposed Ca^{2+} currents were recorded in the absence and presence of the μ agonist DAMGO (1 μM), the δ agonists DADLE, DPDPE, and DELT II (all 1 μM), the κ-agonist U-50,488H (1 μM), and the $GABA_B$ agonist baclofen (50 μM). Neither DPDPE nor DELT II had any effect on Ca^{2+}-current amplitude, as evidenced by indistinguishable currents in the absence and presence of these agonists. Inhibitory responses to DAMGO, DADLE, U-50,488H, and baclofen reversed during the subsequent wash.

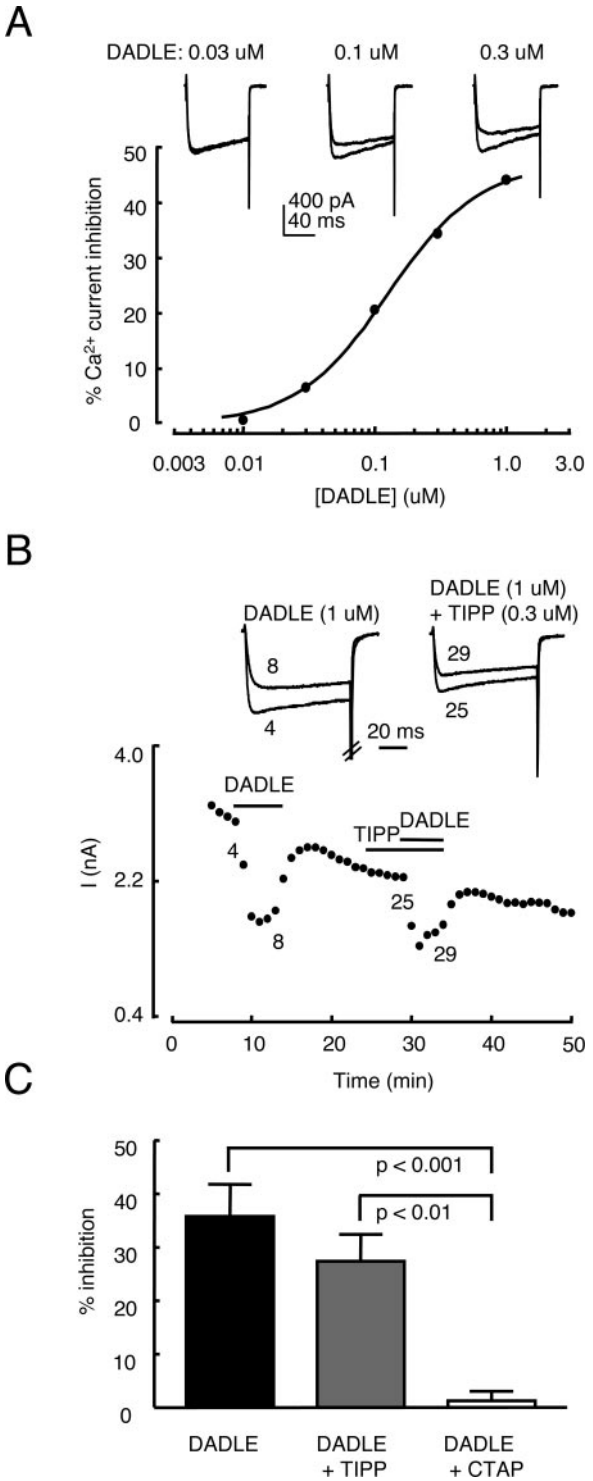

Fig. 2. DADLE inhibits Ca^{2+}-channel activity in dorsal root ganglion neurons through activation of μ receptors. A, DADLE caused a concentration-dependent inhibition of Ca^{2+}-current amplitude. Superimposed Ca^{2+} currents were recorded in the absence and presence of DADLE at the indicated concentrations. The IC_{50} value for DADLE was 0.13 μM and was calculated using a logistic fit to the data points. B, plot of voltage-activated Ca^{2+}-current peak amplitudes against time illustrating the effect of DADLE (1 μM) in the absence and presence of the δ antagonist TIPP (0.3 μM). Exemplar superimposed Ca^{2+} currents were selected at the indicated time points. C, bar graph of the inhibition by DADLE (1 μM) in the absence and presence of either CTAP (0.3 μM) or the δ antagonist TIPP (0.3 μM). Data shown are the mean ± S.E.M. from at least five separate recordings. Statistical significance was determined using ANOVA and the post hoc Tukey test.

(Fig. 3) are caused by changes in trafficking of receptors rather than altered levels of gene expression.

δ Receptors Couple to Ca^{2+} Channels in Dorsal Root Ganglion Neurons of $\mu-/-$ Mice. We hypothesize that δ receptors are expressed at insufficient levels in the membranes of dorsal root ganglion neurons of $\mu+/+$ mice for inhibitory coupling to voltage-activated Ca^{2+} channels. Along these lines, a previous study demonstrated that coupling of δ receptors to Ca^{2+} channels requires a higher level of available membrane receptors than is required for inhibitory coupling to adenylyl cyclase (Prather et al., 2000). We compared opioid receptor coupling with Ca^{2+} channels in dorsal root ganglion neurons from $\mu+/+$, $+/-$, and $-/-$ dorsal root ganglion neurons. As described previously (Walwyn et al., 2004), Ca^{2+} currents recorded from $\mu-/-$ neurons were insensitive to DAMGO (1 μM) ($n = 7$; Fig. 4, A and C). DAMGO was also without effect in $\mu+/-$ dorsal root ganglion neurons ($n = 8$), suggesting that, similar to δ receptors (Prather et al., 2000), there is a critical threshold level of μ receptors required for coupling to Ca^{2+} channels, which presumably exceeds the number of μ receptors in $\mu+/-$ dorsal root ganglion neurons. In contrast, reduction and deletion of μ receptors in $\mu+/-$ and $\mu-/-$ mice, respectively, led to an increase in the ability of the selective δ-ligands DPDPE and deltorphin II to inhibit Ca^{2+}-channel activity recorded from dorsal root ganglion neurons (Fig. 4, A and C). DPDPE (1 μM) and deltorphin II (1 μM) inhibited Ca^{2+}-channel activity by 11 ± 4% ($n = 8$; 63% responded) and 8 ± 3% ($n = 9$; 67% responded) in $\mu-/-$ dorsal root ganglion neurons. When nonresponders were removed from the analysis, DPDPE and deltorphin II inhibited Ca^{2+} currents by 18 ± 4 ($n = 5$) and 12 ± 3 ($n = 6$), respectively. There was no difference in the inhibition of Ca^{2+}-channel activity in $\mu+/+$, $\mu+/-$, and $\mu-/-$ mice by U-50,488H (Fig. 4). In contrast, there was a dramatic increase in the level of Ca^{2+} current inhibition by baclofen (50 μM) in $\mu-/-$ compared with $\mu+/+$ mouse dorsal root ganglion neurons. This is largely explained by an increase in the percentage of neurons responding to baclofen, from 60% ($n = 10$) in $\mu+/+$ neurons to 100% ($n = 5$) in $\mu-/-$ neurons. The μ/δ agonist DADLE (1 μM) inhibited Ca^{2+} channels (by 11%) in two of four of the $\mu-/-$ dorsal root ganglion neurons tested.

We further examined the possibility that μ receptor expression regulates the level of δ receptor coupling to Ca^{2+} channels in dorsal root ganglion neurons by reintroducing the μ receptor into cultured $\mu-/-$ dorsal root ganglion neurons using the Ad-μ receptor construct (Walwyn et al., 2004). Ad-μ receptor also encodes GFP, and successfully infected cells were identified using fluorescence microscopy. Expression of recombinant μ receptors restored the inhibitory effect of DAMGO (1 μM) on Ca^{2+}-channel activity in all fluorescent neurons tested (Fig. 4B). Ca^{2+} channels in $\mu-/-$ cells expressing recombinant μ receptors were resistant to DPDPE (1 μM) and deltorphin II (1 μM) (Fig. 4). These data demonstrate that μ receptor reintroduction abolishes functional coupling of δ receptors to Ca^{2+} channels in dorsal root ganglion neurons.

Taken together, the flow cytometry and electrophysiological data suggest that deletion of the μ receptor gene leads to an up-regulation of the expression of the δ receptor to a level that permits inhibitory coupling to Ca^{2+} channels in dorsal root ganglion neurons.

The Increased Coupling between δ Receptors and Ca^{2+} Channels in Neurons Lacking μ Receptors Is Not Caused by Different Phenotypes of Dorsal Root Ganglion Cultures from μ−/− and μ+/+ Mice. Different proportions of distinct neuronal phenotypes present in these early postnatal cultures could perhaps explain the enhanced inhibitory coupling between δ receptors and Ca^{2+} channels in μ−/− neurons. We therefore used flow cytometry to describe the phenotypic composition of μ−/− and μ+/+ cultures. The percentage of neurons expressing TrkA, IB4, and the pep-

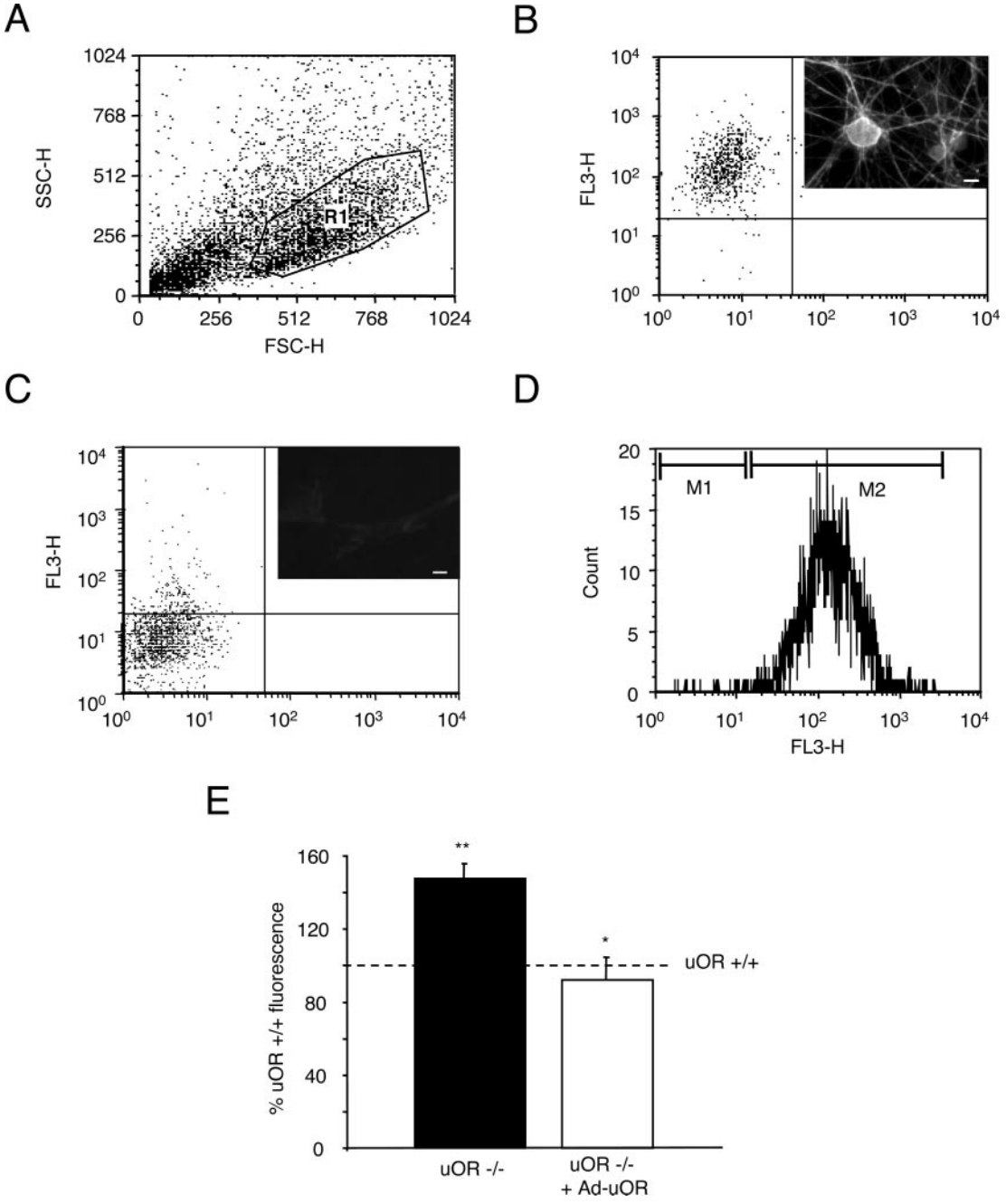

Fig. 3. δ Receptor surface protein is up-regulated in cultured dorsal root ganglion neurons from μ+/+ and μ−/− mice. Neurons were harvested after 3 days in culture. A, size (forward scatter, FSC-H) and granularity (side scatter, SSC-H), determined by flow cytometry, were used to define the population of dorsal root ganglion neurons (R1) labeled with an antibody to the extracellular N terminus of the δ receptor and visualized using PerCP fluorescence. B, the δ receptor-PerCP fluorescence intensity of the R1 population (from A) was acquired from the PerCP channel (FL3-H). Inset, an immunocytochemical example of a dorsal root ganglion neuron similarly labeled (scale bar = 10 μm). C, a scatter plot similar to that in B for dorsal root ganglion neurons lacking the δ receptor from δ−/− mice showing nonspecific labeling of this antibody in FL3-H. The inset shows an immunocytochemical example of the nonspecific labeling of a δ−/− dorsal root ganglion neuron. D, an exemplar histogram of the δ receptor-PerCP fluorescence intensity in FL3-H used to determine the mean RFI of each sample. M1 indicates the typical background fluorescence of a no-primary-antibody control sample and M2 the range of δ receptor-PerCP obtained from a sample labeled with both primary and secondary antibodies. E, normalization of the mean δ receptor-PerCP fluorescence intensity to that of μ+/+ neurons shows that in μ−/− neurons, fluorescence intensity is up-regulated to 149 ± 9% of μ+/+ levels. Returning μ receptor expression using the Ad-μ receptor returned δ receptor fluorescence intensity to μ+/+ levels. **, significant differences of fluorescence intensity compared with that of μ+/+ mice ($p < 0.005$, Student's t test). *, significant difference between the fluorescence intensity of μ−/− neurons and μ−/− neurons after expression of recombinant μ receptors ($p < 0.05$).

tides substance P, CGRP, neuropeptide Y, and somatostatin are shown in Table 1. As expected, these data show these cells to be phenotypically immature with a higher percentage of cells expressing TrkA and CGRP than found in adult dorsal root ganglion neurons. Some cells double-labeled with both IB4 and TrkA (13.4%) would be satellite cells present in these cultures (Walwyn et al., 2004). However, these data show no effect of μ receptor gene deletion on the neuronal subtypes present in these cultures.

Pharmacological Approaches for Boosting δ Receptor Number in Dorsal Root Ganglion Neurons. Pharmacological treatments that up-regulate functional δ receptors on primary afferent neurons may convert the δ receptor into a useful target for analgesic medications. Therefore, we investigated pharmacological strategies for increasing cell-surface δ receptor expression, assayed using flow cytometry, to determine whether such treatments would initiate inhibitory coupling between δ receptors and Ca^{2+} channels. As expected, prolonged (18 h) exposure of cultured $\mu+/+$ dorsal root ganglion neurons to the δ antagonist TIPP (5 μM) increased surface δ receptor expression (Table 2). The stimulatory effect of TIPP on cell-surface δ receptor levels was retained in $\mu-/-$ dorsal root ganglion neurons (Table 2). TIPP, as a strategy for increasing the number of functional δ

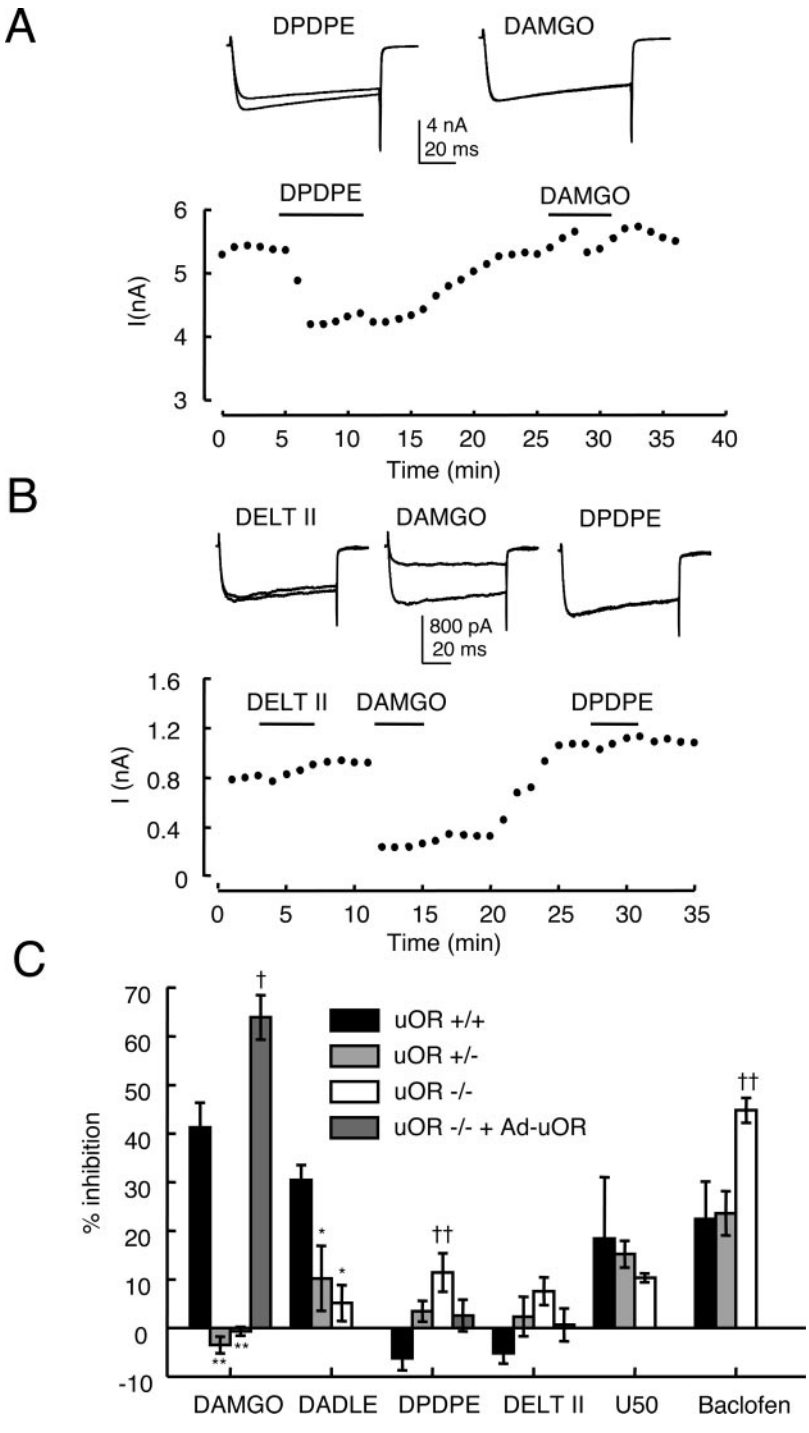

Fig. 4. Activation of δ receptors in $\mu-/-$ dorsal root ganglion neurons inhibits Ca^{2+}-channel activity. A, plot of voltage-activated Ca^{2+}-current amplitude against time, illustrating the effect of DPDPE (1 μM) and DAMGO (1 μM) in a $\mu-/-$ dorsal root ganglion neuron. Superimposed traces represent average Ca^{2+} currents recorded in the absence and presence of DPDPE or DAMGO. B, plot of the Ca^{2+}-current amplitude against time illustrating the effect of DELT II (1 μM), DAMGO (1 μM), and DPDPE (1 μM) in a $\mu-/-$ dorsal root ganglion neuron expressing recombinant μ receptors after transformation using the Ad-μ receptor. Superimposed traces represent average Ca^{2+} currents recorded in the absence and presence of DELT II, DAMGO, and DPDPE. C, bar graph of inhibition of Ca^{2+} currents by μ, δ, and κ agonists (DELT II, DAMGO, DADLE, and DPDPE, all 1 μM) and the $GABA_B$ agonist baclofen (50 μM) in dorsal root ganglion neurons from $\mu+/+$, $\mu+/-$, and $\mu-/-$ mice (with and without recombinant μ receptor expression). Statistical significance was determined using ANOVA and post hoc Tukey test; in all cases significance refers to comparison with control $\mu+/+$ response to that drug: *, reduction in inhibition ($p < 0.01$); **, reduction in inhibition ($p < 0.001$); †, increase in inhibition ($p < 0.05$); ††, increase in inhibition ($p < 0.01$).

receptors, would probably be of little therapeutic value because of the accompanying blockade of δ receptors. Therefore, we sought other methods for δ receptor up-regulation.

Transient exposure of dorsal root ganglion neurons to δ agonists causes a rapid (~50 s) mobilization of surface δ receptors through the insertion of large dense-core vesicles during exocytosis (Bao et al., 2003). If such a mobilization occurred in our electrophysiological experiments, it was insufficient to enable inhibitory coupling δ receptors to Ca^{2+} channels during exposures to either deltorphin II or DPDPE of >100 s (Fig. 1). Furthermore, exposure of cultured μ+/+ and μ−/− dorsal root ganglion neurons to DPDPE (1 μM) for 10 min caused a small reduction in the number of cell-surface δ receptors (Table 2). We therefore shifted our attention to μ ligands, suspecting that the μ receptor may affect δ receptor cell-surface expression, perhaps caused by the existence of μ/δ heterodimers (Gomes et al., 2004) or μ receptor signaling pathways (Eisinger et al., 2002). However an 18-h treatment of μ+/+ dorsal root ganglion neurons with the μ antagonist CTAP (300 nM) had no effect on δ receptor cell-surface expression (Table 2). We examined whether a 10-min exposure to DPDPE (1 μM) would mobilize intracellular δ receptors after 18 h of CTAP exposure. Indeed, this procedure increased the cell-surface expression of δ receptors to 139 ± 5% of levels observed in untreated μ+/+ neurons (Table 2). The stimulatory effects of CTAP and DPDPE treatment on surface δ receptor expression were dependent on the presence of the μ receptor; their combined effect was absent in dorsal root ganglion neurons from μ−/− mice (Table 2).

CTAP Increases Inhibitory Coupling between δ Receptors and Ca^{2+} Channels in Dorsal Root Ganglion Neurons. Treatment of dorsal root ganglion neurons for 18 h with CTAP caused the appearance of inhibitory coupling between δ receptors and Ca^{2+} channels. In most cells, DPDPE (1 μM) or deltorphin II (1 μM) caused a reduction in the Ca^{2+}-current amplitude (Fig. 5A) to 13.6 ± 5.3% (n = 15; 70% responded) and 12.9 ± 12.3% (n = 5; 60% responded) of control, respectively (Fig. 5B). When nonresponding cells were omitted from the analysis, DPDPE (1 μM) caused an inhibition of 18.7 ± 6.1% (n = 7). These data demonstrate that up-regulation of membrane δ receptor expression by 18-h exposure to CTAP (Table 2) initiates inhibitory coupling between δ receptors and Ca^{2+} channels (Fig. 5). CTAP-pretreated cells exhibited a small reduction in their inhibitory response to DAMGO (Fig. 5B). It is possible that there was a low level of residual CTAP that remained bound to μ receptors, despite several minutes of washing with CTAP-free saline before agonist application.

TABLE 1

Phenotypic composition of μ receptor −/− and μ receptor +/+ dorsal root ganglion cultures

Type	Positive Cells	
	μ−/−	μ+/+
	%	
IB4	37.1	29.3
TrkA	87.2	80.5
SP	7.0	4.0
CGRP	59.8	58.3
NPY	14.4	14.8
Somatostatin	13.5	17.6

NPY, neuropeptide Y.

TABLE 2

Pharmacological regulation of membrane δ receptor expression

Dorsal root ganglion neuron cell-surface δ receptor expression measured by flow cytometry after opioid ligand exposure. Naive neurons or neurons treated for 18 h with CTAP or TIPP were treated subsequently for 10 min with DPDPE. The mean δ receptor-PerCP fluorescence labeling intensity of the neuronal population was determined and expressed as a percentage of untreated neurons of the same genotype (± S.E.M.). Data were analyzed by ANOVA for each genotype (μ+/+, df = 5.0, F = 7.45; μOR−/−, df = 5.0, F = 6.84) followed by the least significant difference post hoc test.

	DPDPE (1 μM)	CTAP (0.3 μM)	CTAP (0.3 μM) + DPDPE (1 μM)	TIPP (5 μM)	TIPP (5 μM) + DPDPE (1 μM)
			%		
μ+/+	93 ± 2	102 ± 5	139 ± 5**	128 ± 6**	137 ± 4**
μ−/−	85 ± 7	96 ± 4	102 ± 7	135 ± 14**	153 ± 18**

** $p < 0.005$ versus untreated sample of the same genotype.

Discussion

We examined μ, δ, and κ receptor coupling to Ca^{2+} channels in dorsal root ganglion neurons of μ+/+, +/−, and −/− mice. The μ agonist DAMGO inhibited Ca^{2+}-channel activity recorded from μ+/+ neurons but had no effect on Ca^{2+} currents recorded from μ+/− or −/− neurons. DADLE also inhibited Ca^{2+} currents in μ+/+ neurons, but this occurred through activation of the μ receptor. More selective δ agonists DPDPE and deltorphin II had no effect in μ+/+ neurons. However, δ receptors coupled to Ca^{2+} channels in μ−/− neurons. Coupling of δ receptors to Ca^{2+} channels required up-regulation of receptors in cell-surface membranes either as a consequence of knocking out the μ receptor or through pharmacological means.

Opioid analgesics are primarily μ agonists, and patients may experience side effects, including tolerance and physical dependence (Mason, 1999). Both μ and δ receptors couple to the same effectors and have similar patterns of expression within pain pathways. However, δ agonists have fewer side effects (Porreca et al., 1984). The participation of δ receptors in opioid analgesia has recently been questioned (Scherrer et al., 2004). The lack of selectivity of δ agonists at analgesic concentrations contributes to the confusion. Furthermore, it is unclear from in vitro experiments whether δ receptors on primary afferent neurons are functionally relevant. In agreement with most previous studies in the rat, our data demonstrate that mouse dorsal root ganglion neurons express δ receptors that fail to couple to Ca^{2+} channels (Schroeder et al., 1991; Moises et al., 1994; Liu et al., 1995). One study of rat dorsal root ganglion neurons characterized an inhibitory effect of the δ agonist DADLE on Ca^{2+} channels (Acosta and Lopez, 1999). DADLE inhibits Ca^{2+} channels of mouse dorsal root ganglion neurons through activation of μ receptors and not δ receptors. The inhibition of Ca^{2+}-channel activity by DADLE was resistant to the δ-antagonist TIPP and blocked by the μ-antagonist CTAP in neurons of μ+/+ mice. Furthermore, Ca^{2+}-current inhibition by DADLE was reduced in μ−/+ and μ−/− mice.

Despite evidence for a predominantly intracellular δ receptor distribution in neurons (Zhang et al., 1998), we detected δ receptors on the cell surface of dorsal root ganglion neurons using flow cytometry. There is a critical threshold density of available recombinant δ receptors required to achieve coupling to Ca^{2+} channels in the GH_3 cell line (Prather et al., 2000). This density is greater than that required for coupling of μ receptors to Ca^{2+} channels in the same cells. Thus,

under basal conditions, the δ receptor density in dorsal root ganglion neurons may be below the threshold required for coupling to Ca^{2+} channels. Treatments that cause δ receptor up-regulation could initiate their participation in analgesia. Indeed, several treatments, including long-term pain and prolonged morphine administration, cause δ receptor up-regulation and a corresponding increase in the analgesic efficacy of δ agonists (Cahill et al., 2001, 2003).

We investigated whether δ receptor up-regulation compensates for the absence of μ receptors in μ−/− mice. DAMGO lacks the ability to inhibit Ca^{2+}-channel activity recorded from μ−/− dorsal root ganglion neurons (Walwyn et al., 2004). We found that there was no difference in the level of δ receptor mRNA in μ−/− and μ+/+ neurons, indicating a lack of compensatory up-regulation of δ receptor gene expression. However, there was an increase in the level of surface δ receptors revealed by flow cytometry. Furthermore, δ receptor activation in μ−/− neurons inhibited Ca^{2+}-channel activity. Inhibitory coupling between δ receptors and Ca^{2+} channels in μ−/− dorsal root ganglion neurons may contribute to δ-mediated analgesia in μ−/− mice (Qiu et al., 2000).

There was a striking increase in the number of μ−/− neurons that responded to the $GABA_B$ agonist baclofen compared with μ+/+ neurons. This could be caused by a compensatory increase in $GABA_B$ receptor gene expression, a greater availability of inhibitory G proteins, and/or greater access to other aspects of the Ca^{2+}-channel regulatory complex, in the absence of μ receptors. The latter possibilities could also contribute to the appearance of inhibitory coupling between δ receptors and Ca^{2+} channel in μ−/− neurons. However, this would also increase inhibitory coupling of κ receptors to Ca^{2+} channels in μ−/− neurons, and this did not

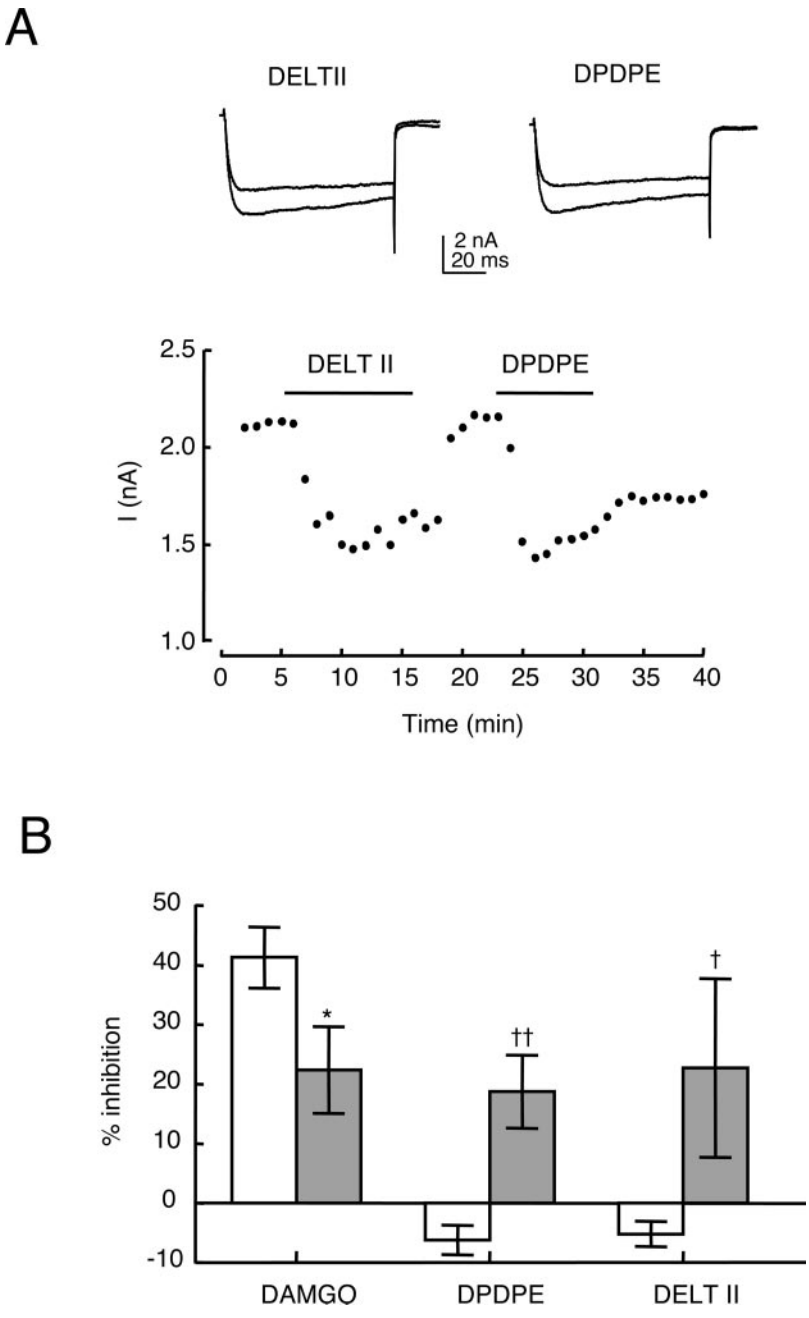

Fig. 5. Pharmacologically induced up-regulation of surface δ receptors initiates inhibitory coupling to Ca^{2+} channels. A, plot of voltage-activated Ca^{2+} current amplitude against time illustrating the effect of DELT II (1 μM) and DPDPE (1 μM) in a μ+/+ dorsal root ganglion neuron after 18 h exposure to CTAP (0.3 μM). Superimposed traces represent average Ca^{2+} currents recorded in the absence and presence of DELT II or DPDPE. B, a graph illustrating the effect of 18 h CTAP exposure on coupling of μ and δ receptors to Ca^{2+} channels in μ+/+ dorsal root ganglion neurons. In control neurons, DPDPE and DELT II cause a small enhancement of Ca^{2+}-current amplitude. CTAP treatment initiates the inhibitory coupling of δ receptors to voltage-activated Ca^{2+} channels. Statistical significance was determined using ANOVA and post hoc Tukey tests; in all cases, significance refers to comparison with control μ+/+ response to that drug: *, reduction in inhibition ($p < 0.01$); †, increase in inhibition ($p < 0.05$); ††, increase in inhibition ($p < 0.001$).

occur. Additional experiments are necessary to investigate the mechanisms underlying the increased baclofen response in μ−/− neurons.

An alternative explanation for altered efficacies of G protein-coupled receptors in μ−/− neurons could be a shift in the proportions of distinct phenotypic subtypes. Adult dorsal root ganglion neurons have been classified into different classes (Snider and McMahon, 1998). Two broad classes of small to intermediate-sized dorsal root ganglion neurons have been described: the TrkA-positive, peptide-rich class, and the IB4-positive, peptide-poor class. The difference in δ receptor and $GABA_B$ function in μ−/− neurons could reflect a difference in the proportion of these cell types (Wu et al., 2004). However, we found no change in composition between μ+/+ and μ−/− cultures (Table 1).

Reintroduction of μ receptors into μ−/− neurons using an adenoviral vector (Walwyn et al., 2004) caused the appearance of robust inhibitory coupling between μ receptors and Ca^{2+} channels. In contrast, DPDPE and deltorphin II had no significant effect on Ca^{2+}-channel activity in infected neurons, demonstrating that up-regulation of μ receptors abolishes functional δ receptor activity. We demonstrated previously that μ adenovirus-infected μ−/− neurons have ~7.5 times as many μ receptors as do uninfected μ+/+ neurons (Walwyn et al., 2004). Despite this high level of expression, κ receptors retained normal inhibitory coupling to Ca^{2+} channels, demonstrating that a failure of δ receptor function is unlikely to be caused simply by μ receptor monopolization of inhibitory G proteins. Together, our data demonstrate that there is a relationship between the level of functional cell-surface μ receptors and δ receptors in dorsal root ganglion neurons.

Elevation of intracellular [Ca^{2+}] or transient δ agonist application rapidly increases the density of δ receptor dorsal root ganglion neuron membranes through insertion by exocytosis of large dense-core vesicles (Bao et al., 2003). Up-regulation of cell-surface δ receptors may initiate inhibitory coupling, and such a phenomenon may convert δ agonists into effective analgesic agents. With this in mind, we used flow cytometry to identify pharmacological treatments that increase surface δ receptor expression. As expected, prolonged exposure of cultured μ+/+ neurons to TIPP increased surface expression of δ receptors. TIPP had similar effects in μ−/− neurons. δ Antagonists do not offer much promise for initiating δ-mediated analgesia because antagonists would render receptors unavailable for agonist activation. Therefore, we examined the effects of μ ligands. Prolonged CTAP treatment alone had little effect on δ receptor membrane expression. However, in parallel electrophysiological experiments, such an approach led to the appearance of inhibitory coupling between δ receptors and Ca^{2+} channels in 70% of μ+/+ neurons. One difference between these two experimental paradigms is the application of DPDPE to neurons during electrophysiological recording. Therefore, we tried prolonged CTAP application followed by a brief exposure to DPDPE and used flow cytometry to observe changes in δ receptor surface expression. This treatment successfully induced δ receptor up-regulation in μ+/+ but not μ−/− neurons. These data may be explained by μ-mediated regulation of δ-agonist-induced internalization (Eisinger and Schulz, 2002). On the other hand, perhaps μ antagonists increase the density of δ receptors on intracellular vesicles, and these are then rapidly mobilized by δ agonist exposure (Bao et al., 2003). Additional experiments are required to elucidate the mechanism of this δ receptor up-regulation.

Although μ and δ receptors are largely localized to different cellular compartments, surface or cytoplasmic (Wang and Pickel, 2001), and are trafficked through different pathways (Tanowitz and von Zastrow, 2003), they form heterodimers in vitro (Martin and Prather 2001; George et al., 2000) and possibly in vivo (Gomes et al., 2004). In dorsal root ganglion neurons, μ and δ receptors may heterodimerize, resulting in μ-dominant coupling to Ca^{2+} channels. A failure of μ/δ heterodimers in μ+/+ neurons to respond to δ ligands could explain the appearance of δ signaling in μ−/− neurons. However, δ receptors couple to Ca^{2+} channels in GH_3 cells expressing both μ and δ receptors (Piros et al., 1996) and δ ligands bind to μ/δ heterodimers inhibiting adenylyl cyclase activity (George et al., 2000).

Perhaps when the μ receptor is no longer available, then the δ receptor couples to Ca^{2+} channels, maintaining the integrity of the antinociceptive pathway. CTAP-pretreated cells show that a functional but not physical absence of μ receptors enables δ receptor up-regulation and initiates coupling to Ca^{2+} channels. CTAP treatment of SH-SH5Y cells up-regulates μ receptors on the cell surface (Zadina et al., 1994). It is possible that CTAP acts as an inverse agonist blocking constitutively active μ receptors. This may provide a signal that causes δ receptor up-regulation. However, CTAP is without intrinsic efficacy (Wang et al., 1994). Thus, it is not clear how CTAP triggers δ receptor up-regulation, but this effect is dependent on μ receptors because it was not observed in μ−/− neurons.

We hypothesize that δ receptors on primary afferent neurons do not contribute to analgesia under basal conditions because of an inadequate density of membrane receptors. Up-regulation of surface receptors initiates their inhibitory coupling to Ca^{2+} channels. Pharmacological approaches that increase surface δ receptor expression may reveal a novel target for opioid analgesia, one less likely to be associated with the side effects accompanying μ receptor-mediated analgesia.

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Address correspondence to: Dr. Tim G. Hales, Department of Pharmacology and Physiology, Medical Center, The George Washington University, 2300 Eye Street NW, Washington, DC 20037. E-mail: phmtgh@gwumc.edu.