**Delta Opioid Receptor Subtypes Activate Inositol-Signaling Pathways in the Production of Antinociception**

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**ABSTRACT**

To analyze the selectivity of delta receptor subtypes to regulate different classes of G proteins, the expression of the α-subunits of Gi2, Gi3, Go1, Go2, Gq and G11 transducer proteins was reduced by administration of oligodeoxynucleotides (ODNs) complementary to sequences in their respective mRNAs. Mice receiving antisense ODNs to Gαo, Gαq, Gα1α and G11α subunits showed an impaired antinociceptive response to all the delta agonists evaluated. An ODN to Gαq specifically blocked the antinociceptive effect of the agonist of delta-1 receptors, [D-Pen²,⁵]enkephalin (DPDPE), without altering the activity of [D-Ala²]deltorphin II or [D-Ser²]-Leu-enkephalin-Thr (DSLET). In mice treated with an ODN to Gαo, the effects of the agonists of delta-2-opioid receptors were reduced, but not those of DPDPE. Thus, Go1 proteins are selectively linked to delta-1-mediated analgesia, and Gq proteins are related to delta-2-evoked antinociception. After impairing the synthesis of Gαq, the effects of DPDPE exhibited an antagonistic activity on the antinociceptive potency of [D-Ala²]deltorphin II. After treatment with ODNs complementary to sequences in Gαo or PLC-β1 mRNAs, the analgesic capacity of [D-Ala²]deltorphin II was diminished. However, the delta-2-agonist did not alter the antinociceptive activity of DPDPE. An ODN complementary to nucleotides 7 to 26 of the murine delta receptor reduced the analgesic potency of [D-Ala²]deltorphin II, but not that observed for DPDPE. In these mice, [D-Ala²]deltorphin II did not antagonize the effect of DPDPE. These results suggest the existence of different molecular forms of the delta opioid receptor, and the involvement of inositol-signaling pathways in the supraspinal antinociceptive effects of delta agonists.

A series of studies on the antinociceptive effects of delta-selective agonists in the mouse has led to the proposed division of delta opioid receptors into subtypes, termed delta-1 and delta-2 (see for review Zaki et al., 1996). Most of the pharmacological evidence favoring the subdivision of delta receptors has come from the use of antagonists (Sofuoglu et al., 1991a; Portoghese et al., 1992; Vanderah et al., 1994). The delta-1 receptor was defined as the site activated by DPDPE and sensitive to antagonism by [D-Ala²,Leu²,Cys⁸]-enkephalin and 7-benzoylidenenaltrexone, the delta-2 receptor binds the agonists [D-Ala²]deltorphin II and DSLET and the antagonist naltrindole S′-isothiocyanate. Other pharmacological approaches, including tolerance and cross-tolerance studies (Mattia et al., 1991; Sofuoglu et al., 1991b), have also supported the existence of subtypes for the delta receptor. Consistent with this proposal, the CXBK inbred strain of mice exhibits a diminished response to i.c.v., but not intrathecal, [D-Ala²]deltorphin II (Raffa et al., 1992). It is proposed that these mice are deficient in the delta-2 subtype of the delta receptor in the brain, but not in the spinal cord. By contrast, a single functional subtype of delta opioid receptor has been described in *in vitro* bioassay systems, such as the mouse vas deferens (Wild et al., 1993).

The primary structure of a unique delta receptor from the adult mouse brain has been obtained by molecular cloning (Yasuda et al., 1994). The mRNA encodes a protein of 372 amino acids that presents a structural homology to G protein-coupled receptors. The ODN strategy has been used to impair delta receptor-mediated functions in *in vivo* studies (Standifer et al., 1994; Lai et al., 1994; Bilsky et al., 1996; Sánchez-Blázquez et al., 1997). After subchronically giving doses of an ODN complementary to sequences in delta opioid receptor, the i.c.v. injection of 125I-IgGs directed to delta receptors demonstrated a reduction of the protein encoded by the targeted mRNA (Sánchez-Blázquez et al., 1997). The *in vivo* administration of an ODN complementary to nucleotides 7 to 29 of the cloned delta receptor did not reduce the antinociceptive potency of all the agonists binding this opioid re-
ceptron (Lai et al., 1994; Bilsky et al., 1996; Sánchez-Blázquez et al., 1997). This observation supports the existence of subtypes for the delta opioid receptor.

It is assumed that, at the cellular level, the effects of opioids involve the inhibition of the Ca++ entry through voltage-sensitive Ca++ channels, and the stimulation of an outward K+ conductance (for review, see Childers, 1991). In addition, recent reports suggest the coupling of opioid receptors to the phosphoinositide cascade (Miyamae et al., 1993; Smart et al., 1994; Jin et al., 1994). Thus, it is of interest to determine whether the stimulatory effect of opioids on inositol lipid turnover also participates in the mechanisms of opioid-induced antinociception. It is well established that delta agonists regulate G proteins to produce supraspinal antinociception; this has been ascertained by the in vivo administration of agents known to interfere directly with the function of G proteins, i.e., pertussis toxin (Sánchez-Blázquez and Garzón 1988, 1991), antibodies administered by the i.c.v. route against G α1 and G α3 subunits (Sánchez-Blázquez and Garzón, 1993; Sánchez-Blázquez et al., 1993, 1995), and in vivo antisense strategies to reduce the synthesis of specific Gα subunits (Raffa et al., 1994; Sánchez-Blázquez et al., 1995; Standifer et al., 1996).

In our work, we have extended our previous studies on the regulation of G proteins by delta receptors in the production of supraspinal analgesia to include G protein classes associated with inositol lipid turnover: Gα1, Gα2, Gq and G11. The participation of PLCβ1 in this opioid activity was also explored. Distinct binding sites and inositol-signaling pathways mediated the supraspinal antinociceptive effects of delta agonists; one was linked to a PTX-sensitive Gα1 protein (delta-1), and the other to a PTX-resistant Gq protein (delta-2). These results provide further support for the existence of subtypes of delta receptors.

**Methods**

**Animals and evaluation of analgesia.** Male albino mice CD-1 (Charles River, Barcelona, Spain) weighing 22 to 25 g, were used throughout this study. Animals were kept at 22°C under a 12-hr light/dark cycle (8 A.M./8 P.M.). Food and water were provided ad libitum. Mice were housed and used strictly in accordance with the guidelines of the European Community about Care and Use of Laboratory Animals. To reduce the possibility of interference from spinal events, all substances were injected i.c.v. into the right lateral ventricle, as previously described (Sánchez-Blázquez et al., 1995, 1997). Briefly, animals were lightly anesthetized with ether, and injections were given with a 10-μL Hamilton syringe at a depth of 3 mm, 2 mm lateral and 2 mm caudal from the bregma. Four μL of each solution were infused at a rate of 1 μL every 5 sec; afterward, the needle was maintained for an additional period of 10 sec. The warm water (52°C) tail-flick test was used to measure the antinociceptive effect. Latencies in seconds were determined both before treatment (basal latency) and also after the administration of the substance under study. Baseline latencies ranged from 1.5 to 2.2 sec and were not affected by ODN administration. A cut-off time of 10 sec was allotted to minimize the risk of tissue damage. Antinociception was expressed as a percentage of the MPE according to the following formula: %MPE = 100 x (test latency-baseline latency)/cut-off time-baseline latency. Dose-response curves were routinely constructed for the opioids under control and treatment conditions; however, for the sake of directness the data are presented in a single dose format. Intracerebroventricular doses of the opioids were given and antinociception was assessed after 90 min for β-endorphin, after 15 min for DPDPPE or DSLET and after 10 min for [d-Ala²,deltorphin II. All compounds were dissolved in distilled water except for [d-Ala²,deltorphin II, which was dissolved in 1% DMSO. Agonist solutions were made up immediately before use. Statistical analysis of the results was accomplished by analysis of variance followed by the Student-Newman-Keuls test. The level of significance was set at P < .05.

**Synthesis of oligodeoxynucleotides.** End-capped phosphorothioate ODNs were synthesized in a CODER 300 DNA synthesizer using phosphorothioate chemistry (Matteucci and Caruthers, 1981). Crude ODNs were purified by reverse-phase chromatography using COP cartridges (Cruachem, Glasgow, UK). The eluted ODNs in 50% acetonitrile-water were then lyophilized (Rouan RC 1009/RCT 90, France). Sequences were as follows: ODN-G α2a: 5’-A*T*GTC- 

AGCCACAGCTCTGCCGATAGGGCC*G*A-3’, corresponding to nucleotides 529-556 of the Gα2a gene sequence (Jones and Reed, 1987). This ODN was also labeled in the final synthetic cycle with fluorescein-CE phosphoramidite at the 5’-end (Cruachen, Glasgow, UK, #22-8409). ODN-G α2a: 5’-G*C*CATCTGCCCATAACGTTGATTACACGCTTCTGAAGGGC*A*C*T-3’, corresponding to nucleotides 554-587 of the Gα2a gene sequence (Jones and Reed, 1987). Both oligos were previously characterized (Raffa et al., 1994; Sánchez-Blázquez et al., 1995). ODN-G α1a: 5’-A*G*GCCAGCTCATCTTATAGGCT*T*T-3’, a 25 base oligo corresponding to nucleotides 882-906 of the murine Gα1a gene sequence; and ODN-G α1a: 5’-G*C*GAGCACATTGTCGTAAGGGC*A*C*T-3’, which corresponds to nucleotides 882-906 of the Gα1a sequence (Strathmann et al., 1990). These two ODNs were identical to those used by Kleuss et al. (1991) in *in vitro* experiments. ODN-G α1: 5’-C*G*GCTACAGGGCGTTACAGT*A*T-3’, corresponding to nucleotides 484-504 of the Gα gene sequence; ODN-G α2a: 5’-C*G*GCTACAGGGCGTTACAGT*A*T-3’, which corresponds to nucleotides 487-507 of the Gα2a sequence; and ODN-G α2a: 5’-C*G*GCTACAGGGCGTTACAGT*A*T-3’, which corresponds to nucleotides 724-744 of the Gα2a sequence (Strathmann and Simon, 1990). ODN-delta3-26: 5’-G*C*GACGG-GGCAAGCAGCCACC*A*G*3’, corresponding to nucleotides 7 to 26 of the murine delta opioid receptor gene. This ODN is identical to that designed by Lai et al. (1994). And finally, ODN-PLCβ1: 5’-G*C*GCTACAGGGCGTTACAGT*A*T-3’, corresponding to nucleotides 49-63 of the PLCβ1 gene sequence (Suh et al., 1989). These sequences displayed no homology to other relevant cloned proteins (GeneBank database).

A random oligo (ODN-RD) with the sequence 5’-C*C*CTTATTATCTACTTCTC*G*C*3’ served as a control (Sánchez-Blázquez et al., 1995).

**Administration of ODNs.** ODN solutions were made up in the appropriate volume of sterile water immediately before use. Animals received either the vehicle, the random sequence ODN or the antisense oligo. These were injected into the right lateral ventricle and subsequent administrations were performed on the same side. Each ODN treatment was performed on a distinct group of 15 to 20 mice using the following schedule: on days 1 and 2, with 2 nmol; on days 3 and 4, with 3 nmol; on day 5, with 4 nmol. On day 6, the opioid agonists were injected i.e.v. and their antinociceptive activity evaluated by the warm water tail-flick test. An interval of 24 hr was selected between ODN administrations to minimize neurotoxic damage (Chasson et al., 1994). With this schedule, the experimental animals displayed no noticeable behavioral changes.

**Visualization at the PAG level of a fluorescence-labeled ODN to G α2a mRNA.** Mice that had received a single i.e.v. injection of 3 nmol of a fluorescence-labeled ODN-G α2a were killed 10, 30 or 120 min later. Brains were removed and frozen on dry ice. Coronal cryostat sections (20 μm) were cut, mounted onto gelatin-subbed slides and coveredslipped in a solution of 0.1 M phosphate buffer-30% glycerol. Sections were analyzed under a Leitz TCS 4D confocal laser-scanning microscope equipped with an argon/krypton-mixed gas laser with excitation peak at 488 nm. The confocal microscope was associated with a Leitz DMRB fluorescence microscope.
Electrophoresis and immunoblotting. Membranes from PAGs of ODN-treated mice were solubilized in a buffer containing 50 mM Tris.HCl, 3% sodium dodecyl sulfate, 10% glycerol, 5% β-mercaptoethanol, pH 6.8, and the proteins (30 to 60 μg protein/lane) were subsequently resolved by polyacrylamide sodium dodecyl sulfate gel electrophoresis [12.5% acrylamide concentration/0.0625% bisacrylamide cross-linker concentration, with a linear gradient from 4 to 8 M urea (Shah and Milligan, 1994)] with 8-cm × 11-cm × 0.15-cm slab gels (Hoefer SE 280) at 20-mA constant current (ISCO power supply, model 595). For detection of PLCβ1, brain PAGs were collected in Tris buffer 50 mM (pH 7.4); 1 mM EDTA, 1 mM benzamidine, 0.1 mM phenylmethanesulfonyl fluoride, 1 μg/μl leupeptin and 1 μg/μl pepstatin, and homogenized. After centrifugation, the supernatant was resolved by electrophoresis (gradient of 5-16% total acrylamide concentration/2.6% bisacrylamide cross-linker concentration). The proteins were transferred (Mini-Trans-Blot electrophoretic transfer cell; Bio-Rad, Hercules, CA) to 0.2-μm polyvinylidene difluoride Trans-Blot membranes (Bio-Rad) using Towing buffer (25 mM Tris.HCl, 192 mM glycerine, 0.04% sodium dodecyl sulfate, 20% methanol), by application of 70 V (200-300 mA) for 120 min. Unoccupied protein sites were blocked with 5% w/v non-fat dry milk (Blocker; Bio-Rad) in Tris-buffered saline for 1 hr at 37°C. The membranes were incubated with antibodies against Ga subunits at 1:1,000 dilution in Tris buffered saline-0.05% Tween 20 at 6°C for 24 hr, and subsequently with a secondary antisera [goat anti-rabbit IgG (H + L) horseradish peroxidase conjugate (BioRad, Hercules, CA #170-6515)] diluted 1:3,000 in Tris-buffered saline-0.05% Tween 20 for 3 hr. Antibody binding was detected using the substrate solution (1 mg/ml 3,3′-diaminobenzidine, 0.02% hydrogen peroxide, 0.04% nickel chloride in 0.1 M Trizma base buffer pH 7.2). A mixture of IgG monoclonal antibodies (Upstate Biotech, Lake Placid, NY, #05-164) diluted to 1:1,000 was used to detect PLCβ1. The IgGs were detected with a secondary antisera [goat anti-mouse IgG (H + L) horseradish peroxidase conjugate (BioRad, #170-6516)] and visualized with Enhanced Chemiluminescence reagents (Amersham Corp., Buckinghamshire, UK.). Immunoblots were analyzed by densitometry using an instrument with reflectance capabilities (BioRad, GS-700 Imaging Densitometer).

Antibodies and chemicals. The antisera used were raised against: anti Gαq internal fragment [115-125: EEQGMPLEDLS] S/1 (Sánchez-Blázquez et al., 1993, 1995). Anti Gαq (371729-Q) and anti Gαq (371752-Q) were obtained from Calbiochem-Novabiochem Corporation (La Jolla, CA). Anti-Gαq (GC/2, NEI-804) and anti Gαq (QL, NEI-809) came from Du Pont-New England Nuclear Research Products (Boston, MA). Anti-PLCβ1 (#05-164) monoclonal antibodies were obtained from Upstate Biotechnology. Human β-endorphin, DSLET, DPDPE and [2-Ala5]Deltorphin II were purchased from Peninsula Laboratories Europe (Merseyside, UK.).

Results

Changes in Ga-like immunoreactivity after in vivo administration of oligodeoxynucleotides. Ten minutes after i.c.v. injection of the fluorescein-labeled ODN, fluorescence was detected in the periaqueductal region (fig. 1). Subsequently, and in a time-dependent fashion, these signals entered from the cerebral aqueduct and spread through the neural tissue. For immunodetection, the mice were killed 6 days after starting the subchronic administration of the ODNs and P2 membranes from PAG were obtained and resolved by polyacrylamide sodium dodecyl sulfate gel electrophoresis. In the absence of urea, the anti Gαq/13, anti Gαq appear to recognize a single, 42 kDa, polypeptide. Similarly, the anti Gαq antisera labeled a single band of 39 kDa (not shown). Gαq and Gα11 subunits could be resolved with a linear gradient of 4 to 8 M urea, Gα11 showing a greater electrophoretic mobility than Gαq subunits (fig. 2, upper panel). An identical approach was utilized to separate Gαq from Gα11 in immunoblots (Mullaney and Milligan, 1990) (fig. 2, upper panel). The ODNs corresponding to mRNA of Ga subunits reduced the extent of labeling in immunoblots. Molecular weight markers are not indicated when urea was used, because this procedure alters the migration of the protein standards used. The diminishing effect of ODNs to mRNAs of Gαq and Gα11 subunits on the target proteins has already been reported (Sánchez-Blázquez et al., 1995).

After resolving the soluble phase of mouse PAG by SDS-PAGE, the mixture of monoclonal antibodies raised against PLCβ1 recognized a protein of about 150 kDa (Suh et al., 1988). When mice had been treated with an ODN-PLCβ1 the intensity of labeling was reduced (fig. 2, bottom). This treatment showed no cross-effect on the immunoreactivity associated to Gαq, Gαq or Gα11 subunits in neural membranes (data not shown).

Effects of in vivo administration of ODNs and antibodies to Ga subunits on supraspinal analgesia induced by opioids binding delta opioid receptors. Mice that received i.c.v. injections of saline, the random oligo or ODNs to Ga subunits, responded in the analgesic test with basal latencies comparable to those of noninjected (naive) mice. The analgesic substances produced similar effects in
mice that received i.c.v. injections of the vehicle, the random oligo, or in naive animals. Thus, the responsiveness of mice in the antinociceptive test was not altered by the experimental procedure alone.

The subchronic administration of ODNs to G\textsubscript{11}α, G\textsubscript{q}α, and G\textsubscript{11}α subunits was followed by a significant decrease in the antinociceptive potency of the delta receptor-agonists DPDPE and [\textit{d-Ala}^2]deltorphin II (fig. 3). The impairment of G\textsubscript{q}α function led to a weaker analgesic response to DPDPE, whereas, the effect of [\textit{d-Ala}^2]deltorphin II was unaltered in these mice. The antisense ODN to G\textsubscript{q}α subunit-mRNA produced opposite effect; DPDPE-induced antinociception was unchanged although the activity of the selective agonists of delta-2-opioid receptors [\textit{d-Ala}^2]deltorphin II and DSLET, as well as that exhibited by the endogenous opioid peptide β-endorphin, clearly diminished (figs. 3 and 4).

In a parallel set of experiments, 2 μg of affinity-purified IgGs to various G\textsubscript{α} subunits were i.c.v.-injected into the mice. Control animals received 2 μg of protein A-purified IgGs (preimmune serum) in the same volume. The supraspinal

Fig. 2. Immunoblots of SDS extracts from mouse PAG with antipeptide antibodies to G\textsubscript{α} subunits and PLC\textsubscript{β}1. Mice received repeated i.c.v. injections of ODNs to G\textsubscript{α} subunits or PLC\textsubscript{β}1 mRNAs on a 5-day schedule. Upper panel, Immunological detection of G\textsubscript{11}α, G\textsubscript{q}α, G\textsubscript{11}α and G\textsubscript{q}α subunits. SDS-solubilized PAG were resolved by polyacrylamide sodium dodecyl sulfate gel electrophoresis (12.5% acrylamide/0.0625% bisacrylamide, with a linear gradient of 4-8 M urea) and Western blotted. Immunodetection of G\textsubscript{q}α was performed with the antiserum 371752-Q. Concurrent immunological detection of G\textsubscript{q}α and G\textsubscript{11}α was achieved with antiserum QL. For G\textsubscript{01}α and G\textsubscript{02}α, the GC/2 antiserum was used. Under these conditions, G\textsubscript{11}α migrated more rapidly than G\textsubscript{q}α, and G\textsubscript{02}α more rapidly than G\textsubscript{01}α. For each comparison, the assay was repeated at least four times using samples from different animals. Lower panel, soluble proteins from PAG were analyzed for PLC\textsubscript{β}1-like immunoreactivity. A linear relationship of the amount of protein to the intensity of signal was attained. Immunoblots were analyzed with optical densitometry by an instrument with reflectance capabilities (BioRad, GS-700 Imaging Densitometer) and Molecular Analyst Software (v 1.5). Data are expressed as the mean ± S.E.M. from four independent assays. RD, Random ODN, ODN: ODN to G\textsubscript{α} or PLC\textsubscript{β}1 mRNAs.

Fig. 3. Effect of subchronic i.c.v. administration of ODNs to G\textsubscript{α} subunits on supraspinal antinociception induced by delta opioid agonists. Animals received injections with increasing amounts of ODNs on a once-daily schedule for 5 consecutive days (see “Methods”). On day 6, the antinociceptive activity of opioids at the supraspinal level was evaluated by the tail-flick test. Preliminary experiments demonstrated the peak effects for analgesia 15 min after DPDPE and 10 min after [\textit{d-Ala}^2]deltorphin II. For each opioid and treatment (saline, ODN-RD, and ODNs), a different group of mice was used. Antinociception is expressed as a percentage of the maximum possible effect (MPE). Values are the mean ± S.E.M. from groups of 10 to 15 mice each. *Significantly different from the control group receiving saline or the random ODN (RD) instead of the ODN to the corresponding G\textsubscript{α} subunit. Analysis of variance, Student-Newman-Keuls test, P < .05.
antinociceptive activity of the opioid agonists was assessed 24 hr later. As previously described, i.c.v. administration of preimmune IgGs did not alter the response of the animals to the analgesic effect of opioids (Sánchez-Blázquez and Garzón, 1993; Sánchez-Blázquez et al., 1993, 1995). Immune IgGs heated at 100°C for 10 min lacked activity on opioid-induced antinociception (data not shown). However, a single administration of the antibodies to the Go subunits reproduced the effects of sub-chronic ODNs. I.c.v. injection of anti-Gioα (S/1), anti-Gi3α (CN-1), anti-Go1/2α (CG/2) and anti-Gq/11α (QL) antibodies reduced the antinociceptive activity of the opioids binding to delta opioid receptors (fig. 5). The administration of anti Gqα (CN-Q) IgGs only impaired the effect of the delta-2-agonist [D-Ala2]deltorphin II.

**Effect of in vivo administration of ODNs to phospholipase Cβ1 on the supraspinal analgesia induced by delta agonists in mice.** The effect of ODN-PLCβ1 was evaluated in vivo. Mice subjected to subchronic administration of the ODN to PLCβ1 showed an impaired analgesic response to [D-Ala2]deltorphin II, DSLET and β-endorphin. Notwithstanding, in these mice the antinociception induced by DPDPE was preserved and [D-Ala2]deltorphin II did not antagonize DPDPE (fig. 7). The effect of agonists binding mu-opioid receptors, DAMGO and morphine, was not altered by treatment with the antisense ODN to phospholipase Cβ1 (not shown).

**Discussion**

Pharmacological studies (Mattia et al., 1991; Sofuoglu et al., 1991a, b; Portoghese et al., 1992; Vanderah et al., 1994)
and antisense ODN strategies (Lai et al., 1994; Bilsky et al., 1996; Sánchez-Blázquez et al., 1997) have convincingly demonstrated the existence of subtypes of the delta opioid rece-

tors. However, only one molecular form for this opioid receptor has been described so far (Evans et al., 1992; Kieffer et al., 1992; Yasuda et al., 1994). In in vivo studies using antibodies (Sánchez-Blázquez and Garzón, 1993; Sánchez-Blázquez et al., 1993, 1995) or antisense ODNs (Raffa et al., 1994; Sánchez-Blázquez et al., 1995; Standifer et al., 1996) directed to Ga subunits of transducer proteins known to inhibit adenylyl cyclase activity, it was found that the blocking of Gi2 or Gi3 subunits, but not Gi1 or Gx/z, significantly reduced delta-mediated supraspinal antinociception in mice. Although these treatments effectively differentiated mu- and delta-mediated antinociception, they did not discriminate between the pharmacologically defined subtypes of delta opioid receptors.

The coupling of delta receptors to various effector systems (e.g., phospholipase C and type II adenylyl cyclase) has been documented in a series of in vitro studies (Tsu et al., 1995). The observed impairment of delta-mediated supraspinal antinociception by antisense ODNs complementary to Go1 and Gq subunits, indicates that distinct signaling pathways associated with phosphoinositide cascades are involved in this opioid effect. The Gi2, Gi3, Go2 and G11 proteins are activated by both subtypes of delta opioid receptors in the production of supraspinal antinociception (Raffa et al., 1994; Sánchez-Blázquez et al., 1995; Standifer et al., 1996; present work). Go1 proteins are regulated selectively by delta-1 receptors whereas Gq seems to be linked solely to the effect of delta-2-agonists (fig. 8).

The selective regulation of different classes of G proteins by the delta-agonists in the production of antinociception, supports the proposed existence of two separate subtypes of delta opioid receptor. It might also be feasible that after binding a unique delta opioid receptor, certain agonists acti-
vate different classes of G proteins, i.e., \( G_\alpha \), \( G_\beta \). Different functional profiles would then be observed. This suggests the pharmacological heterogeneity of the \( \delta \) receptor subtypes described. If this is the case, both antagonists, \([D-Ala^2]\)deltorphin II and DPDPE, ought to bind to this unique \( \delta \) receptor. This is consistent with the finding that after treatment with the ODN to \( G_\alpha \) subunits, DPDPE, which is more effective than \([D-Ala^2]\)deltorphin II in activating Go1 proteins, now antagonized the analgesic effect of \([D-Ala^2]\)deltorphin II. In the single receptor model, \([D-Ala^2]\)deltorphin II, which is more efficient than DPDPE in activating Go1 proteins, should reduce the effect of DPDPE in \( G_\alpha \)-deficient mice. However, in this paradigm, antagonism of \([D-Ala^2]\)deltorphin II on DPDPE was not observed. A single receptor also fails to explain why the selective impairment promoted by ODN-\(\delta\)-29 on the antinoceptive effect of \( \delta \)-agonists (Lai et al., 1994; Bilskey et al., 1996; Sánchez-Blázquez et al. 1997; present work) is not accompanied by \([D-Ala^2]\)deltorphin II antagonism of DPDPE activity. These observations indicate that the \( \delta \)-agonist \([D-Ala^2]\)deltorphin II does not binds to the receptors acted upon by DPDPE, whereas DPDPE might also bind the receptors of \([D-Ala^2]\)deltorphin II.

Similarly, the work of Vanderah et al. (1994), using the antagonists \([D-Ala^2,Leu^6,Cys^8]\)-enkephalin and naltrindole-5′-isothiocyanate selective for the pharmacologically defined \( \delta \)-1 and \( \delta \)-2 receptors, supports the existence of two distinct subtypes of the \( \delta \) receptor. Recent results from work with ODNs corresponding to the cloned opioid receptor gene are also consistent with the existence of closely related molecular forms of this receptor (Rossi et al., 1997; Sánchez-Blázquez et al., 1997). In mice whose \( \delta \) receptor gene has been disrupted by gene targeting, the capacity of both DPDPE and \([D-Ala^2]\)deltorphin II to produce analgesia was strongly but not completely reduced (Zhu et al., personal communication). Therefore, at the present time, it cannot be concluded whether the \( \delta \) receptor subtypes arise as splice variants from a unique gene, or come from separate genes.

In this study, it was shown that besides the Gi family of G-transducer proteins, \( \delta \) receptors are associated with Go and Gq classes in the production of supraspinal antinociception in the mouse. Jin et al. (1994) have reported activation of phosphoinositide cascades via \( \delta \) receptors and the subsequent mobilization of calcium from inositol 1,4,5-triphosphate sensitive stores in neuroblastoma x glioma 108-15 cells. In \( Ltk^- \) fibroblasts transfected with the cloned delta receptor, DPDPE was able to stimulate phosphoinositide-specific phospholipase C through a pertussis toxin-sensitive G protein (Tsu et al., 1995). The inhibition of the \( \alpha \)-subunits of Gi and Go proteins reduced the degree of analgesia evoked by DPDPE. These findings indicate that pertussis toxin-sensitive G proteins are implicated mainly in the physiological effects of this compound. The G proteins activated by the pharmacologically defined \( \delta \)-1 receptor in the production of supraspinal analgesia (fig. 3) are in accordance with observations made when using membranes of human neuroblastoma SH-SY5Y cells. In these cells, which exhibit mu and \( \delta \) receptors, DPDPE, after binding selectively to the receptors acted upon by DPDPE, increased the incorporation of [\( \alpha^{32}P \)]GTP azidoanilide into the \( \alpha \)-subunits of G11, G12, G13, Go1 and Go2 proteins (Laugwitz et al., 1993). There are few in vitro studies on the regulation of G protein classes and effectors by \( \delta \)-selective compounds (Garzón et al., 1997a, b), making direct comparison with the in vivo results difficult. Nevertheless, the data of our study reveal that multiple G proteins, including the pertussis toxin-insensitive Gq which stimulates phosphoinositide breakdown, participate in \( \delta \)-mediated antinociceptive effects.

G protein-mediated activation of phospholipase C\( \beta \) isozymes occurs via at least two mechanisms, each showing different sensitivities to pertussis toxin. Activation of PLC\( \beta 1 \), PLC\( \beta 2 \) and PLC\( \beta 3 \) is achieved with the help of the pertussis toxin-insensitive G\( \alpha \) subunits of Gq transducer proteins (Smrcka et al., 1991; Taylor et al., 1991; Wu et al., 1992, 1993). Also, stimulation of PLC\( \beta \) isozymes is achieved through G\( \beta \gamma \) subunits of pertussis toxin-sensitive G\( \alpha \) subunits of Gq transducer proteins (Moriarty et al., 1990; Camps et al., 1992; Carozzi et al., 1993). In coreconstitution experiments using lipid vesicles, PLC\( \beta 1 \) stimulates hydrolysis of Gq11-bound GTP and acts as a GTPase-activating protein for its physiological regulator, G\( \alpha \)/11 (Berstein et al., 1992). The ODN-induced inhibition of G\( \alpha \) and phospholipase C\( \beta 1 \) leads to a similar impairment in the antinoceptive effects of agonists of \( \delta \)-opioid receptors. This data, supports the idea that there is coupling of this subtype of \( \delta \) receptor to Gq proteins and PLC\( \beta 1 \) effector enzyme. By using DPDPE, the coupling of \( \delta \) opioid receptors in the stimulation of PLC and the generation of phosphoinositides has been shown in NG108-15 cells. This has also been achieved in Xenopus oocytes and \( Ltk^- \) fibroblasts expressing cloned \( \delta \) receptors (Miyamato et al., 1993; Jin et al., 1994; Tsu et al., 1995). The DPDPE-
induced activation of G11 proteins in the production of supraspinal antinociception appears not to be related to PLCβ1 as no reduction was found in this agonist in mice treated with the ODN to this enzyme. Whether DPDPD leads to stimulation of PLCβ2 and/or PLCβ3 isoforms remains to be seen.

In summary, the supraspinal antinociception produced by agonist-activated delta receptors is mediated by G proteins of the Gq/11 family as well as by Gi- and Go-transducer proteins. The present work provides further evidence favoring the existence of delta-1- and delta-2-opioid receptors. Go1 proteins seem to be selectively activated by delta-1 receptors, whereas delta-2 receptors show preference for the pertussis toxin-insensitive Gq proteins in this effect. It might therefore be concluded that delta receptors trigger at least two signaling cascades, adenyl cyclase and phospholipase C, in the production of analgesia.

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References


