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Molecular mechanisms of benzodiazepine-induced down-regulation of $GABA_A$ receptor α_1 subunit protein in rat cerebellar granule cells

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1 Chronic benzodiazepine treatment of rat cerebellar granule cells induced a transient down-regulation of the γ-aminobutyric $acid_A$ ($GABA_A$) receptor α_1 subunit protein, that was dose-dependent (1 nM–1 μM) and prevented by the benzodiazepine antagonist flumazenil (1 μM). After 2 days of treatment with 1 μM flunitrazepam the α_1 subunit protein was reduced by 41% compared to untreated cells, which returned to, and remained at, control cell levels from 4–12 days of treatment. Chronic flunitrazepam treatment did not significantly alter the $GABA_A$ receptor α_6 subunit protein over the 2–12 day period.

2 GABA treatment for 2 days down-regulates the α_1 subunit protein in a dose-dependent (10 μM–1 mM) manner that was prevented by the selective $GABA_A$ receptor antagonist bicuculline (10 μM). At 10 μM and 1 mM GABA the reduction in α_1 subunit expression compared to controls was 31% and 66%, respectively.

3 The flunitrazepam-induced decrease in α_1 subunit protein is independent of GABA, which suggests that it involves a mechanism distinct from the GABA-dependent action of benzodiazepines on $GABA_A$ receptor channel activity.

4 Simultaneous treatment with flunitrazepam and GABA did not produce an additive down-regulation of α_1 subunit protein, but produced an effect of the same magnitude as that of flunitrazepam alone. This down-regulation induced by the combination of flunitrazepam and GABA was inhibited by flumazenil (78%), but unaffected by bicuculline.

5 The flunitrazepam-induced down-regulation of α_1 subunit protein at 2 days was completely reversed by the protein kinase inhibitor staurosporine (0.3 μM).

6 This study has shown that both flunitrazepam and GABA treatment, via their respective binding sites, caused a reduction in the expression of the $GABA_A$ receptor α_1 subunit protein; an effect mediated through the same neurochemical mechanism. The results also imply that the benzodiazepine effect is independent of GABA, and that the benzodiazepine and GABA sites may not be equally coupled to the down-regulation process, with the benzodiazepine site being the more dominant. The biochemical mechanism underlying the benzodiazepine-mediated down-regulation of the α_1 subunit protein seems to involve the activity of staurosporine-sensitive protein kinases.

Keywords: Benzodiazepine; $GABA_A$ receptors; subunit proteins; down-regulation; staurosporine

Introduction

γ-Aminobutyric acid (GABA) is the most abundant inhibitory neurotransmitter in the vertebrate central nervous system, with the majority of neurones in the brain receiving a GABAergic input (Olsen & Tobin, 1990). The $GABA_A$ receptor, a multimeric ion channel complex and a member of the ligand-gated ion channel superfamily, is an important site of modulation by barbiturates, steroids, alcohols and 1,4-benzodiazepines (Macdonald & Olsen, 1994). In the central nervous system, molecular cloning has identified six α (α_{1-6}), four β (β_{1-4}), three γ (γ_{1-3}) and one δ subunit types for the $GABA_A$ receptor (Macdonald & Olsen, 1994), which are thought to make a pentameric structure comprising an integral chloride channel (Nayeem *et al.*, 1994). It has been postulated that the native $GABA_A$ receptor consists of two α, two β, and a γ subunit to be fully functional (Luddens & Wisden, 1991; Macdonald & Olsen 1994), although, due to the isoforms of the various subunits, the potential combinations of subunits in the receptor complex is enormous. The GABA binding site exists on the β subunit, with benzodiazepines binding to the interface of the α and γ subunits (Olsen & Tobin, 1990). The γ subunit is required to be present to enable benzodiazepines to modulate the GABA response (Pritchett *et al.*, 1989). Expression of different subunit isoforms in a given receptor complex leads to a receptor of differential pharmacology, in particular in their sensitivity to GABA and benzodiazepine agonists (Macdonald & Olsen, 1994). One proposed role for differential subunit isoform expression is in the development of tolerance to the effects of benzodiazepines (Hu & Ticku, 1994), whereby the $GABA_A$ receptor becomes less sensitive to certain benzodiazepine actions, despite the continued presence of the drug.

Benzodiazepines bind to the $GABA_A$ receptor complex and allosterically alter its conformation, acting to facilitate GABAergic transmission by increasing the frequency of the choloride channel opening (Study & Barker, 1981). Despite their clinical usefulness as sedatives, anxiolytics, anti convulsants and anaesthetics, the chronic use of benzodiazepines has led to observations of a time-dependent decrease in their therapeutic actions (Greenblatt & Shader, 1978). Further studies have established chronic benzodiazepine-induced tolerance and dependence in behavioural, electrophysiological and biochemical models (Gallager *et al.*, 1984; Rosenberg & Chiu, 1985; Hu & Ticku, 1994).

Benzodiazepine-induced tolerance could be associated with $GABA_A$ receptor desensitization when the receptor itself be-

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comes less responsive to a continued stimulus (Miller *et al.*, 1988). The $GABA_A$ receptor shows rapid and marked desensitization upon exposure to GABA agonists (Numann & Wong, 1984; Schwartz *et al.*, 1986; Cash & Subbarao, 1987) and this desensitization is enhanced by benzodiazepine agonists (Mierlack & Farb, 1988). Potentially, the benzodiazepines may simply enhance the GABA-mediated receptor desensitization effect or they may act by an entirely different mechanism. $GABA_A$ receptor function is diminished after chronic GABA or benzodiazepine treatment (Gallagher *et al.*, 1984; Nutt *et al.*, 1988). However, the mechanisms responsible are still unknown. Uncoupling of the $GABA_A$/benezodiazepine receptor (i.e. reduced ability of the benzodiazepine to be able to potentiate the effect of GABA) is sometimes observed in conjunction with a reduced function (Gallagher *et al.*, 1984; Miller *et al.*, 1990; Roca *et al.*, 1990; Hu & Ticku, 1994).

A number of studies have addressed the molecular basis of benzodiazepine-induced tolerance. These have primarily examined the messenger RNA (mRNA) levels of the $GABA_A$ receptor subunits after chronic benzodiazepine treatment and have shown a decreased expression of the mRNA for α_1 (Heninger *et al.*, 1990; Montipied *et al.*, 1991; Kang & Miller, 1991; Hirouchi *et al.*, 1992), α_5 (O'Donovan *et al.*, 1992b; Zhao *et al.*, 1994a), β_2 and β_3 (Zhao *et al.*, 1994b), and γ_2 (Kang & Miller, 1991; Primus & Gallagher, 1992; Zhao *et al.*, 1994a) subunits. In contrast, an increase in α_3 and α_6, but no alteration of α_1, α_2, β_{1-3}, or γ_2 (O'Donovan *et al.*, 1992a) or α_1 and γ_{2L} (Zhao *et al.*, 1994a) mRNA levels has also been detected after chronic benzodiazepine stimulation. It is very likely that a change in steady-state mRNA levels will lead to an alteration in protein expression, since the expression of cell surface proteins is generally controlled by the regulation of their mRNAs (Goldman & Tami, 1989). However, it is clear that other processes such as efficiency of subunit assembly, receptor insertion into the membrane, protein turnover, or mRNA stability could influence the final level of protein expression (Olson *et al.*, 1983).

The inconsistencies in the effects of benzodiazepines on receptor subunit mRNA levels and $GABA_A$/benzodiazepine radioligand binding, in which the effects observed are often brain area- or drug type-specific (Wu *et al.*, 1994), make it difficult to understand the molecular basis of tolerance. This study has therefore examined the effect of chronic benzodiazepine (flunitrazepam) treatment on the level of $GABA_A$ receptor subunit protein expression in rat cerebellar granule cells and the molecular mechanism thay may underlie the changes in subunit protein levels. An abstract of this work has been published previously (Brown & Bristow, 1995).

Methods

Cell culture

Primary cerebellar granule cell cultures were prepared from post-natal day 8 Sprague-Dawley rats by use of a modification of the technique described by Messer (1977). Cells were isolated by trypsinization (0.25% w/v, 15 min, 37°C) followed by trituration in a DNase solution (0.004% (w/v), 10 min, 37°C) containing a soybean trypsin inhibitor (0.003% (w/v)). Basal modified Eagle's medium (BME), supplemented with 10% (v/v) foetal bovine serum (heat-inactivated), 50 $\mu g\ ml^{-1}$ gentamicin, 100 iu penicillin, 100 $\mu g\ ml^{-1}$ streptomycin and 25 mM potassium chloride was added to the cells, after which they were centrifuged at 123 g_{av} for 90 s, and the supernatant containing the dissociated cells was collected. The pellet was resuspended in 10 ml BME, triturated and centrifuged at 123 g_{av} for 45 s, with the supernatant again being collected and pooled with the previous sample. This stage was repeated twice more and the supernatants pooled in each case.

Cells were seeded at a density of $6-7 \times 10^6$ cells per 25 cm^2 tissue culture flask (coated with 2 $\mu g\ cm^{-2}$ concentration poly-D-lysine) and maintained at 37°C in a humidified incubator (95% air/5% CO_2 (v/v)). Media was changed 18–24 h later and replaced with fresh supplemented BME containing 10 μM cytosine arabinoside, to prevent the division of non-neuronal cells. Cells were maintained in culture (95% air/5% CO_2 (v/v)) for a further 7 days prior to experimental treatment. Studies that have previously characterized the differentiation of cerebellar granule cells in culture have found that by 8 days *in vitro* (DIV), the synthesis and release of glutamate have reached the maximum (Gallo *et al.*, 1982) and that the cells represent mature post-migratory granule cells (Hattan *et al.*, 1984). In addition, the benzodiazepine potentiation of the GABA response has been shown to decline over 14–21 DIV (Mathews *et al.*, 1994). However, in cerebellar granule cell cultures at 20 DIV, we were still able to detect a reduction in α_1 subunit expression after 2 days of flunitrazepam treatment (-26 ± 7% of control, mean ± s.e.mean, $n = 4$), albeit of smaller magnitude than that observed with cells at 8 DIV. For these reasons, 8 DIV was the age of the cerebellar granule cells used in this study.

Experimental treatment of cerebellar granule cells

At 8 DIV, the cells were media changed and replaced with supplemented BME containing 10 μM cytosine arabinoside and one of the following treatments: 1 nM, 10 nM, or 1 μM flunitrazepam alone or in the presence of 10 μM GABA; or GABA alone (5 μM, 10 μM or 1 mM). In addition, either flumazenil (1 μM) or bicuculline (10 μM) was added to certain exposure conditions. Flunitrazepam was dissolved in 99% (v/v) ethanol, the final concentration of ethanol in the medium being 0.1% (v/v). GABA was dissolved in distilled deionized water (DDH_2O) and flumazenil was dissolved in 99% (v/v) ethanol. The cells were then maintained in culture for 2 days, or in the time course experiments (2, 4, 6, 8, 10 or 12 days) (95% air/5% CO_2 (v/v), 37°C) prior to harvesting for protein. Fresh supplemented BME and drug were replenished every 3 days, with every other media change including 10 μM cytosine arabinoside. For the protein kinase experiments, cells were media changed and replaced with supplemented BME containing 1 μM flunitrazepam and DMSO (vehicle) or 1 μM flunitrazepam and 0.3 μM staurosporine. Staurosporine was dissolved in 99% (v/v) dimethyl sulphoxide (DMSO), with the final concentration of DMSO in the medium being 0.1% (v/v). Cells were maintained in culture for 2 days (as above) before harvesting.

Western blotting analysis

Cells were scraped from flasks and dissociated by slow passing up and down through a 25 gauge needle (5 passes). A sample of the cells was removed, diluted 1:1 with trypan blue and counted on a Fuchs Rosenthal counting chamber, with the remainder split into 2 equal amounts and centrifuged at 14000 g_{av} (5 min, 22°C). Cells were then washed in phosphate buffered saline (PBS, composition (mM): NaCl 137, Na_2HPO_4 8.1, KCl 2.7, KH_2PO_4 1.5, pH 7.5), centrifuged at 14000 g_{av} (5 min, 22°C), and one of the 2 samples resuspended in sample reducing and denaturing buffer A (5% (w/v) sodium dodecyl sulphate (SDS), 10% (v/v) β-mercaptoethanol, 20% (v/v) glycerol, 0.1% (w/v) bromophenol blue, 0.5 M Tris-HCl/0.4% (w/v) SDS, pH 6.8, 8 M urea), the other in buffer B (identical to buffer A apart from the absence of 8 M urea). The amount of buffer added was standardized to 200 μl buffer per 1×10^6 cells. Cells were then stored at −20°C prior to gel electrophoresis.

Cell extracts in denaturing buffer (A or B) were passed up and down a 25 gauge needle before being boiled for 10 min and sonicated for 10 min to ensure any DNA had been sheared. Samples were centrifuged at 14000 g_{av} (5 min, 22°C), before being loaded on to a 1.5 mm 5% stacking gel (5% (w/v) acrylamide and 0.13% (w/v) bisacrylamide, 0.1% (w/v) SDS, 1% (v/v) tetramethylethylethylenediamine (TEMED), 0.1% (v/v) ammonium persulphate, 12.5% (v/v) 1.0 M Tris (pH 6.8), and resolved on a 1.5 mm 10% separating gel (10% (w/v) acrylamide and 0.27% (w/v) bisa-

crylamide, 0.1% (w/v) SDS, 25% 1.5 M Tris (pH 8.8), 0.1% (v/v) ammonium persulphate, 0.04% TEMED): 1×10^5 cells were loaded per well, with control and treated samples adjacent to each other. Upon completion of the transfer, gels were silver stained to check equal loading of lanes (data not shown). On each gel a prestained molecular weight standard (range 18.5–106 kDa) was run. The protein extracts were electrophoresed for 45 min at 100 V/500 mA by use of a continuous buffer system (Bio-Rad), (188 mM Trizma base, 188 mM glycine, 0.1% (w/v) SDS). The gels were equilibrated in 4°C transfer buffer (25 mM Trizma base, 193 mM glycine, 20% (v/v) methanol) for 30 min prior to transfer to 0.45 μm pore size nitrocellulose membranes with a Mini Trans-Blot (Bio-Rad) set up for 2 h at 100 V/250 mA. The nitrocellulose membranes were then air dried and stored with desiccant at 4°C.

Nitrocellulose membranes were incubated in blocking buffer (0.2% (v/v) TWEEN-20, 5% (w/v) dried skimmed milk, in Tris-buffered saline (TBS), overnight at 4°C, or for 1 h at 22°C prior to 2 h incubation with the primary antibody of choice (α_1 or α_6). Anti-sera were diluted to 3 mg ml^{-1} for the α_1 subunit (Zimprich *et al.*, 1991), and 1:500 for the α_6 antibody. In each case a control strip of membrane was exposed to an appropriate control. The α_1 antibody (3 μg ml^{-1} in blocking buffer) was preabsorbed with 16 μg ml^{-1} of the immunising peptide for 2 h at 37°C. Since none of the immunising peptide was available for the α_6 antibody, blocking buffer alone was used as a negative control.

Following incubation with the appropriate primary antibody or control, the membranes were washed 3 times during 30 min in approx. 50 ml blocking buffer (22°C). Membranes were then exposed to the secondary antibody. For all subunits, membranes were incubated with gentle agitation (1 h, 22°C) in donkey anti-rabbit horseradish peroxidase (HRP) conjugated antibody (1:5000 dilution with blocking buffer). Membranes were then washed 3 times in 30 min in approx. 50 ml blocking buffer. Enhanced chemiluminescence (ECL) was used to detect the $GABA_A$ receptor subunit proteins. The membranes were then air dried, covered with cling film, placed in a light tight film cassette and left for 15 min to equilibrate. Membranes were then exposed to Hyperfilm ECL, and developed using a Kodak X-OMAT M35 automatic film developer. The ECL signal was calibrated by loading increasing amounts of cells/lane which produced a linear response to ECL over the range $0.5-2 \times 10^6$ cells. All experimental data that was analysed fell within the linear range of the film. Optical density readings were quantitated by an OmniMedia flatbed 600 dpi scanner and IMAGE software. For both subunits, results were calculated as % differences in subunit protein in treated compared to controls.

Materials

Tissue culture media and supplements were purchased from GIBCO BRL, Scotland. HRP conjugated antibodies were purchased from SIGMA Chemical Co. Nitrocellulose membranes were bought from Bio-Rad, U.S.A., with ECL and Hyperfilm ECL bought from Amersham, U.K. The α_1 antibody and peptide were kindly donated by Prof. W. Sieghart, Department of Biochemical Psychiatry, Universitätsklinik für Psychiatrie, Vienna, Austria. The α_6 antibody was kindly donated by Dr R.M. McKernan, Merck Sharp and Dohme. All other chemicals or reagents were purchased from the Sigma Chemical Co. U.K. or BDH, U.K.

Statistical analysis of data

Optical density readings for control and treated conditions were measured and values expressed as % expression compared to controls. Results were analysed by use of Wilcoxon signed ranks test at 5% or 1% significance levels when the numbers of experiments was ≥ 7.

Results

The effect of flunitrazepam treatment of $GABA_A$ receptor α_1 subunit protein expression

Figure 1a shows a representative Western blot of the α_1 subunit protein detected by $GABA_A$ receptor α_1 specific antibody. The α_1 subunit protein can be seen as a dominant band corresponding to 54 ± 3 kDa (mean ± s.e.mean, $n = 10$). This band molecular weight corresponds reasonably with that found in purified receptor preparations from adult bovine cerebral cortex of 51 kDa (Macdonald & Olsen, 1994). In addition, a secondary band of 45 ± 1 kDa (mean ± s.e.mean, $n = 10$) is observed, and it appears to occur in proportion to the dominant band. This anti-α_1 subunit immunoreactive doublet has also been observed by McKernan *et al.* (1991) and the lower molecular weight band is thought to represent a breakdown product of the 54 kDa protein. This is supported by the observation that any agonist-induced alteration in the 54 kDa protein band showed a corresponding change in the level of the 45 kDa band (data not shown). No immunoreactivity was observed when the α_1 antibody was first incubated with its

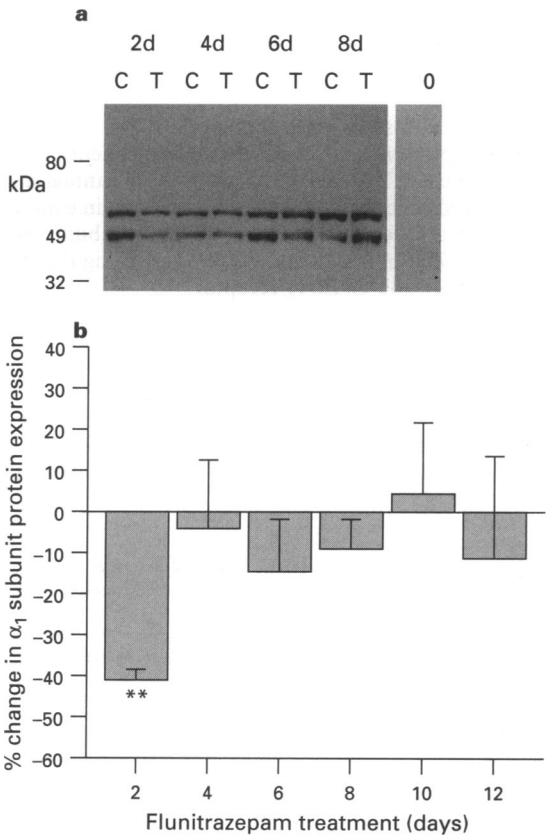

Figure 1 Effect of flunitrazepam on $GABA_A$ receptor α_1 subunit protein expression. Protein extracts from control (vehicle) and flunitrazepam (1 μM)-treated cerebellar granule cells (1×10^5 cells/lane) were separated by 10% SDS-PAGE followed by Western blotting and detected with $GABA_A$ receptor α_1 subunit-specific antibody of ECL as described in Methods. (a) Representative Western blot of $GABA_A$ receptor α_1 subunit protein. The apparent molecular weights were determined by use of prestained molecular weight markers run alongside the samples (shown on the left); d: days of treatment. Lane 0: negative control (α_1 antibody was preabsorbed with immunising peptide); C: control (vehicle) cells; T: flunitrazepam-treated cells. (b) Time course of effect of 1 μM flunitrazepam on α_1 subunit protein. Optical density readings of the protein bands were quantitated and compared between control and treated. Results are expressed as % change in α_1 subunit protein compared to the corresponding controls and given as mean ± s.e.mean (3–11 different cultures). **Significantly different from controls at the 1% level according to the Wilcoxon signed rank test.

immunising peptide. Figure 1b shows that the $GABA_A$ receptor α_1 subunit protein is signficantly reduced ($-41 \pm 3\%$, mean $\pm$ s.e.mean, $n=11$, $P<0.01$) at 2 days 1 μM flunitrazepam treatment compared to vehicle controls. This effect is antagonized by the co-administration of the specific benzodiazepine antagonist, flumazenil (Table 1), confirming an action via the benzodiazepine binding site. Flumazenil does not induce any effects on the α_1 subunit expression alone (Table 1). By 4 days treatment through to 12 days, the expression of the α_1 subunit protein returns to and remains at control values (Figure 1b). The α_1 subunit down-regulation effect induced by 2 days flunitrazepam is dose-dependent (Table 1), with a maximum effect occurring at 10 nM.

The effect of flunitrazepam treatment on $GABA_A$ receptor α_6 subunit protein expression

Figure 2a displays a representative Western blot of the $GABA_A$ receptor α_6 subunit protein. The apparent molecular weight is 58 ± 3 kDa. (mean $\pm$ s.e.mean, $n=6$) which corresponds well with the reported molecular weight of 57 kDa for the α_6 subunit protein (Macdonald & Olsen, 1994). Figure 2b shows that the $GABA_A$ receptor α_6 subunit protein expression is not altered by exposure to 1 μM flunitrazepam treatment over a 12 day treatment period.

Effect of GABA treatment on the expression of the α_1 subunit protein

GABA induced a dose-dependent decrease in the expression of the α_1 subunit protein following 2 days of treatment (Table 1). Bicuculline, the $GABA_A$ receptor site specific antagonist, at 10 μM produced no effect on the α_1 subunit protein expression, but prevented the down-regulation of the α_1 subunit protein induced by 10 μM GABA (Table 1), demonstrating the effect is mediated through the $GABA_A$ receptor.

Effect of a sub-maximal dose of GABA on the flunitrazepam-induced down-regulation of the α_1 subunit protein expression

GABA 10 μM in the presence of 1 μM flunitrazepam produced a significant down-regulation of the α_1 subunit protein following 2 days treatment, which was not significantly different from the effect of 1 μM flunitrazepam alone (Figure 3). At a lower than maximal dose of flunitrazepam (1 and 10 nM), the down-regulation induced by flunitrazepam in the presence of GABA also mirrored the effect of flunitrazepam alone (Figure 3).

Effect of bicuculline and flumazenil on the down-regulation of the α_1 subunit protein induced by flunitrazepam in the presence of GABA

The down-regulation of the α_1 subunit protein induced by 1 μM flunitrazepam was not altered upon co-administration of 10 μM bicuculline (Figure 4). In addition, the down-regulation of the α_1 subunit protein induced by 1 μM flunitrazepam in the presence of 10 μM GABA was not significantly altered upon co-administration of 10 μM bicuculline (Figure 4). In contrast, the co-administration of 1 μM flumazenil appeared to prevent the down-regulation, by 78%, that was induced by the combined effect of flunitrazepam and GABA (Figure 4).

Table 1 The effect of $GABA_A$ /benzodiazepine receptor agonist and /or antagonist treatment on the expression of α_1 subunit protein

Treatment (2 days)	*% change in α_1 subunit protein*
Flunitrazepam 1 nM	-25 ± 4*
Flunitrazepam 10 nM	-58 ± 18
Flunitrazepam 1 μM	-41 ± 3*
Flumazenil 1 μM	8 ± 18
Flunitrazepam 1 μM + Flumazenil 1 μM	-5 ± 27
GABA 5 μM	-10 ± 11
GABA 10 μM	-31 ± 13*
GABA 1 mM	-66 ± 14*
Bicuculline 10 μM	-7 ± 17
GABA 10 μM + Bicuculline 10 μM	2 ± 13

Protein extracts from control (vehicle) and treated (2 days) cerebellar granule cells (1×10^5 cells/lane) were separated by 10% SDS-PAGE followed by Western blotting and detected with $GABA_A$ receptor α_1 subunit-specific antibody and ECL as described in Methods. Optical density readings of the films were quantitated and compared between control and treated. Results are expressed as % change in α_1 subunit protein compared to the corresponding controls and given as mean $\pm$ s.e.mean (3–11 different cultures).** and * denote significantly different from controls at the 1% and 5% level, respectively, according to the Wilcoxon signed rank test.

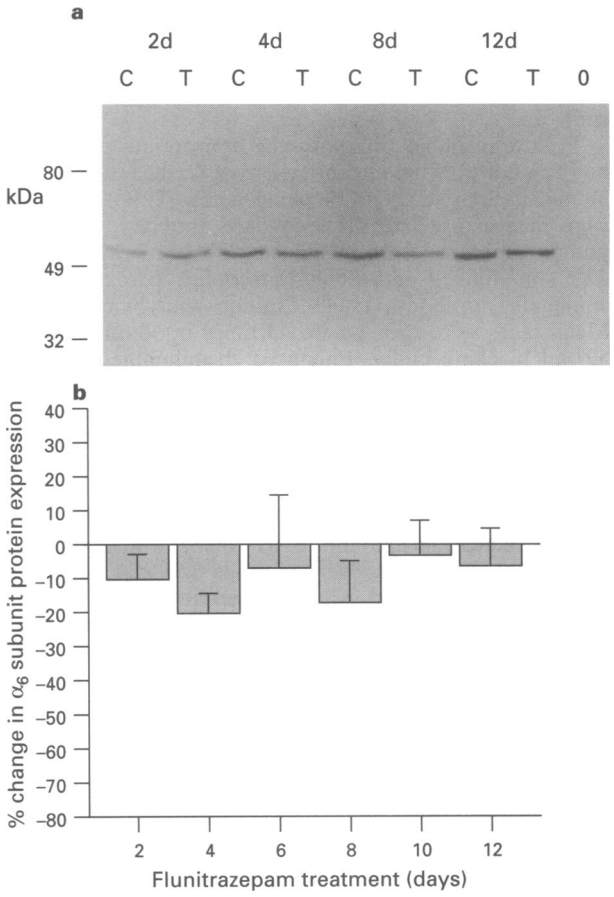

Figure 2 Effect of flunitrazepam on $GABA_A$ receptor α_6 subunit protein expression. Protein extracts from control (vehicle) and flunitrazepam (1 μM)-treated cerebellar granule cells (1×10^5 cells/lane) were separated by 10% SDS-PAGE followed by Western blotting and detected with $GABA_A$ receptor α_6 subunit-specific antibody and ECL as described in Methods. (a) Representative Western blot of $GABA_A$ receptor α_6 subunit protein. The apparent molecular weights were determined by use of prestained molecular weight markers run alongside the samples (shown on the left); d: days of treatment. Lane 0: negative control (α_6 antibody omitted from procedure); C: control (vehicle) cells; T: flunitrazepam-treated cells. (b) Time course of effect of 1 μM flunitrazepam on α_6 subunit protein. Optical density readings of the protein bands were quantitated and compared between control and treated. Results are expressed as % change in α_6 subunit protein compared to the corresponding controls and given as mean $\pm$ s.e.mean (3–7 different cultures).

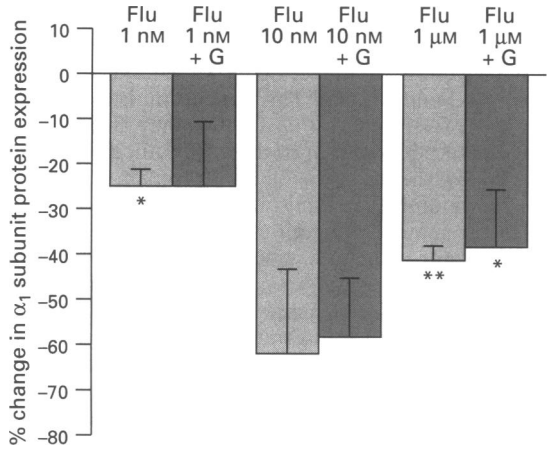

Figure 3 Effect of GABA (10 μM) on the flunitrazepam-induced down-regulation of the α_1 subunit protein. Protein extracts from control (vehicle) and treated cerebellar granule cells (1×10^5 cells/lane) were separated by 10% SDS-PAGE followed by Western blotting and detected with $GABA_A$ receptor α_1 subunit-specific antibody and ECL as described in Methods. Optical density readings of the films were quantitated and compared between control and treated. Results are expressed as % change in α_1 subunit protein compared to the corresponding controls and given as mean+-s.e.mean (3–11 different cultures). ** and * denote significantly different from controls at the 1% and 5% level, respectively, according to the Wilcoxon signed rank test. Flu = flunitrazepam alone; Flu + G = flunitrazepam in the presence of GABA.

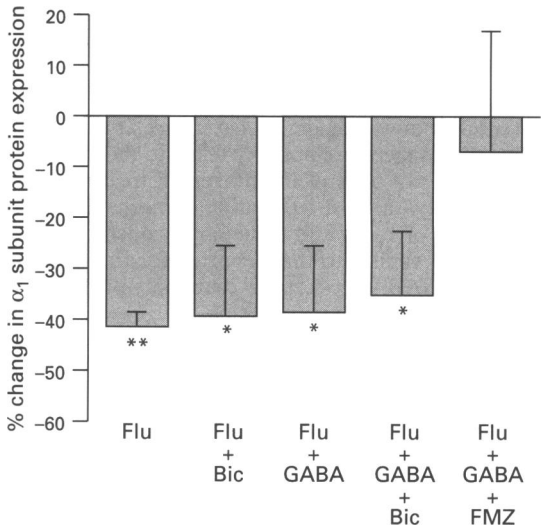

Figure 4 Effect of flumazenil and bicuculline on the combined flunitrazepam and GABA-induced down-regulation of the α_1 subunit protein. Protein extracts from control (vehicle) and treated cerebellar granule cells (1×10^5 cells/lane) were separated by 10% SDS-PAGE followed by Western blotting and detected with $GABA_A$ receptor α_1 subunit-specific antibody and ECL as described in Methods. Optical density readings of the films were quantitated and compared between control and treated. Results are expressed as % change in α_1 subunit protein compared to the corresponding controls and given as mean+s.e.mean (3–11 different cultures). ** and * denote significantly different from controls at the 1% and 5% level, respectively, according to the Wilcoxon signed rank test. Flu = flunitrazepam (1 μM), Bic = bicuculline (10 μM), FMZ = flumazenil (1 μM), GABA (10 μM).

Effect of staurosporine on the flunitrazepam-induced down-regulation of the α_1 subunit protein

Figure 5 shows the effect of 0.3 μM staurosporine on the down-regulation of the α_1 subunit protein induced by 2 days of flunitrazepam (1 μM) treatment. In the presence of vehicle (DMSO), the down-regulation persisted. However, the presence of staurosporine during the pretreatment period prevented the flunitrazepam-induced decrease in α_1 expression whilst having no significant effect compared to vehicle treated cells ($1.7 \pm 15\%$, mean $\pm$ s.e.mean, $n = 3$, staurosporine (0.3 μM) alone compared to vehicle controls).

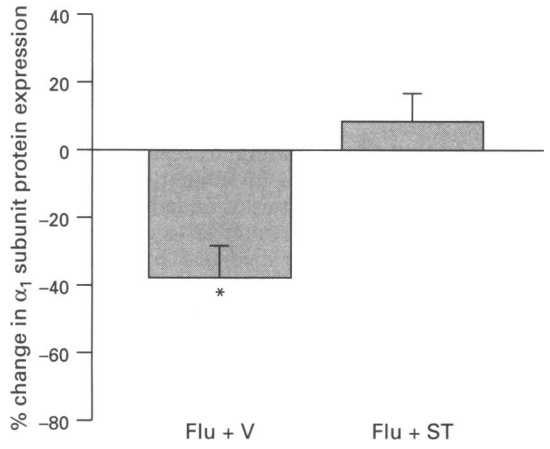

Figure 5 Effect of staurosporine on the flunitrazepam-induced down-regulation of the α_1 subunit protein. Protein extracts from control (vehicle) and treated (flunitrazepam + staurosporine, 2 days) cerebellar granule cells (1×10^5 cells/lane) were separated by 10% SDS-PAGE followed by Western blotting and detected with $GABA_A$ receptor α_1 subunit-specific antibody and ECL as described in Methods. Optical density readings of the films were quantitated and compared between control and treated. Results are expressed as % change in α_1 subunit protein compared to the corresponding controls and given as mean+s.e.mean (3–7 different cultures). * denotes significantly different from controls at the 5% level according to the Wilcoxon signed rank test. Flu = flunitrazepam (1 μM); V (vehicle) = DMSO (0.1% v/v), ST = staurosporine (0.3 μM).

Discussion

Treatment with benzodiazepines causes a decrease in $GABA_A$ receptor function, an uncoupling of the allosteric interactions between the GABA and benzodiazepine recognition sites, a down-regulation of certain $GABA_A$ receptor subunit mRNAs, and in this study we observed a reduced expression of $GABA_A$ receptor α_1 subunit protein. The flunitrazepam-induced down-regulation was transient and concentration-dependent, GABA-independent, and apparently specific for certain benzodiazepine-sensitive receptor combinations, since the α_6 subunit protein levels were unaltered. This study infers that flunitrazepam and GABA use the same mechanism to induce the down-regulation of α_1 protein, although the two binding sites may not be equal in their coupling efficiency, and that intracellular protein kinase(s) appear to underlie the process.

It is unclear at present whether changes in steady state mRNA levels are responsible for the observed decline in α_1 subunit protein expression. Studies in the cerebral cortex have shown a benzodiazepine-mediated reduction in α_1 subunit mRNA levels over the same time course (1 day, Hirouchi *et al.*, 1992), and an appropriate $GABA_A$ receptor turnover rate to account for this reduction of protein has been observed ($t_{1/2}$ 18 h, Borden & Farb, 1988). In addition, the actual mechanism to down-regulate the α_1 subunit mRNA may involve reduced transcription of the gene encoding it, as it has recently been found that chronic lorazepam treatment results in a decreased transcriptional activity of the promoter construct of the α_1 subunit gene (Kang *et al.*, 1994). In contrast, the studies of *in vivo* benzodiazepine treatment which do not detect alterations

in $GABA_A$ receptor mRNA levels in cerebellum (Kang & Miller, 1991; O'Donovan *et al.*, 1992a; Wu *et al.*, 1994), do not support a transcriptional controlled process. However, it is conceivable that these *in vivo* studies may have missed the transient mRNA down-regulation (seen here at the protein level at 2 days), and therefore possibly relate to the unchanged α_1 protein levels at >4 days of flunitrazepam treatment (Figure 1b). Alternatively, a post-translational mechanism for the down-regulation of α_1 subunit protein may operate in the cerebellum, perhaps similar to that reported for the GABA agonist-mediated $GABA_A$ receptor subunit protein down-regulation (Calkin & Barnes, 1994).

The detection procedure employed in this investigation should measure both the cell-surface and internalized proteins, therefore the only two plausible explanations for the loss of α_1 protein immunoreactivity are subunit degradation, or at least destruction of the antibody recognition epitope, and/or reduced protein synthesis. A degradation mechanism seems the most likely, since in earlier studies it was shown that GABA-induced down-regulation of $GABA_A$ receptor binding was not a result of a non-specific decrease in protein synthesis (Roca *et al.*, 1990). Furthermore, a similar degradation mechanism has been proposed to account for GABA agonist-induced down-regulation of $GABA_A$/benzodiazepine receptors in chick cortical neurones (Calkin & Barnes, 1994).

Research on the subunit composition of the $GABA_A$ receptors expressed in cerebellar granule cells suggests the existence of iso-oligomers, including α_1,α_2, $\alpha_1\alpha_3$ and $\alpha_1\alpha_6$ (Duggan *et al.*, 1991; Pollard *et al.*, 1993; 1995). At present it is unknown, with the exception of the α_6 subunit, whether the effect of flunitrazepam is restricted to the α_1 subunit. It seems probable that if the process involves receptor internalization, then other subunits contained in the $GABA_A$/benzodiazepine receptor complex would be removed along with the α_1 protein. Since we do not observe a significant loss of α_6 subunit expression, this may imply that the $\alpha_1\alpha_6$-containing receptors are unaffected by flunitrazepam treatment. Supporting this contention is the observation that $\alpha_1\alpha_6$-containing receptors appear to express the functional pharmacology of the α_6 subunit, that is a receptor insensitive to classical benzodiazepines (Pollard *et al.*, 1995).

Having established that the flunitrazepam-induced down-regulation of $GABA_A$ receptor α_1 subunit protein exists, the question of what are the mechanisms underlying the effect was addressed. It was of particular interest to determine whether the benzodiazepine effect was mediated by potentiating the GABA action, in a manner analogous to the benzodiazepine potentiation of GABA-stimulated channel activity (Study & Barker, 1981), or whether a distinct mechanism was used. Acute and chronic treatment with GABA agonists has previously been shown to manipulate the $GABA_A$ receptor, by inducing desensitization (Numann & Wong, 1984; Schwartz *et al.*, 1986; Cash & Subbarao, 1987; Hablitz *et al.*, 1989), uncoupling GABA and benzodiazepine allosteric interactions (Bristow & Martin, 1989; Roca *et al.*, 1990) and by causing the down-regulation of receptor subunit mRNA levels (Mhatre & Ticku, 1994; Montpied *et al.*, 1991; Hirouchi *et al.*, 1992; Baumgartner *et al.*, 1994) and protein expression (Mhatre & Ticku, 1994; Platt & Bristow, 1994; Spencer & Bristow, 1995). The results presented here confirm the previous observations of a transient loss in α_1 subunit protein after 2 days of GABA agonist treatment (Platt & Bristow, 1994), and extend this work to show GABA concentration-dependence. At a sub-maximal dose of GABA (10 μM), since benzodiazepines can functionally potentiate only sub-maximal doses of GABA (Olsen, 1981), the combination of GABA and flunitrazepam produced the same magnitude of α_1 subunit down-regulation as that of flunitrazepam alone ($-38\pm7\%$, Figure 3). If the down-regulation induced by GABA and flunitrazepam were acting by separate mechanisms, then it would be expected that the extent of α_1 subunit protein reduction seen for flunitrazepam alone ($-41\pm3\%$) and for GABA (10 μM) alone ($-31\pm11\%$) would be additive when in the same treatment regimen. As this is clearly not the case, then the most persuasive argument is that GABA and flunitrazepam act by a common mechanism to induce down-regulation of the $GABA_A$ receptor α_1 subunit protein. This conclusion is consistent with the findings of Hirouchi *et al.* (1992); they showed that the muscimol-induced reduction of the α_1 subunit mRNA was not potentiated by the addition of flunitrazepam. Of important note, is the finding that flunitrazepam-induced down-regulation of the α_1 subunit protein described here does not require $GABA_A$ receptor activation, since bicuculline, a specific $GABA_A$ receptor antagonist, is unable to prevent the flunitrazepam-induced effect (Figure 4). Although the content of GABA in the incubation medium was not measured, we feel this conclusion is justified, since the growth medium does not contain exogenous GABA, and it is unlikely that GABAergic neurones in the culture are releasing appreciable amounts of GABA as the α_1 subunit protein expression after bicuculline treatment (which should prevent any tonic GABA action) is not different from control cells (Table 1).

The antagonist experiments produced some interesting insights into possible binding site interactions. The down-regulation induced by GABA and flunitrazepam together was not inhibited by bicuculline, whereas flumazenil produced inhibition (Figure 4). Coupled with the observation that when in combination, the GABA and flunitrazepam-induced effect mirrored the magnitude of flunitrazepam effect alone (Figure 3), our interpretation is that an allosteric interaction exists between the two sites and that, when both sites are occupied, the benzodiazepine site is dominant. Thus, the studies on the molecular mechanisms of flunitrazepam-induced down-regulation of α_1 subunit proteins, infer a neurochemical process that is very different from that used by the benzodiazepines to faciliate GABAergic transmission.

The evidence supporting the importance of protein kinases in regulating $GABA_A$ receptor function is compelling (reviewed by Macdonald & Olsen, 1994; Krishek *et al.*, 1994). A number of subunits contain consensus sequence sites for phosphorylation by protein kinase C (PKC, the α_4 and α_6, the $\beta_{1\text{-}4}$, and γ_{2L} and γ_{2S}), protein kinase A (PKA, the α_4 and α_6, and $\beta_{1\text{-}4}$) and protein tyrosine kinase (PTK, γ_1, γ_{2L} and γ_{2S}, γ_3) (reviewed by Macdonald & Olsen, 1994). This study investigated the possible role of protein kinases in the flunitrazepam-induced down-regulation mechanism. The results with the protein kinase inhibitor staurosporine are, as far as we are aware, the first evidence that supports an involvement of protein kinases, and therefore most probably of protein phosphorylation, in the flunitrazepam-induced down-regulation of $GABA_A$ receptor subunit proteins. However, one recent study of recombinant $GABA_A$ receptors expressed in stably transfected cells, has examined the effect of staurosporine on the benzodiazepine-induced uncoupling of the GABA and benzodiazepine sites (GABA shift), and ruled out the involvement of protein kinases in this rapid regulatory process (Klein *et al.*, 1994). Staurosporine is a general protein kinase inhibitor with only around a 3 fold selectivity for PKC over PKA (Hidaka & Kobayashi, 1992), thus, at 0.3 μM, the concentration used in this study, it is likely to be inhibiting several protein kinases. There are reports implicating both PKC and PKA (reviewed by Macdonald & Olsen, 1994) in regulating $GABA_A$ receptor function, and therefore a role for protein kinases in the tolerance to benzodiazepines seems plausible. However, although our results support a role for protein kinase(s), further studies with specific inhibitors are needed to confirm the role of particular protein kinases in the flunitrazepam-induced down-regulation of α_1 subunit protein.

The mechanism of action of the protein kinase(s) is unlikely to involve direct phosphorylation of the α_1 subunit, since the α_1 subunit does not contain any known consensus phosphorylation sequences (Macdonald & Olsen, 1994). However, the protein kinase(s) may conceivably act on another subunit, for example the α_4, $\beta_{1\text{-}4}$ or $\gamma_{1\text{-}3}$ subunits, to induce changes in the α_1 subunit expression, because consensus sequences for protein kinases (e.g. PKC, PKA, PTK) are found on these subunits.

Another possibility is that the protein kinase(s) could act to phosphorylate an intermediate protein, which could activate a signal transduction pathway culminating in the down-regulation of the α_1 subunit protein.

In conclusion, this research has shown a chronic flunitrazepam-mediated transient down-regulation of the α_1 subunit protein, with a sparing of the effect on the α_6 subunit. It seems likely that the down-regulation is concomitant with a degradation and/or reduced synthesis of the α_1 subunit protein. The mechanism underlying the process appears to be the same as that employed by GABA agonists to induce down-regulation of $GABA_A$ receptor subunit proteins. However, when both sites are occupied, the benzodiazepine receptor seems to dominate the effect, and supports the existence of an allosteric interaction between the $GABA_A$ and benzodiazepine receptors. The mechanism underlying the benzodiazepine-induced reduction of α_1 protein has been shown to involve staurosporine-sensitive protein kinase action.

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