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European Journal of Pharmacology 452 (2002) 21–33

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Quantitative stoichiometry of G-proteins activated by μ-opioid receptors in postmortem human brain

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Received 8 April 2002; received in revised form 26 July 2002; accepted 9 August 2002

Abstract

Paradoxically, the potencies (EC_{50}) of agonists stimulating [^{35}S]GTPγS binding are several orders of magnitude lower than their affinities in receptor binding assays. We have investigated the quantitative stoichiometry of μ-opioid receptor–G-protein coupling in postmortem human brain. [D-Ala^2,*N*-Me-Phe^4,Gly^5-ol]enkephalin (DAMGO) displaced [^{3}H]naloxone binding in a biphasic pattern. The ratio between $K_{i\text{-}low}$ and EC_{50} of DAMGO stimulating [^{35}S]GTPγS binding was lower than one. The K_A of DAMGO was calculated following μ-opioid receptor alkylation by β-funaltrexamine from [^{35}S]GTPγS binding data using the "nested hyperbolic method", yielding $K_A/EC_{50}>1$. Thus, only 1.2 ± 0.2% of μ-opioid receptors was needed to be occupied to achieve the half-maximal effect of DAMGO. The estimated ratio between the G-proteins activated by 10 μM DAMGO (determined by isotopic dilution curves) and the occupied-μ-opioid receptors was 1304. In conclusion, we have determined the stoichiometric and the kinetic parameters in the μ-opioid receptor–G-protein system.

Keywords: [^{35}S]GTPγS binding; μ-Opioid receptor; G-protein; $GABA_B$ receptor; Brain, human; Receptor reserve; Signal transduction

1. Introduction

The binding of an agonist to G-protein coupled receptor leads to the stabilisation of a ternary complex of agonist–receptor–G-protein (De Lean et al., 1980). In this complex, exchange of GDP for GTP occurs on the G-protein and finally, the ternary complex dissociates yielding α and βγ subunits of the G-protein, which can then alter the activity of effector molecules (Gilman, 1987). This ternary complex model has been extended to include an isomerization of the receptors from inactive (R) to active (R*) state that enables it to couple to the G-protein. This isomerization may occur spontaneously, or be induced by agonists (Lefkowitz et al., 1993).

Biochemical and functional alterations of G-protein coupled receptors have been repeatedly postulated to play a role in the pathophysiology of some neurological and psychiatric diseases. The receptor–G-protein coupling mediated by agonists can be quantified in binding assays by using the non-hydrolizable GTP analogue [^{35}S]GTPγS (Lorenzen et al., 1993; Traynor and Nahorski, 1995). Thus, studies from our laboratory have optimised the [^{35}S]GTPγS binding assay in postmortem human brain samples (González-Maeso et al., 2000, 2002a; Rodríguez-Puertas et al., 2000), and the modulation of this [^{35}S]GTPγS binding in opiate addiction (Meana et al., 2000) and mood disorders (González-Maeso et al., 2002b) has been demonstrated. Nevertheless, little is known about the stoichiometry of G-proteins activated by receptors in normal and pathological human brain. In this regard, the affinities of tritiated agonists for receptors in postmortem human brain are usually several orders of magnitude higher than the potencies (EC_{50}) of these agonists stimulating [^{35}S]GTPγS binding both in rat (Kearn et al., 1999; Selley et al., 1998) and in postmortem human brain membranes (Gabilondo et al., 1994; González-Maeso et al., 2000). This phenomenon would suggest that more than one receptor would have to be occupied to activate a single G-protein, which represents a biological incongruence (Colquhoun, 1998). Previous reports have explained this difference by the composition of the buffers used in the two assays. In this regard, the presence of Na^+ (Pert and Snyder, 1974) and guanine nucleotides (Childers and Snyder, 1980; Gilman, 1987) shift agonist binding into

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PII: S0014-2999(02)02242-2

low-affinity state. Thus, when agonist receptor binding assays have been performed in the presence of added guanine nucleotides (Gardner et al., 1997; Waelbroeck, 2001a) or in buffer conditions similar to that used in [^{35}S]GTPγS binding assays (Lorenzen et al., 1993; Maher et al., 2000; Quock et al., 1997), ratios of agonist affinities in receptor binding to agonist potencies stimulating [^{35}S]GTPγS binding (i.e., K_i/EC_{50}) have been shown to be relatively close to 1 in orders of magnitude. These pharmacological data would suggest a lack of significant G-protein coupled reserve receptors in both mammalian brain (Lorenzen et al., 1993; Maher et al., 2000) and cultured cells (Gardner et al., 1997).

In the present work, we have studied the quantitative stoichiometry of μ-opioid receptor–G-protein coupling in postmortem human brain membranes. We estimated the dissociation constant of the μ-opioid receptor agonist [D-Ala^2,*N*-Me-Phe^4,Gly^5-ol]enkephalin (DAMGO) from functional [^{35}S]GTPγS binding data by using the "nested hyperbolic method" (James et al., 1989). Thus, the analysis of the occupancy–effect relation allowed us to determine the pool of μ-opioid receptor reserve.

Therapeutical strategies at G-protein coupled receptors have been limited primarily to the pharmacological blockage or activation of the receptors, nevertheless, postreceptor components such as G-proteins are also potential therapeutic targets (Freissmuth et al., 1999). If one wishes to alter G-protein coupled receptor signaling pathways in novel ways, it is necessary to understand the dynamics of activation and the limiting factors of each component in the pathway. In the present work, we have examined the pattern of loss of G-protein response to μ-opioid receptor activation (using [^{35}S]GTPγS binding) after blockage of either μ-opioid receptors or G-proteins. The effect of G-protein inactivation was also studied for a structurally different G-protein coupled receptor such as $GABA_B$ receptor, showing a different response to the stoichiometric modification. These two cellular mechanisms to control receptor–G-protein coupling with the limiting components of the pathway may be useful as a model in the development of new therapeutic strategies based on the stoichiometry and compartmentation of the signaling molecules.

2. Materials and methods

2.1. Materials

[^{3}H][D-Ala^2, *N*-Me-Phe^4, Gly^5-ol]enkephalin (50 Ci/mmol; [^{3}H]DAMGO) was purchased from American Radiolabeled Chemicals (St. Louis, USA). [^{3}H]Naloxone (60 Ci/mmol) was from New England Nuclear (Boston, USA). [^{35}S]GTPγS (1250 Ci/mmol) was obtained from DuPont NEN (Brussels, Belgium). Bovine seroalbumine, DAMGO, DL-dithiothreitol, GDP, GTPγS, naloxone and *N*-ethylmaleimide, were purchased from Sigma (St. Louis, USA). Baclofen was from from Tocris Cookson (Bristol, UK). β-Funaltrexamine was purchased from Research Biochemicals International (Massachusetts, USA). All other chemicals were obtained from standard sources and were of the highest purity commercially available.

2.2. Postmortem brain samples

Human brain samples were obtained at autopsy from the Basque Institute of Legal Medicine (Bilbao, Spain). Specimens from prefrontal cortex (Brodmann's area 9) were dissected at the time of autopsy and immediately stored at −70 °C until assay. The collection was performed in accordance with the protocol for postmortem human brain research of the Basque Institute of Legal Medicine. The study was approved by the Research and Ethics Boards of the Medical School of the Basque Country, Spain.

Samples were obtained from subjects without a known history of neurological or psychiatric disorders who had died by sudden and violent causes (motor-vehicle accidents). The samples of subjects with a positive result in toxicological screening for psychotropic drugs and ethanol were excluded from this study. Brain samples of 11 subjects (4 female, 7 male) were included in the study. The age at death of the subjects was 27 ± 4 years. The postmortem delay between death and dissection was 28 ± 4 h and the storage period of the samples at −70 °C was 31 ± 8 months. Not all the brains were used for each set of experiments. However, all the experiments were performed at least in three different brains.

2.3. Preparation of membranes

Membrane preparations were performed as previously reported with minor modifications (González-Maeso et al., 2000). Briefly, tissue samples were homogenized in homogenization buffer A (50 mM Tris–HCl, pH 7.4) for β-funaltrexamine pretreatment and [^{3}H]naloxone binding assays, or homogenization buffer B (1 mM EGTA, 3 mM $MgCl_2$, 1 mM DL-dithiothreitol, and 50 mM Tris–HCl, pH 7.4) for *N*-ethylmaleimide pretreatment and [^{35}S]GTPγS binding assays; both buffers supplemented with 0.25 M sucrose. Cellular P_2 fractions were prepared by sequential centrifugations and resuspensions in homogenization buffer A/B, respectively. Final aliquots were directly used (for β-funaltrexamine pretreatment) or stored at −70 °C until assay.

2.4. [^{3}H]Naloxone binding assays

Competition curves by the selective μ-opioid receptor agonist DAMGO against the binding of the opioid receptor antagonist [^{3}H]naloxone were performed both in "high-affinity conditions" (50 mM Tris–HCl, pH 7.4) and in conditions virtually identical (see below) to [^{35}S]GTPγS binding assays (1 mM EGTA, 3 mM $MgCl_2$, 100 mM NaCl, 0.2 mM DL-dithiothreitol, 50 μM GDP, 0.5 nM unlabelled

GTPγS, and 50 mM Tris–HCl, pH 7.4; TEM-buffer). Membrane aliquots were thawed and resuspended in Tris–HCl or TEM-buffer, respectively. Protein content was measured according to the method of Bradford (1976) using bovine seroalbumine as standard. Binding assays were done by incubating, for 60 min at 25 °C in a total volume of 500 μl, 1 nM [^{3}H]naloxone in the absence or presence of DAMGO as displacer (10^{-10}–10^{-5} M, twelve concentrations). Nonspecific binding was determined in the presence of 10 μM naloxone. Incubations were terminated by dilution and rapid filtration under vacuum through Whatman GF/C glass-fiber filters, followed by three washes with 3 ml of cold 50 mM Tris–HCl, pH 7.4 buffer. The filters were transferred to minivials containing 5 ml of OptiPhase Hisafe II cocktail (Wallac), incubated overnight, and counted for radioactivity by liquid scintillation spectrometry. For [^{3}H]naloxone saturation analysis, membrane preparations were incubated with 0.13 to 16 nM (eight concentrations) in 50 mM Tris–HCl, pH 7.4. The delineation of the nonspecific binding in the presence of 10 μM DAMGO allowed us to selectively determine the pharmacological parameters of μ-opioid receptors. Incubations were performed and radioactivity counted as described above for [^{3}H]naloxone displacement curves. The estimated log K_D and B_{max} values for [^{3}H]naloxone saturation experiments were -8.37 ± 0.11 and 122 ± 9 fmol/mg prot, respectively ($n = 2$ in membrane pools of three different brain samples).

2.5. Pretreatment of the membranes with β-funaltrexamine and [^{3}H]DAMGO binding assays

Pretreatment of membranes with β-funaltrexamine was performed as previously described for rat brain membranes (Clark and Medzihradsky, 1987) with minor modifications. Fresh prepared membrane aliquots of 5 ml (1 mg prot/ml) were incubated in the absence or in the presence of β-funaltrexamine (0.01 μM–1 mM) for 60 min at 25 °C in 50 mM Tris–HCl (pH 7.4). After a three-fold dilution with Tris–HCl (pH 7.4), the suspensions were centrifuged at $40{,}000 \times g$ for 10 min at 4 °C. The pellets were washed and centrifuged as described above three times. Final pellets were suspended in 5 ml of Tris–HCl (pH 7.4). One millilitre was centrifuged and the pellet stored at −70 °C for [^{35}S]GTPγS binding assays (see below). The remaining 4 ml were diluted two-fold (~ 0.5 mg prot/ml) with Tris–HCl (pH 7.4) and directly used for [^{3}H]DAMGO binding assays.

[^{3}H]DAMGO binding assays were performed as previously described for postmortem human brain membranes (Gabilondo et al., 1994) with minor modifications. Control and β-funaltrexamine pretreated membrane suspensions were incubated in Tris–HCl (pH 7.4) in the presence of [^{3}H]DAMGO (0.13–20 nM, eight concentrations) for 60 min at 25 °C. Nonspecific binding was defined in the presence of 10 μM naloxone. Incubations were terminated and radioactivity counted as described above for [^{3}H]naloxone binding assays.

2.6. [^{35}S]GTPγS binding assays

The [^{35}S]GTPγS binding assays were performed as reported in detail previously (González-Maeso et al., 2000). Briefly, membrane protein preparations were thawed, and incubated at 30 °C for 2 h in [^{35}S]GTPγS-incubation buffer (0.5 nM [^{35}S]GTPγS, 1 mM EGTA, 3 mM $MgCl_2$, 100 mM NaCl, 0.2 mM DL-dithiothreitol, 50 μM GDP, and 50 mM Tris–HCl, pH 7.4). The μ-opioid receptor agonist DAMGO (10^{-10}–10^{-4} M, eight concentrations) or the $GABA_B$ receptor agonist baclofen (10^{-9}–10^{-3} M, eight concentrations) were added to determine receptor-stimulated [^{35}S]GTPγS binding. Nonspecific binding was defined in the presence of 10 μM unlabelled GTPγS. Basal binding was assumed to be the specific [^{35}S]GTPγS binding in the absence of agonist.

For [^{35}S]GTPγS saturation analysis, isotopic dilution curves were performed with unlabelled GTPγS (González-Maeso et al., 2000). Control or *N*-ethylmaleimide-pretreated membranes (see below) were incubated, under standard conditions for [^{35}S]GTPγS binding assays, in the absence and in the presence of 10 μM DAMGO or 100 μM baclofen, and in the absence or in the presence of unlabelled GTPγS (10^{-11}–10^{-3} M, sixteen concentrations). If we define the nonspecific binding in the presence of 10 μM GTPγS, the specific binding (binding to heterotrimeric G-proteins, see bellow) was 94.9±0.5%, 96.5±1.6%, 97.1±1.0% for basal, and DAMGO- or baclofen-stimulated curves, respectively.

The reactions were terminated by rapid vacuum and filtration through Whatman GF/C glass fiber filters and the remaining bound radioactivity was measured by liquid scintillation spectrophotometry.

2.7. Pretreatment of the membranes with N-ethylmaleimide

Cortical membranes were pretreated with *N*-ethylmaleimide as previously described (González-Maeso et al., 2000). *N*-ethylmaleimide presents high selectivity alkylating $G_{i/o}$-proteins when the incubations are performed at 4 °C (Ueda et al., 1990). Membrane preparations were preincubated in *N*-ethylmaleimide-incubation buffer (1 mM EGTA, 3 mM $MgCl_2$, 100 mM NaCl, and 50 mM Tris–HCl, pH 7.4) in the absence or presence of *N*-ethylmaleimide at the indicated concentration for 60 min at 4 °C. The reaction was stopped by DL-dithiothreitol (final concentration 20 mM) followed by two membrane wash-outs and subsequent centrifugations. The final pellets were resuspended and used immediately for [^{35}S]GTPγS binding assays.

2.8. Data analysis

The apparent dissociation constants (K_D) and the maximal number of binding sites (B_{max}) for [^{3}H]DAMGO and [^{3}H]naloxone were estimated by saturation curves. The selection between models of the competition curves by

DAMGO against [^{3}H]naloxone was made by the extra sum of squares principle (F test). The more complex model was accepted if the P value resulting from the F test was <0.05. Following the nonlinear curve fitting, $K_{i\text{-high}}$ and $K_{i\text{-low}}$ values for DAMGO in receptor binding assays were calculated from the corresponding IC_{50} values using the Cheng and Prusoff (1973) equation, with -8.37 ± 0.11 as the calculated log K_D for naloxone (see above).

Experimental values of the DAMGO- and baclofen-induced increases of [^{35}S]GTPγS binding were obtained (agonist-stimulated minus basal values) and termed net-stimulations. Further, to display pharmacological parameters of the net-stimulated [^{35}S]GTPγS binding, concentration–response curves for DAMGO and baclofen were performed and their parameters calculated by nonlinear regression analysis (Parker and Waud, 1971). The theoretical maximal effect fitted by the curves (E_{max}) and the concentration of agonist that determines the half-maximal effect (EC_{50}) were obtained.

DAMGO concentration–response curves obtained in membranes pretreated with different concentrations of β-funaltrexamine were analysed by the method described by James et al. (1989). This approach, which has been refereed to as the ''nested hyperbolic method'', is analytically simpler than the classical method of Furchgott (1966) and allows to estimate the DAMGO dissociation constant (K_A), and the fraction of non-inactivated receptors (q) by directly fitting the experimental [^{35}S]GTPγS binding data. Thus, the concentration–response curves obtained after pretreatment with each concentration of β-funaltrexamine were fit to the following equation:

$$E = \frac{E_{max}}{\left(\frac{EC_{50}}{qK_A[A]}(K_A + [A](1-q))\right)^n + 1} \quad (1)$$

where E is the observed effect (expressed as net-stimulation); [A] is the concentration of DAMGO in the β-funaltrexamine pretreated membranes; n represents the slope factor of the function. E_{max} and EC_{50} are obtained from the control (vehicle-pretreated) curve as described above.

The fraction of receptors occupied (f_{occ}) for each concentration of DAMGO ([A]) was calculated by substituting the previously estimated value of K_A (see above) in the following equation derived from the mass action law:

$$f_{occ} = \frac{[A]}{[A] + K_A}. \quad (2)$$

Thus, all experimental sets of data of the occupancy–effect relation were pooled and then fitted by nonlinear regression to the hyperbolic equation described by Black and Leff (1983), as previously reported elsewhere (Pineda et al., 1997).

$$E = E'_{max}\frac{f_{occ}}{f_{occ} + K_E} \quad (3)$$

in which E'_{max} is the maximal effect for this equation; and K_E is the fraction of receptors needed to be occupied to promote the 50% of the maximal effect (the maximal value of K_E would be 1).

Isotopic dilution curves of total, specific, and net-stimulated [^{35}S]GTPγS binding by unlabelled GTPγS were analysed to estimate the apparent dissociation constants of G-proteins for [^{35}S]GTPγS and the maximal number of [^{35}S]GTPγS binding sites (DeBlasi et al., 1989). The selection between models was made by F test (see above).

All mathematical calculations were done by use of nonlinear regression by the computer software GraphPad Prism™. Data are expressed as mean ± S.E.M. of the experimental results. Values obtained from the fitting analyses are expressed as the best fit ± S.E. provided by the computer programme. These S.E. estimates are not real errors of the mean and thus, were not used for further statistical evaluations. Statistical evaluation of differences of the parameters of the fitting analyses between groups was performed by F test (see above). Statistical comparisons of the effects of β-funaltrexamine and N-ethylmaleimide on binding parameters were also determined by one-way analysis of variance (ANOVA) of individual estimations followed by Dunnett's multiple comparison test. Statistical significance between means ± S.E.M. was determined by the unpaired t-test. The level of significance was chosen as $P=0.05$.

3. Results

3.1. [^{3}H]Naloxone binding competition curves by DAMGO

Pharmacological parameters of DAMGO displacing [^{3}H]naloxone binding was determined under "high-affinity conditions" (i.e., Tris–HCl buffer, see Section 2) and under identical buffer conditions to [^{35}S]GTPγS binding assays (i.e., TEM-buffer, see Section 2). In both conditions, competition experiments were best described by a two-site model (Tris–HCl buffer: $F[2,81]=6.64$, $P<0.01$; TEM-buffer: $F[2,75]=3.38$, $P<0.05$) (Table 1, Fig. 1). These two affinities presented by DAMGO ($K_{i\text{-high}}$, $K_{i\text{-low}}$) were

Table 1
Competition curves of DAMGO against [^{3}H]naloxone binding in prefrontal cortex of postmortem human brain

	log $K_{i\text{-high}}$	log $K_{i\text{-low}}$	% High
Tris–HCl buffer	-8.63 ± 0.27	-6.98 ± 0.38	58 ± 13
TEM-buffer	-8.63 ± 0.55	-6.99 ± 0.28	31 ± 14[a]

DAMGO displacement of [^{3}H]naloxone (1 nM) binding were performed in Tris–HCl and TEM-buffers (see Section 2), and the competition curves were analysed by nonlinear regression to derive dissociation constants for the high- ($K_{i\text{-high}}$) and the low- ($K_{i\text{-low}}$) affinity states of the receptor. A two-site model provided a better description of the data. "% High" refers to the percentage of high-affinity sites as calculated from nonlinear fitting. Data are best fit ± S.E. of three assays performed in triplicate in three different brains.

[a] $P<0.0001$, versus Tris–HCl buffer value (F test).

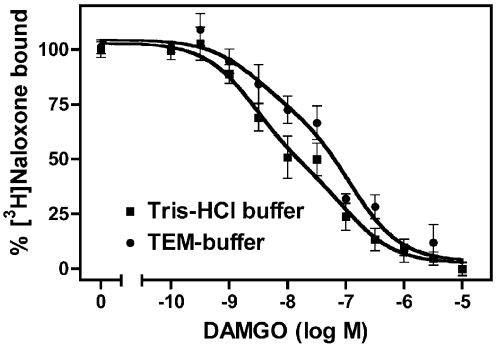

Fig. 1. [^{3}H]Naloxone (1 nM) binding competition curves by DAMGO in postmortem human brain membranes. Experiments were performed as described in Section 2 both in Tris–HCl buffer (■) and under identical buffer conditions to [^{35}S]GTPγS binding assays (TEM-buffer, ●). A two-site model provided a better description of the data (see Section 3 and Table 1). Data are mean ± S.E.M. (bars) values of three experiments performed in triplicate in three different brains.

not different between Tris–HCl- and TEM-buffer conditions (Table 1). In contrast, high-affinity fraction was decreased under TEM-buffer conditions ($F[5,146] = 5.89$, $P < 0.0001$) (Table 1, Fig. 1).

3.2. DAMGO stimulation of [^{35}S]GTPγS binding after μ-opioid receptor alkylation

[^{3}H]DAMGO saturation binding experiments were used to determine the density (B_{max}) and the affinity (K_D) of [^{3}H]DAMGO binding sites after μ-opioid receptor alkylation by different concentrations of the selective μ-opioid receptor alkylating agent β-funaltrexamine. B_{max} value obtained for [^{3}H]DAMGO in vehicle-pretreated membranes (78 ± 9 fmol/mg prot) was decreased in a concentration-dependent manner by β-funaltrexamine pretreatment ($F[2,6] = 22.05$, $P < 0.01$) (Fig. 2A). On the contrary, K_D for [^{3}H]DAMGO in vehicle-pretreated membranes (log K_D: − 8.19 ± 0.20) was not modified by β-funaltrexamine pretreatment ($F[2,6] = 0.81$, $P > 0.05$) (Fig. 2A). After preincubation of the membranes with β-funaltrexamine concentrations above 1 μM, the nonlinear fitting of the curves was not possible (Fig. 2A). The pharmacological parameters (B_{max}, K_D) of [^{3}H]DAMGO binding after β-funaltrexamine pretreatment were obtained as an internal control of μ-opioid receptor alkylation, and were not used in further evaluations.

DAMGO concentration–response curves stimulating [^{35}S]GTPγS binding were performed after μ-opioid receptor alkylation by β-funaltrexamine. In vehicle-pretreated membrane preparations, DAMGO stimulated in a concentration-dependent manner [^{35}S]GTPγS binding (log EC_{50}: − 5.9 ± 0.11; Fig. 2B). Pretreatment of the membranes with β-funaltrexamine did not affect to the basal [^{35}S]GTPγS binding ($F[6,31] = 1.17$, $P > 0.05$). Alkylation of μ-opioid receptors firstly induced a progressive shift to the right of the DAMGO concentration–response curves stimulating [^{35}S]GTPγS binding ($F[4,31] = 12.63$, $P < 0.0001$) (Fig. 2B). This decrease in the potency (increased EC_{50} values) of DAMGO reached the level of statistical significance in membranes pretreated with 1 μM β-funaltrexamine (Dunnett's post-hoc test). Secondly, alkylation of μ-opioid receptors resulted in a reduction of the efficacy (E_{max}) of DAMGO ($F[4,28] = 4.72$, $P < 0.001$) (Fig. 2B). This reduction in the E_{max} of DAMGO reached the level of statistical significance in membranes pretreated with 100 μM β-funaltrexamine

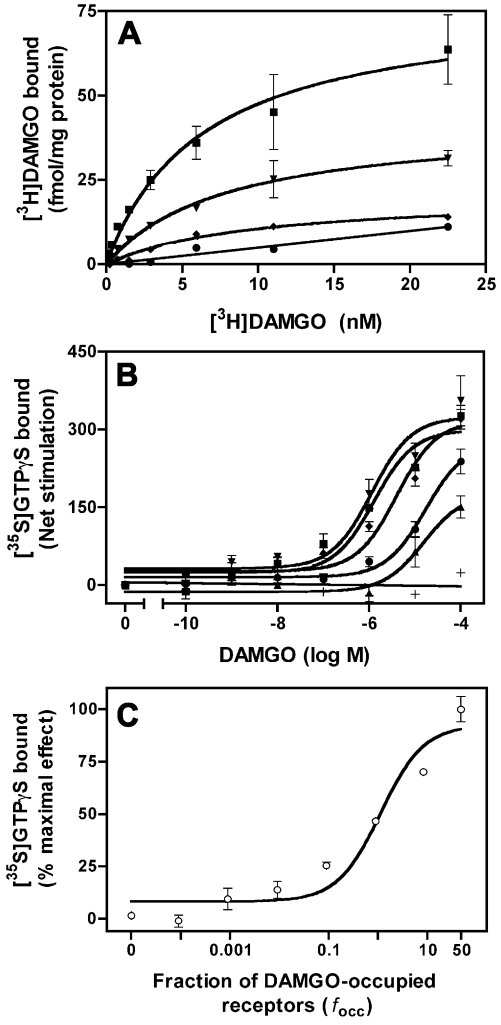

Fig. 2. μ-Opioid reserve receptor analysis of DAMGO in [^{35}S]GTPγS binding assays. Membrane preparations of prefrontal cortex were pre-incubated as shown in Section 2 in the absence (■) and in the presence of 0.1 μM (▼), 1 μM (♦), 10 μM (●), 100 μM (▲), or 1 mM (+) β-funaltrexamine. (A) Specific binding of [^{3}H]DAMGO in control and β-funaltrexamine-pretreated membranes. (B) Concentration–response curves of net-stimulations (agonist stimulated minus basal binding) of [^{35}S]GTPγS binding by DAMGO in control and β-funaltrexamine-pretreated membranes. (C) Occupancy–effect relation of μ-opioid receptors stimulating [^{35}S]GTPγS binding. The fraction of receptors occupied by DAMGO (expressed in percentage and in logarithmic scale) was estimated as shown in Section 2 (Eq. (3)). Incubations were performed as described in Section 2 and data are means ± S.E.M. (bars) values of three experiments performed in duplicate (A) or triplicate (B, C) in three different brains.

(Dunnett's post-hoc test). Preincubation of the membranes with 1 mM β-funaltrexamine completely abolished DAMGO stimulation of [^{35}S]GTPγS binding (Fig. 2B).

The "nested hyperbolic method" was used to estimate both q (fraction of non-inactivated receptors) and K_A (DAMGO dissociation constant). This method was applied to each DAMGO concentration–response curve from β-funaltrexamine-pretreated membranes that presented significant changes from control curve (i.e., 1, 10 and 100 μM β-funaltrexamine, see above). Thus, after pretreatment of the membrane preparations in the presence of 1, 10, and 100 μM β-funaltrexamine, estimated q values (mean ± S.E.) were 0.64 ± 0.14, 0.09 ± 0.02 and 0.03 ± 0.01, respectively. As expected, the K_A values estimated from these three curves were not different (log K_A ± S.E.: 1 μM, −4.03 ± 0.21; 10 μM, −3.94 ± 0.89; 100 μM, −4.28 ± 1.63) and the mean ± S.E.M. of these K_A values was used for further determinations.

The plot of receptor occupancy (f_{occ}) against DAMGO effect resulted in a hyperbolic reaction (Fig. 2C). The analysis of this occupancy–effect curve allowed us to estimate that only a small fraction (1.2 ± 0.2%) of total receptors was needed to be occupied to yield a 50% of the maximal effect of DAMGO (Fig. 2C). In addition, only a fraction (47 ± 2%) of total receptors was calculated to be occupied at the estimated maximal effect achieved by DAMGO (Fig. 2C).

3.3. DAMGO and baclofen stimulation of [^{35}S]GTPγS binding after G-protein alkylation

In order to compare the loss of receptor-mediated responsiveness induced by inactivation of agonist binding sites (β-funaltrexamine pretreatment) with that caused by the inactivation of G-proteins, we examined DAMGO-mediated stimulation of [^{35}S]GTPγS binding in membranes pretreated with different concentrations of the $G_{i/o}$-protein alkylating agent *N*-ethylmaleimide. The effects of *N*-ethylmaleimide on agonist-mediated G-protein activation were also studied for the $GABA_B$ receptor agonist baclofen.

The basal [^{35}S]GTPγS binding was reduced by *N*-ethylmaleimide pretreatment (F[4,10] = 29.91, P < 0.0001) (Fig. 3A). Preincubation of the membrane preparations with increasing concentrations of *N*-ethylmaleimide firstly induced decreases in the potency (increased EC_{50}) of DAMGO activating G-proteins (F[3,32] = 25.1, P < 0.0001) (Fig. 3B). This shift to the right of the curves reached the level of statistical significance at 1 μM *N*-ethylmaleimide (Dunnett's post-hoc test). Higher concentrations of *N*-ethylmaleimide were necessary to reduce the efficacy (E_{max}) of DAMGO. Thus, *N*-ethylmaleimide pretreatment reduced the E_{max} of DAMGO (F[3,32] = 64.11, P < 0.0001) (Fig. 3B), but this reduction reached the level of statistical significance at 10 μM of the alkylating agent (Dunnett's post-hoc test). Baclofen stimulation of [^{35}S]GTPγS binding was affected by *N*-ethylmaleimide in a different way. Thus, Fig. 3C shows that the reduction of $GABA_B$-mediated stimulation was entirely accounted for by a reduction of E_{max} of baclofen (F[5,27] = 340.7, P < 0.0001) with no changes in its EC_{50} (F[3,20] = 0.70, P>0.05). This reduction of baclofen efficacy reached the level of statistical significance at 1 μM *N*-ethylmaleimide (Dunnett's post-hoc test). Preincubation of the membranes with 60 μM (or above) *N*-ethylmaleimide completely abolished baclofen stimulation of [^{35}S]GTPγS binding (Fig. 3C).

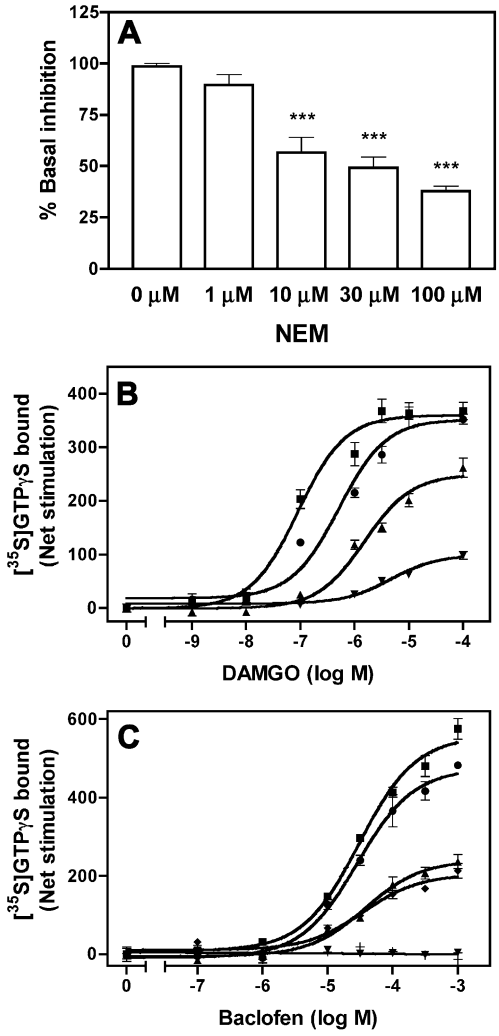

Fig. 3. Effect of $G_{i/o}$-protein alkylation by *N*-ethylmaleimide on DAMGO- and baclofen-stimulation of [^{35}S]GTPγS binding in postmortem human brain membranes. Membranes preparations were preincubated as shown in Section 2 in the absence (■), and in the presence of 1 μM (●), 10 μM (▲), 30 μM (♦), 60 μM (+), or 100 μM (▼) *N*-ethylmaleimide. (A) Effect of the indicated concentration of *N*-ethylmaleimide on the basal [^{35}S]GTPγS binding. Basal [^{35}S]GTPγS binding value in control membranes was 1014 ± 187 fmol/mg protein (* * * P < 0.001, Dunnett's post-hoc test of one-way ANOVA). (B, C) Concentration–response curves of net-stimulated [^{35}S]GTPγS binding (agonist-stimulated minus basal binding) by DAMGO (B) and baclofen (C) in control and *N*-ethylmaleimide-pretreated membranes. Incubations were performed as described in Section 2 and data are mean ± S.E.M. (bars) values of three experiments performed in triplicate in three different brains.

3.4. Isotopic dilution curves of [^{35}S]GTPγS binding

The affinity of G-proteins for [^{35}S]GTPγS (K_D) and the maximal number of [^{35}S]GTPγS binding sites (B_{max}) were estimated by inhibiting [^{35}S]GTPγS binding with unlabelled GTPγS. When isotopic dilution curves of total binding were analysed from 10^{-11} to 10^{-3} M GTPγS as displacer, both basal and agonist-stimulated isotherms produced biphasic plots (a more complex binding model was not performed). Nevertheless, as we have previously reported (González-Maeso et al., 2000), this very low-affinity binding site is not functionally coupled to G-protein coupled receptors, and the delineation of the nonspecific binding as the remaining binding in the presence of 10 μM unlabelled GTPγS (see Section 2) allows us to selectively identify the [^{35}S]GTPγS binding component to heterotrimeric G-proteins. Furthermore, analysis of isotopic dilution curves of specific binding (i.e., total binding minus binding in the presence of 10 μM GTPγS) resulted again in biphasic isotherms both in the absence (basal) and in the presence of DAMGO or baclofen (basal binding: $F[2,27]=26.62$, $P<0.0001$; DAMGO-stimulated binding: $F[2,27]=12.82$, $P<0.0001$; baclofen-stimulated binding: $F[2,26]=18.39$, $P<0.0001$) (Table 2, Fig. 4A). These two binding sites reflect respectively the receptor-activated G-proteins (high-affinity binding sites) and the endogenous GTP/GDP exchange of G-proteins (low-affinity binding sites) (Audinot et al., 2001). The affinities ($K_{D\text{-high}}$ and $K_{D\text{-low}}$) of the binding sites were not affected by the agonists (Table 2, Fig. 4A). On the contrary, both DAMGO and baclofen enhanced the density of the high-affinity binding sites ($B_{max\text{-high}}$) (DAMGO-stimulated binding: $F[5,42]=146.76$, $P<0.001$; baclofen-stimulated binding: $F[5,42]=444.23$, $P<0.001$) without affecting the density of the low-affinity binding sites ($B_{max\text{-low}}$) (Table 2, Fig. 4A).

In order to study the agonist-activated G-proteins, we analysed the isotopic dilution curves of net agonist-stimulated [^{35}S]GTPγS binding (i.e., agonist-stimulated binding minus basal binding). Isotopic dilution curves of net agonist-stimulated [^{35}S]GTPγS binding resulted in monophasic isotherms (Fig. 4B) with affinities ($K_{D\text{-net}}$) in the range of high-affinity binding sites of G-proteins for [^{35}S]GTPγS ($K_{D\text{-high}}$) (Table 2). The amounts of G-proteins activated by the agonists ($B_{max\text{-net}}$) are shown in Table 2. These estimated amounts of G-proteins stimulated by DAMGO and baclofen were not different to that estimated by substracting $B_{max\text{-high}}$ in basal conditions from $B_{max\text{-high}}$ in the presence of the agonist.

To analyse the effect of G-protein alkylation on the density and affinity of receptor-activated G-proteins, membranes were pretreated with a submaximal concentration of *N*-ethylmaleimide (10 μM). Isotopic dilution curves of specific [^{35}S]GTPγS binding in *N*-ethylmaleimide-pretreated membranes presented (in a similar way to control membranes) biphasic isotherms (basal binding: $F[2,21]=12.76$, $P<0.001$; DAMGO-stimulated binding: $F[2,21]=13.28$, $P<0.001$; baclofen-stimulated binding: $F[2,21]=10.08$, $P<0.001$) (Table 2, Fig. 4C). *N*-ethylmaleimide pretreatment of the membranes did not affect either the affinity of high- and low-affinity binding [^{35}S]GTPγS sites ($K_{D\text{-high}}$, $K_{D\text{-low}}$) or the density of the low-affinity [^{35}S]GTPγS binding sites ($B_{max\text{-low}}$) (Table 2, Fig. 4C). On the contrary, $B_{max\text{-high}}$ of basal [^{35}S]GTPγS binding was reduced by *N*-ethylmaleimide pretreatment ($F[5,42]=99.48$, $P<0.001$) (Table 2, Fig. 4C). DAMGO and baclofen also increased the density of high-affinity binding sites ($B_{max\text{-high}}$) in *N*-ethylmaleimide-pretreated membranes (DAMGO-stimulated binding: $F[5,42]=150.59$, $P<0.001$; baclofen-stimulated binding: $F[5,42]=227.07$, $P<0.001$) (Table 2, Fig. 4C). The amount of G-proteins activated by DAMGO

Table 2
Effect of DAMGO and baclofen on [^{35}S]GTPγS binding to control and *N*-ethylmaleimide-pretreated membranes (NEM)

	$B_{max\text{-high}}$ (pmol/mg prot)	$B_{max\text{-low}}$ (pmol/mg prot)	log $K_{D\text{-high}}$	log $K_{D\text{-low}}$	$B_{max\text{-net}}$ (pmol/mg prot)	log $K_{D\text{-net}}$
Basal	16.9 ± 0.8	246 ± 12	− 7.74 ± 0.17	− 6.52 ± 0.17	NA	NA
Basal-NEM	10.1 ± 0.6[a]	324 ± 20	− 7.81 ± 0.19	− 6.27 ± 0.18	NA	NA
DAMGO	30.6 ± 1.3[a]	289 ± 25	− 7.70 ± 0.16	− 6.44 ± 0.23	13.6 ± 0.4	− 7.38 ± 0.08
DAMGO-NEM	20.0 ± 0.8[b]	325 ± 19	− 7.35 ± 0.04	− 6.33 ± 0.42	10.2 ± 0.3[c]	− 7.43 ± 0.11
Baclofen	59.5 ± 1.5[a]	262 ± 22	− 7.60 ± 0.08	− 6.37 ± 0.30	30.7 ± 0.5	− 7.62 ± 0.05
Baclofen-NEM	30.6 ± 0.9[b]	219 ± 16	− 7.70 ± 0.11	− 6.41 ± 0.26	16.6 ± 0.4[d]	− 7.73 ± 0.08

[^{35}S]GTPγS binding to control membranes and membranes pretreated with a submaximal concentration of *N*-ethylmaleimide (10 μM, see Section 2) was displaced by unlabelled GTPγS both in the absence (basal) and in the presence of DAMGO (10 μM) or baclofen (100 μM). Isotherms of specific binding (total minus nonspecific binding) were analysed by non-linear regression providing a two-site model as a better description of the data (see Section 3). Dissociation constants ($K_{D\text{-high}}$, $K_{D\text{-low}}$) and maximal number of binding sites ($B_{max\text{-high}}$, $B_{max\text{-low}}$) were estimated as described in Section 2 for the high- and low-affinity binding sites. Isotherms of net-stimulated [^{35}S]GTPγS binding (agonist-stimulated minus basal binding) were analysed by non-linear regression providing a one-site model a better description of the data (see Section 3). Dissociation constants ($K_{D\text{-net}}$) and maximal number of binding sites ($B_{max\text{-net}}$) were estimated as described in Section 2. Values are best fit ± S.E. values of three experiments performed in triplicate in three different brains.
NA: not applicable.
[a] $P<0.001$ versus basal $B_{max\text{-high}}$.
[b] $P<0.001$ versus basal-NEM $B_{max\text{-high}}$.
[c] $P<0.001$ versus DAMGO $B_{max\text{-net}}$.
[d] $P<0.001$ versus baclofen $B_{max\text{-net}}$ (F test).

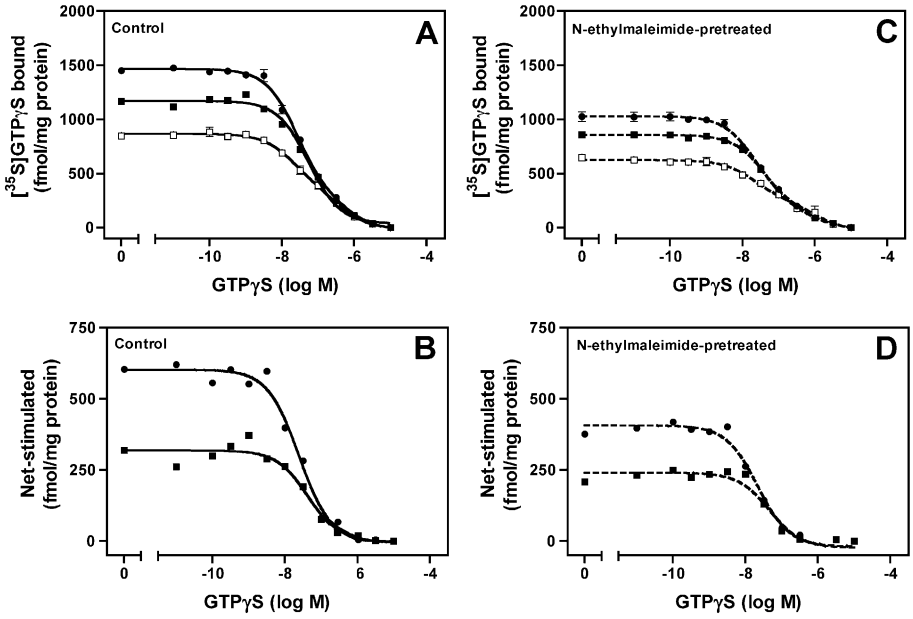

Fig. 4. Isotopic dilution curves of [^{35}S]GTPγS binding to postmortem human brain membranes. Incubations were performed as described in Section 2, and the specific binding (total minus nonspecific binding; A, C) and the net-stimulated binding (agonist-stimulated minus basal binding; B, D) were determined in the absence (□) and in the presence of 10 μM DAMGO (■) or 100 μM baclofen (●) both in control membranes (A, B; solid lines) and in membranes pretreated with a submaximal (10 μM) concentration of *N*-ethylmaleimide (NEM) (C, D: dotted lines). The data fitted best to a two-site model for the specific binding curves and to a one-site model for agonist net-stimulated curves. Data are mean ± S.E.M. (bars) values of a representative experiment in triplicate shown in Table 2.

or baclofen in *N*-ethylmaleimide-pretreated membranes ($B_{max\text{-}net}$) was reduced up to ~ 75% or ~ 50%, respectively, from the amount of G-proteins activated by the agonists in control membranes (= 100%) (Table 2, Fig. 4C and D).

3.5. Stoichiometric parameters of signal transduction

In Table 3, the stoichiometric parameters of signal transduction derived from the data presented in this study are shown. Ratios of affinity constants of DAMGO to its EC_{50} values stimulating [^{35}S]GTPγS binding were calculated as a measure of signal transduction amplification and the pool of receptor reserve. This ratio was calculated both for DAMGO affinity constant estimated by receptor binding assays ($K_{i\text{-}low}$) and for DAMGO dissociation constant estimated by "nested hyperbolic method" (K_A) (Table 3). Note that while $K_{i\text{-}low}/EC_{50} < 1$, $K_A/EC_{50} > 1$.

Table 3
Stoichiometric factors of μ-opioid receptor–G-protein coupling

$K_{i\text{-}low}/EC_{50}$	K_A/EC_{50}	$B_{max\text{-}net}/R_{occ}$
0.081	69.0	1304

$K_{i\text{-}low}$ value in TEM-buffer (see Section 2) for DAMGO is taken from Table 1. EC_{50} is the potency of DAMGO stimulating [^{35}S]GTPγS binding in postmortem cortical membranes (see Section 3). DAMGO dissociation constant (K_A) was estimated by the "nested hyperbolic method" (see Section 3). $B_{max\text{-}net}$ for DAMGO (10 μM) is taken from Table 2. R_{occ} is the number of μ-opioid occupied receptors by 10 μM DAMGO and was estimated from the fraction of occupied receptors (f_{occ}, see Eq. (2)) and the maximal number of specific [^{3}H]naloxone binding sites.

The quantitative stoichiometry of the μ-opioid receptor–G-protein coupling was calculated by the ratio of the G-proteins activated by 10 μM DAMGO ($B_{max\text{-}net}$) to the number of μ-opioid receptors occupied by the same concentration DAMGO (Table 3). The number of μ-opioid receptors occupied by DAMGO (R_{occ}) was estimated from the fraction of occupied receptors (f_{occ}) with 122 ± 9 fmol/mg prot as the calculated B_{max} for [^{3}H]naloxone in these particular experimental conditions (see Section 2).

4. Discussion

As we have previously demonstrated, [^{35}S]GTPγS binding assays provides a functional method to study receptor–G-protein coupling in postmortem human brain (González-Maeso et al., 2000; Rodríguez-Puertas et al., 2000). However, the stoichiometry of receptor–G-protein coupling is not yet well understood. Discrepancies between affinities and potencies of the agonists have been reported in the literature showing lower agonist potencies stimulating [^{35}S]GTPγS binding than their receptor binding affinities.

In the current study, DAMGO displaced [^{3}H]naloxone binding in a biphasic pattern and, as previously reported in rat brain (Maher et al., 2000), the buffer composition of [^{35}S]GTPγS binding assay (i.e., TEM-buffer) decreased, but not eliminated, the fraction of high-affinity binding sites for the agonist. Concentration–response curves of DAMGO stimulating [^{35}S]GTPγS binding presented monophasic plots as previously reported in postmortem human cortical

membranes (González-Maeso et al., 2000). In contrast, Maher et al. (2000) showed biphasic concentration–response curves of DAMGO stimulating [^{35}S]GTPγS binding in rat brain membranes. The monophasic curves of agonist-stimulated [^{35}S]GTPγS binding are not a generalized phenomenon in postmortem human cortex. Thus, the 5-HT_{1A} serotonin receptor agonist 8-OH-DPAT has been shown to induce biphasic concentration–response curves stimulating [^{35}S]GTPγS binding (González-Maeso et al., 2002a,b). The effect of the agonist-occupation of high-affinity binding sites on [^{35}S]GTPγS binding is not clear (Waelbroeck, 2001a). Therefore, it is difficult to explain these discrepancies in μ-opioid receptor stimulation of [^{35}S]GTPγS binding between rat and human cortical membranes, although species or experimental differences may account for them. High-affinity binding sites may correspond to precoupled or spontaneously active forms of the receptors (Chidiac, 1998; Costa et al., 1990; Kenakin, 1997; Selley et al., 1998), and the occupation of these sites by agonists may not increase [^{35}S]GTPγS binding much above basal levels (Maher et al., 2000). Since, in the present work, the occupation of μ-opioid high-affinity binding sites induced non-detectable G-protein activation, it was assumed that, in postmortem human brain and under our experimental conditions, agonists bind to low-affinity binding sites to form active ternary complexes (Strange, 1998). Therefore, concentration–response curves of agonists stimulating [^{35}S]GTPγS binding were analysed with Hill coefficients constrained to unity.

Thus, the $K_{i\text{-}low}/EC_{50}$ ratio obtained for DAMGO in postmortem human cortical membranes was lower than 1, which is a theoretical impossibility as discussed above. Moreover, alkylation of [^{3}H]DAMGO binding sites with β-funaltrexamine to nondetectable levels did not abolish the stimulation of [^{35}S]GTPγS binding by DAMGO. This suggested the existence of μ-opioid reserve receptors in human brain. Therefore, we estimated the dissociation constant of DAMGO (K_A) from [^{35}S]GTPγS binding assays data by the "nested hyperbolic method" (James et al., 1989). Although this analysis also provides an "apparent" K_A (Pineda et al., 1997), it is estimated from directly fitting the [^{35}S]GTPγS binding experimental data. Thus, opposite to $K_{i\text{-}low}/EC_{50} < 1$, the parameter $K_A/EC_{50} \gg 1$ revealed the existence of an important fraction of μ-opioid reserve receptors. These reserve receptors were confirmed by the low estimated K_E value.

Waldhoer et al. (1999) have demonstrated that the activation of the receptor (transition from R to R*) rather than its interaction with G-proteins is the rate-limiting step in the formation of the ternary complex. This kinetics of the ternary complex may explain the observed differences between K_A and $K_{i\text{-}low}$ in the present work. Thus, K_A is obtained from [^{35}S]GTPγS binding data (i.e., from occupied receptors which have activated G-proteins). On the contrary, $K_{i\text{-}low}$ is obtained from receptor binding data. In this regard, because there are different molecular interactions between a particular agonist and the receptor (Strange, 1998), it could be possible that some occupied receptors do not overcome the rate-limiting transition from R to R*. Therefore, the more thermodynamically demanding conformational changes required by the [^{35}S]GTPγS functional binding than by the [^{3}H]agonist binding assays to detect a particular receptor may explain the ratio $K_{i\text{-}low} < K_A$ obtained in the present work. Moreover, the recent evidence that many signaling molecules are enriched in specialized microdomains of the plasma membrane increases the likelihood that G-protein coupled receptor signaling is highly compartmentalized in cells (Neubig, 1994; Strange, 1999; Ostrom et al., 2000). The possible disruption of these microdomains in the preparation of the membranes may also interfere in the receptor–G-protein coupling reducing the affinity estimated for DAMGO from the functional [^{35}S]GTPγS binding experiments (K_A).

The progressive blockage of μ-opioid receptors were performed with the alkylating agent β-funaltrexamine. This irreversible agent has been demonstrated to selectively alkylate μ-opioid receptors in mammalian brain membranes (Clark and Medzihradsky, 1987; Goldstein and James, 1984). Therefore, since DAMGO was used to activate μ-opioid receptors, we monitored the alkylation by performing [^{3}H]DAMGO saturation curves after β-funaltrexamine pretreatment. The reduction obtained in the [^{3}H]DAMGO binding sites density, without affecting affinity values, confirmed the irreversible blockage of the μ-opioid receptors by β-funaltrexamine in postmortem human brain membranes. However, because of its agonist properties, the B_{max} values obtained from [^{3}H]DAMGO saturation curves in control membranes do not correspond with the density of μ-opioid receptor, and was not used in further evaluations. Therefore, the density of μ-opioid receptors was estimated by using the neutral antagonist [^{3}H]naloxone as described in Section 2.

The effect of varying receptor–G-protein stoichiometry by blocking $G_{i/o}$-proteins with *N*-ethylmaleimide on both basal and agonist-stimulated [^{35}S]GTPγS binding was studied. *N*-ethylmaleimide (a sulfhydryl alkylating agent) has been shown to alkylate $G_{i/o}$-proteins in the same cysteine residue that is ADP-ribosylated by pertussis toxin (Winslow et al., 1987). Although it has been reported that *N*-ethylmaleimide is able to alkylate μ-opioid receptors (Shahrestanifar et al., 1996), when incubations are performed under mild conditions (i.e., 4 °C), *N*-ethylmaleimide has been demonstrated to selectively alkylate $G_{i/o}$-proteins without affecting [^{3}H]agonist binding to μ-opioid (Ueda et al., 1990) or $GABA_B$ (Asano and Ogasawara, 1986) receptors. Thus, *N*-ethylmaleimide has been extensively used in mammalian brain to induce a functional uncoupling of G-protein coupled receptors from $G_{i/o}$-proteins (Lorenzen et al., 1993; Odagaki et al., 2000; Olianas and Onali, 1996).

Basal [^{35}S]GTPγS binding (and also basal $B_{max\text{-}high}$, see below) was decreased by $G_{i/o}$-protein alkylation with *N*-ethylmaleimide. Basal [^{35}S]GTPγS binding corresponds

both to the endogenous GTP-ase activity of G-proteins and to G-proteins activated by precoupled receptors (Audinot et al., 2001). Therefore, our results suggest that there is a population of G-protein-precoupled receptors in human frontal cortex. This finding is similar to those described in previous studies in mammalian brain (Lorenzen et al., 1993; Olianas and Onali, 1996). On the contrary, the alkylation of μ-opioid receptors did not produce detectable changes in basal [^{35}S]GTPγS binding. In concordance, no inverse agonism activity on μ-opioid receptors has been detected in rat brain membranes (Sim et al., 1996). On the contrary, in cultured cells, decreases in basal G-protein activity have been reported both by opioid receptor alkylation (Costa et al., 1988) and by μ-opioid receptor–G-protein uncoupling by sodium (Selley et al., 2000). Therefore, different behaviour of receptor–G-protein coupling between cultured cells and mammalian brain membranes may avoid to directly extrapolate conclusions from recombinant to native systems (Kenakin, 1997).

Progressive alkylation of $G_{i/o}$-proteins by *N*-ethylmaleimide firstly induced shifts to the right in DAMGO concentration–response curves stimulating [^{35}S]GTPγS binding followed by decreases in its efficacy (E_{max}). Thus, DAMGO achieved maximal effects after blocking a fraction of G-proteins. These results evidence the presence of "reserve G-proteins" in μ-opioid receptor–G-protein system in human brain. This finding was detected at low *N*-ethylmaleimide concentrations (1 μM), so that *N*-ethylmaleimide treatment presents high specificity for $G_{i/o}$-proteins (see above). In this context, a large quantity of $G_{i/o}$-proteins has been shown in mammalian brain (Neubig, 1994; Ostrom et al., 2000; Sternweis and Robishaw, 1984). Taken together, these results indicate that G-proteins are not a limiting factor for μ-opioid receptor in postmortem human brain membranes.

Activation of G-proteins by baclofen was affected in a different way by *N*-ethylmaleimide. Thus, baclofen efficacy was decreased after $G_{i/o}$-protein alkylation without changes in its EC_{50}. In this way, G-proteins appeared as limiting when blocked by *N*-ethylmaleimide for $GABA_B$ receptor-mediated stimulation of [^{35}S]GTPγS binding. It has been recently demonstrated that while μ-opioid receptors preferably couple to G_o- and $G_{i1/3}$-proteins (Chalecka-Franaszek et al., 2000), G_{i2}-protein is the main transducer of $GABA_B$ receptors (Odagaki and Koyama, 2001). These differences in the selectivity of μ-opioid and $GABA_B$ receptors for G-proteins may account for the different behaviour of the studied receptors after G-protein blockage.

The effect of activated receptors on G-proteins is not still clarified. In this regard, isotopic dilution curves have been repeatedly used to study the G-protein activation with some discrepant results (Breivogel et al., 1998; González-Maeso et al., 2000; Newman-Tancredi et al., 1997; Selley et al., 1997, 1998; Sim et al., 1996). In the present work, three components can be distinguished by isotopic dilution of [^{35}S]GTPγS specific binding as described in Section 2 (Audinot et al., 2001; Newman-Tancredi et al., 2000): First, low-affinity binding sites reflect the endogenous GDP/GTP exchange in G_α-subunits. Second, in the absence of agonists, high-affinity binding sites correspond to G-protein activated by non-occupied receptors (i.e., precoupled receptors). Third, the component of G-protein activated by agonist-occupied receptors. Our data show that agonists increased the density of $B_{max\text{-}high}$ without detectable changes in $B_{max\text{-}low}$. On the contrary, neither $K_{D\text{-}high}$ nor $K_{D\text{-}low}$ were affected by DAMGO or baclofen. These results indicate that G-proteins are present in two detectable states with two different affinities for [^{35}S]GTPγS (high and low). Resting G-proteins show low-affinity for GTP (or [^{35}S]GTPγS) and activated receptors shift G-proteins from resting state to active state (high-affinity), but the characteristic affinity of each state is not altered.

Isotopic dilutions isotherms of basal [^{35}S]GTPγS specific binding also presented biphasic plots and, as reported above, the high-affinity component of basal [^{35}S]GTPγS binding correspond to G-proteins activated by precoupled receptors (Audinot et al., 2001). In this regard, when G-proteins were uncoupled from the receptor by *N*-ethylmaleimide, a decrease in $B_{max\text{-}high}$ was found. This effect is similar to that obtained with inverse agonists in cultured cells (Audinot et al., 2001; Newman-Tancredi et al., 2000). The active coupling of unoccupied receptors in postmortem human brain has been previously suggested (Callado and Meana, 1997; González-Maeso et al., 2000). Therefore, the present data are consistent with the possible presence of precoupled or constitutive active receptors in postmortem human brain. Nevertheless, further work is needed to clarify the presence and relevance of precoupled receptors in G-protein coupled receptor research and drug discovery under physiological conditions.

In order to study the pharmacological parameters (affinity, density) of G-proteins activated by the two different receptors studied in this work, isotopic dilution curves of net agonist-stimulated [^{35}S]GTPγS binding were analysed. The resulting isotherms showed a single class of binding sites ($K_{D\text{-}net}$). This type of analysis is statistically valid (Selley et al., 1997) because it showed no significant differences between $K_{D\text{-}high}$ and $K_{D\text{-}net}$; and between $B_{max\text{-}high}$ (agonist) minus $B_{max\text{-}high}$ (basal) and $B_{max\text{-}net}$. Furthermore, this analysis allowed to estimate $K_{D\text{-}net}$ and $B_{max\text{-}net}$ more precisely from nonlinear regression estimations. The amount of G-proteins activated by baclofen ($B_{max\text{-}net}$) was larger than the activated by DAMGO (as expected, taking into account the higher efficacy (E_{max}) of the former in concentration response curves stimulating [^{35}S]GTPγS binding). Preincubation of the membranes in the presence of a submaximal concentration of *N*-ethylmaleimide decreased a higher percentage of $B_{max\text{-}net}$ for baclofen than for DAMGO. This finding confirmed that, since baclofen reclutes a larger amount (and probably different type, see above) of G-proteins, their blockage by *N*-ethylmaleimide makes G-proteins limiting for $GABA_B$ but not for μ-opioid receptors. Therefore, these results show that the mechanism of activa-

tion of the G-proteins is common in the two studied systems (increasing the amount of G-proteins with high affinity for [^{35}S]GTPγS) but the stoichiometric control of the transduction is different, being G-proteins limiting for $GABA_B$ but not for μ-opioid receptors in the presence of G-protein blockers.

The quantitative stoichiometry of G-protein coupled receptors signal transduction components has been previously studied both in cultured cell lines (Kim et al., 1994; Selley et al., 1998; Waelbroeck, 2001b) and in rat brain membranes (Maher et al., 2000; Selley et al., 1998; Sim et al., 1996). In the present work, densities of both G-proteins (i.e., $B_{max\text{-}high} + B_{max\text{-}low}$) and μ-opioid receptors similar to that obtained in rat brain membranes (Maher et al., 2000; Selley et al., 1998; Sim et al., 1996) are shown. In contrast, the amplification of the signal along receptor–G-protein pathway estimated in the present work is higher than the estimated in previous works for μ-opioid receptors (Maher et al., 2000; Selley et al., 1998; Sim et al., 1996). The simplest explanation for this disparity is the inclusion of f_{occ} in the evaluation of the amplification factor. In this regard, directly comparing B_{max} receptor binding and receptor-activated G-proteins to obtain an amplification factor (Maher et al., 2000; Selley et al., 1998; Sim et al., 1996) might be interpreted as an underestimation of the catalytic activity of the system. Therefore, stoichiometric analysis of the overall cellular expression of receptors and G-proteins may be an overly simplistic approach because such analysis fails to account for the fraction of occupied receptors and for the compartmentalisation of molecules in the membrane (Ostrom et al., 2000). Thus, organization of receptors and G-proteins in the membrane rather than a random collision-coupling model has been demonstrated for μ-opioid receptors (Remmers et al., 2000). The present work, as discussed above, demonstrates in human brain tissue that maximal G-protein stimulations can be achieved by submaximal receptor occupancy. Therefore, comparing the number of G-proteins activated by occupied-receptors ($B_{max\text{-}net}$) to the number of agonist occupied-receptors (R_{occ}) is a more precise estimation of the signal transduction mechanisms, and considers not all the μ-opioid receptor population but only those activated by the agonist.

The great influence of experimental conditions such as Mg^{2+}, GDP or Na^{+} concentrations on the steady-state of [^{35}S]GTPγS binding (Breivogel et al., 1998; González-Maeso et al., 2000) makes difficult to obtain an absolute quantification of the receptor–G-protein system. Furthermore, inherent variables to postmortem studies such as age, postmortem delay, and storage time of the samples have also been shown to alter the obtained results (González-Maeso et al., 2002a). This forces us to consider all the obtained pharmacological parameters as apparents, and constrained to the particular experimental conditions. Nevertheless, the estimated $B_{max\text{-}net}/R_{occ}$ ratio used in the present work as an estimation of receptor–G-protein coupling may be useful in the study of the molecular mechanisms of agonists efficacies when compared in the same system. The presented results are consistent with the notion that occupied receptors catalytically activate a larger population of G-proteins (Waelbroeck, 2001b).

In conclusion, in the present work we have estimated the number of activated G-proteins by μ-opioid occupied receptors. Identifying the components that determine potency (EC_{50}) and efficacy (maximal response) can lead to insights as how to best enhance or suppress a dysregulated system (Ostrom et al., 2000). The limiting factors for G-protein activation have also been studied in two structurally different receptor systems. In this regard, we have studied the effect of varying μ-opioid and $GABA_B$ receptor–G-protein stoichiometry on the pharmacological parameters of the agonists stimulating receptor–G-protein coupling. These findings could be useful in the research of G-protein coupled receptors in pathophysiological conditions, and in the study of important concepts in molecular pharmacology such as agonist efficacy.

Acknowledgements

This study was supported by grants from the Basque Government (PI98/8), FIS (01/0358) and the University of the Basque Country (00026.125-13588/2001). JG-M was a recipient of a predoctoral fellowship from MEC. The authors wish to thank the staff members of the Basque Institute of Legal Medicine (Dr. R. Alcaraz) for their cooperation in the study. Dr. J. Pineda is thanked for useful discussions. Dr. S.C. Sealfon is thanked for critical reading of the paper.

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