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SPECIAL REPORT

Flunitrazepam rapidly reduces $GABA_A$ receptor subunit protein expression *via* a protein kinase C-dependent mechanism

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Acute flunitrazepam (1 μM) exposure for 1 h reduced $GABA_A$ receptor α1 (22$\pm$4%, mean$\pm$s.e.mean) and β2/3 (21$\pm$4%) subunit protein levels in cultured rat cerebellar granule cells. This rapid decrease in subunit proteins was completely prevented by bisindolymaleimide 1 (1 μM), an inhibitor of protein kinase C, but not by N-[2-((*p*-bromocinnamyl)amino)ethyl]-5-isoquinolinesulfonamide (H-89, 4.8 μM), an inhibitor of protein kinases A and G. These results suggest the existence of a benzodiazepine-induced mechanism to rapidly alter $GABA_A$ receptor protein expression, that appears to be dependent on protein kinase C activity.

Keywords: Cerebellar granule cells; $GABA_A$ receptor; protein down-regulation; protein kinase C; benzodiazepines

Introduction Chronic exposure to certain 1,4-benzodiazepine agonists is known to regulate $GABA_A$ receptor subunit mRNA (Sieghart, 1995) and protein (Brown & Bristow, 1996; Impagnatiello *et al*., 1996; Johnston & Bristow, 1998) expression. The changes in receptor subunit levels are believed to alter the pharmacology of the expressed $GABA_A$ receptor and thereby account for the appearance of benzodiazepine tolerance and physical dependence (Costa & Guidotti, 1996). However, an understanding of the neurochemical events resulting from benzodiazepine exposure is incomplete, particularly regarding the time course of benzodiazepine-induced effects and the biochemical pathways underlying the changes in $GABA_A$ receptor subunit expression.

Protein phosphorylation is an important mechanism of regulation of amino acid-gated ion channel receptors (Moss & Smart, 1996). The $GABA_A$ receptor is phosphorylated on the β and γ2 subunits, and receptor function appears to be controlled by a balance of phosphorylation of the receptor proteins by different protein kinases (Moss & Smart, 1996). In addition, we have recently reported that the chronic (48 h) flunitrazepam-induced reduction in α1 subunit protein levels can be completely prevented by staurosporine, implying the involvement of a protein kinase-dependent mechanism (Brown & Bristow, 1996). However, staurosporine is a non-selective protein kinase inhibitor and therefore unable to identify the protein kinase(s) responsible for the effect. The use of the more selective inhibitors, bisindolylmaleimide 1 (Toullec *et al*., 1991) and N-[2-((*p*-bromocinnamyl)amino)ethyl]-5-isoquinolinesulfonamide (H-89, Chijiwa *et al*., 1990), to discriminate between the involvement of protein kinase C (PKC) and protein kinase A and G, respectively, is necessary to further clarify the molecular mechanism of flunitrazepam-mediated reduction of $GABA_A$ receptor subunit proteins.

This study has investigated the effects of acute benzodiazepine exposure on $GABA_A$ receptor subunit protein and the biochemical process underlying the down-regulation of protein expression. The results demonstrate that benzodiazepine exposure rapidly reduces $GABA_A$ receptor α1 and β2/3 subunit expression in cerebellar granule cells through a PKC-dependent pathway.

Methods Rat primary cerebellar granule cell cultures were prepared as described previously (Brown & Bristow, 1996). At 8 days *in vitro*, the cells were pretreated (30 min, 37°C) with either both vehicles ethanol (0.1% v/v) and dimethyl sulphoxide (DMSO, 0.1% v/v), or ethanol (0.1% v/v) and bisindolylmaleimide 1 (1 μM, Calbiochem), or DMSO (0.1% v/v) and N-[2-((*p*-bromocinnamyl)amino)ethyl]-5-isoquinolinesulfonamide (H-89, 4.8 μM, Calbiochem). The cells were then exposed to flunitrazepam (1 μM, Sigma) or vehicle (ethanol 0.1% v/v) and the incubation continued for 10 min or 1 h (37°C). The cells were lysed in denaturing buffer and the α1 and β2/3 subunit protein detected in whole cell extracts as described previously (Platt *et al*., 1996; Johnston & Bristow, 1998), measuring the protein immunoreactivity in grey-scale units by densitometry. Increase in protein, over the range $0-1.5\times10^5$ cells, produced proportional increases in immunoreactivity, measured in grey-scale units, up to saturation of the photographic film (data not shown). All results were obtained from the linear portion of the film. Ponceau S (5%, v/v) staining of nitrocellulose membranes was used to check for equal loading of the total protein between control and treated samples. Results were analysed using the Wilcoxon Signed Rank Test when the number of experiments was $\geqslant$7. All treated cells were compared with those exposed to both DMSO and ethanol (vehicle pretreated control). Bisindolylmaleimide 1 and H-89 were dissolved in DMSO and ethanol (99% v/v), respectively.

[^{3}H]Flunitrazepam (0.4 and 0.9 nM) binding studies were based on a protocol described elsewhere (Bristow & Martin, 1987), but using Tris-HCl buffer (50 mM, pH 7.4) and at either 4 or 37°C. Bisindolylmaleimide 1 was used at 1 and 10 μM and compared to DMSO (0.1% v/v) controls. Whole rat brains were homogenised (Ultra-Turax, 20 s) in Tris-HCl, followed by centrifugation (1000 $\times g$, 5 min, 4°C). The supernatant was removed and centrifuged (20,000 $\times g$, 30 min), the pellet resuspended and centrifuged (20,000 $\times g$, 30 min), and the final pellet resuspended in Tris-HCl buffer (to approx. 2 mg protein ml^{-1}) and stored at -20°C until use. The protein concentration in the incubation was approx. 0.1 mg.

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Results The α1 specific antibody detected proteins with a dominant α1 band of 52±0.5 kDa (mean±s.e.mean, $n=6$) and a minor band of 43±0.5 kDa. After flunitrazepam treatment, the 43 kDa protein was reduced in proportion to the α1 (52 kDa) band, and therefore did not contribute to the loss of the α1 signal. The β2/3 specific antibody detected a protein of 55±0.9 kDa (means±s.e.mean, $n=6$). The sizes of the immunoreactive bands are all consistent with them labelling the appropriate subunit proteins (Brown & Bristow, 1996; Platt *et al*., 1996).

In vehicle pretreated cells flunitrazepam exposure for 1 h caused a significant decrease in both α1 and β2/3 subunit protein expression (Figure 1). At an earlier time of 10 min exposure to flunitrazepam, α1 protein levels are not reduced (99±3%, mean % of control±s.e.mean, $n=5$). Following pretreatment of the cells with H-89, at a concentration likely to inhibit both protein kinase A and G inhibitor (Chijiwa *et al*., 1990), flunitrazepam exposure reduced the expression of the α1 and β2/3 subunit proteins to a similar level as the vehicle pretreated controls (Figure 1). However, pretreatment with the PKC inhibitor, bisindolylmaleimide 1 (Toullec *et al*., 1991) at 1 μM, abolished the flunitrazepam-induced reduction of both α1 and β2/3 subunit protein levels (Figure 1).

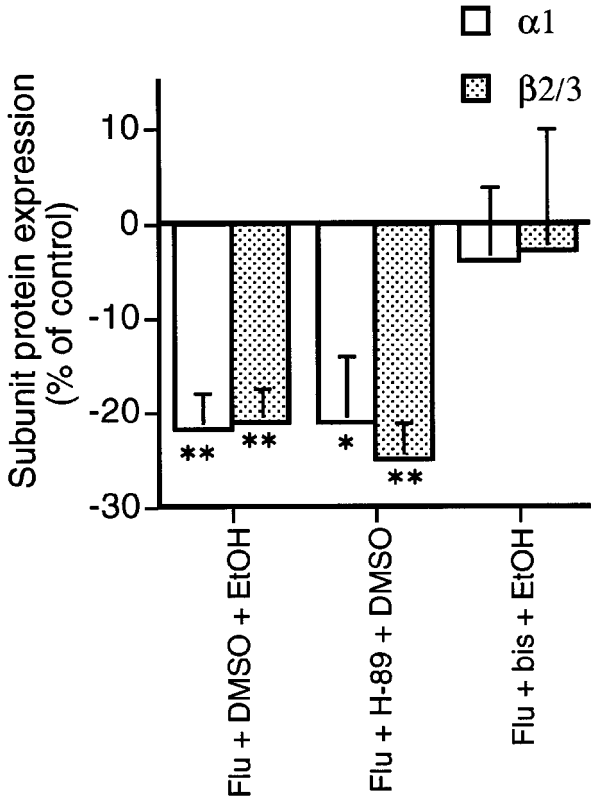

Figure 1 Bisindolylmaleimide 1, but not H-89, blocks flunitrazepam-induced decrease in α1 and β2/3 subunit protein expression. Primary cultures of cerebellar granule cells were pretreated for 30 min with DMSO and ethanol (each 0.1% (v/v)), H-89 (4.8 μM) and DMSO (0.1% v/v)) or bisindolylmaleimide 1 (1 μM) and ethanol (0.1% (v/v)). The cells were then treated with either 1 μM flunitrazepam or 0.1% (v/v) ethanol for 1 h. Samples were analysed by Western blotting and relative α1 and β2/3 subunit protein levels were determined by comparison of the intensity of immunoreactive bands from control and treated samples. The results are expressed as mean % change from control values±s.e.mean ($n=5-9$). * and ** indicate significant difference $P<0.05$ and $P<0.01$, respectively, from corresponding control cells (Wilcoxon signed rank test). Flu: flunitrazepam; bis: bisindolylmaleimide; EtOH: ethanol; DMSO: dimethyl sulphoxide.

Specific [^{3}H]flunitrazepam binding (0.9 nM, 4°C) to rat brain homogenates was not inhibited by bisindolylmaleimide 1 at 1 or 10 μM (93±13% (3), 90±17% (3), respectively, mean %±s.e.mean (n) of specific binding), nor was specific [^{3}H]flunitrazepam binding (0.4 nM) inhibited (108±10% (3) of specific binding (n)) by 1 μM bisindolylmaleimide 1 at physiological temperatures (37°C).

Discussion It is generally accepted that $GABA_A$ receptor function and expression are affected by chronic benzodiazepine exposure (Sieghart, 1995), but it is unclear how rapidly the effects of benzodiazepines occur and the molecular mechanisms involved. The results presented here show a significant decrease in α1 and β2/3 subunit protein expression in cerebellar granule cells after 1 h of exposure to flunitrazepam, suggesting the existence of a benzodiazepine-induced mechanism which can rapidly alter $GABA_A$ receptor protein levels. Furthermore, this rapid benzodiazepine-induced regulation of protein expression appears to be a protein kinase C-dependent process.

The rapid reduction in $GABA_A$ receptor α1 and β2/3 protein expression following benzodiazepine treatment is a novel observation. Previous research has tended to concentrate on chronic (days–weeks) benzodiazepine treatment times, with the earliest loss in $GABA_A$ receptor α1 protein expression noted within 2 days of benzodiazepine agonist exposure (Brown & Bristow, 1996; Johnston & Bristow, 1998). Clearly, the speed of protein reduction in this study, occurring within 10–60 min of exposure, has implications for the likely underlying mechanism, and must favour a degradation process rather than reduced synthesis. This is consistent with the unchanged α1 mRNA levels after 2 h diazepam treatment (Heninger *et al*., 1990). Interestingly, GABA exposure to chick cortical neurones also decreases $GABA_A$ receptor subunit protein expression within 2 h, and this is believed to occur by a degradation process (Calkin & Barnes, 1994).

The biochemical pathway underlying flunitrazepam-induced decrease of cellular $GABA_A$ receptor proteins is poorly understood, but chronic flunitrazepam-mediated α1 protein down-regulation seems to involve protein kinase(s) (Brown & Bristow, 1996). This study has shown that pretreatment with bisindolylmaleimide 1, a PKC inhibitor used at an appropriate and effective concentration ($100\times IC_{50}$ concentration, Toullec *et al*., 1991), was able to prevent the acute flunitrazepam-induced decrease in α1 and β2/3 protein expression. In contrast, H-89, at a concentration likely to inhibit both protein kinases A and G ($100\times IC_{50}$ at protein kinase A and $10\times IC_{50}$ at protein kinase G, Chijiwa *et al*., 1990), was ineffective.

Previous work has shown that the flunitrazepam-induced decrease in α1 subunit protein occurs in the presence of the $GABA_A$ receptor antagonist, bicuculline, and is blocked by the benzodiazepine antagonist, flumazenil, implying that the down-regulation is due to occupancy of the benzodiazepine receptor (Brown & Bristow, 1996). Bisindolylmaleimide 1 did not inhibit [^{3}H]flunitrazepam binding to rat brain membranes, even at a 10 fold greater concentration than that used here to block PKC activity in cerebellar granule cells. This suggests that the prevention of the protein loss by bisindolylmaleimide 1 is not due to a direct effect on the $GABA_A$/benzodiazepine receptor, which has been reported with other hydrophobic drugs used to modulate protein kinase activity (Moss & Smart, 1996). These observations therefore suggest a PKC-dependent pathway underlying the loss of $GABA_A$ receptor proteins, although at present the site of action is unknown.

In conclusion, we describe a decrease in $GABA_A$ receptor proteins after 1 h of flunitrazepam exposure that appears to involve a PKC-dependent mechanism. The causal role of these rapid biochemical adaptations in the development of benzodiazepine tolerance awaits further investigation.

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