Differential G-protein activation by alkaloid and peptide opioid agonists in the human neuroblastoma cell line SK-N-BE

Stéphane ALLOUCHE^{*1}, Jocelyne POLASTRON^{*}, Ahmed HASBI^{*}, Vincent HOMBURGER[†] and Philippe JAUZAC^{*}

^{*}Laboratoire des Neurosciences, Université de Caen, CNRS UMR 6551, BP 5229, 14074 Caen Cedex, France, and [†]CCICE, CNRS UPR 9023, rue de la Cardonille, 34094 Montpellier cedex 5, France

Differences in the specificity of coupling of δ -opioid receptor with G-protein have been reported in the literature. We have observed a differential desensitization of δ -opioid receptors, endogenously expressed in the neuroblastoma cell line SK-N-BE, induced by peptide and alkaloid agonists. By combining photo-affinity labelling of receptor-activated G-proteins with [α -³²P]-azidoanilide-GTP and an anti-sense oligodeoxynucleotide strategy, we examined whether the chemical nature of opioid agonists, alkaloid or peptide, has a critical role in determining a $G_{i\alpha}/G_{o\alpha}$ -protein-selective activation by the human δ -opioid receptors. Etorphine, a non-selective alkaloid agonist, was shown to stimulate the incorporation of [α -³²P]azidoanilide-GTP into $G_{i\alpha}1$, $G_{i\alpha}2$, $G_{i\alpha}3$ and pertussis-toxin-insensitive G_{α} subunits. In contrast, [D-Pen²,D-Pen⁵]enkephalin (DPDPE; Pen is penicillamine)

and Tyr-D-Ala-Phe-Asp-Val-Val-Gly-NH₂ (deltorphan I), selective peptide agonists, mainly activated $G_{i\alpha}2$ and $G_{o\alpha}2$ subunits. The 'knock-down' of $G_{o\alpha}2$ subunits by anti-sense oligodeoxynucleotides selectively decreased the inhibition of adenylate cyclase induced by DPDPE and deltorphan I, whereas anti-sense oligodeoxynucleotides directed against $G_{i\alpha}2$ subunits only decreased the potency of etorphine in inhibiting cAMP accumulation. These results suggest that the nature of the agonist, peptide or alkaloid is critical in determining the interaction between human δ -opioid receptors and G_{α} subunits.

Key words: alkaloid agonist, anti-sense oligodeoxynucleotide, $G_{i\alpha}/G_{o\alpha}$ proteins, δ -opioid receptor, peptide agonist.

INTRODUCTION

The μ -, δ - and κ -opioid receptors belong to the superfamily of heptahelical receptors interacting with GTP-binding proteins [1]. These receptors produce biological responses by selectively activating pertussis-toxin (PTX)-sensitive and PTX-insensitive G-proteins, which regulate intracellular effectors, including adenylate cyclase [2,3], K⁺ channels [4], Ca²⁺ channels [5], phospholipase C [6,7] and intracellular Ca²⁺ levels [8,9]. Cholera-toxin-catalysed ADP-ribosylation [10] and photolabelling with [α -³²P]azidoanilide-GTP ([α -³²P]AA-GTP) [11,12] indicated that δ -opioid receptors interact with multiple G_{α} subunits, which in turn can couple to one or more effectors [5,13]. There is little information about the mechanisms that govern the interactions between receptors and G-proteins, which are influenced largely by a variety of factors including compartmentalization and the expression levels of the signalling components [14]. The cytoplasmic domains of the opioid receptors corresponding to the third intracellular loop and the C-terminal tail have been demonstrated to be responsible for effective G-protein coupling [15,16]. However, no consensus sequence in the opioid receptors for the activation of a selective G_{α} subunit has been defined. Therefore the factors that might influence this selectivity still remain to be determined.

Opioid receptors are activated both by endogenous opioid peptides and by alkaloid agonists, including morphine [17]. We recently observed that the desensitization of δ -opioid receptors, endogenously expressed in the human neuroblastoma cell line SK-N-BE [18], occurred at different rates depending on the nature, alkaloid or peptide, of the agonist [19]. This might be

related to different coupling between receptors and G-proteins. To address this issue, we first characterized the PTX-sensitive G-proteins, which are known to be coupled to δ -opioid receptors, expressed in this neuroblastoma cell line. Using agonist-stimulated [α -³²P]AA-GTP photolabelling into G-proteins and also experiments with anti-sense oligodeoxynucleotides (AS-ODN), we showed that the peptide agonists [D-Pen²,D-Pen⁵]enkephalin (DPDPE) and Tyr-D-Ala-Phe-Asp-Val-Val-Gly-NH₂ (deltorphan I) exhibited a different pattern of G-protein activation from the alkaloid agonist etorphine.

MATERIALS AND METHODS

Cell culture

The neuroblastoma cell lines SK-N-BE and NG 108-15 were cultured as described previously [18].

Preparation of membrane fractions

SK-N-BE, NG 108-15 and human colon carcinoma HT29 cells (kindly provided by Dr. H. Paris) were harvested in a 50 mM Tris/HCl (pH 7.4)/5 mM EDTA buffer, centrifuged at 21000 *g* for 15 min at 4 °C; pellets containing membranes were stored at –80 °C. Membranes prepared from Sprague–Dawley rat brain (minus cerebellum, brainstem and olfactory bulbs) were homogenized in 10 vol. of 50 mM Tris/HCl, pH 7.4. The homogenate was centrifuged at 100000 *g* for 35 min at 4 °C; the pellet was then washed twice with 50 mM Tris/HCl, pH 7.4, and suspended in the same buffer by three 20 s pulses with a Polytron. Membrane fractions were finally pelleted at 21000 *g* for 15 min at 4 °C and stored at –80 °C until use.

Abbreviations used: AA-GTP, azidoanilide GTP; AS, anti-sense; deltorphan I, Tyr-D-Ala-Phe-Asp-Val-Val-Gly-NH₂; DMEM, Dulbecco's modified Eagle's medium; DPDPE, [D-Pen²,D-Pen⁵]enkephalin, where Pen is penicillamine; ODN, oligodeoxynucleotides; PTX, pertussis toxin; S, sense.

¹ To whom correspondence should be addressed. Present address: CHU côte de Nacre, Laboratoire de Biochimie A, 14033 Caen Cédex, France (e-mail allouche-s@chu-caen.fr).

Immunoblotting

The frozen membrane fractions of SK-N-BE, NG 108-15, HT29 cells and rat brain membranes were thawed and suspended in 50 mM Tris/HCl buffer containing 5 mM EDTA for protein determination [20]. After centrifugation (18000 *g* for 20 min at 4 °C), final pellets were solubilized in 100 mM Tris/HCl, pH 6.8, containing 10% (w/v) SDS, 10% (v/v) 2-mercaptoethanol, 20% (v/v) glycerol and 0.075% Bromophenol Blue. Proteins were separated by SDS/PAGE [21] on gels made with 10% (w/v) acrylamide, 0.26% bisacrylamide and 6 M urea. Proteins were then transferred to nitrocellulose membranes (0.2 µm; Bio-Rad) for 20–22 h at a constant voltage of 30 V. Nitrocellulose filters were incubated overnight at 4 °C in the presence of primary antibodies directed against G α subunits. These antibodies, AS/7 (selective for C-terminal sequences of G α 1 and G α 2), EC/2 (selective for the C-terminal sequence of G α 3 with a cross-reactivity with G α), anti-G α 1 (directed against residues 291–302 of G α 1 with a cross-reactivity with G α 3) and anti-G α 2 (directed against residues 291–302 of G α 2), were used at a final dilution of 1:500. Membranes were rinsed with Tris-buffered saline (TBS)/Tween 20 [10 mM Tris/HCl (pH 7.4)/150 mM NaCl/0.1% (v/v) Tween 20] and incubated with the secondary antibody (peroxidase-conjugated anti-rabbit IgG at 1:1000 dilution, or biotin-conjugated anti-rabbit IgG and peroxidase-conjugated streptavidin used at 1:500 dilution) for 6 h at room temperature or overnight at 4 °C. Immune complexes were revealed by using 0.5 mg/ml 4-chloro-1-naphthol and 0.05% (v/v) H₂O₂ in 10 mM Tris/HCl, pH 7.4.

Photoaffinity labelling of G α subunits

Synthesis of the photoactivatable [α -³²P]AA-GTP was performed as described [22] and separation from [α -³²P]GTP was conducted by TLC on a polyethyleneimine-cellulose plate in 0.8 M triethylammonium bicarbonate ('TEA') buffer, pH 7.5. Radioactivity was determined by counting 1 µl of each aliquot; TLC on polyethyleneimine-cellulose assessed the purity, as described [23].

Frozen SK-N-BE cell membrane fractions were thawed and dispersed in 50 mM Tris/HCl (pH 7.4)/5 mM EDTA and homogenized by sonication for protein determination [20]. After centrifugation (18000 *g* for 20 min at 4 °C), the pellet was resuspended in buffer A [2 mM Hepes/0.3 mM MgCl₂/0.1 mM EDTA (pH 7.4)]. Photolabelling of G α subunits was performed in a final volume of 50 µl with 70–100 µg of membrane protein, in the presence or the absence of the opioid agonist at the desired concentration with or without 1 µM GDP. After 3 min of incubation at 30 °C, 1 µCi of [α -³²P]AA-GTP was added and the incubation was continued for a further 3 min. Reactions were stopped by centrifugation (18000 *g* for 20 min at 4 °C). Pellets were suspended in 60 µl of buffer A supplemented with 0.2 mM dithiothreitol and samples were then irradiated for 3 min at 4 °C in the dark with a UV lamp (254 nm, 6 W, distance 2 cm). Samples were collected, centrifuged (18000 *g* for 20 min at 4 °C) and pellets were resuspended in 30 µl of sample buffer, heated for 5 min at 95 °C and subjected to SDS/PAGE [10% (w/v) gel] in the presence of 6 M urea. Labelled proteins were identified by autoradiography.

Two-dimensional gel electrophoresis

The two-dimensional gels were prepared by the method of O'Farrell [24]. In brief, [α -³²P]AA-GTP-labelled membranes were solubilized in 9.5 M urea/2% (v/v) ampholines (1.6% pH 4–6 and 0.4% pH 3–10)/2% (v/v) Nonidet P40/5% (v/v) 2-mercaptoethanol at room temperature. After prefocusing, samples

were applied to the basic side of the rod gels. Isoelectric focusing was performed in two steps (15 h at 400 V followed by 1 h at 1000 V) with 0.05 M NaOH and 0.01 M H₃PO₄ as the upper and lower buffers respectively. The pH gradient, measured by slicing the gel in 10 mM KCl, was linear between 6.6 and 4.1. Before SDS/PAGE [10% (w/v) gel], rod gels were equilibrated in 2% (w/v) SDS/10% (v/v) glycerol/250 mM Tris/HCl (pH 6.8) for 45 min.

Treatment of cells with ODN

SK-N-BE cells were split at a density of 10⁵ cells per well in 24-well plates or at 3 × 10⁵ cells per well in six-well plates in Dulbecco's modified Eagle's medium (DMEM) supplemented with fetal calf serum. The culture medium was then replaced with DMEM containing a low concentration (1%, v/v) of fetal calf serum either alone or supplemented with 8 µg/ml lipofectin (Gibco) in the presence or the absence of ODN.

The medium was removed and replaced with fresh medium containing ODN every 3 days for up to 6 days. On the fifth day, 0.6 µCi of [³H]adenine was added to each well for 15 h to determine the intracellular cAMP accumulation. Phosphorothioate ODN directed against G α 1, G α 2, G α 3 and G α subunits were kindly provided by Professor Philippe de Mazancourt (INSERM CJF 94-02, Paris, France). The sequences are as follows: sense α ₁₁, 5'-GCGACGCTCGGCCACCATGGGA-3'; anti-sense α ₁₁, 5'-TCCCATGGTGGCCGAGCGTCGC-3'; sense α ₁₂, 5'-GCCGGCGGACGGCAGGATGGGC-3'; anti-sense α ₁₂, 5'-GCCCCATCCTGCCGTCCGCCGGC-3'; sense α ₁₃, 5'-CTCTCCGGCCGCCGTCATGGGC-3'; anti-sense α ₁₃, 5'-GCCCCATGACGGCGGCCGGAGAG-3'; sense α _o, 5'-CAGGGAAGGGGCCACCATGGGA-3'; anti-sense α _o, 5'-TCCCATGGTGGCCCCCTTCCCTG-3'.

Intracellular cAMP determination

Measurement of intracellular cAMP accumulation was performed as described previously [9].

RESULTS

Characterization of G α proteins labelled with [α -³²P]AA-GTP

In a previous paper [9] we observed that the inhibition of adenylate cyclase by δ -opioid receptors in the SK-N-BE cell line was mediated by one or more PTX-sensitive G-protein(s). To identify the PTX substrates in this cell line, we used antisera directed against G α 1 and G α 2 (AS/7), G α 3 (EC/2), G α 1 and G α 2 and compared their electrophoretic mobilities with other G α /G α subunits already well characterized in the cell line NG 108-15 [13], in a human colon carcinoma HT29 [25] and in rat brain membrane [13] (results not shown). As shown in Figure 1(A), G α 1 and G α 2 subunits were identified by AS/7 antiserum at positions of 41 and 40 kDa respectively. The G α 3 protein with an apparent molecular mass of 41 kDa was intensely labelled in the SK-N-BE membrane preparation (Figure 1A). A lower band of 38–39 kDa was also revealed by EC/2 antibody, which is known to cross-react with G α . Using antibodies directed against G α 1 subunits, we were unable to detect the presence of G α 1 proteins in SK-N-BE cell membranes, whereas this protein was present in NG 108-15 cells and rat brain membranes (results not shown). In a last series of experiments, Western blots were performed with specific antisera directed against G α 2 proteins; a representative blot is shown in Figure 1(A), demonstrating that SK-N-BE cell membranes contained G α 2 protein.

Figure 1 Identification of $[\alpha\text{-}^{32}\text{P}]\text{AA-GTP}$ -labelled membrane proteins of SK-N-BE cells in the presence of alkaloid and peptide agonists

(A) $[\alpha\text{-}^{32}\text{P}]\text{AA-GTP}$ -labelled membranes were separated on SDS/PAGE [10% (w/v) gel/6 M urea] and transferred to nitrocellulose membrane. The filter was cut into strips, autoradiographed (AA-GTP) and then incubated with the different antisera indicated. (B) Photolabelling of G α subunits in the absence (0) or in the presence of agonists (Ago) at 0.1 and 10 μM : etorphine (Eto), DPDPE (Dp) and deltorphin I (Del). (C) Specificity was assessed by incubating SK-N-BE membranes in the absence of ligand (lane a), in the presence of agonist (lane b), in the presence of both agonist and antagonist (lane c) or in the presence of antagonist alone (lane d): 100 nM etorphine and 10 μM naloxone (Eto), 10 μM DPDPE and 100 μM naltrindole (Dp) and 10 μM deltorphin I and 100 μM naltrindole (Del). Similar results were obtained in two other independent and similar experiments. Abbreviations: $\alpha i1,2$, G $\alpha 1$ /G $\alpha 2$; $\alpha i3$, G $\alpha 3$; $\alpha o2$, G $\alpha 2$.

Identification of G $_i$ /G $_o$ proteins activated by the δ -opioid receptors in the SK-N-BE cells was realized with a photoreactive GTP analogue, $[\alpha\text{-}^{32}\text{P}]\text{AA-GTP}$, in the presence and in the absence of different agonists. In the absence of agonist and GDP (to allow a better incorporation of $[\alpha\text{-}^{32}\text{P}]\text{AA-GTP}$ into G α subunits than in the presence of GDP), we observed the labelling of three distinct bands of 45, 41 and 38–40 kDa with a more pronounced incorporation of radioactivity in the fastest migrating band (Figure 1A). Because the δ -opioid receptors are negatively coupled to adenylate cyclase, the identity of the 45 kDa proteins, probably corresponding to G α proteins, was not further studied. We focused on the 38–41 kDa radiolabelled G α proteins, which were analysed by Western blotting. Figure 1(A) shows that the slower-migrating band labelled with $[\alpha\text{-}^{32}\text{P}]\text{AA-GTP}$ corresponded to both G $\alpha 1$ and G $\alpha 3$ subunits whereas the faster migrating band was composed of both G $\alpha 2$ and G $\alpha 2$ subunits.

Identification of G α proteins coupled to δ -opioid receptors by using $[\alpha\text{-}^{32}\text{P}]\text{AA-GTP}$

Photolabelling of G α /G α proteins was performed in the presence of etorphine (a non-selective alkaloid agonist), DPDPE and deltorphin I (selective peptide agonists) at two concentrations (100 nM and 10 μM), chosen from binding experiments (results not shown), and in the presence of 1 μM GDP to allow the best ratio between agonist-stimulated and basal photolabelling of G α subunits. Whereas etorphine clearly induced the incorporation of radioactivity into both bands, 38–40 and 41 kDa (Figures 1B and 1C, Eto), DPDPE and deltorphin I stimulated the photoaffinity labelling of only the 38–40 kDa band at both concentrations (Figures 1B and 1C, Dp and Del). We can also note that the alkaloid agonist seems to activate G $\alpha 2$ /G $\alpha 2$ proteins to a greater extent than G $\alpha 1$ /G $\alpha 3$ proteins.

The specificity of opioid agonist-induced $[\alpha\text{-}^{32}\text{P}]\text{AA-GTP}$ incorporation into G α subunits was controlled by using a non-selective antagonist, naloxone, and a selective opioid antagonist, naltrindole. Data showed that naloxone blocked the incorporation of radioactivity stimulated by the non-selective agonist etorphine (Figure 1C Eto, lanes b and c). The effects of DPDPE and deltorphin I were also reversed by the addition of 100 μM naltrindole (Figure 1C, Dp and Del, lanes b and c). Naloxone and naltrindole by themselves had no effect on photolabelling (Figure 1C, Eto, Dp and Del, lanes a and d).

Two-dimensional electrophoresis of $[\alpha\text{-}^{32}\text{P}]\text{AA-GTP}$ -labelled G α proteins

To separate G $\alpha 1$ from G $\alpha 3$ and G $\alpha 2$ from G $\alpha 2$, radiolabelled subunits were separated by two-dimensional electrophoresis. Membrane proteins were incubated with $[\alpha\text{-}^{32}\text{P}]\text{AA-GTP}$, in the absence of GDP to allow the labelling of all G α subunits, and were subjected to isoelectrofocusing in a linear pH gradient (6.6–4.1) followed by SDS/PAGE [10% (w/v) gel]. The identification of the different G α subunits was performed by Western blotting with antibodies directed against G $_i$ /G $_o$ proteins.

As shown in Figure 2(A), $[\alpha\text{-}^{32}\text{P}]\text{AA-GTP}$ -labelled proteins, ranging from 38 to 41 kDa, were resolved into five major different spots with pI values of 6.6, 5.8, 5.7 and 5.2. G $\alpha 3$ and G $\alpha 2$ subunits were immunodetected at pI 6.6 but G $\alpha 2$ subunits displayed a slightly greater electrophoretic mobility than G $\alpha 3$. AS/7 antiserum, directed against G $\alpha 1$ and G $\alpha 2$, recognized two proteins with pI values of 5.8 and 5.2. A close comparison between autoradiograms obtained from one- and two-dimensional electrophoresis revealed that the fastest migrating band (G $\alpha 2$ /G $\alpha 2$) in the former and the proteins with pI values of 6.6

Figure 2 Identification of opioid agonist-induced [α - 32 P]AA-GTP labelling of G α subunits by two-dimensional electrophoresis

(A) Radiolabelled membrane proteins of SK-N-BE cells were subjected to isoelectric focusing (IEF) in a first dimension on a linear pH gradient between 6.6 and 4.1 and in a second dimension on SDS/PAGE [10% (w/v) gel]. Proteins were then transferred to nitrocellulose and subjected to autoradiography [32 P]AA-GTP, followed by Western blot analysis with anti-G α 3 (anti-G α 3), anti-G α 2 (anti-G α 2) and anti-(G α 1/G α 2) (anti-G α 1,2) antisera. (B) Membrane proteins of SK-N-BE cells were photolabelled with [α - 32 P]AA-GTP with no additions (Cont) or in the presence of 100 nM etorphine (Eto), 100 nM DPDPE (Dp) and 10 nM deltorphin I (Del) and separated by two-dimensional electrophoresis. The gels were dried and then autoradiographed. The different G α subunits and the position of a 45 kDa molecular mass marker are indicated at the bottom and at the left respectively. Results are representative of three different and identical experiments. Abbreviations: α _{i1}, G α 1; α _{i2}, G α 2; α _{i3}, G α 3; α _{o2}, G α 2; α _{PTX-ins}, PTX-insensitive G α .

and 5.8 in the latter were strongly labelled in the absence of both agonist and GDP. Because G α 2 was detected at the most basic side of the gel, proteins with pI values of 5.8 and 5.2 should therefore correspond to G α 2 and G α 1 respectively. In Figure 1(A) we observed that the expression level of G α 1 protein was higher than that of G α 2 protein; Figure 3(A) shows that the intensity of the protein with a pI of 5.2 was greater than the intensity of the protein with a pI of 5.8. These observations confirm that the protein with a pI of 5.2 corresponded to G α 1. The protein with a pI of 5.7 was not detected by antisera directed against G α /G α proteins and thus might correspond to a PTX-insensitive G α subunit (G α _{PTX-ins}).

Differential activation of G α /G α subunits by alkaloid and peptide agonists

In the absence of agonist and in the presence of 1 μ M GDP, four spots were detected, a major one at pI 5.8 and three others at pI 6.6, 5.7 and 5.2 (Figure 2B, Cont). When 100 nM etorphine, a

concentration inducing a maximal inhibition of adenylate cyclase (results not shown), was added to the reaction medium, an increase in the photolabelling of four different proteins (pI 6.6, 5.8, 5.7 and 5.2) was observed, corresponding to G α 3, G α 2, the PTX-insensitive G α subunit and G α 1 respectively, but not G α 2 (Figure 2B, Eto). The activation of δ -opioid receptors by 100 nM DPDPE resulted in the stimulation of G α 2 and G α 2 subunits (Figure 2B, Dp). The incorporation of [α - 32 P]AA-GTP was also increased in the pI 5.7 spot (PTX-insensitive) (Figure 2B, Dp). In the presence of 10 nM deltorphin I, we observed a strong increase in photolabelling of the pI 6.6 (G α 2) and the pI 5.8 (G α 2) spots but no modification of the intensity of the other spots (Figure 2B, Del).

Identification of G α subunits involved in the agonist-induced inhibition of adenylate cyclase by AS-ODN

To confirm the differential activation of PTX-sensitive substrates by etorphine and peptide agonists, phosphorothioate AS-ODN

Figure 3 Effects of AS-ODN directed against G α subunits on the opioid agonist-induced inhibition of cAMP accumulation

Immunoblots with G α 1 (G α 1) (A) and G α 2 (G α 2) (B) antisera of SK-N-BE membranes from cells exposed for 6 days either to DMEM (Cont) or to S-ODN plus Lipofectin® (S) or AS-ODN plus Lipofectin® (AS). Inhibition of the forskolin-stimulated adenylate cyclase by 100 nM DPDPE (Dp-7), 10 nM deltorphin I (Del-8) (C) or 100 nM etorphine (Eto-7) (D) was measured in SK-N-BE cells exposed to DMEM (white bars), to S-ODN plus Lipofectin® (black bars) or to AS-ODN plus Lipofectin® (hatched bars) directed against G α 1 (G α 1), G α 2 (G α 2), G α 3 (G α 3) or G α 2 (G α 2) proteins. Results are means $\pm$ S.E.M. for three different experiments performed in triplicate. * P < 0.05 (analysis of variance, Bonferroni test).

Table 1 G α 2 AS-ODN decrease the potency of etorphine to inhibit the forskolin-stimulated accumulation of cAMP

SK-N-BE cells were pretreated for 6 days in the presence of DMEM (control), AS-ODN or S-ODN directed against G α 1, G α 2 or G α 3. We determined the ability of various concentrations of etorphine (10 pM to 100 nM) to inhibit the forskolin-stimulated accumulation of cAMP. $E_{\max}$ (percentage inhibition of stimulation by forskolin) and IC_{50} were determined by computer curve fitting with GraphPad Prism software. Results are means $\pm$ S.E.M. for three different experiments performed in duplicate. *Significantly different from control and from G α 2 S-ODN (P < 0.05; analysis of variance, Bonferroni test).

Parameter	Control	G α 1		G α 2		G α 3	
		AS-ODN	S-ODN	AS-ODN	S-ODN	AS-ODN	S-ODN
$E_{\max}$ (%)	64 $\pm$ 5	61 $\pm$ 4	62 $\pm$ 7	50 $\pm$ 6	52 $\pm$ 6	66 $\pm$ 2	65 $\pm$ 4
IC_{50} (nM)	0.6 $\pm$ 0.08	0.54 $\pm$ 0.02	0.75 $\pm$ 0.04	2.3 $\pm$ 0.14*	0.14 $\pm$ 0.04	0.44 $\pm$ 0.1	0.72 $\pm$ 0.09

complementary to regions overlapping the translation initiation sites of G α , G α 1, G α 2 and G α 3 subunits were used to inhibit the expression of each protein in the SK-N-BE cells. The specificity of AS-ODN was controlled by using the sequence of the sense strand (S-ODN) for each G α subunit over the same regions. We used 8 μ g/ml Lipofectin®, a cationic lipid preparation that has been reported to enhance the uptake of ODN into the cell [26]. The effects of treatment with AS-ODN were monitored on the G α subunit levels by Western blotting and in experiments with adenylate cyclase.

Preliminary experiments were first conducted to determine the optimal conditions (time and concentrations) for pretreatment by AS-ODN. We observed that a treatment of SK-N-BE cells with 5 μ M AS-ODN directed against G α subunits in the presence of 8 μ g/ml Lipofectin® for 6 days resulted in a maximal decrease in the expression of these proteins; higher concentrations of AS-ODN seemed cytotoxic (results not shown).

Under these conditions, AS-ODN targeting to G α 2 and G α 12 subunits greatly decreased the expression of these proteins at the plasma membrane compared with control and S-ODN treated

cells (Figures 3A and 3B). The treatment of SK-N-BE cells by 8 $\mu\text{g}/\text{ml}$ Lipofectin® had no effect on $G\alpha$ subunit levels (results not shown).

We then investigated the effects of the 'knock-down' of G_{i1} , G_{i2} , G_{i3} and G_{o2} on the inhibition of cAMP accumulation produced by etorphine, DPDPE and deltorphin I. In control cells, the inhibition elicited by the maximal effective concentration of etorphine ranged from 45% to 70%, whereas DPDPE and deltorphin I inhibited cAMP accumulation by 40–50%. For more clarity, the inhibition of adenylate cyclase elicited by the different agonists was taken as 100%. When SK-N-BE cells were pretreated with AS-ODN targeting either G_{i2} or G_{o2} proteins and then challenged with peptide agonists, the blockade of G_{o2} subunits led to a significant decrease (65–75%) in the inhibition produced by both DPDPE and deltorphin I (Figure 3C). This decrease in the inhibitory action of peptide agonists was not observed when SK-N-BE cells were pretreated with S-ODN directed against G_{o2} proteins (Figure 3C). In contrast, the decrease in the expression level of G_{i2} proteins, which have been demonstrated to couple the murine δ -opioid receptors to adenylate cyclase [13], did not modify the inhibitory action of the peptide agonists (Figure 3C).

Because we observed in photolabelling experiments that etorphine could activate G_{i1} , G_{i2} and G_{i3} subunits, studies with anti-sense oligodeoxynucleotides directed against these proteins were conducted; the results are shown in Figure 3(D). The decrease in the expression level of G_{i1} , G_{i2} , G_{i3} or G_{o2} proteins by AS-ODN did not modify the inhibition of cAMP accumulation produced by the maximally effective concentration of etorphine (Figure 3D). We also tested the ability of the alkaloid agonist to inhibit cAMP accumulation in concentration–response experiments after pretreatment with anti-sense oligodeoxynucleotides directed against G_{i1} , G_{i2} or G_{i3} proteins. In these conditions, we were able to observe a significant decrease in the potency of etorphine, as shown by an increase in the IC_{50} (2.3 ± 0.14 nM compared with 0.6 ± 0.08 nM for the control), only when SK-N-BE cells were exposed to G_{i2} AS-ODN for 6 days (Table 1). The change in etorphine potency in inhibiting adenylate cyclase was not observed when SK-N-BE cells were treated with G_{i2} S-ODN (Table 1). When SK-N-BE cells were pretreated with either G_{i1} or G_{i3} AS-ODN, no modification of the potency of etorphine to inhibit adenylate cyclase was detected (Table 1).

DISCUSSION

Here we present evidence that the chemical nature of agonists, alkaloid or peptide, can influence the interactions between the human δ -opioid receptors and $G\alpha$ subunits in the neuroblastoma cell line SK-N-BE. The coupling of δ -opioid receptors to $G\alpha$ subunits was determined by monitoring the incorporation of [α - ^{32}P]AA-GTP into these proteins in the presence of either a non-selective alkaloid agonist, etorphine, or selective peptide agonists, DPDPE and deltorphin I.

The pattern of activation of $G\alpha$ proteins was clearly different between etorphine on the one hand and DPDPE and deltorphin I on the other hand, as shown by one-dimensional and two-dimensional electrophoresis. The separation and identification of the different radiolabelled $G\alpha$ subunits were performed on two-dimensional gels rather than by immunoprecipitation because we observed cross-reactivity between G_{i1}/G_{i2} and G_{i3}/G_{o2} with AS/7 and EC/3 antisera in Western blotting experiments (Figure 1). Etorphine-induced photolabelling of G_{i1} , G_{i2} and G_{i3} subunits and one PTX-insensitive $G\alpha$ subunit was observed but was not detectable in the G_{o2} subunit. The ability of the δ -

opioid receptor to interact with multiple G-proteins has already been observed in different experimental models [11,12]. The protein with a pI value of 5.7, which was not recognized by antisera directed against the PTX-sensitive $G\alpha$ proteins, was clearly activated by etorphine, and to a smaller extent by DPDPE, as shown on two-dimensional electrophoresis. We have previously demonstrated in the SK-N-BE cell line that etorphine triggered a Ca^{2+} mobilization from a ryanodine-sensitive intracellular store and that this effect was not dependent on a PTX substrate [9]. This PTX-insensitive $G\alpha$ protein, activated by etorphine, could be responsible for the increase in cellular Ca^{2+} concentration. The nature and the regulatory role of this $G\alpha$ protein are currently being investigated.

The selective peptide agonists DPDPE and deltorphin I increased the incorporation of radioactivity into G_{o2} and G_{i2} . As shown by one-dimensional and two-dimensional electrophoresis, deltorphin I clearly activates G_{o2} and G_{i2} to a greater extent than DPDPE. In the mouse periaqueductal grey matter and with the use of immunoelectrophoresis, deltorphin has previously been shown to activate several $G\alpha$ subunits with a greater efficacy than DPDPE [27]. One could argue that these differences in stimulation of $G\alpha$ proteins between non-selective and selective agonists might reflect the activation of other opioid receptors than the δ receptors by etorphine, which interacts with μ -, δ - and κ -opioid receptors [28]. However, pharmacological characterization has demonstrated that this cell line expressed only the δ -opioid receptor [18]. Moreover, if SK-N-BE cells were to express other opioid receptors than the δ receptors, etorphine would activate the same $G\alpha$ proteins as DPDPE and deltorphin I. Nevertheless, we were unable to detect any activation of G_{o2} proteins by etorphine.

Recent results obtained with chimaeric receptors and site-directed mutagenesis revealed that peptide and non-peptide (alkaloid) ligands interact at different sites on δ -opioid receptors [29] as well as on μ -opioid receptors [30] and κ -opioid receptors [31]. With regard to the δ -opioid receptor, the binding of peptide agonists involves several regions of the receptor (transmembrane domains I, II and VI, and extracellular loops I and III) [29], whereas alkaloid ligands, which are smaller molecules, interact with a more limited region between transmembrane domain VI and extracellular loop III [32]. The idea of different binding domains for peptide and alkaloid ligand was strengthened by mutagenesis data obtained with the mouse δ -opioid receptor showing a selective loss of peptide agonist binding [33] and potency in inhibiting cAMP accumulation [34], whereas the properties of alkaloid agonists were unaffected. Differences between peptide and alkaloid agonists have also been reported [35] in NG 108-15 cells, in which etorphine pretreatment was shown to induce changes in the δ -opioid receptors that were characterized by a loss of peptide agonist binding, whereas the binding of alkaloid ligands was not altered.

Agonist binding to distinct regions of the receptor could then promote different conformational changes, resulting in the exposure of various sequences of the intracellular loops for $G\alpha$ subunit interaction. These regions, and especially the third intracellular loop and the C-terminal domain of the δ -opioid receptor, are known to be directly involved in the activation of G-proteins [15]. Several studies have been conducted to identify the structural determinants of receptors involved in interactions with G-proteins, but no consensus sequence has been defined for the selective $G\alpha$ subunit activation. However, a segment of 14 residues derived from the human IGF-II/mannose-6-phosphate receptor, which is also present in muscarinic and α -adrenergic receptors, was found to preferentially activate G_{i2} subunit compared with G_{i1} , G_{i3} and G_{o2} subunits [36]; this result

suggests that different sequences are involved in the activation of the various $G_i\alpha/G_o\alpha$ subunits. One can assume that the binding of etorphine would expose several determinants leading to the activation of various $G\alpha$ subunits, whereas peptide agonists would promote the exposure of distinct intracellular regions leading to the activation of only $G_o\alpha2$ and $G_i\alpha2$ subunits. The slight difference between the patterns of G-protein activation (i.e. the PTX-insensitive $G\alpha$ subunit) elicited by the two peptide agonists in our experiments could be explained by the different abilities of DPDPE and deltorphin I to bind regions that are located in extracellular loop II of the δ -opioid receptor [29]. The difficulties in characterizing an amino acid sequence for the activation of a specific $G\alpha$ subunit suggests that several intracellular domains might form a three-dimensional binding site of the receptor for α -subunit interaction.

The AS-ODN strategy was used to characterize precisely the α -subunit(s) involved in the agonist-induced inhibition of adenylate cyclase because it permits a specific inactivation of a given $G\alpha$ protein. The exposure of SK-N-BE cells to AS-ODN directed against $G_o\alpha2$ subunits for 6 days resulted in a decrease in both DPDPE- and deltorphin I-induced inhibition of adenylate cyclase by 65–75 %, whereas this treatment had no effect on the inhibitory action of etorphine. These results corroborate the data that we obtained in photolabelling experiments, in which we observed that etorphine could not activate $G_o\alpha2$ subunits. This decrease is clearly specific because pretreatment with S-ODN did not modify the properties of all agonists in inhibiting the adenylate cyclase activity and was directly linked to a decrease in $G_o\alpha2$ levels at the plasma membrane. We were unable to observe a complete decrease in $G_o\alpha2$ protein expression even after 6 days of pretreatment by AS-ODN; this indicates that the inhibitory effect of DPDPE and deltorphin I was not totally abolished. When the expression of $G_i\alpha2$ proteins was blocked by treatment with AS-ODN, no significant modification of the maximal inhibition level of any agonist was detected. These results suggest that the $G_o\alpha2$ subunit is the only G-protein that mediates the inhibition of cAMP accumulation induced by DPDPE and deltorphin I.

Concerning the alkaloid agonist, we tested the ability of AS-ODN directed against $G_i\alpha1$, $G_i\alpha2$, $G_i\alpha3$ and $G_o\alpha$ to impair their coupling to adenylate cyclase but we were unable to detect any decrease in the maximal inhibition produced by etorphine. However, in dose-response experiments we did observe a significant increase in the IC_{50} of etorphine when SK-N-BE cells were pretreated with $G_i\alpha2$ AS-ODN. These results suggest that etorphine probably activates at least two different $G\alpha$ proteins; nevertheless, $G_i\alpha2$ proteins would preferentially interact with δ -opioid receptors in the presence of etorphine to inhibit adenylate cyclase, as shown by the absence of any effect of treatment with $G_i\alpha1$ and $G_i\alpha3$ AS-ODN. To identify the other α -subunit(s) activated by etorphine, a combination of two different AS-ODN (including $G_o\alpha2$ AS-ODN) to block etorphine-induced cAMP accumulation inhibition was tested. However, this treatment was too cytotoxic for further functional experiments. The finding that $G_i\alpha2$ AS-ODN did not decrease the inhibition produced by the maximal effective concentration of etorphine probably reflects the excess of $G\alpha$ proteins over δ -opioid receptors [37]. Consequently, the blockade of one of the α -subunits that mediates etorphine action on adenylate cyclase would not be sufficient for the observation of any significant decrease on the maximal inhibition level, because the other δ -opioid-receptor-activated $G\alpha$ protein(s) could ensure the coupling between opioid receptors and adenylate cyclase. In the neuroblastoma cell line SH-SY 5Y, the three antisera directed against $G_i\alpha1$, $G_i\alpha2$, $G_i\alpha3$ and $G_o\alpha$ impair the coupling of δ -opioid receptors to adenylate cyclase,

demonstrating that the mediation of the inhibition of adenylate cyclase involves several $G\alpha$ subunits [38]. We showed previously that a sustained stimulation of δ -opioid receptors by etorphine led to a different heterologous desensitization between opioid, D_2 -dopaminergic and α_2 -adrenergic receptors [39]. To explain these differences we hypothesized that the inhibition of adenylate cyclase in SK-N-BE cells by etorphine was mediated by two different $G\alpha$ proteins: a $G\alpha$ subunit shared by opioid and α_2 -adrenergic receptors, and another member of the G_i/G_o family interacting only with the opioid receptor.

Our results provide further evidence that δ -opioid receptors can couple to several GTP-binding proteins, as demonstrated in other experimental models, including NG 108-15 cells [10], SH-SY 5Y cells [40], transfected Chinese hamster ovary cells [11], mouse brain [7] and smooth muscle of guinea pig intestine [41]. It seems also obvious that the same $G\alpha$ subunit can regulate different effectors [13,42] and conversely that the same effector (i.e. adenylate cyclase) can be regulated by different $G\alpha$ subunits [13,38]. It was suggested that these differences observed in the different cellular models were related only to the opioid receptors and the $G\alpha$ expression levels. However, our results also show that the coupling of the human δ -opioid receptors in the SK-N-BE cells to different $G\alpha$ subunits is clearly dependent on the chemical nature of the agonist. Etorphine, an alkaloid molecule derived from morphine, activates $G_i\alpha1$, $G_i\alpha2$, $G_i\alpha3$ and one PTX-insensitive $G\alpha$ protein; the peptide agonists DPDPE and deltorphin I stimulate mainly $G_i\alpha2$ and $G_o\alpha2$ subunits. With the use of an AS-ODN strategy, we have demonstrated that $G_o\alpha2$ and $G_i\alpha2$ subunits mediate the inhibitory action of peptide and alkaloid agonists respectively on adenylate cyclase. These substantial differences in the pattern of G-protein activation could have important implications in the understanding of the physiological actions of these opioid ligands *in vivo*.

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