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Prolonged exposure to γ-aminobutyric acid up-regulates stably expressed recombinant α1β2γ2s $GABA_A$ receptors

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Abstract

The aim of this study was to better understand the mechanisms that underlie adaptive changes in $GABA_A$ receptors following their prolonged exposure to drugs. Exposure (48 and/or 96 h) of human embryonic kidney (HEK 293) cells stably expressing recombinant α1β2γ2s $GABA_A$ receptors for γ-aminobutyric (GABA, 1 mM) and muscimol (100 μM), but not for diazepam (1 μM), enhanced the maximum number (B_{max}) of [^{3}H]flunitrazepam binding sites without affecting their affinity (K_d). The GABA-induced enhancement in B_{max} was reduced by the GABA receptor antagonist, bicuculline (100 μM), and by cycloheximide (10 μl/ml), a protein synthesis inhibitor. GABA (100 μM) enhanced the affinity of [^{3}H]flunitrazepam binding to vehicle- and GABA-pretreated, but not to diazepam-pretreated, HEK 293 cells. The results suggest that chronic GABA treatment up-regulates stably expressed $GABA_A$ receptors, presumably by stimulating their synthesis. Unlike chronic diazepam, which produced functional uncoupling of GABA and benzodiazepine binding sites, chronic GABA failed to produce this effect.

Keywords: $GABA_A$ receptor; HEK 293 cell; GABA (γ-aminobutyric acid); Diazepam; Muscimol; Radioligand binding

1. Introduction

γ-Aminobutyric acid (GABA), the major inhibitory neurotransmitter in the brain, fulfils its physiological actions via three major classes of GABA receptors ($GABA_A$, $GABA_B$ and $GABA_C$). $GABA_A$ receptors mediate most of the fast inhibitory neurotransmission in the central nervous system. These receptors belong to the superfamily of ligand-gated ion channels (Barnard, 1996), which includes the nicotinic acetylcholine receptor, the 5-HT_3 and the glycine receptor. $GABA_A$ receptors appear in different forms. Their heterogeneity results from the association into a functional complex of various polypeptide subunits (α1-6, β1-3, γ1-3, δ, ∈, π, θ, ρ1-3), which are products of different genes (Mehta and Ticku, 1999). The subunit composition of the most common type of $GABA_A$ receptors found in the brain is 2α:2β:1γ, although it appears that the pentameric $GABA_A$ receptor assembly can also be formed from a combination of two, four, or even five different subunits (Mehta and Ticku, 1999). $GABA_A$ receptors possess binding sites for a variety of drugs, such as anxiolytics, anticonvulsants, general anesthetics, barbiturates, ethanol and neurosteroids, which are known to elicit at least some of their pharmacological effects via $GABA_A$ receptors (Mehta and Ticku, 1999; Korpi et al., 2002).

Prolonged occupancy of $GABA_A$ receptors by agonists and drugs facilitating the action of GABA leads to regulatory changes generally resulting in down-regulation of receptor levels and function. Thus, chronic exposure of cultured cerebral cortical neurons to GABA or muscimol decreases the number of benzodiazepine binding sites without affecting their affinity (Tehrani and Barnes, 1988; Mehta and Ticku, 1992; Lyons et al., 2001). Chronic GABA treatment also decreases $GABA_A$ receptor subunit protein (Mhatre and Ticku, 1994; Brown and Bristow, 1996) and GABA-stimulated Cl-uptake (Tehrani and Barnes, 1988). Reduction of mRNAs encoding different subunits of $GABA_A$ receptors was also found in cultures of chick and mammalian neurons exposed to GABA or muscimol (Montpied et al., 1991; Hirouchi et al., 1992; Mhatre and Ticku, 1994). Prolonged treatment of cortical cultures with GABA also induces an allosteric uncoupling of GABA and benzodiazepine binding sites (Roca et al., 1990a; Mehta and Ticku, 1992), characterised by a decrease in the GABA

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enhancement of benzodiazepine binding. Uncoupling is also induced by prolonged treatment with benzodiazepines, barbiturates and steroids (Gallager et al., 1984; Roca et al., 1990b; Friedman et al., 1996). It has been suggested that this phenomenon may be related to the development of tolerance and physical dependence (Gallager et al., 1984; Roca et al., 1990b; Klein and Harris, 1996), which appear in animals and humans following prolonged treatment with these drugs.

The functional and pharmacological properties of $GABA_A$ receptors, including the potency and efficacy of the neurotransmitter itself, depend on their subunit composition (Korpi et al., 2002). Whereas certain combinations of subunits are widely distributed in the brain, the expression of others is restricted to specific brain regions. The co-occurrence of multiple subtypes of GABA receptors in small regions, or even on a single neuron (Barnard et al., 1998), makes the study of pharmacological properties of a particular receptor subtype rather complex.

Use of transient and stably transfected cells offers some advantages over the studies on the native receptors. The advantages of cultured cells include a relatively homogenous cell population that can be well characterised with respect to receptor subunit composition, structure and function. Culture conditions can be tightly controlled, which facilitates the study of the mechanism of neurotransmitter receptor regulation (Klein and Harris, 1996). However, reports related to prolonged exposure of these cells to GABA and/or its agonists are few (Klein et al., 1995; Klein and Harris, 1996). More numerous are the reports (Wong et al., 1994; Klein et al., 1995; Primus et al., 1996; Ali and Olsen, 2001) of prolonged effects of drugs such as benzodiazepines. Given acutely, benzodiazepines facilitate GABA-mediated transmission at the $GABA_A$ receptor, while chronic treatment with these drugs leads to development of tolerance and physical dependence. In spite of many in vivo and in vitro studies, the mechanisms involved in the development of tolerance and dependence following chronic treatment with benzodiazepines are not clear (Ali and Olsen, 2001; Costa et al., 2002).

In this study, stably transfected human embryonic kidney (HEK) 293 cells (Besnard et al., 1997) were used as a model to study the effects of prolonged GABA exposure on the recombinant $\alpha1\beta2\gamma2s$ $GABA_A$ receptors, the most common type of $GABA_A$ receptor found in the brain (McKernan and Whiting, 1996), with the aim to better understand the mechanisms that underlie adaptive changes in $GABA_A$ receptors following their prolonged exposure to drugs. The experiments in which HEK 293 cells were exposed to chronic diazepam were also included. We could thus compare the effects produced by GABA with those produced by diazepam, and estimate whether recombinant $GABA_A$ receptors in stably expressed HEK 293 cells following chronic exposure to benzodiazepines mainly behave like receptors in other transfected cells (PA3, WSS-1, Sf9) as well as in vivo (Gallager et al., 1984).

2. Materials and methods

2.1. Cell culture

HEK 293 cell line expressing the $\alpha1\beta2\gamma2s$ subtype of $GABA_A$ receptor was kindly donated by Dr. François Besnard (Synthélabo Recherche, Bagneux Cedex, France). The cells were maintained at 37 °C in 5% CO_2 in Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 units/ml penicillin G and 100 μg/ml streptomycin in 75 cm^2 Falcon flasks according to standard cell culture techniques.

2.2. Drugs

GABA, diazepam, bicuculline and cycloheximide were from Sigma (St. Louis, MO), and muscimol from RBI (Natick, MA). Unlabeled TBOB was a gift from Prof. H.I. Yamamura. [^{3}H]flunitrazepam (specific activity 85 Ci/mmol) was purchased from Du Pont, NEN. [^{3}H]TBOB (*t*-[^{3}H]butylbicycloorthobenzoate, specific activity 24 Ci/mmol) was purchased from Amersham. Culture medium, antibiotics and fetal bovine serum were from Sigma.

2.3. Drug treatment

Three days prior to exposure to drugs, the cells were transferred to new flasks and grown in the above medium. Three days after seeding, the medium was removed and replaced with fresh medium containing drugs or vehicles. GABA (final concentration 1 mM), diazepam (final concentration 1 μM), bicuculline (final concentration 100 μM) and cycloheximide (10 μg/ml) were used. GABA, cycloheximide and muscimol were dissolved in distilled water, diazepam in 0.1 N HCl and bicuculline in warm 0.1 N HCl. Control cells were treated with the corresponding vehicles. The cells were treated with GABA or diazepam for 48 and 96 h, with bicuculline for 96 and with cycloheximide for 12 h. When treatment lasted for 96 h, the drugs were replaced after 2 days in culture.

2.4. Preparation of the membranes

Membranes from stably transfected HEK 293 cells were prepared mainly as described by Fuchs et al. (1995). The cells were washed, harvested by scraping into phosphate-buffered saline and centrifuged at 12,000 × *g* for 12 min. The cell pellet was homogenised in 50 mM Tris–citrate buffer, pH 7.4, using 10 strokes (up and down) in a glass/teflon homogeniser at 1250 rpm, and then centrifuged at 200,000 × *g* for 20 min. The pellet was resuspended, centrifuged at 200,000 × *g* for 20 min twice more, resuspended again and stored at −20 °C. On the day of assay, the suspension was centrifuged once more at 200,000 × *g* for 20 min and used for binding studies.

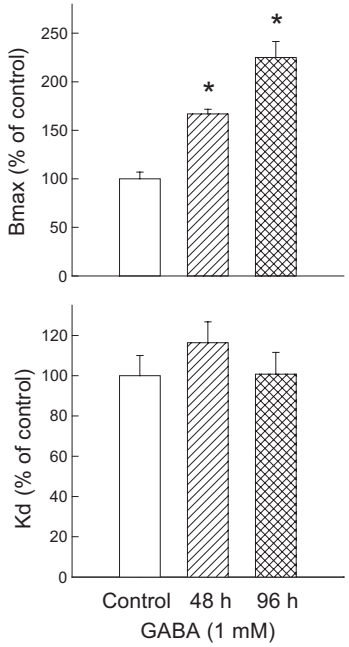

Fig. 1. The effect of chronic GABA treatment on [^{3}H]flunitrazepam binding to membranes from HEK 293 cells stably transfected with $\alpha1\beta2\gamma2s$ subunits of $GABA_A$ receptors. The cells were treated with 1 mM GABA for 48 or 96 h. Cell membranes from GABA-treated and control (vehicle-treated) cells were prepared as described in Materials and methods, and incubated with increasing concentrations of [^{3}H]flunitrazepam (0.2–16 nM). Binding in the presence of diazepam (100 μM) was subtracted from total [^{3}H]flunitrazepam binding to give specific [^{3}H]flunitrazepam binding. To obtain B_{max} and K_d values, the data were subjected to Scatchard analysis. Mean B_{max} value for control cells was 5.64 ± 0.39 pmol/mg protein ($n = 21$), while the K_d value was 2.69 ± 0.27 nM. Treated samples were always compared with matched controls that were cultured and assayed the same day as the treated samples. B_{max} and K_d values obtained from the treated groups were then expressed as percentages of controls. Data for treated groups are derived from 7 (GABA 48 h) and 16 (GABA 96 h) separate experiments. *$P < 0.01$ vs. control (ANOVA followed by the Newman–Keuls test).

2.5. [^{3}H]flunitrazepam binding assay

Aliquots of the cell membrane preparation (~ 100 μg protein) were incubated in 50 mM Tris–citrate buffer (pH = 7.4) containing 150 mM NaCl at 4 °C for 90 min with [^{3}H]flunitrazepam (0.2–16 nM) in the presence or absence of GABA (100 μM). The total assay volume was 0.5 ml. Nonspecific [^{3}H]flunitrazepam binding, defined in the presence of 100 μM diazepam, was less than 5% of the total binding (at the concentration of [^{3}H]flunitrazepam 1 nM). Radioactivity bound to membranes was determined after rapid filtration on Whatman GF/C filters.

2.6. [^{3}H]TBOB binding assay

Aliquots of the cell membrane preparation (~ 90 μg protein) were incubated in 50 mM Tris–citrate buffer (pH = 7.4) containing 200 mM NaCl at 25 °C for 90 min. Data for Scatchard plots were obtained by adding varying concentrations of nonradioactive TBOB to a fixed concentration (8 nM) of [^{3}H]TBOB so that 10 final concentrations (8–200 nM) were obtained. The total assay volume was 0.5 ml. Nonspecific [^{3}H]TBOB binding, defined in the presence of 100 μM picrotoxin, was < 23% of the total binding.

2.7. Protein determination

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

2.8. Data analysis

The binding data were analysed using a computer-based equilibrium binding data analysis (EBDA) program (McPherson, 1983). EBDA calculates the apparent dissociation constant (K_d) and the maximum density of binding sites by Scatchard transformation of the saturation binding data. The GABA shift was defined as the K_d of [^{3}H]flunitrazepam binding measured in the absence of GABA divided by K_d measured in the presence of 100 μM GABA.

Results are expressed as means ± S.E.M. Statistical analysis of results was by one-way analysis of variance (ANOVA) followed by the Newman–Keuls multiple comparison test, and by two-way ANOVA, when the effects of two different treatments were studied in the same experiment. Student *t*-test was also used where appropriate. *P*-values of < 0.05 were considered significant.

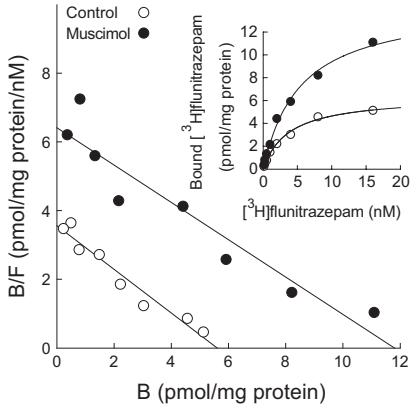

Fig. 2. Scatchard analysis of [^{3}H]flunitrazepam binding to membranes from control and muscimol pre-treated HEK 293 cells expressing recombinant $\alpha1\beta2\gamma2s$ $GABA_A$ receptors - representative experiment. The cells were treated with muscimol (100 μM, 96 h) or vehicle. Membranes from treated and control cells were incubated with increasing concentrations of [^{3}H]flunitrazepam (0.2–16 nM). Binding in the presence of diazepam (100 μM) was subtracted from total [^{3}H]flunitrazepam binding to give specific [^{3}H]flunitrazepam binding. Inset: saturation isotherms. B_{max} and K_d values were obtained by Scatchard analysis. Mean B_{max} values were: control: 5.80 ± 0.39; muscimol: 11.31 ± 0.55 pmol/mg protein ($n = 3$ for both groups; $P < 0.01$, Student's *t*-test). K_d values were: control: 3.45 ± 0.06; muscimol: 3.39 ± 0.14 nM.

3. Results

3.1. The effect of chronic drug treatment on [^{3}H]flunitrazepam binding to membranes from HEK 293 cells stably transfected with α1β2γ2s subunits of GABA$_A$ receptors

Prolonged exposure of HEK 293 cells, stably transfected with α1β2γ2s subunits of GABA$_A$ receptors, to 1 mM GABA, enhanced [^{3}H]flunitrazepam binding to cell membranes in a time-dependent manner. As shown in Fig. 1 and indicated by one-way ANOVA ($F(2,41)=33.78$; $P<0.0001$), the enhancement was due to an increase in B_{max}. The exposure for 48 and 96 h enhanced by 67% and 125%, respectively, the maximum number of [^{3}H]flunitrazepam binding sites ($P<0.01$, Newman–Keuls test), whereas the K_d of these sites was unchanged.

Chronic exposure of HEK 293 cells, transfected with α1β2γ2s subunits of GABA$_A$ receptors, to muscimol (100 μM, 96 h), as shown by a representative Scatchard plot (Fig. 2), also enhanced the maximum number of [^{3}H]flunitrazepam binding sites without affecting their affinity. The enhancement of binding sites obtained from three independent experiments was 96.1 ± 11.7%, and this number was significantly greater than that obtained from the control group of cells ($P<0.01$, paired Student's *t*-test).

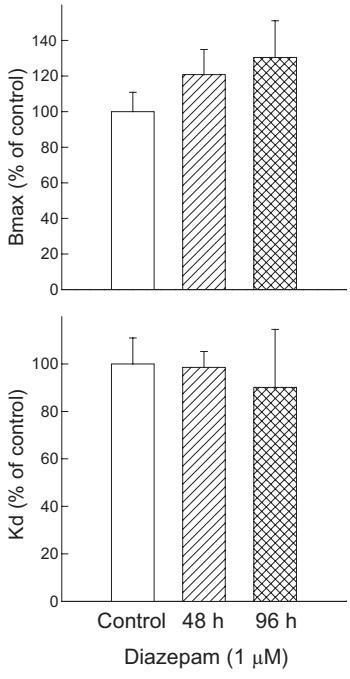

Fig. 3. Lack of effect of chronic diazepam treatment on [^{3}H]flunitrazepam binding to membranes from HEK 293 cells stably transfected with α1β2γ2s subunits of GABA$_A$ receptors. The cells were treated with 1 μM diazepam for 48 or 96 h. Cell membranes from treated and control cells were prepared as described in Materials and methods, and incubated with increasing concentrations of [^{3}H]flunitrazepam (0.2–16 nM). Binding in the presence of diazepam (100 μM) was subtracted from total [^{3}H]flunitrazepam binding to give specific [^{3}H]flunitrazepam binding. To obtain B_{max} and K_d values, data were subjected to Scatchard analysis. Treated samples were always compared with matched controls that were cultured and assayed the same day as the treated samples. B_{max} and K_d values obtained from the treated groups were then expressed as percentages of controls. Data for the control group are derived from six, and data for treated groups from three, separate experiments.

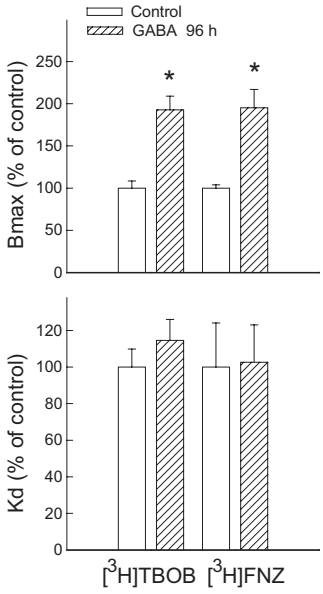

Fig. 4. The effect of prolonged GABA treatment on the maximum number (B_{max}) and affinity (K_d) of [^{3}H]TBOB (*t*-[^{3}H]butylbicycloorthobenzoate) and [^{3}H]flunitrazepam binding sites on the membranes of HEK 293 cells stably transfected with α1β2γ2s subunits of GABA$_A$ receptors. HEK 293 cells were treated with 1 mM GABA for 96 h. Preparations of cell membranes and binding assays were done as described in Materials and methods. [^{3}H]TBOB and [^{3}H]flunitrazepam binding was done on identical membrane preparations. To obtain B_{max} and K_d values, the data were subjected to Scatchard analysis. Treated samples were always compared with matched controls that were cultured and assayed the same day as the treated samples. B_{max} and K_d values obtained from the treated groups were then expressed as percentages of controls and shown as means ± S.E.M. Data for treated and control groups are derived from three separate experiments performed in duplicate. B_{max} value obtained in [^{3}H]TBOB binding assay in control was 5.31 ± 0.45 pmol/mg protein, and the K_d value was 29.17 ± 2.87 nM ($n=3$). *$P<0.01$ vs. the corresponding control (Student's *t*-test).

Prolonged exposure (48 or 96 h) of the same cells to 1 μM diazepam failed to affect the maximum number of [^{3}H]flunitrazepam binding sites ($F(2,9)=0.73$). The K_d value of these sites ($F(2,9)=0.12$) was also unchanged (Fig. 3).

3.2. The effect of GABA treatment on [^{3}H]TBOB] binding to membranes from HEK 293 cells stably transfected with α1β2γ2s subunits of GABA$_A$ receptors

To test whether a 96-h GABA exposure of HEK 293 cells stably transfected with α1β2γ2s subunits of GABA$_A$ receptors also augments the number of other binding sites at the GABA$_A$ receptor, we determined in the same membrane preparation the kinetic properties of binding sites for convulsants and benzodiazepines. Following exposure to GABA (1 mM, 96 h) the number of binding sites labelled

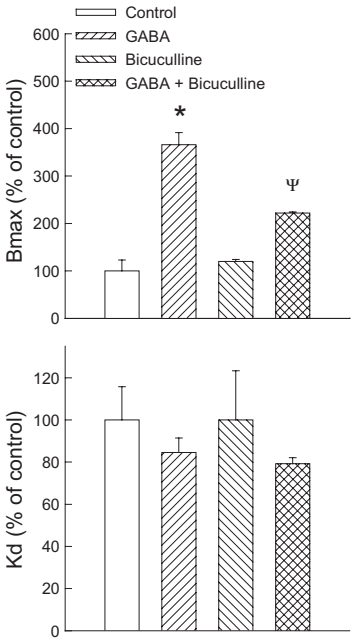

Fig. 5. The ability of bicuculline to counteract the enhancement of [^{3}H]flunitrazepam binding induced by GABA (1 mM, 96 h) in HEK 293 cells stably transfected with α1β2γ2s subunits of $GABA_A$ receptors. HEK 293 cells were treated for 96 h with 1 mM GABA or 100 μM bicuculline, the combination of both drugs, or with their vehicles. After 2 days in culture, the medium was removed and replaced with fresh medium containing drugs. Cell membranes were prepared as described in Materials and methods, and incubated with increasing concentrations of [^{3}H]flunitrazepam (0.2–16 nM). Binding in the presence of diazepam (100 μM) was subtracted from total [^{3}H]flunitrazepam binding to give specific [^{3}H]flunitrazepam binding. To obtain B_{max} and K_d values, the data were subjected to Scatchard analysis. B_{max} and K_d values obtained from the treated groups are expressed as percentages of controls and are shown as means ± S.E.M. The data are derived from two or three separate experiments performed in duplicate. *$P<0.01$ vs. control; $^{\Psi}P<0.01$ vs. all other groups (ANOVA followed by the Newman–Keuls test).

with the convulsant [^{3}H]TBOB was enhanced by 93%. This alteration was accompanied by an almost identical enhancement (95%) of [^{3}H]flunitrazepam binding sites ($P<0.01$ for both enhancements, Student's *t*-test). The K_d of the same binding sites was unaffected by prolonged exposure to GABA (Fig. 4).

3.3. The effect of bicuculline on GABA-induced up-regulation of [^{3}H]flunitrazepam binding to membranes from HEK 293 cells stably transfected with α1β2γ2s subunits of $GABA_A$ receptors

To test whether GABA binding sites were responsible for the enhancement of [^{3}H]flunitrazepam binding following treatment with 1 mM GABA for 96 h, we treated one group of cells simultaneously with GABA and bicuculline, the competitive antagonist of GABA binding sites. As shown in Fig. 5 and indicated by one-way ANOVA ($F(3,6)=45.14$; $P<0.0001$), the maximum number of [^{3}H]flunitrazepam binding sites between the four treated groups (control, GABA, bicuculline, GABA + bicuculline) was different. GABA (1 mM) again produced a great enhancement of [^{3}H]flunitrazepam binding sites. Bicuculline (100 μM) decreased the effect of GABA ($P<0.01$), but bicuculline alone had no effect on the control expression levels. Two-way ANOVA also indicated a very significant effect of GABA ($F(1,6)=97.643$, $P<0.0001$) and bicuculline ($F(1,6)=18.493$, $P<0.005$), as well as a significant GABA × bicuculline interaction ($F(1,6)=19.291$, $P<0.005$). The affinity of benzodiazepine binding sites was affected by neither of these treatments.

3.4. The effect of cycloheximide on GABA-induced up-regulation of [^{3}H]flunitrazepam binding to membranes from HEK 293 cells stably transfected with α1β2γ2s subunits of $GABA_A$ receptors

The ability of GABA (1 mM) to enhance, after 48 h of treatment, the density of [^{3}H]flunitrazepam binding sites in HEK 293 cells stably transfected with α1β2γ2s subunits of $GABA_A$ receptors was also tested in the presence of cycloheximide (10 μg/ml), an inhibitor of protein synthesis. One-way ANOVA ($F(3,8)=19.73$) indicated significant differences ($P<0.0005$) between the four treated groups (control, GABA, cycloheximide, GABA + cycloheximide). The GABA effect was again significant ($P<0.01$, Newman–Keuls test). Cycloheximide abolished the effect of GABA ($P<0.01$), whereas the effect of cycloheximide alone was not significantly different from that in the control (Fig. 6). Two-way ANOVA also indicated a significant effect of GABA ($F(1,8)=38.260$, $P<0.0003$), and cyclo-

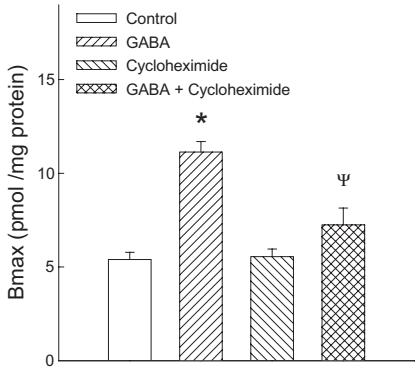

Fig. 6. The ability of cycloheximide to counteract the enhancement of [^{3}H]flunitrazepam binding induced by GABA (1 mM, 48 h) in HEK 293 cells stably transfected with α1β2γ2s subunits of $GABA_A$ receptors. HEK 293 cells were treated with 1 mM GABA, cycloheximide (10 μg/ml; present in cell culture for the last 12 h), the combination of both drugs, or with their vehicles. Cell membranes were prepared as described in Materials and methods, and incubated with increasing concentrations of [^{3}H]flunitrazepam (0.2–16 nM). Binding in the presence of diazepam (100 μM) was subtracted from total [^{3}H]flunitrazepam binding to give specific [^{3}H]flunitrazepam binding. To obtain B_{max} and K_d values, data were subjected to Scatchard analysis. B_{max} values are expressed as means ± S.E.M. Data are derived from three separate experiments performed in duplicate. *$P<0.01$ vs. control; $^{\Psi}P<0.01$ vs. GABA-treated group (ANOVA followed by the Newman–Keuls test).

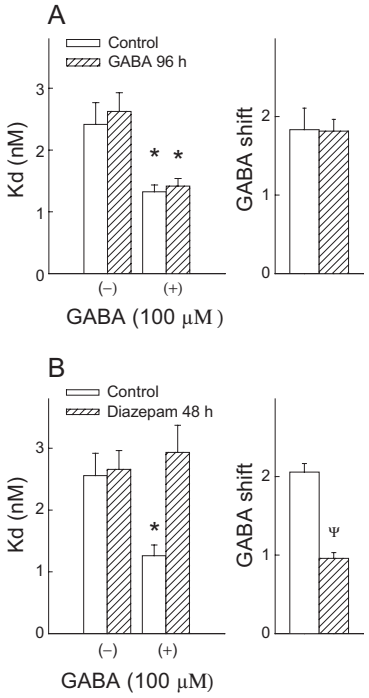

Fig. 7. Effects of chronic GABA and diazepam pre-treatment on GABA-enhanced affinity of [^{3}H]flunitrazepam binding to HEK 293 cells expressing recombinant α1β2γ2s $GABA_A$ receptors. HEK 293 cells were treated with 1 mM GABA for 96 h (A), with 1 μM diazepam for 48 h (B) or with the corresponding vehicles. Cell membranes from treated and control cells were prepared as described in Materials and methods, and incubated with increasing concentrations of [^{3}H]flunitrazepam (0.2–16 nM) in the absence or presence of 100 μM GABA. Binding in the presence of diazepam (100 μM) was subtracted from total [^{3}H]flunitrazepam binding to give specific [^{3}H]flunitrazepam binding. To obtain K_d values, the data were subjected to Scatchard analysis. The results are expressed as means ± S.E.M. from five (GABA) and seven (diazepam) separate experiments. GABA shift was obtained by dividing K_d of [^{3}H]flunitrazepam binding measured in the absence of GABA by K_d measured in the presence of 100 μM GABA. *$P<0.02$ or $P<0.01$ vs. the corresponding group incubated in the absence of GABA; $^{\Psi}P<0.001$ vs. GABA shift obtained in control (Student's *t*-test).

heximide ($F(1,8)=9.672$, $P<0.01$), as well as a significant GABA × cycloheximide interaction ($F(1,8)=11.256$, $P<0.01$). As indicated by one-way ANOVA ($F(3,8)=0.96$), neither of these treatments affected the K_d of [^{3}H]flunitrazepam binding (data not shown).

3.5. Effects of chronic GABA and diazepam exposure on GABA-enhanced [^{3}H]flunitrazepam binding

As expected, the addition of GABA (100 μM) enhanced the affinity of [^{3}H]flunitrazepam binding to membranes from control HEK 293 cells, i.e., the K_d value in the presence of GABA was significantly lower than that in the absence of 100 μM GABA (GABA shift = 1.83 ± 0.27, $n=5$; $P<0.02$). GABA (100 μM) added to membranes from HEK 293 cells pretreated for 96 h with 1 mM GABA also enhanced the affinity of [^{3}H]flunitrazepam binding (GABA shift = 1.81 ± 0.15, $n=5$; $P<0.01$), i.e., the GABA shift determined in membranes of HEK 293 cells chronically pretreated with GABA was not different from that obtained with control cells (Fig. 7A).

On the other hand, GABA (100 μM) added to membranes from HEK 293 cells pre-treated for 48 h with 1 μM diazepam failed to reduce the K_d value of [^{3}H]flunitrazepam binding, i.e., chronic diazepam abolished the GABA shift (control: 2.06 ± 0.29; diazepam: 0.96 ± 0.07; $n=7$ in both groups; $P<0.001$, Student's *t*-test (Fig. 7B).

Differences between the membranes from control and diazepam (1 μM, 48 h)-pre-treated HEK 293 cells after the addition of 100 μM GABA in one typical Scatchard analysis are shown in Fig. 8. A reduction in K_d value with no apparent effect on B_{max} following addition of 100 μM GABA can be seen in the control (Fig. 8A), but not in diazepam-pretreated (Fig. 8B) HEK 293 cells.

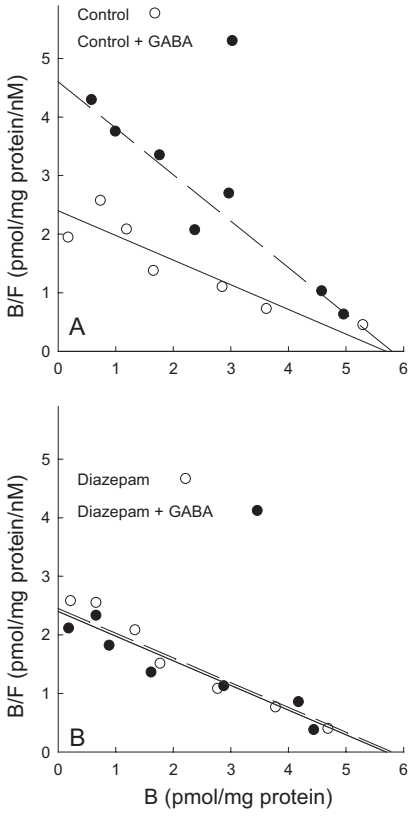

Fig. 8. Effect of chronic diazepam pre-treatment on the ability of GABA to enhance the affinity of [^{3}H]flunitrazepam binding to HEK 293 cells expressing recombinant α1β2γ2s $GABA_A$ receptors. HEK 293 cells were treated with 1 μM diazepam for 48 h or with the corresponding vehicle (control). Cell membranes from treated and control cells were prepared as described in Materials and methods, and incubated with increasing concentrations of [^{3}H]flunitrazepam (0.2–16 nM) in the absence or presence of 100 μM GABA. Binding in the presence of diazepam (100 μM) was subtracted from total [^{3}H]flunitrazepam binding to give specific [^{3}H]flunitrazepam binding. Representative Scatchard plots for vehicle (A) and diazepam pre-treated (B) HEK 293 cells in the absence and presence of 100 μM GABA.

4. Discussion

The present study demonstrated that prolonged exposure of stably transfected HEK 293 cells, expressing recombinant α1β2γ2s $GABA_A$ receptors, to GABA enhances the number of [^{3}H]flunitrazepam binding sites. The up-regulation of benzodiazepine binding sites was accompanied by an analogous enhancement of binding sites for the convulsant TBOB, suggesting that prolonged exposure to GABA up-regulates the number of $GABA_A$ receptors. Chronic treatment of stably transfected HEK 293 cells with the GABA receptor agonist, muscimol, also enhanced the number, while chronic treatment with diazepam affected neither the number nor the affinity of [^{3}H]flunitrazepam binding sites. Consistent with our previous study (Peričić et al., 1998), stably transfected HEK 293 cells (Besnard et al., 1997) possess a relatively high density of $GABA_A$ receptors.

The observed up-regulation of $GABA_A$ receptors obtained in radioligand binding studies with HEK 293 cells is in agreement with the report of Klein et al. (1995), who observed an enhanced (45%) binding of [^{3}H]flunitrazepam to recombinant bovine α1β1γ2L $GABA_A$ receptors stably expressed in mouse Ltk$^-$ (PA3) cells following prolonged exposure to GABA (50 μM, 96 h). However, these data obtained for the recombinant receptors are in contrast with most of the data obtained for neurons demonstrating down-regulation of receptors after their prolonged exposure to GABA or GABA receptor agonists (Tehrani and Barnes, 1988; Hablitz et al., 1989; Roca et al., 1990a; Montpied et al., 1991; Mehta and Ticku, 1992; Lyons et al., 2001).

It is also consistent with the reported down-regulation of $GABA_A$ receptors that there are data demonstrating that GABA treatment of chick or mammalian brain neurons in culture down-regulated the mRNAs encoding different subunits of the $GABA_A$ receptor (Montpied et al., 1991; Hirouchi et al., 1992; Mhatre and Ticku, 1994). Down-regulation of $GABA_A$ receptor subunits has also been observed (Calkin and Barnes, 1994; Brown and Bristow, 1996), although treatment of rat cerebellar granule cells for 8 days increased the expression of several subunit proteins, suggesting a biphasic effect of GABA on $GABA_A$ receptor subunit protein expression (Platt et al., 1996). This increase is consistent with results of experiments in which rats chronically treated with ethanolamine *O*-sulphate (a GABA transaminase inhibitor) showed up-regulation of GABA binding sites (Sykes et al., 1984). The enhanced expression of subunit proteins observed by Platt et al. (1996) was not accompanied by changes in mRNA levels, while in immature cerebellar granule cells, GABA, presumably due to its trophic effects (Meier et al., 1983; Schousboe and Redburn, 1995) induced up-regulation of GABA binding sites and mRNAs for α1 and β2 $GABA_A$ receptor subunits (Kim et al., 1993). Similar enhancement of the number of cerebellar $GABA_A$ receptors has been observed in vivo during postnatal development (Meinecke and Rakic, 1990). Trophic effects of GABA are probably mediated by both synaptic and nonsynaptic mechanisms (Owens and Kriegstein, 2002).

To find whether an increased synthesis and not a decreased degradation of receptor protein, an enhanced rate of receptor incorporation into membranes or some other factors (Maksay and Ticku, 1984) were responsible for the GABA-induced up-regulation of $GABA_A$ receptors, we co-treated stably transfected HEK 293 cells with cycloheximide, a protein synthesis inhibitor. As shown in Fig. 6, cycloheximide abolished the GABA-induced enhancement of benzodiazepine binding sites without affecting the maximum number of these sites in control cells. A significant GABA × cycloheximide interaction obtained in the two-way ANOVA also indicated a different effect of cycloheximide on the receptor protein in the control and the GABA-treated group of cells. These results might suggest that prolonged GABA exposure up-regulates stably expressed $GABA_A$ receptors by stimulating their synthesis. Further studies are needed to confirm this suggestion and to determine whether this stimulation occurs at the translation or the transcription and translation level. In our experiments, a general trophic effect of GABA in stably transfected HEK 293 cells could presumably be excluded, since neither total cellular RNA nor total cellular proteins in GABA-treated cells were increased (data not shown). Additionally, in our control experiments (when binding was performed under conditions used for transfected cells), membranes from untransfected HEK 293 cells failed to bind [^{3}H]TBOB or [^{3}H]flunitrazepam after chronic GABA treatment, excluding the likelihood that up-regulated, possibly endogenously present, $GABA_A$ receptor subunits (Fuchs et al., 1995; Davies et al., 2000) interfere with stably expressed recombinant receptors.

The GABA-induced up-regulation of [^{3}H]flunitrazepam binding sites now observed was inhibited by bicuculline, a competitive $GABA_A$ receptor antagonist, indicating that the effect is mediated through the GABA recognition site. Simultaneous treatment with bicuculline alone failed to modify [^{3}H]flunitrazepam binding.

Unlike treatment with GABA, that with diazepam, in agreement with results of other in vivo (Gallager et al., 1984) and in vitro studies (Hu and Ticku, 1994; Wong et al., 1994; Primus et al., 1996) failed to modify the kinetic parameters (B_{max} and K_d) of [^{3}H]flunitrazepam binding.

Several studies have shown that chronic treatment of $GABA_A$ receptors with agonists results in uncoupling or decreased coupling of allosteric interactions between GABA and benzodiazepine binding sites, as evidenced by a decrease in the enhancement of benzodiazepine binding by GABA. This change in the so-called GABA shift (a measure of allosteric coupling) has been observed in chick (Roca et al., 1990a; Lyons et al., 2001) and mouse cerebral cortical cultured neurons (Mehta and Ticku, 1992). Prolonged treatment with GABA also reduced the GABA enhancement of [^{3}H]flunitrazepam binding to mouse Ltk$^-$ cells stably transfected with bovine α1β1γ2L $GABA_A$

receptor subunits (Klein et al., 1995), but not to WSS-1 cells stably transfected with α1γ2 subunits (Wong et al., 1994). As shown in Results, in our study the Scatchard analysis of [^{3}H]flunitrazepam binding to membranes of control and GABA (1 mM, 96 h)-pre-treated cells, performed in the absence and presence of 100 μM GABA, failed to reveal differences in GABA shift, suggesting the existence of functional coupling between GABA and benzodiazepine binding sites. On the other hand, 100 μM GABA failed to enhance the affinity of [^{3}H]flunitrazepam binding to membranes from diazepam (1 μM, 48 h)-pre-treated cells, suggesting an allosteric uncoupling of GABA and benzodiazepine binding sites.

Accordingly, although it is generally accepted that the benzodiazepine action is mediated by modulation of the GABA effect at $GABA_A$ receptors, under our experimental conditions, chronic diazepam treatment of stably transfected HEK 293 cells produced uncoupling in the absence of GABA and neuronal elements, while chronic GABA failed to produce uncoupling. Our results with transfected HEK 293 cells following chronic diazepam thus agree with results for other transfected cells such as WSS-1 (Wong et al., 1994), PA3 (Klein et al., 1994; Klein and Harris, 1996) and Sf9 (Primus et al., 1996; Ali and Olsen, 2001). All these data demonstrate that prolonged occupation of benzodiazepine binding site by an agonist is sufficient to produce functional alterations resembling those produced in vivo (Gallager et al., 1984) or in neuronal cultures (Roca et al., 1990b; Hu and Ticku, 1994).

After studying the possible mechanisms involved, Ali and Olsen (2001) proposed that chronic benzodiazepine occupation of the receptor favours a conformation of receptor that is a substrate for internalization and uncoupling. The authors suggest that surface $GABA_A$ receptors become internalised into intracellular compartments where normal benzodiazepine binding can occur, but not potentiation by GABA. Internalisation of surface receptors was not observed by Primus et al. (1996). It remains to be determined whether post-translational modifications, such as dephosphorylation of protein kinase C sites on $GABA_A$ receptor or associated proteins (Leidenheimer et al., 1993; Klein and Harris, 1996), dephosphorylation of proteins phosphorylated by protein kinase A (Ali and Olsen, 2001) or some other adaptation mechanisms, are related to uncoupling. Namely, for transfected cells, which express receptors with a defined subunit composition, other possible explanations, e.g., replacement of subunits, leading to expression of receptors with reduced coupling, as proposed for neurons, can presumably be excluded.

Our results show clearly that, in the same cell line, chronic occupancy of the GABA binding site by GABA or its agonist, muscimol, produces effects different from those of chronic occupancy of benzodiazepine binding sites by diazepam.

Whether a different expression milieu or the intensive up-regulation of receptors observed in our study explains why we could not see the change in GABA shift following chronic GABA exposure remains to be determined.

When comparing our results with those obtained with neurons (Roca et al., 1990a; Lyons et al., 2001), however, it has to be emphasized that the expression mechanisms in HEK 293 cells might be different due to a lack of endogenous GABA and of a normal neuronal environment. One should not neglect either the fact that these cells are embryonic, and that perhaps due to this fact, the $GABA_A$ receptors expressed on the membranes of HEK 293 cells respond to chronic GABA exactly as do the receptors in immature cerebellar granule cells (Schousboe and Redburn, 1995). Actually, it has recently been demonstrated that HEK 293 cells have an unexpected relationship to neurons (Shaw et al., 2002).

In conclusion, prolonged exposure of HEK 293 cells stably expressing recombinant α1β2γ2s $GABA_A$ receptors for GABA and muscimol, but not for diazepam, results in up-regulation of receptor number. The enhanced number of $GABA_A$ receptors appears to be due to increased synthesis rather than decreased degradation of receptor proteins. Unlike chronic diazepam, which produced functional uncoupling of GABA and benzodiazepine binding sites in the absence of GABA and neuronal elements, chronic GABA failed to produce allosteric uncoupling. Further studies are needed to determine the relevance of these results to the phenomena of tolerance and dependence produced by prolonged treatment with benzodiazepines and other positive allosteric modulators of $GABA_A$ receptors.

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