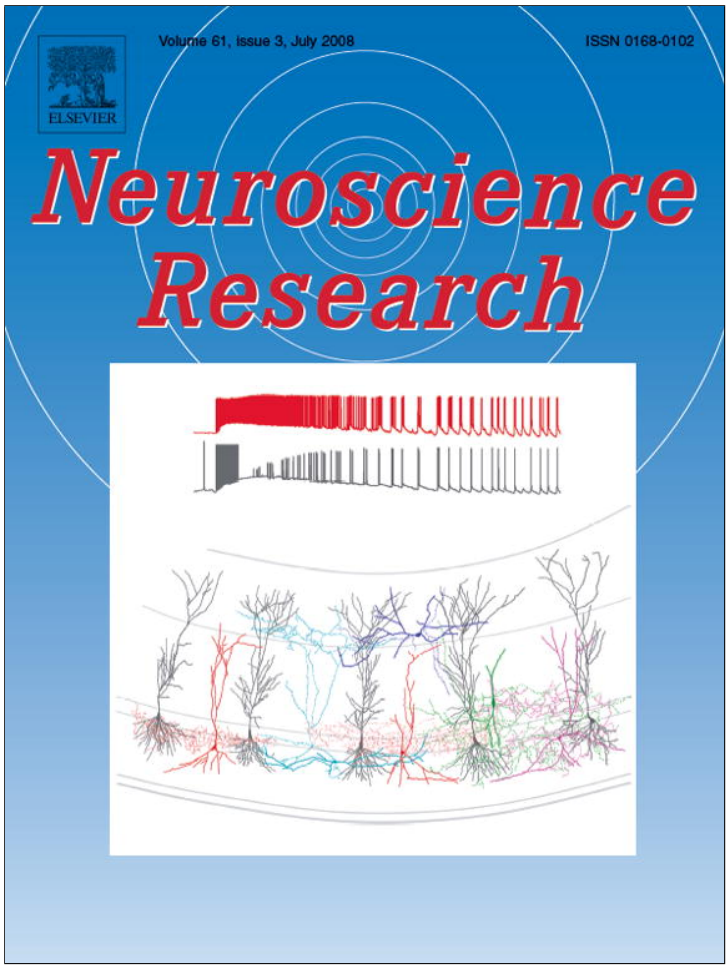

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The role of transcriptional and translational mechanisms in flumazenil-induced up-regulation of recombinant $GABA_A$ receptors

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Abstract

The aim of this study was to further elucidate the mechanisms involved in adaptive changes of $GABA_A$ receptors following prolonged exposure to flumazenil, the antagonist of benzodiazepine binding sites on $GABA_A$ receptors. The effects of prolonged flumazenil treatment were studied on recombinant $\alpha_1\beta_2\gamma_{2S}$ $GABA_A$ receptors stably expressed in human embryonic kidney (HEK 293) cells. Using radioligand binding experiments we found enhancement in the maximum number of [^{3}H]muscimol labeled binding sites in different preparations of HEK 293 cells. The parallel increase of [^{3}H]flunitrazepam binding sites in the membranes was reduced in the presence of actinomycin D and cycloheximide, inhibitors of RNA and protein synthesis, respectively. Chronic flumazenil also raised the steady-state level of mRNA encoding α_1 receptor subunit. The results suggest that the up-regulation of $GABA_A$ receptors, observed after prolonged flumazenil treatment is at least partly due to increased *de novo* synthesis of receptor proteins at both transcriptional and translational level.

Keywords: Chronic flumazenil; $GABA_A$ receptor; Recombinant; HEK 293 cells; Inhibitors of RNA and protein synthesis; [^{3}H]muscimol and [^{3}H]flunitrazepam binding; α_1 gene expression

1. Introduction

γ-Aminobutyric acid (GABA) is the main inhibitory neurotransmitter in the mammalian brain. GABA exerts most of its effects through ionotropic $GABA_A$ receptors. These receptors consist of various polypeptide subunits (α1–6, β1–3, γ1–3, δ, ε, π, θ) that are encoded by different genes (Sieghart et al., 1999; Korpi et al., 2002). Additional diversity of receptor structure is generated by alternative splicing of some of these subunit mRNAs, e.g., γ_2 has two alternatively spliced variants termed γ_{2L}, and γ_{2S}. However, most $GABA_A$ receptors are comprised of α, β, and γ subunits (Barnard et al., 1998), and the most prevalent form of native $GABA_A$ receptors appears to be the combination of two α, two β and one γ subunit (Tretter et al., 1997).

A variety of clinically important substances (such as anxiolytics, anticonvulsants, general anesthetics, barbiturates, ethanol and neuroactive steroids) achieve at least some of their pharmacological actions after binding at different binding sites on $GABA_A$ receptor complex (Mehta and Ticku, 1999; Korpi et al., 2002). Occupancy of $GABA_A$ receptors by these drugs generally modulates effects of GABA on GABAergic transmission. From the pharmacological and clinical point of view, the benzodiazepine binding site is one of the most important modulatory sites on the $GABA_A$ receptor. Various ligands acting at benzodiazepine binding site can enhance (agonists) or reduce (inverse agonists) the actions of GABA on GABAergic transmission with a different range of efficacy (from full to partial). A number of compounds are devoid of intrinsic activity (antagonists), but can inhibit the effects of both agonists and inverse agonists at this modulatory site (Korpi et al., 2002).

Many studies have revealed that long-term exposure to positive modulators of $GABA_A$ receptor function induces changes responsible for the reduction in receptor number and/or function (Gallager et al., 1984; Roca et al., 1990; Hu and Ticku, 1994; Klein et al., 1994; Primus et al., 1996; Ali and Olsen, 2001). Long-term exposure to neutral modulators of $GABA_A$ receptor function (antagonists) usually has opposite effects (Miller et al., 1989; Urbancic and Marczynski, 1989; Kulkarni and Ticku, 1990; Peričić et al., 2004, 2005a,b). Exact

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molecular mechanisms of these changes are still not completely understood.

The effects on kinetic and pharmacological properties of $GABA_A$ receptors after long-term exposure to antagonists of benzodiazepine binding sites have not been investigated in detail. Possible long-term use of one drug from this group, flumazenil, is considered for the treatment of hepatic encephalopathy patients (Goulenok et al., 2002; Dursun et al., 2003), and for chronic pain therapy in combination with opioids (Holtman et al., 2003).

Although flumazenil is classified as the benzodiazepine antagonist, a number of studies have suggested an intrinsic agonist enhancing effect on GABAergic transmission (Follesa et al., 2001; Weiss et al., 2002; Biggio et al., 2003; McMahon and France, 2006). For further pharmacological studies, a recent finding of McMahon and France (2006) can be interesting. These authors have demonstrated that the positive modulatory actions of some otherwise-silent, low efficacy benzodiazepines (e.g., flumazenil) may become evident when combined with positive $GABA_A$ modulators acting at a nonbenzodiazepine site.

Characterization of interactions between a specific drug and a homogeneous population of specific receptor subtype is a prerequisite for understanding the complex nature of interactions between that drug and heterologous population of native $GABA_A$ receptors. Generally, it is assumed that stably transfected cell lines with a defined subunit composition represent a suitable model for the assessment of specific drug-receptor interactions, despite the fact that they do not reflect possible changes in the neuronal environment. Furthermore, one study has shown that human embryonic kidney (HEK 293) cells have an unexpected relationship to neurons (Shaw et al., 2002).

There are only a few groups of authors that report the effects of chronic flumazenil exposure on the properties of recombinant $GABA_A$ receptors (Wong et al., 1994; Primus et al., 1996). In these studies chronic flumazenil produced either no significant change (Primus et al., 1996) or a reduction in GABA enhanced [^{3}H]flunitrazepam binding (Wong et al., 1994). However, even a brief exposure of recombinant $GABA_A$ receptors expressed in Sf9 cells to flumazenil was able to reverse the uncoupling of GABA and benzodiazepine recognition sites induced by chronic diazepam treatment (Primus et al., 1996; Ali and Olsen, 2001). This uncoupling was explained by internalization of surface $GABA_A$ receptors into an intracellular acidic compartment where normal benzodiazepine binding can occur, but not potentiation by GABA (Ali and Olsen, 2001).

As an extension of our previous work (Peričić et al., 2004, 2005a,b), we examined the effects of prolonged flumazenil exposure on the number and cellular localization of GABA binding sites in the culture of HEK 293 cells, stably transfected with the $\alpha_1\beta_2\gamma_{2S}$ subtype of $GABA_A$ receptor, the predominant subtype in mammalian brain (McKernan and Whiting, 1996). Experiments in the presence of the protein synthesis inhibitors, cycloheximide and actinomycin D, as well as the semi-quantitative RT-PCR analysis were performed to find out which mechanisms at the cellular and molecular level are implicated in the observed enhancement of $GABA_A$ receptors following exposure to a chronic benzodiazepine antagonist.

2. Materials and methods

2.1. Cell culture

The human embryonic kidney 293 cell line stably transfected with the rat $GABA_A$ receptor α_1, β_2 and γ_{2S} subunit cDNA was a generous gift from Dr. David Graham (Sanofi-Synthélabo Research, France).

The HEK 293 cell line was generated by transformation of normal human embryonic kidney cells with sheared fragments of adenovirus (Ad)5 DNA (Graham et al., 1977). As a result, these cells contain an insert of the viral genome, comprising the early region (E1) transforming sequences, incorporated into human chromosome 19. Those adenoviral genes increase the expression of proteins encoded by cDNAs artificially incorporated into plasmid vectors under the control of a strong cytomegalovirus (CMV) promoter. Such plasmids were used in the development of HEK 293 cell line stably expressing $\alpha_1\beta_2\gamma_{2S}$ subtype of $GABA_A$ receptors. The cloned cDNAs encoding α_1, β_2 and γ_{2S} subunits were subcloned into the expression vectors pcDNA3, pCDM8 and pCISD22, respectively, which were further used for transfection of HEK 293 cells. Using a two-step strategy, the colony that expressed the highest level of $\alpha_1\beta_2\gamma_{2S}$ $GABA_A$ receptor combination was selected (Besnard et al., 1997). Pharmacological and functional characterization of this cell line was performed (Besnard et al., 1997; Peričić et al., 1998, 2001).

HEK 293 cells were cultured in Dulbecco's modified Eagle's medium (Sigma, St. Louis, MO, USA) supplemented with 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine, 10 mM HEPES, 100 units/ml penicillin G and 100 μg/ml streptomycin in humidified air with 5% CO_2 at 37 °C. Fetal bovine serum and antibiotics for cell culture were supplied by Gibco (Scotland, UK).

2.2. Drugs

Flumazenil (Ro 15–1788) was obtained from Hoffman–La Roche Ltd. (Basel, Switzerland). Cycloheximide and actinomycin D were from Sigma (St. Louis, MO, USA). [^{3}H]flunitrazepam (specific activity 96 Ci/mmol) was purchased from Amersham Biosciences UK Ltd. and [^{3}H]muscimol (specific activity 36.5 Ci/mmol) from PerkinElmer (Boston, MA, USA). Flunitrazepam (Hoffman–La Roche Ltd.) and diazepam (Sigma) were used in binding studies to determine non-specific binding.

2.3. Drug treatment

Each batch of cultured cells was divided into control and drug treated groups. Three days after seeding, HEK 293 cells with stable expression of $\alpha_1\beta_2\gamma_{2S}$ recombinant $GABA_A$ receptors were exposed to 5 μM flumazenil for 48 h, or to 5 μM flumazenil in combination with cycloheximide (5 μg/ml) or actinomycin D (7.5 μg/ml) which were present in the cell culture for the last 12 h. The concentration of flumazenil was in the range of those used in the previous studies (Roca et al., 1990; Primus et al., 1996; Biggio et al., 2003). The concentrations of cycloheximide and actinomycin were according to Filtz et al. (1994). Flumazenil and actinomycin D were dissolved in ethanol, cycloheximide in bidestillated water. Control HEK 293 cells were treated with appropriate vehicles (0.05% and 0.14% ethanol for flumazenil and actinomycin, respectively).

2.4. Cell proliferation assay

To find out whether flumazenil treatment affects the viability and proliferation of HEK 293 cells, the colorimetric 4-[3-(4-Iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate (WST-1) assay was used. The estimation of cell proliferation is based on the ability of cells to cleave tetrazolium salt WST-1 to formazan dye by activity of mitochondrial dehydrogenases. The amount of produced color is directly proportional to the number of metabolically active cells. Assays were performed according to manufacturer's instructions (Roche). Exponentially growing cells were plated in 96-well culture plates

at a density 12.5×10^3 cells/well and allowed to adhere in 100 μl of culture medium for 24 h. After that period, additional 100 μl of culture medium containing double concentration of flumazenil was added (final concentration of 1 and 5 μM were achieved), and incubated for a further 48 h. At the end of incubation period, 20 μl of WST-1 reagent was added to all wells and incubated 4 h in humidified atmosphere. The absorbance of the red colored formazan dye was measured with a microplate spectrophotometer at 450 nm.

2.5. Radioligand binding studies

[^{3}H]Flunitrazepam binding studies were performed on cell membranes, and [^{3}H]muscimol binding studies on three different preparations: intact cells, cell homogenate and cell membranes.

Cell membranes were prepared mainly as described by Fuchs et al. (1995). The cells were washed twice with 5 ml of phosphate-buffer saline (PBSA), scraped with a plastic cell scraper into ice-cold PBSA and centrifuged at 12,000 × *g* for 10 min. The cell pellet was homogenised in 50 mM Tris–citrate buffer, pH 7.4, by 10 strokes (up and down) at 1250 rpm, and then collected by centrifugation at 200,000 × *g* for 20 min. The pellet was resuspended using a Teflon pestle and a glass homogeniser. The centrifugation–resuspension procedure was repeated two more times. The final pellet was resuspended in 50 mM Tris–citrate buffer, pH 7.4, aliquoted and frozen at −20 °C.

On the day of assay, the suspension was thawed, centrifuged once more at 200,000 × *g* for 20 min and used for [^{3}H]flunitrazepam binding assay, or prepared further for [^{3}H]muscimol binding by a modification of the method previously described (Peričić and Tvrdeić, 1993). Briefly, the membrane suspension was incubated for 15 min in ice-cold 50 mM Tris–citrate buffer, pH 7.4, containing 0.05% Triton X-100, and then centrifuged at 200,000 × *g* for 20 min. The pellet was washed from Triton by three resuspension–centrifugation cycles at 17,000 × *g* for 10 min in 50 mM Tris–citrate and finally resuspended in the same buffer at approximate concentration of 1 mg/ml.

Saturation analysis of [^{3}H]flunitrazepam binding to cell membranes was performed at 4 °C for 90 min. Increasing concentrations of cold flunitrazepam (0.4–100 nM) were added to a fixed concentration (1 nM) of [^{3}H]flunitrazepam. Non-specific binding was determined in the presence of 100 μM diazepam.

Saturation analysis of [^{3}H]muscimol binding to cell membranes was performed at 4 °C for 60 min. Increasing concentrations of cold muscimol (0.2–100 nM) were added to 4 nM of labeled [^{3}H]muscimol so that 10 increasing [^{3}H]muscimol concentrations were achieved. Non-specific binding was determined in the presence of 1 mM GABA.

The [^{3}H]muscimol binding to the cell surface of *intact* (viable) *cells* was performed by a modification of the method described by Primus et al. (1996). For saturation binding studies, viable HEK 293 cells were collected in ice-cold PBSA, centrifuged at 500 × *g* for 5 min at 4 °C and resuspended in 1:1 volume of PBSA and 50 mM Tris–citrate buffer.

Cell homogenate was obtained with an automatic glass/Teflon homogeniser (ten strokes up and down at 1250 rpm).

Aliquots of the cell homogenate or of the intact cell suspension (∼650 μg of membrane proteins in 400 μl) were incubated with a fixed concentration of [^{3}H]muscimol (4 nM) and increasing concentrations of cold muscimol (3–420 nM) for 60 min at 4 °C.

In all binding studies the total assay volume was 0.5 ml. Each assay tube was run in duplicate. The binding reaction was terminated by vacuum filtration on Whatman GF/C filters, and the radioactivity retained on them was measured in β-scintillation counter (PerkinElmer, Wallace 1409DSA).

2.6. Protein concentration determination

Protein content was determined in 10 μl of membrane suspension according to method of Lowry et al. (1951) using bovine serum albumin as a standard.

2.7. Determination of mRNA levels by semi-quantitative RT-PCR

Total cellular RNA was extracted from 1×10^6 control and flumazenil treated HEK 293 cells using High Pure RNA Isolation Kit (Roche) according to the manufacturer's instruction and quantified at 260 nm using a spectrophotometer. RNA (1 μg) together with random hexadeoxynucleotide primers (2.5 μM) was denaturated at 65 °C for 5 min and the first strand of cDNA was synthesized in a reaction volume of 20 μl by adding the following reagents: reverse transcription buffer (50 mM Tris–HCl, pH 8.3; 40 mM KCl; 6 mM $MgCl_2$, 10 mM DTT), 10 mM dNTPs, 40 units RNase-Inhibitor and 40 units Murine-Moloney Leukemia virus (M-MLV) reverse transcriptase (RT). The enzyme and all reagents were supplied from Roche. After primer annealing (25 °C, 10 min), the reaction mixture was incubated at 37 °C for 1 h and heated to 95 °C for 5 min. Each RT reaction included two negative controls: RNA sample without M-MuLV RT, and M-MuLV RT without the RNA template to test for contamination with genomic DNA. Housekeeping gene β-actin was used as an internal standard. The resulting cDNA (dilution 1:5 in nuclease free water) was amplified by PCR with 1U of *Taq* DNA polymerase (Eppendorf) in 20 μl of standard buffer containing 0.2 μM of each sense and antisense primers, 1.5 mM $MgCl_2$ and 200 μM of each deoxynucleoside triphosphate. The reaction was performed in a PerkinElmer 9600 thermocycler. PCR primers used in the study are listed in Table 1. Cycle parameters included an initial denaturation step (95 °C for 5 min) followed by denaturation (95 °C, 45 s), annealing (60 °C, 45 s) and extension (72 °C, 1 min). Preliminary experiments were performed with each set of primers to assess linearity of PCR product formation with respect to cycle number, and the PCR products of both the α_1 and β-actin gene, were measured during the log phase of the reaction, i.e. before saturation was reached. Samples of the reaction products (8.5 μl) were electrophoretically separated on a 1.5% agarose gel and stained with ethidium-bromide (0.5 μg/ml). Maximal optical density of detected bands was obtained with Image Master VDS software 1.0 (Pharmacia). Expression of α_1 mRNA was normalized to β-actin mRNA expression.

2.8. Data analysis

The analysis of [^{3}H]flunitrazepam and [^{3}H]muscimol binding data was performed using GraphPad Prism version 4.00 for Windows (GraphPad Software, San Diego, CA, USA).

The values K_d and B_{max} were obtained by nonlinear regression using the equation for a hyperbola (one binding site): $Y = B_{max} \times X/(K_d + X)$, where Y is the specific binding, X is the concentration of the ligand, B_{max} is the maximum number of binding sites, and K_d is the equilibrium dissociation constant, i.e. the concentration of ligand required to reach half-maximal binding. Data are presented as means ± S.E.M. of at least three independent experiments performed in duplicate. Statistical analysis of results was performed by Student's *t*-test (for the comparison of two means) and by two-way analysis of variance (ANOVA), when the effects of two different factors were studied in the same experiment. Post hoc analysis was done by Newman–Keuls multiple comparison test. *P*-Values of < 0.05 were considered significant.

3. Results

3.1. The effect of prolonged flumazenil exposure on [^{3}H]muscimol binding to preparations from HEK 293 cells stably transfected with $\alpha_1\beta_2\gamma_{2S}$ subunits of $GABA_A$ receptors

To investigate the effect of chronic flumazenil exposure on the number and cellular distribution of GABA recognition sites

Table 1
Primer sequences used for PCR amplification

Gene	Primer sequence (5′ → 3′)	Product length (bp)	Primer reference
β-Actin	f: TCA CCA ACT GGG ACG ACA TG r: TTC GTG GAT GCC ACA GGA CT	602	Gou et al. (2001)
α1	f: AGC TAT ACC CCT AAC TTA GCC AGG r: AGA AAG CGA TTC TCA GTG CAG AGG	304	Devaud et al. (1995)

in the culture of HEK 293 cells stably transfected with the $\alpha_1\beta_2\gamma_{2S}$ subtype of $GABA_A$ receptors, we carried out a series of saturation receptor binding assays by using [^{3}H]muscimol as a ligand. We studied the binding of [^{3}H]muscimol in three different preparations: intact cells, cell homogenate and cell membranes (see Section 2). On intact cells [^{3}H]muscimol labels only the cell-surface GABA binding sites (Mizokami et al., 2007). As shown in Fig. 1, the exposure to 5 μM flumazenil (48 h) increased profoundly the maximum number of [^{3}H]muscimol binding sites. The observed increases ranged from 124% ($P < 0.01$) in the cell homogenate (Fig. 1b) to 163% ($P < 0.02$) in the suspension of intact (viable) cells (Fig. 1a).

While the type of preparation failed to show a considerable effect on the number, it affected significantly the affinity of [^{3}H]muscimol binding. As expected, the greatest affinity, i.e., the lowest dissociation constant (K_d) was observed in cell membranes (Fig. 1c). As shown on Fig. 1a–c, in none of these preparations was the K_d value of GABA binding sites for [^{3}H]muscimol binding affected by flumazenil treatment ($P > 0.05$, Student's t-test).

3.2. The effect of prolonged flumazenil exposure on viability and proliferation ability of HEK 293 cells

Our further intention was to elucidate if the stimulation in the expression of GABA binding sites is a result of a general proliferative effect of the chronic flumazenil exposure on the growth of HEK 293 cells. We performed tetrazolium WST-1 assay and tested the effects of 1 and 5 μM flumazenil on the proliferation ability of HEK 293 cells stably transfected with $\alpha_1\beta_2\gamma_{2S}$ subunits of $GABA_A$ receptors. The results of the proliferation assay presented in Table 1 show that the prolonged (48 h) exposure to flumazenil had no influence on the cell proliferation or viability. This implies that the up-regulated expression of GABA binding sites observed on recombinant $\alpha_1\beta_2\gamma_{2S}$ $GABA_A$ receptors following chronic flumazenil treatment is not the result of the stimulated growth of HEK 293 cells.

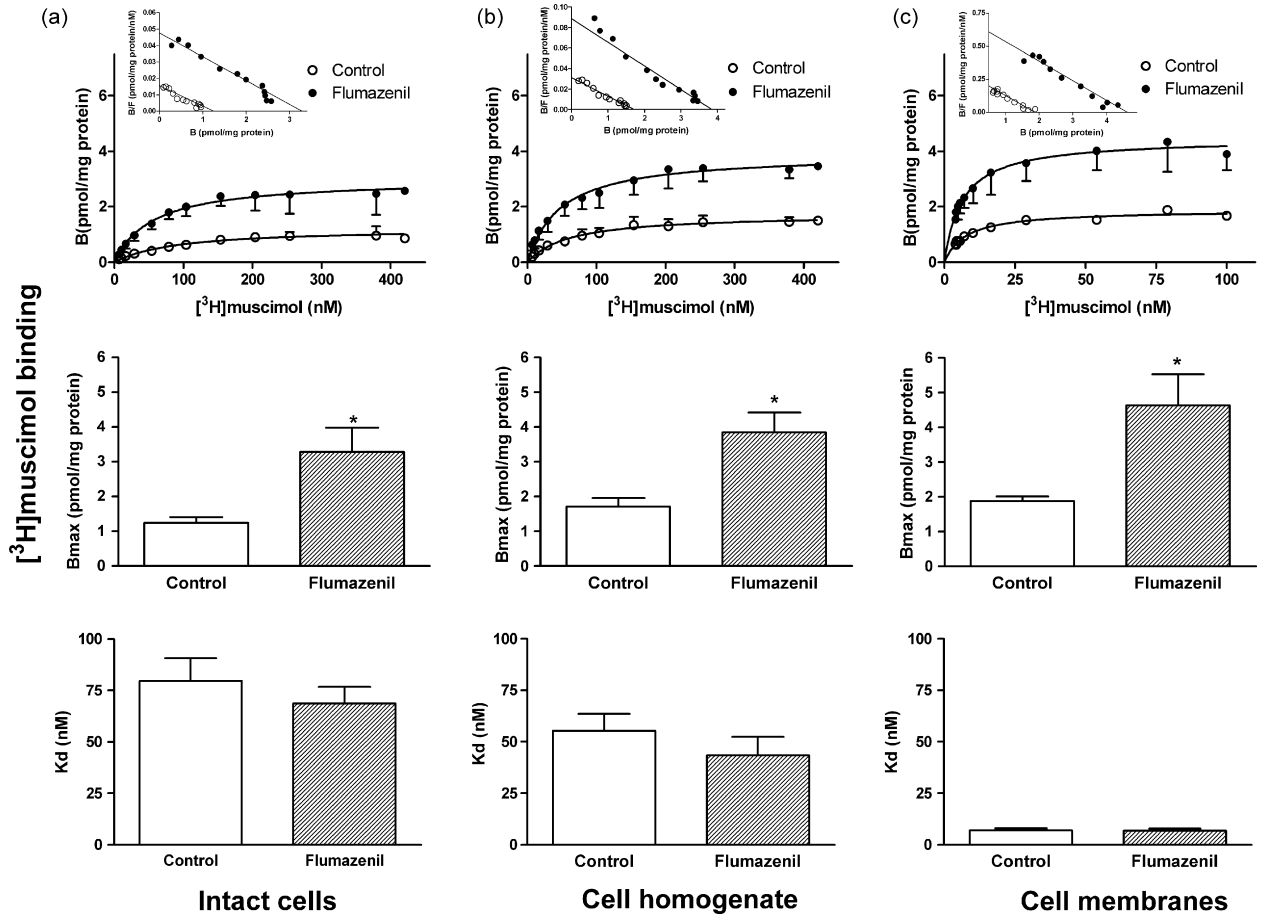

Fig. 1. The effect of prolonged flumazenil exposure on [^{3}H]muscimol binding to three different preparations from HEK 293 cells stably transfected with $\alpha_1\beta_2\gamma_{2S}$ subunits of $GABA_A$ receptors. HEK 293 cells with stable expression of $\alpha_1\beta_2\gamma_{2S}$ subtype of $GABA_A$ receptors were treated with 5 μM flumazenil or with appropriate vehicle (control) for 48 h. The maximum number (B_{max}) and dissociation constant (K_d) of [^{3}H]muscimol binding sites were determined on the surface of intact cells (a), in cell homogenate (b) or on membrane preparations from HEK 293 cells (c). Preparations from HEK 293 cells (prepared as described in "Section 2") were incubated with increasing concentrations of nonradioactive muscimol in the presence of a fixed (4 nM) concentration of [^{3}H]muscimol. The values of B_{max} and K_d were obtained by nonlinear regression. Results are means ± S.E.M. from at least three independent experiments performed in duplicate. *$P < 0.01$ or $P < 0.02$ versus the corresponding control (Student's t-test).

3.3. The effect of inhibitors of RNA and protein synthesis on the enhancement of [^{3}H]flunitrazepam binding sites induced by chronic flumazenil treatment

Our previous studies demonstrated that chronic exposure of HEK 293 cells to flumazenil stimulates the expression of benzodiazepine binding sites at the recombinant $\alpha_1\beta_2\gamma_{2S}$ $GABA_A$ receptors (Perićić et al., 2004, 2005a). To investigate whether this change is mediated by alterations at the transcriptional and/or translational level, we examined the intensity of flumazenil effect in the presence of mRNA (actinomycin D) and protein synthesis (cycloheximide) inhibitors. The experiment, as shown on Fig. 2a and b consisted of HEK 293 cells treated with: flumazenil (5 μM), flumazenil in combination with the inhibitor, the inhibitor alone, and the corresponding vehicles (control). Actinomycin D binds to DNA at the transcription initiation complex and inhibits RNA synthesis, while cycloheximide is an inhibitor of protein synthesis, which blocks translational elongation on ribosomes. The concentrations of both inhibitors (7.5 μg/ml for actinomycin D; 5 μg/ml for cycloheximide) and the time of exposure (the last 12 of the 48 h exposure to flumazenil) were selected in such a manner as not to induce changes in the expression of benzodiazepine binding sites in vehicle treated control cells.

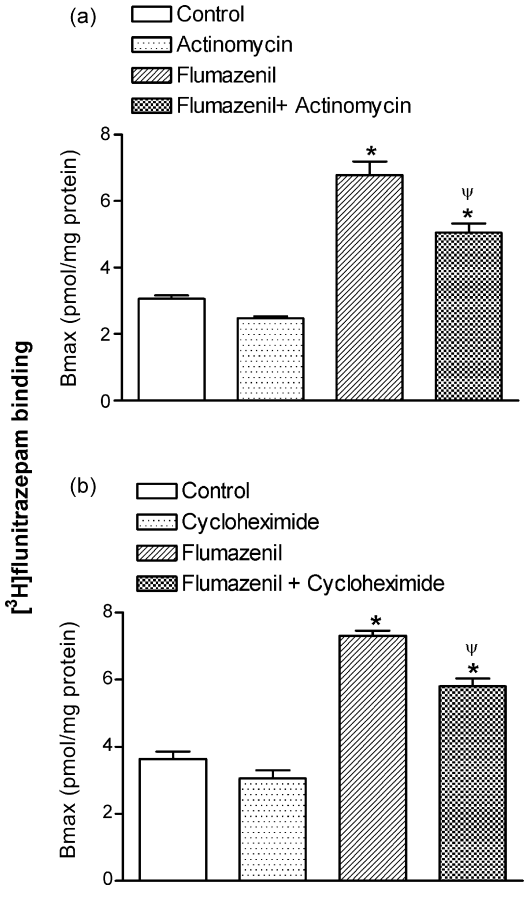

Fig. 2. The effect of actinomycin D and cycloheximide on flumazenil induced enhancement in the expression of [^{3}H]flunitrazepam binding sites. Cells were treated: (a) with flumazenil 5 μM, actinomycin D 7.5 μg/ml (present in the cell culture for the last 12 h), the combination of both drugs, or with their vehicles; b) with flumazenil 5 μM, cycloheximide 5 μg/ml (present in the cell culture for the last 12 h), the combination of both drugs, or with their vehicles. Results are means ± S.E.M. from five (a) and six (b) independent experiments performed in duplicate. $^*P < 0.001$ versus control and inhibitor treated group; $^{\psi}P < 0.001$ versus flumazenil-treated group (ANOVA and Newman–Keuls test).

As indicated by two-way ANOVA, flumazenil, as expected, enhanced ($P < 0.0001$) the maximum number of [^{3}H]flunitrazepam binding sites in both, the experiment with actinomycin [$F(1,16) = 152.9$] (Fig. 2a) and cycloheximide [$F(1,20) = 237.6$] (Fig. 2b). The cells treated with actinomycin [$F(1,16) = 20.6$; $P < 0.0003$] and cycloheximide [$F(1,20) = 24.9$; $P < 0.0001$] showed differences in the number of [^{3}H]flunitrazepam recognition sites in comparison to cells without inhibitors. However, actinomycin and cycloheximide diminished significantly ($P < 0.001$) the number of benzodiazepine binding sites in flumazenil-treated but not in control vehicle-treated cells (post hoc Newman–Keuls test). A significant flumazenil × actinomycin [$F(1,16) = 5.2$; $P < 0.04$] and flumazenil × cycloheximide [$F(1,20) = 4.9$; $P < 0.04$] interaction, obtained by two-way ANOVA, suggested a different action of inhibitors in flumazenil-treated than in vehicle-treated cells.

3.4. The effect of long-term flumazenil treatment on the expression of $GABA_A$ receptor α_1 subunit mRNA

To study in further detail if the stimulating effect of prolonged flumazenil exposure could be explained by an enhanced transcription of the receptor gene, a semi-quantitative RT-PCR analysis was performed (Fig. 3). After isolation of total RNA from vehicle- and flumazenil-treated (5 μM, 48 h) HEK 293 cells stably transfected with $\alpha_1\beta_2\gamma_{2S}$ $GABA_A$ receptors, the expression of the α_1 subunit mRNA was examined. Amplification of cDNA from control and flumazenil-treated cells with the primers specific for β-actin and $GABA_A$ receptor α_1 subunit resulted in DNA products with expected molecular size (see Section 2). Abundance of α_1 mRNA (represented as maximal optical density) was normalized to the values of house-keeping gene β-actin to account for eventual inaccuracy in the spectrophotometric measurements of RNA used for cDNA synthesis, loading of RNA samples, quality of samples and for possible differences in reaction efficiency (Thellin et al., 1999; Suzuki et al., 2000). The expression level of β-actin mRNA stayed unchanged after flumazenil treatment (data not shown). The negative control, where cDNA was replaced by water, did not show amplification, ruling out any contamination in PCR preparations. As shown in Fig. 3, 48 h-exposure of cells to flumazenil induced a significant increase (71%) in the amount of the α_1 subunit mRNA ($P < 0.001$, Student t-test), further suggesting the transcriptional mechanism of flumazenil-induced changes in the expression of $GABA_A$ receptors.

4. Discussion

Our previous studies have shown that chronic occupancy of benzodiazepine binding sites at the recombinant $GABA_A$

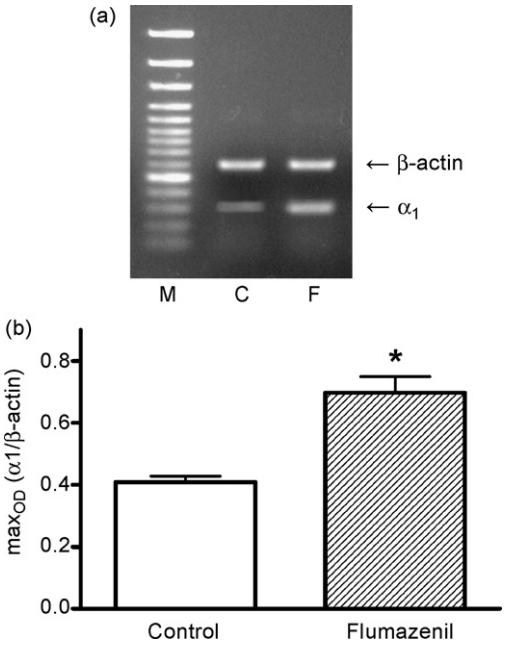

Fig. 3. The effect of long-term flumazenil treatment on the expression of $GABA_A$ receptor α_1 subunit mRNA. (a) Representative agarose gel electrophoresis; (b) Results of RT-PCR analysis. HEK 293 cells stably transfected with $\alpha_1\beta_2\gamma_{2S}$ subunits of $GABA_A$ receptor were treated with 5 μM flumazenil for 48 h and the amount of $GABA_A$ receptor α_1 subunit mRNA was determined by semi-quantitative RT-PCR analysis. Abbreviations denote: size marker (M), products from control cells (C), products from flumazenil-exposed cells (F). Amplified products were separated in a 1.5% agarose gel, stained with ethidium bromide (EtBr) and visualized under UV light. Densitometric quantification of bands stained with EtBr was performed using Image Master VDS software 1.0. The maximal optical density of the α_1 subunit band was normalized to the expression of house keeping gene β-actin. The data are expressed as means ± S.E.M. from 6 independent RT-PCR analyses (with PCR performed in triplicate) after three separate preparations of total RNA. $^*P < 0.001$ versus control (Student's *t*-test).

receptors by the benzodiazepine antagonist flumazenil produced an enhancement of [^{3}H]flunitrazepam and [^{3}H]TBOB binding sites (Peričić et al., 2004, 2005a,b). In this study, by using the same $\alpha_1\beta_2\gamma_{2S}$ subtype of $GABA_A$ receptors stably expressed in HEK 293 cells, we demonstrate that chronic flumazenil treatment enhanced the expression of GABA binding sites, suggesting an up-regulation of $GABA_A$ receptors.

As shown in Fig. 1, chronic flumazenil produced a very marked increase in the expression of [^{3}H]muscimol binding sites in the suspension of intact HEK 293 cells, in their homogenate and in cell membranes. Because [^{3}H]muscimol labels cell surface GABA binding sites (Mizokami et al., 2007), the absence of a significant variation in the number of these binding sites between intact and homogenised control cells suggests that the majority of recombinant $\alpha_1\beta_2\gamma_{2S}$ $GABA_A$ receptors are expressed on the cell surface, implying that flumazenil up-regulates functionally relevant receptors. In the culture of Sf9 cells stably expressing the recombinant $\alpha_1\beta_2\gamma_2$ $GABA_A$ receptors, Primus et al. (1996) also found that almost all of the benzodiazepine binding sites were located on the cell surface. Similar results, showing a small fraction of intracellular $GABA_A$ receptors in the total pool of cellular receptors were obtained on the preparations from bovine and chick cortical neurons (Calkin and Barnes, 1994; Tehrani et al., 1997).

The affinity of GABA binding sites for [^{3}H]muscimol in the suspension of intact HEK 293 cells and in their homogenate (Fig. 1a and b) was relatively low, while the affinity obtained on membrane preparations treated with Triton X-100 (Fig. 1c) was high and similar to that observed by other authors on the same type of $GABA_A$ receptors stably expressed in HEK 293 cells (Søgaard et al., 2006).

There are several potential mechanisms that might contribute to up-regulated expression of cell surface recombinant $GABA_A$ receptors after prolonged drug exposure. One possibility is that flumazenil treatment increases the expression of recombinant $GABA_A$ receptors through an exocytosis of intracellular receptors. However, our results (Fig. 1) suggesting that intracellular $GABA_A$ receptors represent only a small proportion in the overall population of cellular receptors, imply that the enhanced expression of $GABA_A$ receptors on the cell surface is not achieved by changes in exocytosis. Another possibility, which cannot be excluded, is that chronic flumazenil decreases receptor endocytosis and degradation. The results of WST-1 proliferation assay (Table 2) excluded the possibility of a general trophic effect of flumazenil on the growth of HEK 293 cells.

Changes in mRNA and protein stability, as well as variations in *de novo* synthesis of receptor subunits at the level of mRNAs and/or functional proteins, could also contribute to changes in the expression of recombinant $GABA_A$ receptors after chronic drug exposure. As shown in Fig. 2, both actinomycin D, a drug that inhibits RNA synthesis, and cycloheximide, a potent inhibitor of protein synthesis, applied in concentrations that failed to modify [^{3}H]flunitrazepam binding in control cells, reduced flumazenil-induced increase in the maximum number of [^{3}H]flunitrazepam binding sites. These results suggest that the observed up-regulation was at least partly due to increased *de novo* synthesis of receptor subunits at both transcriptional and translational level. The contribution of transcriptional mechanism in flumazenil-induced up-regulation of $GABA_A$ receptors was additionally suggested by our results demonstrating that the prolonged flumazenil exposure produced an up-regulation of the α_1 subunit mRNA (Fig. 3). As described in Section 2, HEK 293 cells (as well as other engineered cells used

Table 2
The effect of prolonged flumazenil exposure (48 h) on HEK 293 cell growth

Treatment	Absorbance of formazan dye at 450 nm	*n*
Vehicle	2.39 ± 0.08	3
Flumazenil 1 μM	2.40 ± 0.03	3
Vehicle	2.38 ± 0.07	3
Flumazenil 5 μM	2.44 ± 0.03	3

HEK 293 cells with stable expression of recombinant $\alpha_1\beta_2\gamma_{2S}$ subtype of $GABA_A$ receptors (12.5×10^6 cells/well) were exposed to 1 μM and 5 μM flumazenil for 48 h, and WST-1 assay was performed for measuring cell growth. Cell viability was determined by the absorbance of formazan dye, 4 h after addition of WST-1 reagent to culture medium. Results are expressed as means ± S.E.M. of three independent experiments performed in hexaplicates.

in similar studies) apparently do not have a normal promoter control of $GABA_A$ receptor subunit expression. Thus, although these cells express many proteins typically found in neurons (Shaw et al., 2002), transcription of genes encoding receptor subunits is not regulated in the same way as it is *in vivo* or in primary neuronal culture in which endogenous genes are involved. However, our result showing flumazenil-induced elevated expression of α_1 subunit mRNA is in accordance with the results of Zheng et al. (1996) obtained in the primary culture of cerebellar granule cells. Moreover, as discussed in our previous papers (Peričić et al., 2004, 2005a,b), our results, which demonstrate that chronic flumazenil up-regulates in a bicuculline-sensitive manner benzodiazepine binding sites at stably expressed $GABA_A$ receptors, are in line with the up-regulation of benzodiazepine binding sites observed *ex vivo* following chronic flumazenil administration (Miller et al., 1989; Urbancic and Marczynski, 1989; Kulkarni and Ticku, 1990; Flaishon et al., 2003).

Subunit substitution (Zheng et al., 1996; Pesold et al., 1997; Follesa et al., 2001; Sanna et al., 2005) and alterations in the conformation and phosphorylation of receptor subunits (Harris et al., 1998) have also been proposed as possible mechanisms involved in the regulation of $GABA_A$ receptor function after chronic drug exposure. Nevertheless, the transfected cell lines exclude the possibility of subunit switching.

Potential long-term use of flumazenil is considered for the treatment of patients with hepatic encephalopathy, a multifactorial syndrome that is accompanied by an increased GABAergic tone. Previous studies have suggested that the allosteric interactions between binding sites as well as the functional state of $GABA_A$ receptors are not affected by chronic flumazenil treatment (Roca et al., 1990; Hu and Ticku, 1994; Primus et al., 1996; Peričić et al., 2004, 2005b). Hence, it seems possible that the stimulated expression of $GABA_A$ receptors on the cell surface, if it happens *in vivo*, would lead to intensified activity of GABAergic system. This implies that the long-term use of flumazenil would even be contraindicated for the treatment of hepatic encephalopathy patients who already have an augmented GABAergic tone. On the other hand, if the allosteric uncoupling of $GABA_A$ receptor binding sites is related to the development of tolerance and physical dependence (Bateson, 2002; Wafford, 2005), it might be expected that chronic occupancy of benzodiazepine binding sites by benzodiazepine antagonist flumazenil will not develop tolerance to drug effects.

In conclusion, our results demonstrate that prolonged exposure of HEK 293 cells stably expressing $\alpha_1\beta_2\gamma_{2S}$ $GABA_A$ receptors to flumazenil, an antagonist of benzodiazepine binding sites, induces an enhanced expression of GABA binding sites. These results further confirm our previous suggestion that chronic flumazenil up-regulates $GABA_A$ receptors. Several lines of evidence suggested that transcriptional and translational mechanisms are at least partly responsible for this effect.

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