Opioids Binding Mu and Delta Receptors Exhibit Diverse Efficacy in the Activation of $G_{i2}$ and $G_{x/z}$ Transducer Proteins in Mouse Periaqueductal Gray Matter

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ABSTRACT

A nonisotopic, immunoelectrophoretic technique was used to analyze the characteristics of opioid-evoked activation of $G_{i2}$/$G_{x/z}$ transducer proteins of mouse periaqueductal gray matter membranes. In the presence of picomolar concentrations of guanosine 5’-O-(3-thiotriphosphate), the opioid agonists promoted concentration-dependent increases of immunoreactivity associated with free $G_{i2,0}$ and $G_{x/z,0}$ subunits. [$\alpha$-Ala$^2$,N-MePhe$^4$, Gly-$\beta$-ol$^5$]enkephalin and morphine (preferential agonists at mu opioid receptors) and $\beta$-endorphin-(1–31) (an agonist at $\mu$/$\delta$ opioid receptors) activated $G_{i2,0}$ proteins. In contrast, the agonists of delta opioid receptors, [D-Ala$^2$]deltorphin II and [D-Pen$^2$-$\gamma$]-enkephalin, displayed little or no activity on this pertussis toxin resistant regulatory protein. Although exhibiting diverse efficacy, all the opioids studied activated $G_{i2}$ transducer proteins. [$\alpha$-Ala$^2$,N-MePhe$^4$,Gly-$\beta$-ol$^5$]enkephalin and [D-Ala$^2$]-deltorphin II were more potent at $G_{i2,0}$ subunits than at $G_{x/z,0}$ subunits. The opioid antagonist naloxone displayed a competitive profile in reducing the activation of G proteins promoted by morphine. Moreover, [D-Pen$^2$-$\gamma$]-enkephalin antagonized the releasing effect exerted by [D-Ala$^2$]deltorphin II on $G_{i2,0}$ and $G_{x/z,0}$ subunits. $N,N$-diallyl-Tyr-Alb-Alb-Phe-Leu (ICI-174864) reduced the $G_{i2}$-related immunosignals promoted by agonists of delta opioid receptors. Therefore, it is suggested that opioids exhibit marked differences in efficacy and/or potency in the activation of $G_{i2}$ and $G_{x/z}$ transducer proteins in mouse periaqueductal gray matter.

Opioid receptors belong to the superfamily of receptors that regulates GTP-binding proteins (G proteins). The ability of a receptor to regulate different classes of G proteins causing the productive activation of multiple G protein signaling pathways has been convincingly documented (Kenakin, 1995). Much work has been performed to characterize transduction regulated by opioid receptors, not only in vitro but also in vivo systems, e.g., the production of antinociception. The i.c.v. injection of pertussis toxin into rodents demonstrated the regulation of $G_i/G_o$ families by mu/delta opioid receptors in the mediation of analgesia (Parenti et al., 1986; Sánchez-Blázquez and Garzón, 1988, 1991; Lutfy et al., 1991). To gain information about the transduction system implicated in analgesia mediated by $G_{i2}$ and $G_{x/z}$ opioid receptors, the authors’ group pioneered in vivo studies with antibodies to $G_{i/o}$ subunits. Possible neural processes facilitating the access of IgGs to G proteins have been discussed (Garzón, 1995). Supraspinal antinociception produced by agonists of delta receptors was reduced in mice receiving antibodies to $G_{i2,0}$ and $G_{i3,0}$ subunits by i.c.v. injection (Sánchez-Blázquez and Garzón, 1993; Sánchez-Blázquez et al., 1993). However, the analgesic profile of mu opioid receptor agonists appeared greatly diminished after injecting antibodies to pertussis toxin-sensitive $G_{i2,0}$ and pertussis toxin-insensitive $G_{x/z,0}$ subunits (Sánchez-Blázquez et al., 1993, 1995). The assignment of G proteins to $\mu$ and delta opioid receptors has been substantiated by an alternative approach; the in vivo administration of antisense oligodeoxynucleotides complementary to portions of mRNAs expressing different subtypes of $G_{i/o}$ subunits (Raffa et al., 1994; Rossi et al., 1995; Sánchez-Blázquez et al., 1995; Tang et al., 1995; Standifer et al., 1996).

Of potential interest was the finding that after reducing the availability of a single class of $G_{i/o}$ subunits, agonist-antagonist properties of opioids were revealed (Garzón et al., 1994; Sánchez-Blázquez and Garzón, 1988). Because the various classes of $G_{i/o}$ subunits exhibit structural differences in their C-terminal peptide sequences, it is feasible that agonist-bound receptors should display diverse efficacy in the

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ABBREVIATIONS: GTP-$\gamma$S, guanosine 5’-O-(3-thiotriphosphate); DAMGO, [D-Ala$^2$,N-MePhe$^4$,Gly-$\beta$-ol$^5$]enkephalin; DPDPE, [D-Pen$^2$-$\gamma$]-enkephalin; ICI-174864, $N,N$-diallyl-Tyr-Alb-Alb-Phe-Leu; PAG, periaqueductal gray matter. TTBS, Tris-buffered saline plus 0.05% Tween 20; SDS, sodium dodecyl sulfate; i.c.v., intracerebroventricular; GTP, guanosine 5’-triphosphate.
activation of the subtypes of G proteins (Roerig et al., 1992; Chabre et al., 1994; Law et al., 1994; Lui et al., 1994; Offermanns et al., 1994). To obtain further insight into receptor-mediated effects, it is important to determine the classes of G-proteins regulated and their order of activation. The efficacy of activation of all, or some, of the G proteins regulated by a given receptor should shed some light on the agonist-antagonist properties of the ligands.

A number of laboratories have described a regulated association of G protein subunits with cytoskeletal proteins (see references in Neubig, 1994). In immunocytochemical studies, G proteins are often viewed as clusters distributed along the plasma membrane (Lewis et al., 1991). The finding that Triton X-100 solubilization of cell membranes provides insoluble complexes enriched in different types of proteins, receptors and GTP-binding proteins included, suggests that this constrained distribution corresponds to that of noncoated pits or caveolae-sites enriched in signal transducing elements (Neubig, 1994; Sargiacomo et al., 1993). In agreement with these findings, heterologous aggregates of proteins containing Gα-like immunoreactivity have been isolated after mild solubilization of cell membranes (Coulter and Rodbell, 1992; Jahangeer and Rodbell, 1993). Further, disaggregation was achieved using guanine nucleotides or extensive solubilization of the membranes (Nakamura and Rodbell, 1990). In the present investigation the characteristics of the GTP-dependent opioid-induced activation of different G proteins in the cell membrane were investigated using the rocket immunoelectrophoretic technique described by Laurell (1966).

An investigation was made of the activation of Gαα and Gααα subunits by agonists binding mu and/or delta opioid receptors in membranes from mouse PAG. This neural structure plays a major role in mediating the supraspinal analgesic effect of opioids when given by i.c.v. injection (Yakhsh et al., 1976; Jensen and Yaksh, 1986). The results of this study indicate that mu opioid receptors couple with the Gαα and Gααα types, whereas delta opioid receptors prefer Gαα over the Gααα type. The observation that opioids displayed dissimilar “efficacy” at mu/delta opioid receptors in the activation of these Gαα/Gααα transducer proteins is of potential interest.

Methods

Membrane Preparation

Experimental tissue was provided by albino male mice CD-1 (Charles River, Barcelona) weighing 22 to 25 g. P2 fractions from PAG were prepared as previously described by Sánchez-Blázquez and Garzón (1989). Membranes were diluted in Tris.HCl buffer, pH 7.7, to a final protein concentration of 2 μg μl⁻¹.

Antisera, SDS-PAGE and Immunoblotting

Anti-Gα sera were raised in New Zealand White rabbits (Biocentrum, Barcelona, Spain). The corresponding synthetic peptides were conjugated to bovine thyroglobulin (Sigma no. T-1001, Sigma Chemical Co., St. Louis, MO) by means of (1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (Sigma no. E-6383), or m-maleimido benzoyl-N-hydroxysuccinimide ester (Sigma no. M-2786). The antigenic sequences used were: Gαα internal fragment [115–125: EEQGMPLEDS] S/1, and the Gααα internal fragment [111–125: C-TGPAEKSIEPTPELL] W/1 of the cDNA-predicted sequence of these proteins (Jones and Reed, 1987; Matsuoka et al., 1990). The antisera (S/1 anti-Gααα, W/1 anti-Gααα) immunoreacted with proteins of 39 to 41 KDa in neural structures of mouse CNS (Sánchez-Blázquez et al., 1993).

Membranes from mouse PAG were solubilized in a buffer containing 50 mM Tris.HCl, 5% SDS, 10% glycerol, 5% 2′-mercaptoethanol, pH 6.8, and boiled for 5 min at 100°C. Approximately 40 μg of protein were loaded on each lane of a 8 cm × 11 cm × 0.15 cm gel slab [7–18% acrylamide/1.9% bis-acrylamide (w/v)] (Hoefer Vertical Slab Unit SE 280). A 20 mA constant current was applied (ISCO Power Supply 595). Proteins were transferred (Mini Trans-Blot Electrophoretic Transfer Cell, BioRad) to polyvinylidene difluoride microporous (0.2 μm) membranes (BioRad) using Towing buffer (25 mM Tris.HCl, 192 mM glycin, 0.04% SDS and 20% methanol, pH 8.3) applying 70V (from 200 to 300 mA) for 120 min.

Unoccupied protein binding sites were blocked with non-fat dry milk (BioRad, no. M7439) in tris-buffered saline (50 mM Tris.HCl, 500 mM NaCl, pH 7.7) for 1 hr at 37°C. Primary antisemur (1:3000 dilution) in TTBS, was added and incubation allowed overnight (Hoefer, Deca-Probe Incubation Manifold PR 150). After removing the antisemur the blot was washed with TTBS. Secondary antiserum (goat anti-rabbit IgG (FC) alkaline phosphatase conjugate (Promega, no. S373B)) in TTBS was added at 1:3000 dilution and left for 3 hr. The secondary antisemur was removed and the membrane washed with TTBS. Western Blue (Promega no. S384B) was used as a substrate (nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate in dimethylformamide) (fig. 1). Positive specificity of W1 antisemur was determined by labeling recombinant Gααα subunits (generously provided by Drs. T. Fields/P. J. Casey), but not Gααα/Gααα.
subunits (Calbiochem). The S/I antibodies labeled the recombinant Gαα/α subunits, but not Gαα, Gααα, Gααα or Gαααα subunits (fig. 1). An identical pattern of selectivity was obtained when recombinant Ga subunits were studied in agarose gels containing either the S/I or W/I antibodies (fig. 1).

**Incubation of P2 Membranes with GTPγS and Opioids**

Concentrations of GTPγS ranging from 1 mM to 300 μM were incubated with 40 to 80 μg protein from mouse PAG in 30 to 50 μl (25 mM Tris.HCl, pH 7.7) for 60 min at 25°C. The nucleotides GDP, GDPβS and ATPγS were used at 100 μM. The opioid agonists, morphine, β-endorphin-(1–31), DAMGO, DPDPDE, [d-Ala2]deltorphin II and the opioid antagonists, naloxone and ICI-174864, were incubated in this buffer supplemented with 1 mM GTPγS. The interaction between opioids was allowed by adding a fixed concentration of the first ligand 10 min before a range of concentrations of the second ligand, followed by incubation for 50 min. The samples, membranes incubated medium, were SDS-solubilized (boiled for 3 min at 100°C) and the final ratio being 2 μg SDS μg⁻¹ membrane protein. For immunoelectrophoresis, 2 to 4 μg protein were transferred to each sample well in volumes of 4 to 6 μl.

In a series of assays, the membranes were incubated for 60 min at 25°C with 1 μM morphine in the presence of 100 μM guanosine 5′-[γ-35S]triphosphate (New England Nuclear, Boston, MA, NEG-030H, 1244 Ci/mmol). Samples were centrifuged at 20,000 × g for 20 min. The precipitate and the supernatant were SDS-solubilized and immunoelectrophoresis was performed in agarose plates loaded with anti Gαα antisera (fig. 1). After this the dried plates were exposed to X-OMAT film (Kodak). Films were developed in Kodak LX-24 developer and Kodak AL-4 fixer.

**ADP-Ribosylation of Mouse PAG Membranes Catalyzed by Pertussis and Cholera Toxin**

The method described by Wong et al., (1988) was followed with minor modifications. Pertussis toxin, 25 μg ml⁻¹, was preactivated in 50 mM dithiothreitol for 1 hr at 25°C. Cholera toxin, 100 μg ml⁻¹, was preactivated in 20 mM dithiothreitol for 1 hr at 25°C. PAG membranes were incubated in 100 mM Tris.HCl, pH 7.5 (final volume of 100 μl), 1 mM NAD, 1 mM EDTA, 1 mM ATP, 100 μM GTP, 10 mM thymidine, 75 μg protein of membranes, in the presence or absence of 2.5 μg preactivated pertussis toxin or 10 μg cholera toxin. After 60 min of incubation at 25°C the membranes were pelleted by centrifugation at 20,000 × g for 20 min and resuspended in the appropriate medium for incubation with or without GTPγS.

**Immunoelectrophoresis**

**Sample preparation.** After GTP-dependent activation of G proteins a significant fraction of Ga subunits is released into the supernatant (McArdle et al., 1988; fig. 2, bottom right). Therefore, after incubating the guanine nucleotides and/or opioids, the membranes plus the supernatant were studied. Mouse PAG membranes, 80 to 100 μg protein in 50 μl of 25 mM Tris.HCl, pH 8.6, were SDS-solubilized using a ratio of 2 μg SDS per μg protein and centrifuged at 11,000 × g for 10 min. After precipitation with 0.15% deoxycholic acid (w/v) and 72% trichloroacetic acid (w/v), protein content of the solubilized samples was ascertained by the method of Lowry et al. (1951).

**Immunoelectrophoresis.** The method used was that described by Laurell (1966) with minor modifications (Dunbar, 1987). This nonisotopic procedure is based on the differential migration of the antigen and immunoglobulins in an appropriate buffer system. The electrophoretic migration of IgGs is very low at pH 8.6 whereas the Ga subunits with isoelectric point values in the range 5.4 to 6.1 (Goldsmith et al., 1988; Spicher et al., 1992) migrate rapidly in the electric field. As the antigen moves to the anode the content of IgGs inside the migrating track becomes reduced and the insoluble antigen-IgG complexes construct the sides of the rocket-shaped immunoprecipitate. When no antigen is available the immunoprecipitate ends in a sharp peak. The Ga subunits in complexes with other proteins or included in the aggregates, are bound very poorly by specific antibodies. The accessibility of the IgGs is enhanced after promoting the release of Ga subunits by either aggressive solubilizations, high concentrations of guanine nucleotides or agonist-ligated receptors (Nakamura and Rodbell, 1990). The final effect would be as if the amount of antigen had increased, thus producing greater immunosignals; the stoichiometric ratio Gaα/IgG was reduced.

**Agorase gel preparation.** A solution of 1.5% (w/v) agarose (FMC no. 80002, LE) was prepared in electrode buffer: 20 mM Tris.HCl, 25 mM sodium barbital, 5 mM barbital, 0.85 mM calcium lactate, 0.03% (w/v) sodium azide (pH 8.6). Agarose was dissolved by constant stirring in the heated buffer (90°C, microwave oven). When cooled to 45°C, immune serum was added at 1:100 dilution. Once the solution became clear and thickened, 12 ml of the molten agarose were poured over the whole surface of leveled 85 × 100 mm plastic plates (GelBond FMC no. 53734). The gelled casts were first cooled to room temperature and then placed in a humidity chamber at 4°C for at least 2 hr before use. Samples were applied to the gel in 2.5-mm round wells of approximately 7 μl capacity cut with a gel punch connected to a vacuum source. The gel minus the well area was covered with a plastic film and immunoelectrophoresis performed in a humid atmosphere on a cooling plate (LKB 2117 Multiphor II Electrophoresis System) connected to a thermostatic circulator bath (Haake G/3D) set at 10°C. Samples containing the antigen were loaded into the punched holes on the cathode side. A constant current of 7 mA per agarose gel plate was applied for 18 hr (Isoco Power Supply 452). Antigen molecules migrated toward the anode and the insoluble immunocomplexes formed the rocket-shaped precipitate. A relationship between the height of the “rocket” and the amount of antigen applied was obtained (Laurell, 1966; Dunbar, 1987).

**Staining and quantification.** The agarose gel was separated from the plastic support. To remove the unreacted proteins the gel was placed between sheets of chromatography paper (Whatman 3 mm CHR) soaked in 100 mM NaCl and pressed (1 kg) for 2 × 30 min. The gel was then immersed and gently agitated in this saline solution for 20 min. After a second pressing for 30 min, the gel was dried under a warm air stream before staining. Immunocomplexes, containing the Ga-like immunoreactivity plus the specific IgGs, were stained with Coomassie Brilliant Blue R-250 (BioRad). Silver staining of the “rockets” was also performed with reagents suitable for agarose gels (Silver Stain Plus, BioRad), although increases in the signal were accomplished by higher backgrounds. The length of the immunoprecipitates—distance from the center of the well to the peak of the rocket—was assessed by optical densitometry (IsoGel Scanner 1312/UA-5).

**Drugs**

Human β-endorphin-(1–31), DAMGO, DPDPDE and [d-Ala²]deltorphin II were purchased from Peninsula Laboratories Europe (Merseyside, England). ICI-174864 was from CRB (Cambridge, England). Morphine sulfate came from Merck (Darmstadt, Germany), naloxone hydrochloride, GTPγS, GDP, GDPβS and ATPγS from Sigma. Pertussis and cholera toxin were obtained from LIST Biological Labs (Campbell, CA). Synthetic peptides: EEQQMLPDEL5 (Peninsula), and C-TGAPAESKGTTPEL (Bio-Synthesis, Madrid, Spain). SDS biotechnology grade came from Amresco (Solon, OH). Recombinant Gαα and Gααα subunits were purchased from Calbiochem (San Diego, CA).

**Statistics**

The data are expressed as the mean ± S.E.M. of at least three independent experiments. Statistical significance was assessed using the Student’s paired t test. P < .05 were considered to be significant. Calculations were performed using the SigmaStat computer program (Jandel Scientific Software, Erkrath, Germany).
Results

Detection of Ga subunits by immunoelectrophoresis.

Mouse PAG membranes solubilized with SDS in the absence of thiol-reducing agents were loaded into agarose gel plates containing anti Gαi2 or anti Gαia serum at 1:100 dilution (Fig. 2A). The length of the immunoprecipitates “rockets” increased linearly with the amount of protein undergoing electrophoresis. The curves correlating protein concentration with the amount of protein undergoing the GTP/GTP exchange reaction are shown (fig. 2A). The concentration of negatively charged detergent SDS in the solubilizing buffer influenced the size of the immunosignals. Increases in the ratio SDS/protein extended the thresholds and parallel curves could be observed (fig. 2A). Samples solubilized with ratios SDS/protein less than 0.5 hardly entered the agarose gel and the stained proteins were detected in the sample wells (not shown). For the purpose of the study, a constant ratio of 2 μg SDS μg⁻¹ protein was selected. Immunodetection was achieved at low micrograms of membrane protein (1–6 μg) indicating that Go subunits are detected in the nanogram range. The method is highly reproducible, the S.E.M. of the computed immunoprecipitates is less than ± 2% and the accuracy of the measurements increases with the length of the rockets (Laurell, 1966). In assays where differences are expressed as a percentage of the control, the threshold “blank” was subtracted from the length of the rockets.

Proteins other than the Gαi2/Gαia subunits, provide no immunoprecipitates, e.g., bovine serum albumin. Moreover, in agarose gels containing preimmune sera, or immune sera boiled at 100°C for 10 min, the immunogens did not produce the rocket-shaped precipitates (data not shown).

GTPγS produces a concentration-dependent increase of Gα-like immunoreactivity.

Incubation of samples with GTP/GTPγS produced an enhancement of the Ga-related immunosignals (fig. 2). The increases in immunoreactivity observed after raising the amount of SDS in the solubilizing buffer masked the effect of 100 μM GTPγS (fig. 2A, bottom left). Samples incubated with GTPγS and solubilized with 2 μg SDS μg⁻¹ protein gave steeper curves than those produced by the corresponding controls. However, the threshold was practically maintained (more antigen being available for the IgGs) (fig. 2A). The time-course for the enhancing effect of GTP and GTPγS on Gα-like immunoreactivity is shown (fig. 2B). A concentration of 10 μM GTP/GTPγS, was incubated with mouse PAG membranes at several intervals before stopping the reaction (SDS-solubilization). The GTPγS raised the Gα-like-related immunoreactivity over the basal values (obtained in the absence of the nucleotide). The pattern showed a fast rise during the first 15 min followed by a steady plateau that extended beyond 120 min a result in agreement with that reported by Milligan and Unson (1989) who used membranes of rat glioma C6 BU1 cells and Gpp(NH)p. A concentration of 10 μM GTPγS promoted a steady increase of Gα-like-related immunoreactivity after 40 to 50 min of incubation (not shown). An interval of 60 min was therefore selected to carry out further assays with GTPγS. The effect of GTP showed a biphasic pattern on Ga-like immunoreactivity: an initial rise lasting up to 30 min followed by a pronounced decrease that practically returned to the basal levels. The metabolism suffered by GTP was responsible for the reversible activation of the Go subunits. At the end of the incubation period the fraction of activated Ga subunits depends on the concentration of GTP that remains. Considerable loss of sensitivity and experimental variation follows.

Incubation of mouse PAG membranes with GTPγS brought about concentration-dependent increases of the immunoprecipitates. A concentration of 6.5 ± 0.6 μM GTPγS produced a 50% increase of the basal Gαi2-like immunoreactivity of PAG membranes.
membranes (fig. 2C). For \( \text{G}_{2 \alpha \alpha} \)-related immunoreactivity the computed ED\(_{50} \) was 800 ± 12 nM GTP\(_{\gamma} \)S (fig. 3D). This simple and sensitive method permitted immunodetection of GTP-activated Ga subunits. The potency (ED\(_{50} \)) of GTP\(_{\gamma} \)S to increase \( \text{G}_{2 \alpha \alpha} \)-like immunoreactivity compares satisfactorily with that reported by Milligan and Unson (1989) using Gpp(NH)p. These authors report that 0.1 to 1 mM Gpp(NH)p produced the greatest activation of Ga subunits. In our study 0.1 to 0.3 mM GTP\(_{\gamma} \)S achieved this effect.

After incubating membranes with 1 \( \mu \)M morphine and 100 \( \mu \)M guanosine 5'-[\( \gamma \)-thio]triphosphate \([\text{35S}]\) the labeled nucleotide was detected bound to \( \text{G}_{2 \alpha \alpha} \)-like immunoreactivity in both fractions, membranes and supernatant. This was not observed when incubation was conducted without the opioid (fig. 2, bottom right).

**Specificity of the GTP\(_{\gamma} \)S effect on Ga-like immunoreactivity.** Incubation of PAG membranes with 100 \( \mu \)M ATP\(_{\gamma} \)S, a nonrelevant nucleotide in G protein activation, did not increase Ga immunoreactivity. GDP/GDP\(_{\beta} \)S, after stabilizing the associated conformation of the G proteins (Wong et al., 1985; Mcardle et al., 1988), reduced the basal immunosignals associated with \( \text{G}_{2 \alpha \alpha} \) subunits in mouse PAG (fig. 3A). After removing 100 \( \mu \)M GDP from the incubation media, 100 \( \mu \)M GTP\(_{\gamma} \)S produced a significant increase of Ga-like immunoreactivity. In the presence of 100 \( \mu \)M GDP\(_{\beta} \)S, GTP\(_{\gamma} \)S failed to increase this signal (fig. 3A). Pertussis toxin, which produces the ADP-riboylation of trimeric Gi/Gs proteins (Wong et al., 1985; Milligan, 1987) and inhibits the receptor-activated GTPase activity of Ga subunits (Van Dop et al., 1984; Aktories et al., 1993), reduced the basal immunosignals and also prevented GTP\(_{\gamma} \)S (100 \( \mu \)M) from increasing Ga-like immunoreactivity (fig. 3A). Cholera toxin did not suppress the enhancing activity of the nucleotide on \( \text{G}_{2 \alpha \alpha} \)-like immunoreactivity of PAG (fig. 3A). After adding 2'-mercaptopropanol to the SDS-solubilizing buffer, increases of Ga-like immunoreactivity could be observed (fig. 3B). This effect was maximal for 2% v/v of the reagent (data not shown). In this experimental protocol, GTP\(_{\gamma} \)S failed to enhance the resulting immunosignals (fig. 3B). In agarose plates containing anti-\( \delta \)-opioid receptor serum (Garzón et al., 1994), incubation of PAG membranes with 100 \( \mu \)M GTP\(_{\gamma} \)S did not modify immunoreactivity. However, the thiol-reducing agent significantly expanded the magnitude of the immunosignals (fig. 3C).

**Opioid-induced GTP\(_{\gamma} \)S-dependent activation of G transducer proteins.** The opioid agonists, morphine, \( \beta \)-endorphin-(1–31), DAMGO, DPDPE, [\( \alpha \)-Ala\(^2 \)]\( \delta \)-endorphin II, or the antagonists, naloxone and nicipox and ICI-174864, when incubated in 25 mM Tris.HCl, pH 7.5, in the absence of GTP\(_{\gamma} \)S, did not modify Ga-related immunosignals. Concentrations of GTP\(_{\gamma} \)S in the range 1 \( \mu \)M to 1 \( \mu \)M were subeffective or produced a limited increase of the \( \text{G}_{2 \alpha \alpha} \)-like immunoreactivity from PAG membranes (figs. 2C and 4A). Samples preincubated with combinations of 1 \( \mu \)M morphine or [\( \beta \)-endorphin-(1–31)] plus GTP\(_{\gamma} \)S ranging from 10 \( \mu \)M to 1 \( \mu \)M, significantly increased \( \text{G}_{2 \alpha \alpha} \)-related immunoreactivity (fig. 4A). In the presence of GTP\(_{\gamma} \)S of less than 10 \( \mu \)M, the opioids did not alter the immunoprecipitates. Incubation of opioid agonists with 1 \( \mu \)M GTP\(_{\gamma} \)S (subeffective concentration), provided concentration-related increases of Ga-related immunosignals (figs. 4 and 5). Thus, GTP\(_{\gamma} \)S was required by opioid agonists to augment Ga-related immunosignals. The enhancement could be ob-
served even in the presence of nonactivating concentrations of the nucleotide. This attribute preserved most of the trimetric G proteins for the study of agonist-mediated increases of Gα-like immunoreactivity. Therefore, the correlation of agonist concentration (gradual occupation of receptors) with the fractional activation of G transducer proteins was feasible.

Whereas certain opioids displayed a weak activity, e.g., DPDPE and [D-Ala²]deltorphin II on Gₐ₂α proteins (fig. 5C), the basal immunoreactivity was almost doubled by morphine, β-endorphin-(1–31) and DAMGO (fig. 4C). By way of comparison, the apparent ED₅₀s (mean ± S.E.M. from three independent determinations) needed for these opioids to produce one-half of their corresponding maximal effect were estimated (table 1).

The selective agonist of mu opioid receptors, DAMGO, activated the G₁₂ and Gₓ/z classes of transducer proteins in a sequential fashion. [D-Ala²]deltorphin II and DPDPE selective agonists of delta opioid receptors activated G₁₂ better than they did Gₓ/z proteins. DPDPE was particularly weak in activating G₁₂α subunits and inactive on the Gₓ/z type. However, this opioid appears to display greater efficacy in the activation of other classes of G proteins regulated by delta receptors (Lauwertz et al., 1993; Sánchez-Blázquez and Garzón, 1993).

In the presence of 1 pM GTPγS, 1 nM DPDPE significantly reduced (P < .05, Student’s t test) the ability of
The involvement of opioid receptors in the observed opioid-evoked activation of G proteins was demonstrated. Naloxone, a competitive antagonist of opioid receptors reduced the enhancing activity of opioids on Gα-like immunoreactivity in a concentration-dependent fashion. ICI174864, antagonist of delta opioid receptors, displays negative intrinsic activity or inverse agonist activity on basal GTPase activity of, and binding of [35S]GTPγS to, Gα subunits (Costa and Herz, 1989; Georgoussi and Zioudrou, 1993; Mullaney et al., 1996). The concentration of GTPγS selected did not activate (bind) Ga subunits. Therefore, ICI174864 exhibited no reducing effect on basal Gα-related immunosignals. Interestingly, this antagonist exhibited high potency to reduce the activating effect of [d-Ala²]deltorphin II and DPDPE on Gα-like immunoreactivity. DPDPE showed even a greater potency to impair the effect of [d-Ala²]deltorphin II on Gα-like immunoreactivities. The antagonism of DPDPE on [d-Ala²]deltorphin II-evoked effects has already been documented for analgesia (Vanderah et al., 1994). Thus, it seems that ICI174864 and DPDPE, after binding to delta opioid receptors, reduce the affinity of certain classes of G proteins toward GTP/GTPγS nucleotides. As a result of this inverse agonist activity, the efficacy of agonists in the activation of delta receptor-coupled G proteins is impaired. Further studies are in progress to explore this possibility.

It is established that G receptors display selectivity toward certain classes of G-transducer proteins, e.g., muscarinic acetylcholine receptors (Offermanns et al., 1994), somatostatin receptors (Law et al., 1994) or dopamine receptors (Lui et al., 1994). With respect to opioid receptors, evidence supporting the existence of a similar phenomenon is accumulating. The mu, delta and kappa opioid receptors exhibit differences in the classes of G proteins they regulate in the production of antinociception: mu opioid receptors regulate Gα₁ and Gα₁, delta opioid receptors regulate Gα₁ and Gα₁ types and kappa opioid receptors regulate Gα₁ and Gα₁ (Sánchez-Blázquez and Garzón, 1993; Sánchez-Blázquez et al., 1993, 1995; Raffa et al., 1994; Standafer et al., 1996). The results of this study show that mu opioid receptors in PAG couple with the Gα₁ and Gα₁ types, whereas delta opioid receptors prefer Gα₁ over Gα₁/α₁ proteins.

The i.c.v. injection of pertussis toxin into mice reduces the efficacy of opioids in invoking supraspinal analgesia (Sánchez-Blázquez and Garzón, 1988). The antinociception elicited by the preferential ligands of mu opioid receptors, DAMGO and FK 33824, of mu/delta receptors, [d-Ala², Met⁵]-enkephalinamide and [d-Ala², d-Leu⁵]enkephalin, and of
**delta receptors, [-Ala]deltorphin II and DPDPE, appeared largely resistant in these mice. Most notably, the activities of morphine, β-casomorphin (1–4) amide and human β-endorphin were much more resistant to the bacterial toxin (Sánchez-Blázquez and Garzón, 1988, 1991). The results of our study and those of previous reports, confirm the involvement of pertussis toxin-resistant G proteins in the analgesic effects of certain opioids when acting at the supraspinal level (Sánchez-Blázquez et al., 1993; Garzón et al., 1994, 1995; Sánchez-Blázquez et al., 1995).

In pertussis toxin-treated mice, opioids for which activity was greatly reduced by this toxin behaved as antagonists of morphine-evoked analgesia (Sánchez-Blázquez and Garzón, 1988). Antagonism was also observed in mice that had received a quantifiable method allowed the detection of opioid-activated G proteins of mouse PAG membranes. Opioids showed different potency and efficacy in the activation of Gi2 and G proteins in rats (Sanchez-Blaquez and Garzon, 1988). The results of our study and those of previous reports, confirm the involvement of pertussis toxin-resistant G proteins in the analgesic effects of certain opioids when acting at the supraspinal level (Sánchez-Blázquez et al., 1993; Garzón et al., 1994, 1995; Sánchez-Blázquez et al., 1995).

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