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Characterization of receptor-mediated [^{35}S]GTPγS binding to cortical membranes from postmortem human brain

Javier González-Maeso, Rafael Rodríguez-Puertas, Ane M. Gabilondo, J. Javier Meana *

Departamento de Farmacología, Universidad del País Vasco / Euskal Herriko Unibertsitatea, E-48940 Leioa, Bizkaia, Spain

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Abstract

The [^{35}S]GTPγS binding assay represents a functional approach to assess the coupling between receptors and G-proteins. The optimal conditions for [^{35}S]GTPγS binding to human brain homogenates were established in postmortem samples of prefrontal cortex. The influence of protein content, incubation time, GDP, Mg^{2+}, and NaCl concentrations on the [^{35}S]GTPγS binding were assessed in the absence and presence of the α_2-adrenoceptor agonist UK14304 5-bromo-*N*-(4,5-dihydro-1*H*-imidazol-2-yl)-6-quinoxalinamine). In conditions of 50 μM GDP and 100 mM NaCl, UK14304 increased the apparent affinity of the specific [^{35}S]GTPγS binding without changing the apparent density. Concentration–response curves to agonists of α_2-adrenoceptors, μ-opioid, 5-HT_{1A}, cholinergic muscarinic, and $GABA_B$ receptors displayed, in the presence of NaCl, maximal stimulations between 24% and 61% with EC_{50} values in the micromolar range. Selective antagonists shifted to the right the agonist-induced stimulation curves. The G_i/G_o-protein alkylating agent *N*-ethylmaleimide decreased basal [^{35}S]GTPγS binding in a concentration-dependent manner and inhibited the stimulation induced by the different agonists. In cortical sections, [^{35}S]GTPγS binding to gray matter was stimulated by the agonist UK14304. The present study demonstrates that functional studies of the receptor coupling to G_i/G_o-proteins can be performed in postmortem human brain samples.

Keywords: [^{35}S]GTPγS binding; Brain, human; α_2-Adrenoceptor; 5-HT_{1A} receptor; μ-Opioid receptor; G-protein

1. Introduction

Many transmembrane signalling processes of extracellular hormones and neurotransmitters are mediated by receptor interaction with heterotrimeric guanine nucleotide binding proteins (G-proteins) (Gilman, 1987). It is well accepted that receptor activation by specific agonists alters the conformation of the G-proteins and leads to the exchange of GDP by GTP at an specific site on the α subunit (Gilman, 1987; Birnbaumer et al., 1990). This conformational change promotes the dissociation of the heterotrimeric G-protein into α-GTP and βγ subunits, both of which can regulate several effector molecules within the cell (Clapham and Neer, 1997).

A direct evaluation of the receptor-dependent G-protein activity can be obtained by measuring the stimulation of guanine nucleotide exchange on G-proteins through the use of radiolabelled GTP analogues. Thus, the modulation of guanine nucleotide binding by the presence of G-protein-coupled receptor agonists represents a functional information on receptor status and a valuable method for the monitoring of agonist and antagonist drugs properties. In particular, the non-hydrolizable GTP analogue guanosine 5′-(γ-[^{35}S]thiotriphosphate) ([^{35}S]GTPγS) has been used to evaluate, among others, α_2-adrenoceptors in reconstituted systems of purified proteins (Cerione et al., 1986), muscarinic cholinergic and μ-opioid receptors in membrane homogenates (Hilf et al., 1989; Selley et al., 1997), formyl peptide, muscarinic, 5-HT_{1A}, and α_2-adrenoceptors in lysate of cells transfected with the cloned receptors (Gierschik et al., 1989; Lazareno and Birdsall, 1993; Newman-Tancredi et al., 1996; Peltonen et al., 1998), μ-opioid, cannabinoid, $GABA_B$, and 5-HT_1 receptors in autoradiography (Sim et al., 1995; Dupuis et al., 1998), and muscarinic and β-adrenoceptors in immunoprecipitation assays (Wang and Friedman, 1994). A common fact for almost all the G-protein-coupled receptors tested by the [^{35}S]GTPγS binding assay is their coupling to pertussis-toxin sensitive

* Corresponding author. Fax: +34-94-480-01-28.
E-mail address: kfpmemaj@lg.ehu.es (J.J. Meana).

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G_i/G_o-proteins. However, up to now, there is not a deep study of [^{35}S]GTPγS binding developed in either membrane preparations or autoradiographic sections obtained from postmortem human brain. Two previous reports restricted to [^{35}S]GTPγS binding stimulation by a single concentration of muscarinic agonists have been published in human brain (Ferrari-DiLeo et al., 1995; Cowburn et al., 1996). A very recent autoradiographic study has evaluated the [^{35}S]GTPγS binding stimulation by 5-HT_{1A} serotonin receptor agonists (Dupuis et al., 1999).

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. Other lines of evidence suggest the existence of specific abnormalities of G-protein expression and/or function in the brain of subjects with neuropsychiatric disorders (Manji, 1992; Young et al., 1993; Cowburn et al., 1996; Friedman and Wang, 1996; Pacheco et al., 1996). Therefore, the quantification of the [^{35}S]GTPγS binding to human brain tissue, and its stimulation induced by selective receptor agonists could represent a powerful tool for the study of CNS disorders.

The aim of the present study was to establish the optimal conditions for the [^{35}S]GTPγS binding in postmortem human brain preparations. The stimulation of [^{35}S]GTPγS binding by several G-protein-coupled receptor agonists was also analysed. In addition, a preliminary autoradiographic assay was done to delineate anatomical resolution and to draw optimal conditions for a further study in human tissue slices. Abstracts of this work were given at the 1998 Forum of European Neuroscience (González-Maeso et al., 1998) and the 1998 Society for Neuroscience (Rodríguez-Puertas et al., 1998).

2. Methods

2.1. Materials

[^{35}S]GTPγS (1250 Ci/mmol) was purchased from DuPont NEN (Brussels, Belgium). Atropine, bovine seroalbumine (BSA), carbachol, [D-Ala^2, *N*-Me-Phe^4, Gly^5-ol]-enkephalin (DAMGO), (±)-8-hydroxy-2-(di-*n*-propylamino)tetralin (8-OH-DPAT), DL-dithiothreitol (DTT), GDP, GTP, GTPγS, naloxone, and *N*-ethylmaleimide (NEM) were purchased from Sigma (St. Louis, USA). Baclofen, 5-bromo-*N*-(4,5-dihydro-1*H*-imidazol-2-yl)-6-quinoxalinamine (UK14304) and phaclofen were obtained from Tocris Cookson (Bristol, UK). 2-Methoxyidazoxan (RX821002) was synthesized at Lasa Laboratorios (Barcelona, Spain); *N*-[2-[4-(2-methoxyphenyl)-1-piperazinyl]ethyl]-*N*-(2-pyridinyl)cyclohexanecarboxamide (WAY100635-A-5) was from Wyeth-Ayerst (Princeton, 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 from subjects who had died by sudden and violent causes (motor-vehicle accidents). Samples 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 approved protocols of the Instituto Anatómico Forense, Bilbao, Spain for postmortem human studies. Toxicological screening for psychotropic drugs and alcohol was carried out in tissue samples, and subjects with a positive test were excluded from the study. Eight male subjects (age at death 37 ± 6 years) were definitively included. The postmortem delay between death and dissection was 32 ± 5 h and the storage period at −70°C was 43 ± 9 months. Not all the brains were used for each set of experiments because of the limited availability of tissue. However, all the experiments were performed at least in two, mainly three, different brains. A block of prefrontal cortex from one subject (23 years, 14 h of postmortem delay, and 1 month of storage time at −70°C) was dissected for autoradiographic assay.

2.3. Preparation of membranes and tissue sections

Tissue samples of each subject (~1 g) were homogenized using a Teflon-glass grinder (10 up-and-down strokes at 1500 rpm) in 30 volumes of homogenization buffer (1 mM EGTA, 3 mM $MgCl_2$, 1 mM DTT, and 50 mM Tris–HCl, pH 7.4) supplemented with 0.25 M sucrose. The crude homogenate was centrifuged for 5 min at 1000 × *g* (4°C) and the supernatant was recentrifuged for 10 min at 40,000 × *g* (4°C). The resultant pellet was washed twice in 20 volumes of homogenization buffer and recentrifuged in similar conditions. Aliquots of 0.4–0.6 mg protein were stored at −70°C until assay. Protein content was measured according to the method of Bradford (1976) using BSA as standard, and were similar in the different brain samples (10.9 ± 0.8 mg protein/g tissue).

For autoradiography, sections of 20 μm were cut at −25°C using a cryostat, mounted on gelatin coated slides, and stored at −25°C (González et al., 1994).

2.4. [^{35}S]GTPγS binding assays

Unless otherwise indicated, the incubation buffer for measuring [^{35}S]GTPγS binding to brain membranes contained, in a total volume of 500 μl, 1 mM EGTA, 3 mM $MgCl_2$, 100 mM NaCl, 0.2 mM DTT, 50 μM GDP, 50 mM Tris–HCl at pH 7.4 and 0.5 nM [^{35}S]GTPγS (about 700,000 cpm). For the binding studies, protein aliquots were thawed and resuspended in a buffer containing 1 mM EGTA, 3 mM $MgCl_2$, 100 mM NaCl, and 50 mM Tris–HCl, at pH 7.4. The incubation was started by addition of the membrane suspension (40 μg of membrane proteins) to the previous mixture and was performed at 30°C for 120

min with shaking. Optimization studies were carried out by modifying protein content, incubation time, and GDP, GTPγS, Mg^{2+}, and NaCl concentrations. In order to evaluate the influence of selective drugs on [^{35}S]GTPγS binding, several G-protein-coupled receptor agonists (10^{-11} to 10^{-3} M) and antagonists (10^{-6} or 10^{-4} M) were included in the incubation buffer. Incubations were terminated by adding 3 ml of ice-cold resuspension buffer followed by rapid filtration through Whatman GF/C filters presoaked in the same buffer. The filters were rinsed twice with 3 ml of ice-cold resuspension buffer, transferred to vials containing 5 ml of OptiPhase HiSafe II cocktail (Wallac, UK) and the radioactivity trapped was determined by liquid scintillation spectrometry (Packard 2200CA). The [^{35}S]GTPγS bound was about 7–14% of the total [^{35}S]GTPγS added. Nonspecific binding of the radioligand was defined as the remaining [^{35}S]GTPγS binding in the presence of 10 μM unlabelled GTPγS, and amounted to 0.2–0.5% of the total [^{35}S]GTPγS added. Nonspecific binding was substracted from total bound radioactivity to determine [^{35}S]GTPγS specific binding. Preliminary assays of presoaking the glass-fiber filters with 0.5% polyethylenimine (Bruns et al., 1983) increased nonspecific binding by ~220 fold, which prevented detection of specific [^{35}S]GTPγS binding.

A preliminary autoradiographic study of [^{35}S]GTPγS binding to human frontal cortex was also carried out as described by Sim et al. (1995) in rat brain. Cortical sections were incubated by immersion in a buffer containing 1 mM EGTA, 3 mM $MgCl_2$, 100 mM NaCl, 0.2 mM DTT, 2 mM GDP, 50 mM Tris–HCl at pH 7.4 and 0.05 nM [^{35}S]GTPγS during 120 min at room temperature. After the incubation, the slides were rinsed in cold 50 mM Tris–HCl buffer (pH 7.4, 4°C, 2 × 15 min) and dried in a cold-air stream. Nonspecific binding and responses to G-protein-coupled receptor agonists and antagonists were defined in similar conditions to assays in brain membranes. A single consecutive 20-μm-thick section was used for every different condition. Sections were apposed to a β-radiation-sensitive film (Hyperfilm β-max film, Amersham, UK) in a closed cassette at 4°C for 48 h. ^{14}C Standards were included on the film, scanned and a standard curve was prepared. The densities were converted to nCi/g tissue equivalent.

2.5. Membrane treatment with NEM

NEM is a sulfhydryl-alkylating agent that selectively uncouples receptor from G_i/G_o-proteins (Ueda et al., 1990; Olianas and Onali, 1996). Cortical membranes were preincubated (1 ml) in the absence or presence of NEM (1 μM–2 mM) for 60 min at 4°C. The reaction was stopped by 100 μl of DTT (final concentration 20 mM) followed by two membrane wash-outs with resuspension buffer and subsequent centrifugations at 20,000 × *g* for 20 min. The final pellets were resuspended in the same buffer and used immediately for binding studies. [^{35}S]GTPγS binding assays in standard conditions were performed with NEM-treated membranes and their respective non-treated controls.

2.6. Data analysis

Experimental values of the agonist-induced increases of [^{35}S]GTPγS binding were obtained (agonist-stimulated minus basal values) and termed net stimulations. Under such conditions, the maximal net stimulation of each receptor agonist could be estimated. Further, to display pharmacological parameters of the agonist-induced stimulation of [^{35}S]GTPγS binding, concentration–response curves were performed and their parameters were calculated by nonlinear regression analysis. The theoretical maximal effect fitted by the curves (E_{max}) and the concentration of the agonist that determines the half-maximal effect (IC_{50} or EC_{50}) were obtained by using the Prism™ software. In some cases (Fig. 5), and in order to allow better comparisons between agonists, the concentration–response curves are displayed as relative stimulations over basal values ([stimulated values − basal values] × 100/basal values). The isotopic dilution curve of the [^{35}S]GTPγS binding by unlabelled GTPγS was analysed by using the EBDA-LIGAND software (Munson and Rodbard, 1980; McPherson, 1985) to calculate the apparent equilibrium dissociation constants (K_D) and the apparent maximal number of binding sites (B_{max}). The selection between binding models and the statistical comparison between curves was made by the extra sum of squares principle (*F*-test), as outlined by Munson and Rodbard (1980). Data are expressed as the mean ± SEM of the results or the best fit ± SE obtained from the nonlinear analysis of the curves. Statistical significance of the difference between means was determined by the Mann–Whitney *U*-test with a level of significance at $p = 0.05$.

3. Results

3.1. Influence of protein, GDP and Mg^{2+} concentrations. Effect of α_2-adrenoceptor-mediated stimulation

The specific binding of [^{35}S]GTPγS (1 nM) to human brain cortical membranes was initially evaluated with increasing amounts of proteins (up to 100 μg) at different concentrations of GDP. The radioligand [^{35}S]GTPγS bound to membrane proteins linearly at GDP concentrations higher than 10 μM ($r^2 > 0.9$) (Fig. 1). However, at lower concentrations of GDP, [^{35}S]GTPγS binding fitted to an hyperbolic curve ($r^2 > 0.9$) that saturated when membrane protein concentrations were increased (Fig. 1). In order to obtain an appropriate [^{35}S]GTPγS bound/free ratio (~1/10), 40 μg protein (0.08 mg/ml) was chosen as a suitable concentration. When proteins were denatured by

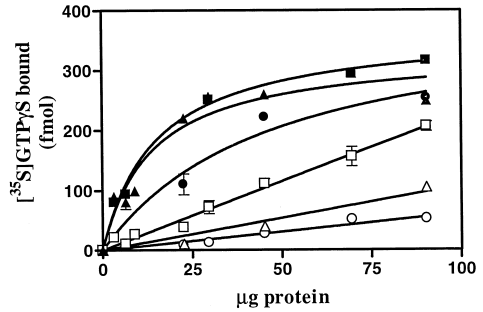

Fig. 1. Specific binding of [^{35}S]GTPγS to postmortem human brain cortical membranes as function of the amount of proteins at different concentrations of GDP: 0 μM (■), 0.1 μM (▲), 1 μM (●), 10 μM (□), 50 μM (△), and 100 μM (○). The incubations were performed in the presence of [^{35}S]GTPγS (1 nM), and NaCl (100 mM) at 30°C for 60 min. Data are mean ± SEM (bars) values from three experiments performed in different brains.

heating the membrane preparations at 70°C for 30 min, [^{35}S]GTPγS binding lowered to nonspecific levels.

The addition of GDP (0.1 μM–1 mM) diminished in a concentration-dependent manner, the [^{35}S]GTPγS specific binding with a IC_{50} value of 24.8 ± 0.2 μM ($n = 3$) in the absence of any receptor agonist (Fig. 2). In the presence of the α_2-adrenoceptor agonist UK14304 (10 μM), the GDP inhibition curve of [^{35}S]GTPγS binding shifted to the right ($F[3,34] = 15.94$, $p < 0.0001$), with a IC_{50} value of 37.5 ± 0.2 μM (Fig. 2). The maximal relative stimulation of [^{35}S]GTPγS binding induced by UK14304 at 30°C for 120 min was observed between 50 μM and 1 mM GDP (Fig. 2, inset). As consequence for subsequent assays, 50 μM GDP concentration was chosen to get an appropriate basal [^{35}S]GTPγS binding at the same time that an optimal agonist-induced stimulation.

The basal binding of [^{35}S]GTPγS to human brain membranes increased at concentrations above 10 μM free Mg^{2+}. The stimulation induced by UK14304 (10 μM) was absolutely dependent on the presence of higher levels than 100 μM free Mg^{2+}. A concentration of 3 mM Mg^{2+}, which increased basal [^{35}S]GTPγS binding by ~7-fold, was assumed for all subsequent experiments.

3.2. [^{35}S]GTPγS binding saturation parameters. Effect of α_2-adrenoceptor-mediated stimulation

The saturation binding parameters (K_D, B_{max}) were determined by dilution experiments of [^{35}S]GTPγS (0.5 nM) with increasing concentrations of unlabelled GTPγS (Fig. 3). Under steady-state conditions, the nonlinear analysis displayed a two-site binding model as the best fit of the total [^{35}S]GTPγS bound ($F[2,17] = 3.94$, $p < 0.05$). When calculated at 0.5 nM concentration of [^{35}S]GTPγS, the high-affinity component ($K_D = 54.2 \pm 25.2$ nM) accounted for the 95% of the total [^{35}S]GTPγS binding whereas the low-affinity component (estimated K_D values between 0.5 and 50 μM) represented the remaining 5%. According to these findings, for subsequent experiments, the nonspecific binding was chosen as the [^{35}S]GTPγS binding in the presence of 10 μM unlabelled GTPγS. Under these conditions, the low-affinity component was assumed to be nonspecific binding and the experimental values of this nonspecific binding represented 5.1 ± 0.5% of the total [^{35}S]GTPγS binding.

Further, an analysis of the influence of the α_2-adrenoceptor agonist UK14304 (10 μM) on these saturation binding parameters was performed. The inclusion of UK14304 in the assay increased the [^{35}S]GTPγS binding to the high-affinity population ($F[3,28] = 5.94$, $p < 0.01$) (Fig. 3). Nonlinear analysis of the curves showed that the increase was due to an enhanced affinity (best fit for K_D values: basal, 54.9 ± 2.0 nM; UK 14304-stimulated, 38.7 ± 9.7 nM; $F[2,30] = 9.47$, $p < 0.001$) without significant variations in the apparent maximal density of binding sites (best fit for B_{max} values: basal, 61 ± 22 pmol/mg protein;

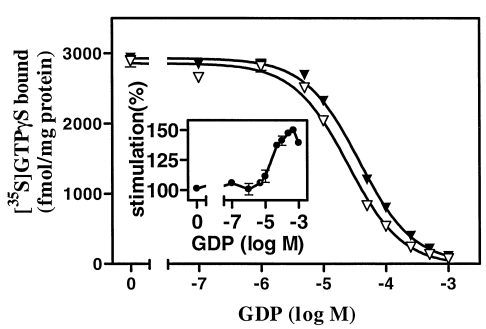

Fig. 2. Influence of GDP on [^{35}S]GTPγS specific binding to postmortem human brain cortical membranes in the absence (▽) or in presence (▼) of the α_2-adrenoceptor agonist UK14304 (10 μM). The incubations were performed with [^{35}S]GTPγS (0.5 nM), and NaCl (100 mM) at 30°C for 120 min. Data are mean ± SEM (bars) values from three experiments performed in different brains. Bars are not presented when SEM values are lower than the symbol size. Inset: Stimulation of [^{35}S]GTPγS binding by UK14304 expressed as the percentage of increase over basal binding values determined at each concentration of GDP.

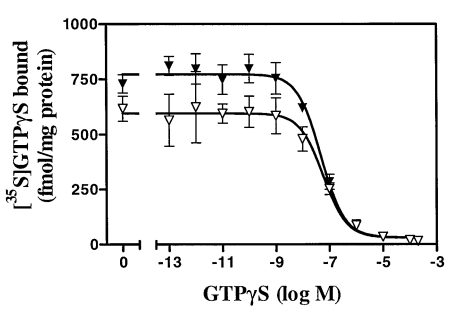

Fig. 3. Isotopic dilution curve of [^{35}S]GTPγS binding to postmortem human brain cortical membranes. Increasing concentrations of unlabelled GTPγS indicated on the abscissa were added to a fixed concentration of [^{35}S]GTPγS (0.5 nM), and the total radioligand binding was determined in the absence (▽) or presence (▼) of the α_2-adrenoceptor agonist UK14304 (10 μM). The incubations were performed with GDP (50 μM), and NaCl (100 mM) at 30°C for 120 min. Data are mean ± SEM (bars) values from two experiments performed in different brains.

UK14304-stimulated, 62 ± 15 pmol/mg protein). The [^{35}S]GTPγS binding parameters to the low-affinity population (i.e., nonspecific binding under the present conditions) were not affected by the presence of the agonist.

3.3. Time course of [^{35}S]GTPγS binding. Effect of receptor-mediated stimulations

In order to elicit the best conditions to get the maximal differences between receptor-stimulated and basal [^{35}S]GTPγS binding, the time course of the radioligand binding was analysed under both situations (Fig. 4). The [^{35}S]GTPγS specific binding increased over time without reaching equilibrium within 150 min of evaluation. The relative stimulations of the binding by the α_2-adrenoceptor agonist UK14304, the μ-opioid receptor agonist DAMGO, the 5-HT$_{1A}$ serotonin receptor agonist 8-OH-DPAT, the cholinergic muscarinic agonist carbachol, and the GABA$_B$ receptor agonist baclofen (all of them at 10 μM) reached top in 15 min and remained stable until 150 min (Fig. 4). The optimal incubation time was chosen as 120 min.

3.4. Concentration–response of receptor-mediated stimulations of [^{35}S]GTPγS binding

The receptor-stimulated binding of [^{35}S]GTPγS was pharmacologically characterized in the presence of NaCl (100 mM) by establishing concentration–response curves to the agonists UK14304, DAMGO, 8-OH-DPAT, carbachol (Fig. 5) and baclofen. Baclofen and DAMGO displayed maximal stimulatory effects higher than UK14304, carbachol or 8-OH-DPAT (Table 1) (Fig. 5). In preliminary assays, other agonists such as bethanecol (cholinergic muscarinic receptor agonist) and morphine (μ-opioid receptor agonist) produced lower stimulations (data not shown). Under similar conditions, the β-adrenoceptor agonist isoprenaline did not stimulate [^{35}S]GTPγS binding at all. The potencies (EC$_{50}$ values) of the different agonists

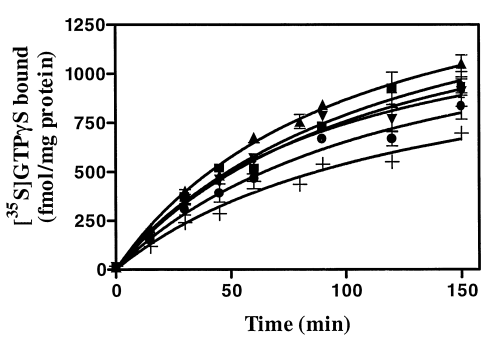

Fig. 4. Time course curves of [^{35}S]GTPγS specific binding to postmortem human brain cortical membranes in basal conditions (absence of agonist, +) or in presence (10 μM) of UK14304 (▼), DAMGO (▲), 8-OH-DPAT (♦), carbachol (■) and baclofen (●). The incubations were performed with [^{35}S]GTPγS (0.5 nM), GDP (50 μM), and NaCl (100 mM) at 30°C for 120 min. Data are mean ± SEM (bars) values from three experiments performed in different brains.

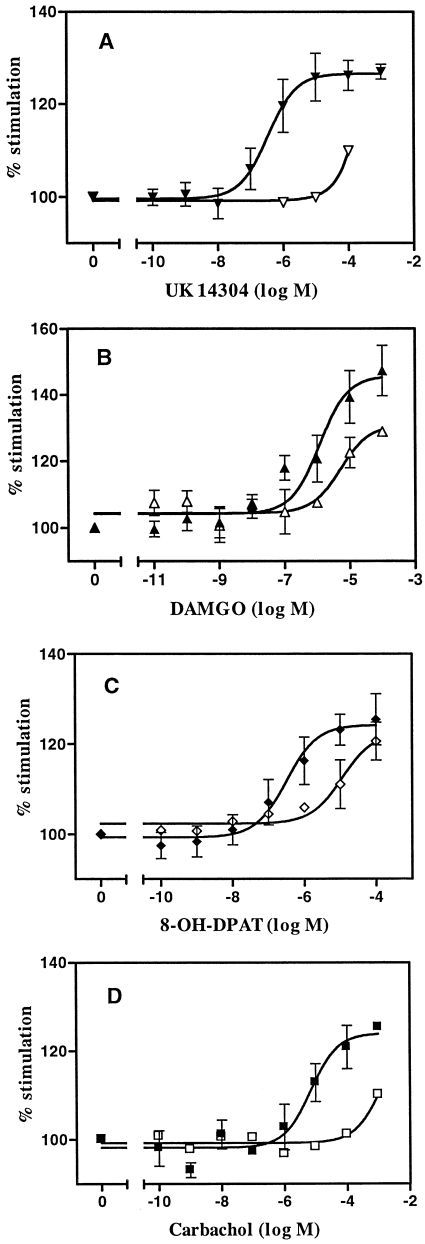

Fig. 5. Concentration–response curves of the stimulation of [^{35}S]GTPγS specific binding to postmortem human brain cortical membranes by different agonists in the absence (▼, ▲, ♦, ■) or in presence (▽, △, ◇, □) of selective antagonists (1 μM). Membranes were incubated with [^{35}S]GTPγS (0.5 nM), GDP (50 μM), NaCl (100 mM) at 30°C for 120 min and the addition of (A) UK14304 (▼) or UK14304 and RX821002 (▽), (B) DAMGO (▲) or DAMGO and naloxone (△), (C) 8-OH-DPAT (♦) or 8-OH-DPAT and WAY100635-A-5 (◇), and (D) carbachol (■) or carbachol and atropine (□). Data are mean ± SEM (bars) values from three experiments performed by triplicate in different brains.

were in the micromolar range (Table 1) with UK14304 being the most potent and baclofen the weakest agonist (Table 1). The inclusion in the assay of specific antagonists such as RX821002 (1 μM) for the α_2-adrenoceptor, naloxone (1 μM) for the μ-opioid receptor, WAY100635-A-5 (1 μM) for the 5-HT$_{1A}$ serotonin receptor, atropine (1 μM) for the cholinergic muscarinic receptor and phaclofen

Table 1
Pharmacological parameters of the stimulation of [^{35}S]GTPγS binding by different agonist drugs in postmortem human brain cortex
Maximal net stimulation data were obtained from the differences between maximal values of stimulation induced by the agonist and their respective basal value. E_{max} and EC_{50} values were obtained from the concentration–response curves. In such conditions, the experimental basal levels of the [^{35}S]GTPγS binding were 916 ± 14 fmol/mg protein (in presence of NaCl) and 1937 ± 18 fmol/mg protein (in absence of NaCl). In the absence of NaCl, carbachol and baclofen did not produce a significant stimulation over the basal values to allow the delineation of E_{max} and EC_{50} values (ND = not detectable). Data are mean ± SEM values or the best fit ± SE (nonlinear analyses of the curves) of three assays performed in triplicate in three different brains.

Agonist drug	Maximal net stimulation (fmol/mg protein)		E_{max} (fmol/mg protein)		EC_{50} (μM)	
	NaCl 100 mM	NaCl absence	NaCl 100 mM	NaCl absence	NaCl 100 mM	NaCl absence
UK14304	230.4 ± 52.1[a]	309.2 ± 44.3[b]	1163 ± 34	2246 ± 33	0.23 ± 0.01	0.12 ± 0.07
DAMGO	375.8 ± 29.6[a]	295.7 ± 70.5[a]	1337 ± 96	2110 ± 93	1.15 ± 0.12	0.15 ± 0.03
8-OH-DPAT	241.2 ± 42.9[a]	349 ± 77.3[a]	1159 ± 58	2226 ± 43	0.82 ± 0.08	0.44 ± 0.03
Carbachol	187.2 ± 15.4[b]	30.5 ± 8.5[c]	1145 ± 180	ND	5.99 ± 1.38	ND
Baclofen	548.8 ± 50.2[a]	123.5 ± 68.7[c]	1482 ± 106	ND	29.1 ± 2.70	ND

[a] Significantly different from basal values (Mann–Whitney *U*-test, $p < 0.05$).
[b] Significantly different from basal values (Mann–Whitney *U*-test, $p < 0.01$).
[c] Significantly different from net stimulation values in the presence of NaCl 100 mM (Mann–Whitney *U*-test, $p < 0.01$).

(at the higher concentration of 100 μM because of its low potency) for the $GABA_B$ receptors, inhibited the stimulation of [^{35}S]GTPγS binding by their respective agonists. The concentration–response curves to agonists were shifted to the right by the inclusion of their selective antagonists confirming the presence of an interaction of the agonist drugs with the respective receptor (Fig. 5).

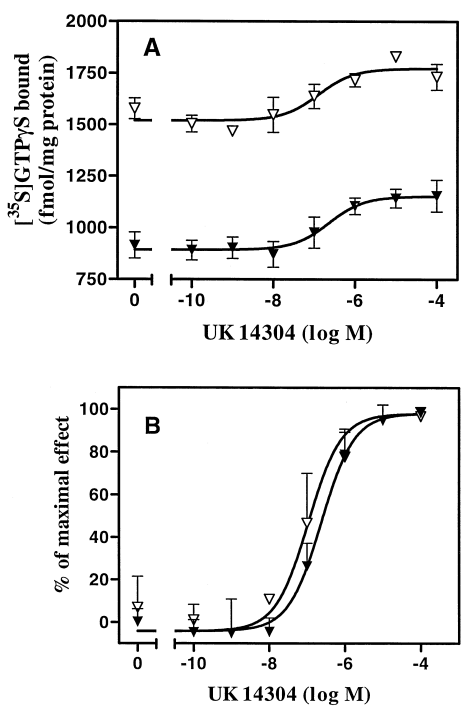

Fig. 6. Concentration–response curves of the stimulation of [^{35}S]GTPγS specific binding to postmortem human brain cortical membranes by the α_2-adrenoceptor agonist UK14304 in the absence (▽) or in presence (▼) of NaCl (100 mM). (A) Absolute values showing the net effects of NaCl and UK14304. (B) Percentage of the respective maximal stimulation for each curve. The incubations were performed with [^{35}S]GTPγS (0.5 nM), and GDP (50 μM) at 30°C for 120 min. Data are mean ± SEM (bars) values from three experiments performed in different brains.

3.5. Influence of NaCl on receptor-mediated stimulations of [^{35}S]GTPγS binding

The influence of NaCl on the binding of [^{35}S]GTPγS was evaluated in the absence and presence of agonist drugs. The addition of NaCl (100 mM) to the assay buffer reduced to half the basal [^{35}S]GTPγS binding (Table 1) (Fig. 6). In the absence of NaCl, the maximal stimulations were displayed by the agonists UK14304, DAMGO and 8-OH-DPAT, whereas the stimulations induced by carbachol or baclofen were abolished (Table 1). The net maximal stimulations (in fmol/mg protein) induced by each agonist were not statistically different between those in absence and those in presence of NaCl, except for the agonists carbachol and baclofen (Table 1). In the absence of NaCl, the concentration response curves for UK14304, DAMGO and 8-OH-DPAT displayed slight increases of potency (lower EC_{50} values) when compared with curves in the presence of NaCl (Table 1). However, the nonlinear analysis of the curves did not show any statistically signifi-

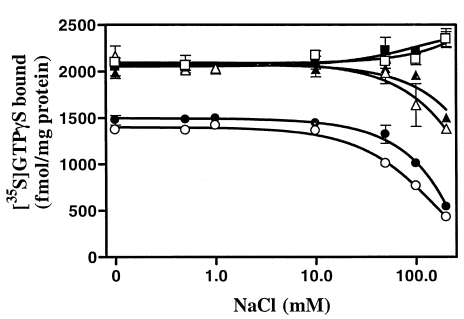

Fig. 7. Influence of NaCl and GDP on the [^{35}S]GTPγS specific binding to postmortem human brain cortical membranes in the absence (□, △, ○) or presence (■, ▲, ●) of the agonist UK14304 (10 μM). The incubations were performed with [^{35}S]GTPγS (0.5 nM) in the absence (□, ■) or presence of GDP at 5 μM (△, ▲) or 50 μM (○, ●) concentrations at 30°C for 120 min. Data are mean ± SEM (bars) values from three experiments performed in different brains. Bars are not present when SEM values are lower than the symbol size.

Table 2
Effect of preincubation with NEM on the stimulation by different agonists of the specific [^{35}S]GTPγS binding in postmortem human brain cortex
Membrane preparations were preincubated in the absence (control) or presence (50 μM) of NEM for 60 min at 4°C. After stopping the reaction and membrane wash-out, each set of membranes was resuspended and incubated with [^{35}S]GTPγS (0.2 nM) in 50 μM GDP and 100 mM NaCl conditions for 120 min at 30°C. Basal and stimulation of [^{35}S]GTPγS binding induced by the different agonist drugs were performed in control and NEM-pretreated membranes. Stimulation data were obtained from the difference between agonist-induced stimulation values and the respective basal values. Experimental basal levels under the present conditions were 360.4±29.3 fmol/mg protein in control membranes and 144.6±8.1 fmol/mg protein in NEM-pretreated membranes.
Data are mean±SEM values of two or three assays performed in triplicate in two or three different brains.

Agonist (concentration)	Control	NEM
	Net stimulation (fmol/mg protein)	Net stimulation (fmol/mg protein)
UK14304 (10 μM)	87.9±22.9	12.7±1.8[a]
DAMGO (10 μM)	152±6.1	19.4±4.5[a]
8-OH-DPAT (10 μM)	122±8.8	37.2±2.6[a]
Carbachol (100 μM)	108±9.6	2.5±1.7[a]
Baclofen (100 μM)	254±19	9.9±1.9[a]

[a] Significantly different from control values (Mann–Whitney *U*-test, $p < 0.001$).

cant difference between them (UK14304: $F[3,71] = 0.31$, $p = 0.97$; DAMGO: $F[3,58] = 8.02$, $p = 0.06$; 8-OH-DPAT: $F[3,54] = 1.94$, $p = 0.33$) (Fig. 6).

3.6. Interaction between NaCl and GDP on [^{35}S]GTPγS binding

A similar approach was applied to test the combined influence of GDP and NaCl on the [^{35}S]GTPγS binding to human brain cortical membranes. Thus, the influence of NaCl (0.1–200 mM) on the binding of the radioligand was investigated in the absence and presence of 5 and 50 μM of GDP evaluating simultaneously the possible stimulation elicited by UK14304 (10 μM). Three features of the combined effect became evident as shown in Fig. 7. First, both GDP and NaCl decreased the [^{35}S]GTPγS binding in a concentration-dependent manner. However, the presence of GDP was necessary to produce the NaCl effects. Second, both compounds enhanced the relative agonist stimulation when the NaCl concentration was increased up to 100 mM. Third, NaCl concentrations higher than 100 mM produced a reduction of the stimulations induced by the agonist at 5 and 50 μM of GDP ($p < 0.05$; 100 vs. 200 mM NaCl values; Mann–Whitney *U*-test).

3.7. Subtypes of G-protein involved in the [^{35}S]GTPγS binding

In order to verify that the recorded stimulations by receptor agonist drugs were dependent of the [^{35}S]GTPγS binding to G_i/G_o proteins, the membrane preparations were exposed to NEM in a new set of assays. After incubation with NEM (1 μM–2 mM), basal [^{35}S]GTPγS

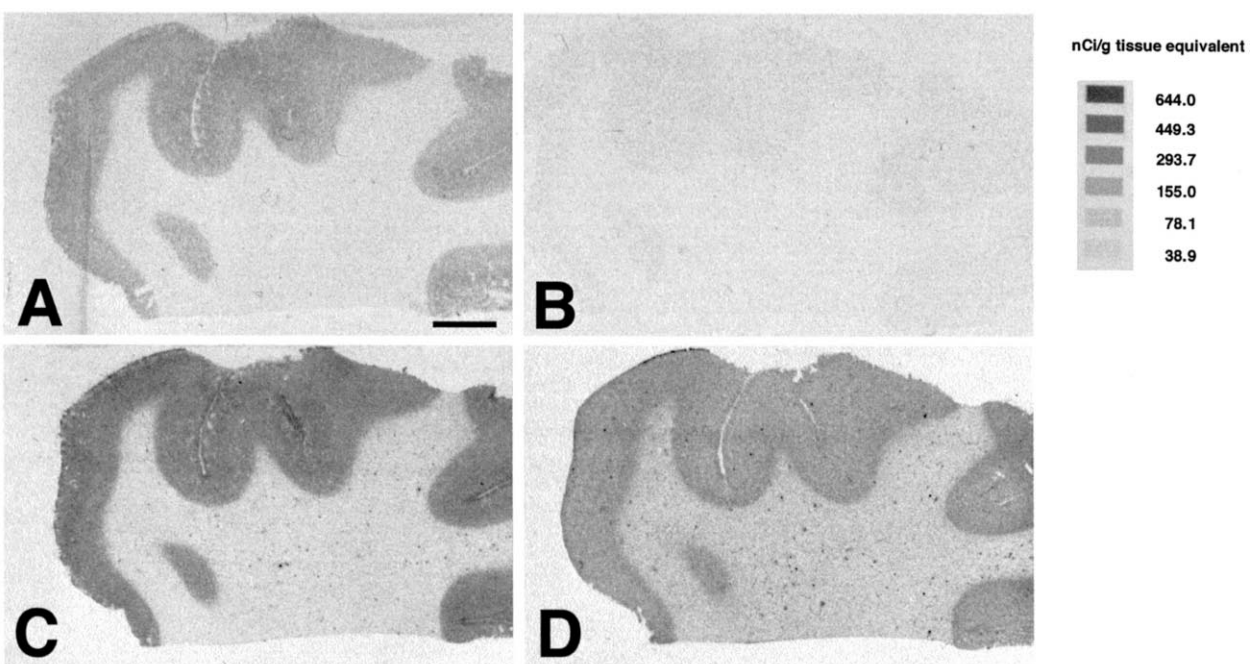

Fig. 8. Autoradiograms showing the binding of [^{35}S]GTPγS to tissue sections of postmortem brain cortex (Brodmann's area 9) from a representative subject. Sections were incubated with [^{35}S]GTPγS at the appropriate concentration, followed by exposure to Hyperfilm β-max. (A) Basal [^{35}S]GTPγS binding. (B) Nonspecific [^{35}S]GTPγS binding defined in the presence of unlabelled GTPγS (10 μM). (C) [^{35}S]GTPγS binding sites in the presence of the α_2-adrenoceptor agonist UK14304 (10 μM). (D) [^{35}S]GTPγS binding sites in the presence of UK14304 (10 μM) and the α_2-adrenoceptor antagonist RX821002 (1 μM). Note the inhibition of the [^{35}S]GTPγS binding stimulation when the tissue section was incubated in the presence of the antagonist (C vs. D). Scale bar 5 mm.

binding decreased in a concentration-dependent manner by 68% ($p < 0.01$; Mann–Whitney *U*-test) with a IC_{50} value of 22.7 ± 2.2 μM. The maximal inhibitory effect on basal levels was obtained at 100 μM of NEM. The previous exposure of the membranes to NEM also produced a progressive reduction of the net stimulatory response of [^{35}S]GTPγS binding to the agonist UK14304 (10 μM) reaching a full inhibition at 50 μM NEM. In subsequent experiments with different G-protein-coupled receptor agonists, the stimulatory effect on [^{35}S]GTPγS binding (0.2 nM) showed a clear reduction after preincubation of membranes with NEM (50 μM) (Table 2).

3.8. Autoradiographic distribution of [^{35}S]GTPγS binding. Effect of α_2-adrenoceptor-mediated stimulation

In tissue sections, specific [^{35}S]GTPγS binding to frontal cortex gray matter could be observed (Fig. 8A and B). The α_2-adrenoceptor agonist UK14304 (10 μM) induced an stimulatory effect on [^{35}S]GTPγS binding over all the frontal cortex layers (Fig. 8C). This stimulatory effect was inhibited in the presence of the α_2-adrenoceptor antagonist RX821002 (1 μM) (Fig. 8D).

4. Discussion

The present study establishes conditions and requirements for the quantification of [^{35}S]GTPγS binding in cortical membranes from postmortem human brain. The receptor-mediated stimulation of [^{35}S]GTPγS binding by several agonists was used to demonstrate that the functional coupling between receptors and G-proteins remains activable in postmortem human tissue. The results also reveal that the reported conditions allow to evaluate the GDP/GTP exchange on the G_i/G_o type of G-proteins in postmortem human brain membranes.

The effects on the [^{35}S]GTPγS binding exerted by GDP, Mg^{2+}, incubation time and NaCl were analysed under basal conditions and following receptor activation by the α_2-adrenoceptor agonist UK14304. The impacts of DTT and incubation temperature were not evaluated and were obtained from previously published data. Temperature in the range 25–37°C does not affect the relative stimulation or the EC_{50} values of [^{35}S]GTPγS binding stimulation by 5-HT_{1A} receptor agonists (Alper and Nelson, 1998). In the human brain, the α_2-adrenoceptor is abundant in prefrontal cortex (Meana et al., 1989) and corresponds mainly to the α_{2A}-adrenoceptor subtype (Grijalba et al., 1996). The α_{2A}-adrenoceptor represents a G_i/G_o-protein-coupled receptor that in human brain has been implicated in several neuropsychiatric disorders, as depression (Meana et al., 1992a; González et al., 1994; Ordway et al., 1994; De Paermentier et al., 1997; Callado et al., 1998), opiate addiction (Gabilondo et al., 1994) or Alzheimer's disease (Meana et al., 1992b). Previous studies in cultured cell membranes have demonstrated the pharmacological profile of selective drugs for the different α_2-adrenoceptor subtypes on the [^{35}S]GTPγS binding (Tian et al., 1994; Jasper et al., 1998; Peltonen et al., 1998). In fact, UK14304 acts as a full agonist at the α_{2A}-adrenoceptor subtype in CHO cells (Peltonen et al., 1998). More recently, the ability of the α_{2A}-adrenoceptor subtype to modulate the [^{35}S]GTPγS binding in mammalian brain membranes has been established (Happe et al., 1999). Therefore, the technical possibility of a functional monitoring of α_2-adrenoceptors based on the [^{35}S]GTPγS binding assay and the involvement of α_2-adrenoceptors in neuropsychiatric disorders provide rational support for conducting studies of [^{35}S]GTPγS binding modulation by α_2-adrenoceptors in normal and pathological human brain.

The GDP concentration in the incubation buffer has been demonstrated to be a critical factor for the [^{35}S]GTPγS binding in membrane of rat CNS (Sim et al., 1995; Olianas and Onali, 1996; Alper and Nelson, 1998). A linear correlation between the radioligand specifically bound and the amount of proteins was observed only when the GDP concentration was higher than 10 μM (Fig. 1). The finding confirms the sensitivity of the [^{35}S]GTPγS binding to protein concentration (Lorenzen et al., 1993; Sim et al., 1996). Subsequently, the GDP concentration necessary to obtain an optimal rate of agonist-mediated stimulation of the [^{35}S]GTPγS specific binding over basal values was determined. GDP reduced in a concentration-dependent manner the basal [^{35}S]GTPγS binding, presumably by displacing the equilibrium of the nucleotide exchange to the GDP-bound state of the G-protein (Birnbaumer et al., 1990). The stimulation of [^{35}S]GTPγS binding in human cortical membranes by the α_2-adrenoceptor agonist UK14304 was found to be highly dependent on the presence of micromolar concentrations of GDP (Fig. 2). UK14304 at 10 μM elicited maximal relative stimulation at GDP concentrations from 50 μM to 1 mM (Fig. 2). Similar concentrations of GDP have been found to be required for the stimulation of [^{35}S]GTPγS binding in rodent and bovine brain membranes by adenosine A_1 receptor agonists (Lorenzen et al., 1993), μ-opioid receptor agonists (Sim et al., 1995; Selley et al., 1997), cholinergic muscarinic receptor agonists (Olianas and Olani, 1996), 5-HT_{1A} receptor agonists (Alper and Nelson, 1998), and in PC-12 cells expressing the α_{2A}-adrenoceptor subtype (Tian et al., 1994). In the present study, the addition of exogenous GDP seems to be absolutely necessary to maintain the G-proteins in a GDP-bound state, the inactive state. The receptor activation by agonists would induce the exchange of guanine nucleotides, shifting the α subunit of the G-protein to a lower affinity state for GDP and promoting the [^{35}S]GTPγS binding (Birnbaumer et al., 1990; Wieland and Jakobs, 1994). In agreement, the stimulation of [^{35}S]GTPγS binding by the α_2-adrenoceptor agonist UK14304 involves an increase in the apparent affinity of the radioligand (decreased K_D values) with no changes in

the apparent maximal binding capacity (B_{max} values). The observed decrease of K_D values induced by UK14304 would reflect the receptor-stimulated guanine nucleotide exchange on G-proteins that favours the binding of [^{35}S]GTPγS over GDP by modifying their relative affinities. Similar results have been shown in rodent brain membranes (Olianas and Onali, 1996; Sim et al., 1996) and cultures of cell lines (Tian et al., 1994; Traynor and Nahorski, 1995; Williams et al., 1997) when α_2-adrenoceptors and other G-protein-coupled receptors were evaluated. An important issue in the determination of [^{35}S]GTPγS binding parameters is the proposed irreversibility of the process, which precludes the possibility of obtaining parameters ''at equilibrium'' (Fig. 4) (Breivogel et al., 1997). However, under the present assay conditions, the stimulation of [^{35}S]GTPγS binding by cannabinoid receptor agonists in rat brain has been shown to be readily dissociable, reaching steady-state levels within 2 h (Breivogel et al., 1998). This finding allows the data to be analysed in the manner of traditional radioligand binding although the [^{35}S]GTPγS binding parameters (K_D, B_{max}) should always be considered as apparent more than absolute estimates. Therefore, differences between binding parameters should be considered in the context of similar GDP and [^{35}S]GTPγS concentrations. The isotopic dilution curves of [^{35}S]GTPγS binding by unlabelled GTPγS fitted to a two-site model with a high- and a low-affinity binding populations. The agonist UK14304 selectively increased the affinity of [^{35}S]GTPγS binding to the high-affinity population, whereas the low-affinity population was not affected. Parallel observations have been reported for μ-opiate receptors (Selley et al., 1997) and cannabinoid receptors (Breivogel et al., 1998). The low-affinity binding sites of [^{35}S]GTPγS seem to be functionally non-coupled to receptors and could represent proteins with very low-affinity for GTP and analogs (present results) and a high-K_m GTPase intrinsic activity (Aktories et al., 1981). The delineation of the nonspecific binding of [^{35}S]GTPγS as the remaining binding in the presence of 10 μM unlabelled GTPγS allows to selectively identify the high-affinity population of the [^{35}S]GTPγS binding, which represents the binding component to receptor-coupled G-proteins (Fig. 3).

Another important condition for the optimal detection of the agonist [^{35}S]GTPγS binding stimulation was the presence of NaCl. The addition of NaCl diminished the basal levels of [^{35}S]GTPγS bound, probably by induction of the uncoupling of receptors from G-proteins (Gierschik et al., 1989; Tian et al., 1994) (Fig. 6A). Despite the Na^+ ion site is probably located on the receptor protein, the cation modifies the GDP:GTP equilibrium exchange on the α subunit of the G-protein by promoting the uncoupling of pre-coupled receptors and, thus, favouring the formation of the GDP–G-protein complex (Lorenzen et al., 1993). Therefore, it could be postulated that occurrence of NaCl effects require the presence of GDP to bring about the formation of the G-protein in the GDP–G-protein conformation complex.

In the absence of NaCl, the agonist responses were weaker than in the presence of NaCl (Table 1) (Fig. 6). It is well known that Na^+ ions are inhibitors of agonist binding to many G-protein-coupled receptors such as α_2-adrenoceptors (Michel et al., 1980), μ-opioid receptors (Pert and Snyder, 1974), 5-HT_{1A} receptors (Hall et al., 1985), and cholinergic muscarinic receptors (Rosenberger et al., 1980). Therefore, it is likely that a highly homologous protein domain confers Na^+ sensitivity of agonist binding to G-protein-coupled receptors. In fact, many G-protein-coupled receptors possess an aspartate residue in their second membrane-spanning domain that is directly involved in the Na^+ regulation of receptor–ligand interactions (Horstman et al., 1990).

The receptor-mediated stimulation of [^{35}S]GTPγS binding to postmortem human brain membranes by the different agonists reached maximal effects of 25–60% above basal values (Fig. 5). A similar rate of stimulation over basal values (21–25%) has been reported in human brain for the stimulation of the [^{35}S]GTPγS binding by 1 mM of the cholinergic muscarinic agonist carbachol (Ferrari-DiLeo et al., 1995) or 10 μM of the 5-HT_{1A} receptor agonist 8-OH-DPAT (Dupuis et al., 1999). These maximal effects in human brain were lower than those previously reported in other mammalian CNS areas (Lorenzen et al., 1993; Sim et al., 1995; Waeber and Moskowitz, 1997) and could be attributed to intrinsic characteristics of human tissues or to methodological conditions (postmortem delay, freezing, storage period, etc.) (Happe et al., 1999). In the present study, clear influences of these conditions on basal and agonist-stimulated [^{35}S]GTPγS binding could not be established, due to the small number of brain samples. The feature requires further studies with a larger sample size. The finding that the stimulation induced by several agonists on the [^{35}S]GTPγS binding can be effectively quantified in postmortem human brain, adds major advantages over the GTPase assay (Wieland and Jakobs, 1994). Thus, when the high-affinity GTPase assay has been used in human frontal cortex, receptor agonists produced smaller maximal effects than in the present study or, even failed to modulate the GTPase enzymatic activity (Cutler et al., 1994; Odagaki et al., 1998). The concentration–response curves of the agonist-stimulated [^{35}S]GTPγS binding in human brain yielded EC_{50} values in the micromolar range (Table 1) (Fig. 5). The slight reduction of potency as compared with previously reported EC_{50} values in mammalian tissues (Sim et al., 1995, 1996; Olianas and Onali, 1996; Waeber and Moskowitz, 1997; Alper and Nelson, 1998; Dupuis et al., 1998; Jasper et al., 1998) could represent differences in the receptor:G-protein ratio in humans (Newman-Tancredi et al., 1997), differences in the methodological conditions (notably the GDP concentration), or could be intrinsic to study conditions in postmortem human brain (postmortem delay, freezing and

storage) (Happe et al., 1999). Baclofen elicited the lowest potency in stimulating [^{35}S]GTPγS binding (Table 1) which agrees with results in rat brain sections where 100-fold more baclofen than DAMGO was used to obtain significant [^{35}S]GTPγS binding stimulations (Sim et al., 1995). Under the same binding conditions, the G_s-protein-coupled β-adrenoceptor agonist isoprenaline did not stimulate [^{35}S]GTPγS binding. This phenomenon could be explained by the low GTPase activity of G_s-proteins (Katada et al., 1984) and the higher relative abundance of G_i/G_o-proteins in human brain when compared with G_s (Sternweis and Robishaw, 1984; Sastre and García-Sevilla, 1994; Laitinen and Jokinen, 1998).

The anatomical distribution of [^{35}S]GTPγS binding in sections of human frontal cortex displayed a prominent basal binding to G-proteins in the gray matter that was increased by the α_2-adrenoceptor agonist UK14304 (Fig. 8). This preliminary finding is a demonstration that [^{35}S]GTPγS binding stimulation by α_2-adrenoceptor agonists can be performed in sections from postmortem human brain. Complementary studies are being currently developed to examine the [^{35}S]GTPγS binding in sections of different postmortem human brain areas and the modulation exerted by different receptor agonist drugs on this autoradiographic binding.

NEM, at micromolar concentration, induces a selective functional uncoupling of receptors from G_i/G_o-proteins by alkylation of the same cysteine residue that is ADP-ribosylated by pertussis toxin (Asano and Ogasawara, 1986; Ueda et al., 1990). The exposure of human brain membranes to NEM decreased the basal [^{35}S]GTPγS binding and reduced the stimulations induced by the different receptor agonists (Table 2). The finding is similar to those described in previous studies (Lorenzen et al., 1993; Olianas and Onali, 1996) and suggests the active coupling to G_i/G_o-proteins of unoccupied receptors in human frontal cortex. Furthermore, the inactivation induced by NEM technically confirms the preservation in postmortem human tissue of the ability for the coupling of the different receptors to the G_i/G_o-proteins (Table 2).

In conclusion, the present study demonstrates that in human frontal cortex, [^{35}S]GTPγS binds specifically with high affinity to G_i/G_o proteins. The [^{35}S]GTPγS specific binding can be modulated by agonist and antagonist drugs through the interaction with their respective G-protein-coupled receptor. The technique of [^{35}S]GTPγS binding provides a functional method to study both biochemical and anatomically the transmission of an extracellular signal to an intracellular response in a postmortem human tissue.

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