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Short communication

Regulation of $GABA_A$ receptor α_1 protein is a sensitive indicator of benzodiazepine agonist efficacy

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Abstract

The effect of benzodiazepine agonists of varying efficacy on γ-aminobutyric $acid_A$ receptor α_1 subunit protein expression was determined in primary cultured cerebellar granule cells. After 48 h exposure to 1 μM drug concentrations, flunitrazepam, diazepam, and the partial agonists Ro 19-8022 and bretazenil, but not the partial agonists Ro 42-8773, Ro 41-7812 or imidazenil, decreased α_1 subunit protein expression. The grading of effect of the benzodiazepine partial agonists on α_1 subunit protein expression is consistent with their agonist efficacies. This model, therefore, appears to act as a sensitive indicator of benzodiazepine agonist efficacy with the ability to differentiate between partial agonists.

Keywords: Cerebellar granule cell; Benzodiazepine; Partial agonist; Protein regulation

1. Introduction

There are three classes of ligand at the benzodiazepine binding site on the γ-aminobutyric $acid_A$ ($GABA_A$) receptor, positive modulators, high potency/no efficacy antagonists and negative modulators (Ehlert, 1986). The positive modulator class is divided into three further categories which all exhibit high potency but differing efficacies (Ducic et al., 1993). Full allosteric modulators (FAMs, e.g., triazolam) are high efficacy benzodiazepine agonists at many $GABA_A$ receptor subtypes. Selective allosteric modulators (SAMs, e.g., diazepam) are high efficacy benzodiazepine agonists at selected $GABA_A$ receptor subtypes. Partial allosteric modulators (PAMs, e.g., bretazenil) are low efficacy benzodiazepine agonists at many $GABA_A$ receptor subtypes.

The clinical use of FAM and SAM benzodiazepines is limited by many undesirable side effects including tolerance, physical dependence, ataxia, sedation and potentiation of ethanol (Costa and Guidotti, 1996). However, several PAMs seem to induce less pronounced side effects whilst retaining anxiolytic and anticonvulsant activity and, therefore, may have important therapeutic potential (Haefely et al., 1990; Costa and Guidotti, 1996). Additionally, PAMs have been shown to antagonise side effects produced by FAM and SAM benzodiazepines in animal models (Martin et al., 1993; Costa and Guidotti, 1996).

Regulation of $GABA_A$ receptor subunit mRNA (Sieghart, 1995) and, more recently, subunit protein (Brown and Bristow, 1996; Impagnatiello et al., 1996; Pesold et al., 1997) has been reported following administration of FAM and SAM benzodiazepines. Contrastingly, administration of the PAM imidazenil fails to induce such changes (Impagnatiello et al., 1996; Pesold et al., 1997). These observations are consistent with the hypothesis that benzodiazepine tolerance, and perhaps dependence, are caused by compensatory changes in $GABA_A$ receptor structure that reduce the sensitivity of the receptor to modulation by benzodiazepines (Costa and Guidotti, 1996).

Previous work from this laboratory has demonstrated that the SAM flunitrazepam reduces $GABA_A$ receptor α_1 subunit protein expression in primary cultured cerebellar granule cells (Brown and Bristow, 1996). This study used the same model system to assess the ability of a range of benzodiazepine agonists with different efficacies to alter α_1 subunit protein expression.

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2. Materials and methods

Primary cerebellar granule cell cultures from 8 day postnatal rats were prepared, maintained and, at 8 days in vitro, media changed as described previously (Brown and Bristow, 1996). The cells were then treated with either flunitrazepam, diazepam, Ro 19-8022, bretazenil, Ro 41-7812, Ro 42-8773 (final concentration 1 μM), imidazenil (final concentration 10 nM, 100 nM, or 1 μM), or co-treated with flunitrazepam and either flumazenil, imidazenil, Ro 42-8773, or Ro 41-7812 (final concentration 1 μM for each drug). All treated cells were matched with the appropriate vehicle control(s). Cells were maintained in culture for a further 2 days before harvesting.

Cells were harvested, counted, lysed in denaturing buffer and the α_1 subunit protein detected as described previously (Brown and Bristow, 1996), measuring α_1 subunit immunoreactivity in grey-scale units using densitometry. Whole cell protein extracts from 1×10^5 cells were loaded per lane of the electrophoresis gel, and Ponceau S (5% v/v) staining of nitrocellulose membranes was used to check for equal loading of total protein between control and treated samples. Increases in protein, corresponding to $0–1.5 \times 10^5$ cells, produce proportional increases in α_1 subunit immunoreactivity, as measured in grey-scale units, up to saturation of the photographic film (unpublished observations). All results were obtained from the linear portion of the film. Immunoreactivity values for treated conditions were expressed as mean $\pm$ S.E.M. of controls. Results were analysed using the Wilcoxon signed rank test at 5% or 1% significance levels, when the number of experiments was ≥ 7.

Imidazenil, bretazenil, Ro 19-8022, Ro 41-7812, Ro 42-8773 and flumazenil were generous gifts from Dr. Grayson Richards, Hoffmann-La Roche, Basel, Switzerland. Ro 41-7812 was dissolved in dimethyl sulphoxide (DMSO). All other drugs were dissolved in 99% (v/v) ethanol. The α_1 subunit antibody was a generous gift from Prof. W. Sieghart, Universitatsklinik fur Psychiatrie, Vienna, Austria.

3. Results

The α_1 specific antibody detected proteins with a dominant α_1 protein band of 52 ± 0.5 (6) (mean $\pm$ S.E.M. (n)) kDa and a minor band of 43 ± 0.5 (6) kDa, which correlate with bands produced in previous studies (McKernan et al., 1991; Brown and Bristow, 1996). The 43 kDa protein changed in proportion to, and produced a much lower immunoreactive signal than, the 52 kDa band and, therefore, did not contribute to any of the measured changes in α_1 subunit protein expression.

Expression of α_1 subunit protein in cerebellar granule cells was significantly reduced following 48 h exposure to 1 μM flunitrazepam ($20 \pm 3.0\%$, (7)) (mean $\pm$ S.E.M. (n)), diazepam ($19 \pm 4.4\%$, (8)), Ro 19-8022 ($19 \pm 5.2\%$, (7)) and bretazenil ($11 \pm 4.4\%$, (9)) (Fig. 1). In contrast, no significant change in α_1 subunit protein level was induced by 1 μM Ro 42-8773, Ro 41-7812 or imidazenil (Fig. 1). In addition, imidazenil also failed to reduce α_1 subunit protein expression at concentrations of 10 nM ($5 \pm 6.2\%$, (7)) and 100 nM ($1 \pm 5.1\%$, (6)).

The reduction in α_1 subunit protein expression induced by 1 μM flunitrazepam combined with either ethanol (0.1% (v/v)) or DMSO (0.1% (v/v)) vehicle was prevented completely by co-incubation with either 1 μM

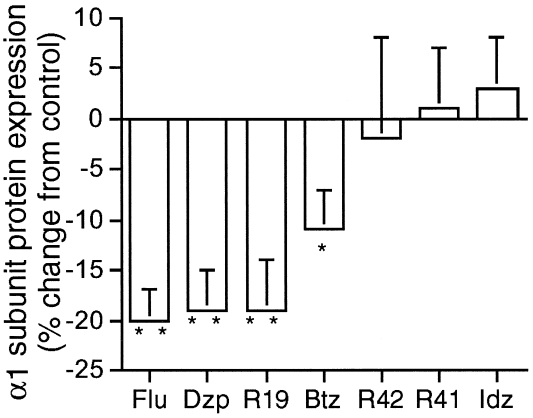

Fig. 1. The effect of a range of benzodiazepine agonists on $GABA_A$ receptor α_1 subunit protein expression. Primary cultures of cerebellar granule cells were exposed to the above drugs (1 μM final concentration) for 48 h. Samples were analysed by Western blotting and relative α_1 subunit protein levels were determined by comparison of the intensity of immunoreactive bands from control and treated samples, measured in grey-scale. The results are expressed as mean % change from control values $\pm$ S.E.M. ($n = 3–9$). $*$ Indicates $P < 0.05$, $**$ indicates $P < 0.01$ (Wilcoxon signed rank test). Flu: flunitrazepam; Dzp: diazepam; R19: Ro 19-8022; Btz: bretazenil; R42: Ro 42-8773; R41: Ro 41-7812; Idz: imidazenil.

Table 1
The effect of combinations of benzodiazepine ligands on the expression of $GABA_A$ receptor α_1 subunit protein

Treatment	α_1 subunit protein (% of control)
Flu + ethanol (0.1% (v/v))	$-17 \pm 4*$
Flu + flumazenil	-2 ± 7
Flu + imidazenil	2 ± 6
Flu + Ro 42-8773	-1 ± 10
Flu + DMSO (0.1% (v/v))	-27 ± 7
Flu + Ro 41-7812	-12 ± 15

Primary cultures of cerebellar granule cells were exposed to the above drugs, each at 1 μM final concentration, for 48 h. Samples were analysed by Western blotting and relative α_1 subunit protein levels were determined by comparison of the intensity of immunoreactive bands from control and treated samples, measured in grey-scale. The results are expressed as mean $\pm$ S.E.M. ($n = 3–7$). $*$ Indicates $P < 0.05$ (Wilcoxon signed rank test). Flu: flunitrazepam.

flumazenil, imidazenil, or Ro 42-8773 and partially by coincubation with 1 μM Ro 41-7812 (Table 1).

4. Discussion

Chronic exposure of cerebellar granule cells to flunitrazepam, diazepam, Ro 19-8022 or bretazenil, but not Ro 42-8773, Ro 41-7812 or imidazenil, reduced $GABA_A$ receptor α_1 subunit protein expression. In addition, flunitrazepam-induced reduction in α_1 subunit protein expression was prevented by co-incubation with 1 μM flumazenil, imidazenil or Ro 42-8773. A clear distinction between the ability to reduce α_1 subunit protein expression was seen with the PAMs used in this study, in a manner consistent with their reported agonist efficacies (Facklam et al., 1992; Martin et al., 1993; Serra et al., 1994). These results suggest that there may be a threshold benzodiazepine agonist efficacy which, when exceeded, causes regulation of $GABA_A$ receptor protein.

Initial work compared the ability of flunitrazepam and imidazenil to induce changes in $GABA_A$ receptor α_1 subunit protein expression. Whereas flunitrazepam caused a significant reduction of α_1 subunit protein expression, consistent with previous results from this laboratory (Brown and Bristow, 1996), imidazenil, at 10 nM, 100 nM, or 1 μM, failed to induce such a change. The lack of effect of imidazenil in this model is consistent with its inability to change [^{3}H]flumazenil binding sites (Zanotti et al., 1996) and $GABA_A$ receptor subunit expression (Impagnatiello et al., 1996; Pesold et al., 1997), or produce in vivo side effects (Costa and Guidotti, 1996). Furthermore, imidazenil was able to prevent the flunitrazepam-induced effect (Table 1), which is consistent both with them acting at a common binding site and the reported antagonism of side effects of FAM / SAM benzodiazepines in vivo (Martin et al., 1993; Costa and Guidotti, 1996). These results infer that the reduction of α_1 subunit protein expression in cultured cerebellar granule cells may be a predictor of in vivo benzodiazepine effects.

Subsequent experiments tested the hypothesis that the effects of benzodiazepine agonists with different efficacies would have a range of effects on α_1 subunit expression. The SAM diazepam (Ducic et al., 1993) and the PAMs Ro 19-8022 (Jenck et al., 1992) and bretazenil (Haefely et al., 1990) induced significant reductions of α_1 subunit protein, but the PAMs Ro 41-7812 and Ro 42-8773 (Martin et al., 1993) were ineffective (Fig. 1). Thus, we report a full spectrum of effects induced by PAMs, ranging from no effect to the same magnitude of effect induced by flunitrazepam and diazepam. This grading of effect induced by the PAMs correlates with previous published observations that show Ro 19-8022 to have a higher efficacy (Facklam et al., 1992) and Ro 41-7812 and Ro 42-8773 to have lower efficacy (Martin et al., 1993) than bretazenil. In addition, imidazenil has been shown to antagonise the effects of bretazenil on $GABA_A$ receptor function (Serra et al., 1994), which also implies that it has a lower efficacy. It therefore appears that the ability of benzodiazepine agonists to induce a reduction in α_1 subunit protein in cerebellar granule cells is dependent on their efficacy and indicates that there is an efficacy threshold between that of bretazenil and Ro 42-8773 that must be exceeded in order to induce an effect. Similarly, the magnitude of change in both GABA sensitivity (Hernandez et al., 1989) and behavioural effects (Martin et al., 1993) following administration of benzodiazepines can be correlated with benzodiazepine efficacy.

In vivo studies using animal models have consistently shown that PAMs, at therapeutic doses, induce very few side effects and exhibit low tolerance and dependence liability (Haefely et al., 1990; Costa and Guidotti, 1996). However, there is some discrepency between results obtained from animal and human studies. Benzodiazepine tolerance in animals only predicts tolerance in man to a certain extent (Fleischhacker, 1988). Early clinical experience of bretazenil showed that, although it shows better separation of therapeutic and adverse effects than diazepam, it can still cause sedation, ataxia, psychomotor and cognitive impairment (Delini-Stula, 1992). Furthermore, a recent study found no dissociation of the sedative and anxiolytic effects of bretazenil in man (Van Steveninck et al., 1996). Thus, the pharmacological requirement for a benzodiazepine with low side effect liability in man appears to be more complex than merely being designated a PAM in animal models.

The in vitro cerebellar granule cell model is capable of distinguishing between PAMs of differing efficacies. For example, it shows bretazenil to have a significant, yet submaximal, effect and therefore appears to be a more sensitive predictor of PAM pharmacology in man than existing animal models. The reason for this increased sensitivity may be related to the high benzodiazepine concentration used in this study, which gives each drug greater opportunity to induce an effect. Furthermore, a drug which is unable to reduce α_1 subunit protein expression under these conditions is unlikely to do so under any conditions. It is unlikely that the effects produced by such high benzodiazepine concentrations are due to nonspecific actions, since flumazenil, a specific antagonist of the benzodiazepine binding site, prevents the flunitrazepam-induced effect (Table 1; Brown and Bristow, 1996). Furthermore, the importance of testing a high drug concentration is emphasised by reports of cross tolerance (Bronson, 1995), precipitated withdrawal (Martin, 1993) and mild sedation (Haefely et al., 1990) following administration of high bretazenil doses in vivo.

In conclusion, the cerebellar granule cell model appears to be a sensitive neurochemical indicator of the in vivo pharmacology of benzodiazepine agonists. It discriminates between PAMs of differing efficacies and, therefore, may be a sensitive predictor of the extent of their side effect

profile in man, with a place as an adjunct to animal behavioural models in preclinical studies of PAMs with therapeutic potential.

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