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Benzodiazepine agonist and inverse agonist coupling in $GABA_A$ receptors antagonized by increased atmospheric pressure

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Abstract

Past work found that exposure to 12 times normal atmospheric pressure (ATA) of helium–oxygen gas (heliox) selectively antagonizes (uncouples) and differentiates allosteric coupling in $GABA_A$ receptors initiated by benzodiazepines versus neurosteroids. The present study tested the hypothesis that pressure can differentiate coupling initiated by a spectrum of benzodiazepine receptor ligands by measuring the effects of pressure on benzodiazepine ligand modulation of GABA-activated $^{36}Cl^-$ uptake in mouse brain membranes. 12 ATA completely antagonized allosteric modulation by: benzodiazepine receptor agonists diazepam and flunitrazepam; Type-1 selective benzodiazepine receptor agonist zolpidem and the benzodiazepine receptor partial inverse agonist ethyl-8-azido-5,6-dihydro-5-methyl-6-oxo-4H-imidazo[1,5-α][1,4]benzodiazepine-3-carboxylate (Ro15-4513). The similar, non-competitive-like characteristics of pressure antagonism of these ligands suggest common structural/functional elements underlying their coupling. Pressure also antagonized allosteric modulation by the benzodiazepine receptor inverse agonist methyl 6,7-dimethoxy-4-ethyl-β-carboline-3-carboxylate (DMCM), but the antagonism was not complete and appeared to be surmountable (competitive-like) suggesting unexpected differences in coupling for DMCM versus Ro15-4513. These studies represent the first attempt to use pressure as a tool to dissect benzodiazepine receptor coupling. The results suggest that there is a common, pressure antagonism sensitive structural/functional element underlying coupling for benzodiazepine receptor ligands and that coupling for the full inverse benzodiazepine receptor agonist DMCM differs from coupling for benzodiazepine receptor agonists and benzodiazepine receptor partial inverse agonists.

Keywords: (Swiss Webster mouse); $GABA_A$ receptor; Benzodiazepine coupling; Diazepam; DMCM; Benzodiazepine inverse agonist; Benzodiazepine Type-1 receptor agonist

1. Introduction

$GABA_A$ receptors are allosterically modulated by several classes of compounds acting at distinct recognition binding sites including sites for benzodiazepines, barbiturates and neuroactive steroids (Macdonald and Olsen, 1994; McCauley et al., 1994; Sieghart, 1995; Smith and Olsen, 1995). Allosteric modulation involves three distinct events: (1) ligand binding to the benzodiazepine recognition site, (2) transduction of the signal (coupling) to the GABA effector site and (3) alteration in GABA-gated currents (Macdonald and Olsen, 1994; McCauley et al., 1994; Smith and Olsen, 1995; Davies et al., 2001).

The benzodiazepine binding region of $GABA_A$ receptors is pharmacologically unique in that benzodiazepine receptor ligands can be arranged on a continuum according to their efficacy ranging from full agonists to full inverse agonist (Smith and Olsen, 1995; Lüddens and Korpi, 1996). Positive benzodiazepine receptor ligands (e.g., diazepam) induce their effects by increasing frequency of Cl^- channel opening without a change in the single-channel conductance, whereas negative (inverse) agonist benzodiazepine receptor ligands (e.g., methyl 6,7-dimethoxy-4-ethyl-β-carboline-3-carboxylate [DMCM]) induce their effects by decreasing channel open frequency (Rogers et al., 1994; Bianchi and Macdonald, 2001). Benzodiazepine ligands do not alter Cl^- channels in the absence of GABA (Rogers et al., 1994; Smith and Olsen, 1995).

The molecular architecture and function of benzodiazepine ligand binding regions on $GABA_A$ receptors have been

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extensively investigated, but differences in the benzodiazepine binding site do not appear to explain differences in the functional effects of benzodiazepine receptor ligands (Olsen and Tobin, 1990; Pritchett and Seeburg, 1991; Macdonald and Olsen, 1994; Smith and Olsen, 1995; Sieghart, 1995; Kelly et al., 2002). Only recently have studies begun to focus on the structural and functional bases mediating allosteric coupling induced by ligands binding to the benzodiazepine receptor site. Overall, these studies, using molecular manipulations, suggest that functional differences between the effects of benzodiazepine receptor agonists and inverse agonists may reflect subtle differences in the structural determinants underlying coupling for respective ligands (Mihic et al., 1994; Buhr et al., 1996; Boileau et al., 1998; Boileau and Czajkowski, 1999; Carlson et al., 2000; Williams and Akabas, 2000; Kelly et al., 2002).

Despite these advances, progress in understanding the role coupling plays in mediating functional differences in benzodiazepine ligands is slowed by the difficulties in manipulating coupling directly, since available pharmacological tools act via interference with binding (e.g., flumazenil), by blocking the GABA effector (e.g., bicuculline) or by blocking the channel (e.g., picrotoxin). Hence, pharmacological agents do not act directly on coupling and cannot be used in the classical manner to tease apart differences in mechanisms and structures mediating coupling.

Recent findings suggest that increased atmospheric pressure is a direct, selective antagonist of allosteric coupling that can help fill this void. These studies found that exposure to 12 times normal atmospheric pressure (12 ATA) of helium–oxygen gas (heliox) antagonized flunitrazepam and pentobarbital potentiation of GABA-activated Cl^- uptake, but did not antagonize potentiation by the neuroactive steroid, 3α-hydroxy-5β-pregnan-20-one (Davies et al., 1999, 2001). These biochemical studies also found that exposure to 12 ATA heliox did not affect benzoodiazepine receptor affinity (K_d) or the number of benzodiazepine receptors (B_{max}), thus providing evidence that pressure does not antagonize allosteric modulators by altering binding (Davies et al., 2001). Behavioral studies found similar selectivity of pressure (Davies et al., 1996, 1999). Exposure to 12 ATA heliox did not alter the effects of GABA on $GABA_A$ receptor function in the absence of allosteric modulators (Davies and Alkana, 1998; Davies et al., 1999). Collectively, these findings indicate that: (1) Pressure directly antagonizes BZ-induced allosteric modulation by uncoupling the benzodiazepine receptor binding site from the GABA effector (Davies et al., 2001) and (2) allosteric modulation of $GABA_A$ receptor function can be sub-categorized on the basis of sensitivity to pressure antagonism (Davies et al., 2001).

The present study begins to test the hypothesis that pressure can differentiate coupling initiated by a spectrum of benzodiazepine receptor ligands with different functional properties. This was accomplished by testing the effects of 12 ATA heliox versus coupling initiated by: (1) non-selective benzodiazepine receptor agonists (diazepam and flunitrazepam); (2) imidazopyridine Type-1 selective benzodiazepine receptor agonist (zolpidem); (3) imidazobenzodiazepine benzodiazepine receptor partial inverse agonist (ethyl-8-azido-5,6-dihydro-5-methyl-6-oxo-4H-imidazo[1,5-α][1,4]benzodiazepine-3-carboxylate [Ro15-4513]) and 4) β-carboline full benzodiazepine receptor inverse agonist (DMCM). The results suggest that there is a common, pressure antagonism sensitive structural/functional element underlying coupling for benzodiazepine agonist and inverse agonist ligands. Differences in characteristics of the antagonism between DMCM and the other benzodiazepine receptor ligands tested suggest that there may be subtle differences in coupling of benzodiazepine receptor full inverse agonists versus coupling for benzodiazepine receptor partial inverse agonists and agonists.

2. Materials and methods

2.1. Animals

Adult, drug naïve male Swiss Webster mice (SW mice, Simonsen Laboratories, Gilroy, CA) were housed four per cage on a 12-h light–dark cycle (0700 h on) in a room maintained at 22 ± 1 °C for at least 1 week before testing. Food (Harlan Rodent Laboratory Chow, Harlan Rodent Laboratory, Indianapolis, IN) and water were freely available until experimentation was initiated. The studies reported herein were carried out in accordance with the Declaration of Helsinki and/or with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the National Institute of Health, USA.

2.2. Experimental overview and design

On test day, mice were acclimated to the laboratory for at least 2 h prior to initiation of the experiment. The mice were decapitated, their brains were removed and a brain (less cerebellum) homogenate consisting of membrane vesicles (microsacs) was prepared. The microsacs were mixed with a $^{36}Cl^-$ reaction mixture, containing ± GABA ± benzodiazepine receptor ligand as appropriate, and were exposed to 1 ATA air (control) or 12 ATA heliox (experimental) atmospheric conditions using individual, temperature controlled, hyperbaric chambers (Davies et al., 2001). The reaction was terminated, the chamber was decompressed, followed by rapid filtration and washing of the assay preparation. Radioactivity associated with tissue retained on the filter was determined using liquid scintillation spectroscopy. Experiments were designed to minimize variability in the chloride assay procedure and to insure that order effects within an experiment did not bias the results (Davies et al., 2001). We used 12 ATA heliox in the present studies because this pressure–gas combination was used in the initial in vitro studies upon which the present experiments are based and

because extensive evidence has shown that the mechanism of antagonism at this pressure is direct (Davies and Alkana, 1998; Davies et al., 1999, 2001). We did not include a 1 ATA heliox control based on prior behavioral and biochemical studies that found no significant difference between 1 ATA air and 1 ATA heliox (Davies and Alkana, 1998; Davies et al., 1999, 2001). Ligand concentrations were selected to produce similar moderate and near maximal degrees of receptor modulation by respective ligands under control conditions (Mehta and Ticku, 1988; Harris et al., 1988; Morrow and Paul, 1988; Allan et al., 1992a; Davies et al., 2001).

2.3. Microsac preparation

The methods for preparing the microsacs were modified from those of Allan and Harris (1986b) and have been described elsewhere (Davies et al., 2001). Briefly, two unanesthetized mice were euthanized by decapitation and their brains were removed on ice and pooled. The cerebellum and white matter were dissected and discarded and the remaining portion of the brain was homogenized in 20 ml of ice-cold Tris–HEPES assay buffer (145 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 10 mM D-glucose, 1 mM $CaCl_2$, 10 mM 4-(2-hydroxyethyl)-1-piperazine-ethane-sulfonic acid, adjusted to pH = 7.5 with Tris base), centrifuged at 1000 × *g* for 15 min at 4 °C. The pellet was washed with 20 ml of ice cold buffer and again centrifuged at 1000 × *g* for 15 min at 4 °C. The final pellet was suspended in 7.5–8.5 ml ice cold Tris–HEPES buffer, which yielded a protein concentration of 3–4 mg/ml as determined later by the method of Lowry (Lowry et al., 1951).

2.4. Cl^- uptake and hyperbaric conditions

$GABA_A$ receptor function was measured using a chloride flux filtration assay, developed by Harris and Allan (Harris and Allan, 1989), and modified for use under hyperbaric conditions as described elsewhere (Davies et al., 2001). Briefly, separate 200 μl aliquots of microsacs and 200 μl aliquots of assay buffer [$^{36}Cl^-$ (1.6 μCi/ml; specific activity 14 mCi/g Cl^-) ± GABA ± diazepam or zolpidem] were pre-incubated at 35 °C for 10 min. Ro15-4513 and DMCM were pre-incubated in the microsac aliquots, rather than the assay buffer aliquots, for the final 5 min of pre-incubation. This protocol for inverse agonists was based on prior work and pilot studies in our laboratory which found that inverse agonists and antagonists require pre-incubation with the microsacs for activity (Allan and Harris, 1986a; Allan et al., 1992b; Davies and Alkana, 1998). Cl^- uptake was initiated by combining the microsacs with the $^{36}Cl^-$ assay buffer in a 75-ml hyperbaric chamber under control or experimental atmospheric conditions using premixed, certified compressed gases (SoCal Airgas, Los Angeles, CA) (Davies and Alkana, 1998). The heliox mixture consisted of 1.7% oxygen and 98.3% helium. This mixture provided a relatively normal oxygen partial pressure at 12 ATA. After 5 s, the reaction was terminated by addition of 4 ml ice-cold Tris–HEPES assay buffer containing 100 μM picrotoxin followed by decompression, when necessary, and rapid filtration of the assay preparation (within 20 s of picrotoxin addition) through a Whatman GF/C filter. The filters were washed twice and filter-bound radioactivity was measured.

2.5. Drugs and chemicals

Drug solutions were prepared daily. All BZ ligands were dissolved in dimethyl sulfoxide (DMSO) and diluted with assay buffer up to a maximum of 0.5% DMSO. GABA (ICN Biomedicals, Aurora, OH) was dissolved in Tris–HEPES buffer (145 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 10 mM D-glucose, 1 mM $CaCl_2$, 10 mM 4-(2-hydroxy ethyl)-1-piperazine-ethane-sulfonic acid, adjusted to pH = 7.5 with Tris base). DMSO to a concentration of 0.5% was added to GABA when tested in the absence of benzodiazepine receptor ligands. $^{36}Cl^-$ (specific activity 13.98 mCi/g Cl^-) was purchased from NEN Life Science Products (Boston, MA). All other drugs and reagents used were analytical grade and were supplied by Sigma (St. Louis, MO).

2.6. Statistical analysis

The data were analyzed using Prism (GraphPad Software, San Diego, CA). Two-way analysis of variance (ANOVA) was utilized to determine the effects and interactions of drug and atmospheric condition on GABA-activated Cl^- uptake. GABA-activated Cl^- uptake was defined as the amount of Cl^- taken up when 10 μM GABA was present in the medium minus the amount of Cl^- taken up when agonist was not present (Davies et al., 2001). Further analyses were conducted using one-way ANOVA followed by Bonferroni Multiple Comparisons Tests. Uptake data presented in the text and figures are expressed as mean ± S.E. The level of statistical significance was set at $P < 0.05$ for all analyses.

3. Results

3.1. Positive benzodiazepine receptor ligands

Previous in vitro work measured the effects of pressure versus flunitrazepam enhancement of $GABA_A$ receptor function in Long Sleep and C57 mice (Davies et al., 2001). To determine whether pressure antagonism of benzodiazepine receptor ligands extends to other positive benzodiazepine receptor ligands, we measured the effects of 12 ATA heliox versus diazepam and zolpidem. We first tested the effects of 12 ATA heliox versus diazepam enhancement of $GABA_A$ receptor function in SW mice. Diazepam is a commonly prescribed "classical" non-selective benzodiaze-

pine receptor agonist (Pritchett et al., 1989; Benke et al., 1996). The primary purpose of this study was to determine if the characteristics of pressure antagonism versus diazepam enhancement of $GABA_A$ receptor function are similar to those of previous in vitro findings with flunitrazepam, another non-selective benzodiazepine receptor agonist (Davies et al., 2001). A separate study testing the effects of 12 ATA heliox versus 0.5 μM flunitrazepam in SW microsacs was also conducted as a positive control. As illustrated in Fig. 1, diazepam (0.1 and 1.0 μM) significantly enhanced GABA-activated Cl^- uptake in a concentration-dependent manner compared to 10 μM GABA alone when tested under 1 ATA air control conditions. Exposure to 12 ATA heliox completely antagonized diazepam enhancement of GABA-activated Cl^- uptake (Fig. 1). In contrast, exposure to 12 ATA heliox did not significantly alter the effects of 10 μM GABA-activated Cl^- uptake, nor did pressure significantly affect basal levels of Cl^- uptake. The effects of pressure on flunitrazepam in SW microsacs were the same (data not shown) as seen with diazepam. Overall, the diazepam and flunitrazepam findings agree with previous flunitrazepam in vitro studies (Davies et al., 1999, 2001).

The effects of 12 ATA heliox versus zolpidem enhancement of $GABA_A$ receptor function are shown in Fig. 2. This study was undertaken, in part, to determine whether there

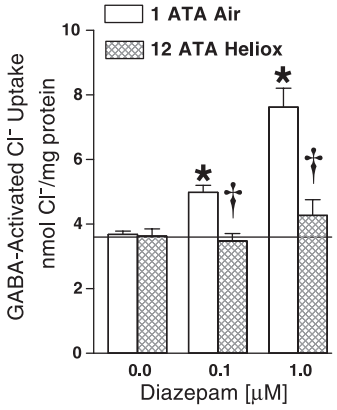

Fig. 1. Antagonism of diazepam by 12 ATA heliox. Two-way ANOVA revealed a significant main effect of atmospheric condition on GABA-activated Cl^- uptake [$F(1,26)=26.88$, $P<0.0001$]. There was also a significant main effect of drug condition [$F(2,26)=22.60$, $P<0.0001$] and a significant interaction between main effects [$F(2,26)=9.97$, $P<0.001$]. Post-hoc analyses revealed that diazepam (0.1 and 1.0 μM) potentiation of GABA-activated Cl^- uptake measured at 12 ATA heliox was not significantly different than that of diazepam (0.0 μM) potentiation of GABA-activated Cl^- uptake measured at 1 ATA air (Bonferroni multiple comparison test). Thus exposure to 12 ATA heliox completely antagonized diazepam-induced potentiation of GABA-activated Cl^- uptake. The horizontal line represents the effects of 10 μM GABA when tested at 1 ATA Air. The concentration of GABA was 10 μM for all conditions. A 5-s incubation of microsacs with $^{36}Cl^-$ (1.6 μCi/ml) was used. Basal uptake (3–5 nmol chloride/mg protein) was subtracted from the measured uptake in all conditions. Each point represents the mean ± S.E. for $n=6$ independent determinations each done in triplicate (*$P<0.05$, agonist action compared with respective 0 μM diazepam control, †$P<0.05$, antagonist action compared to respective 1 ATA Air control).

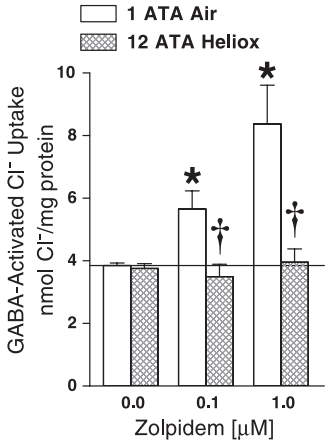

Fig. 2. Antagonism of zolpidem by 12 ATA heliox. Two-way ANOVA revealed a significant main effect of atmospheric condition on GABA-activated Cl^- uptake [$F(1,26)=16.66$, $P<0.001$]. There was also a significant main effect of drug condition [$F(2,26)=6.83$, $P<0.01$] and a significant interaction between main effects [$F(2,26)=5.43$, $P<0.01$]. Post-hoc comparisons revealed that zolpidem significantly increased GABA-activated Cl^- uptake when tested at 1 ATA air, but not when tested at 12 ATA heliox. The horizontal line represents the effects of 10 μM GABA when tested at 1 ATA Air. The other figure details are the same as presented in Fig. 1 (*$P<0.05$, agonist action compared with respective 0 μM zolpidem control, †$P<0.05$, antagonist action compared to respective 1 ATA Air control; $n=6$ per condition).

are differences in the characteristics of pressure antagonism among benzodiazepine receptor full agonists with different receptor subtype specificity's. Zolpidem is a selective benzodiazepine receptor Type-1 agonist. Zolpidem (0.1 and 1.0 μM) significantly enhanced GABA-activated Cl^- uptake in a concentration-dependent manner under 1 ATA air control conditions (Fig. 2). Exposure to 12 ATA heliox completely antagonized zolpidem enhancement of GABA-activated Cl^- uptake. As in the diazepam experiment, exposure to 12 ATA heliox did not significantly alter the effects of 10 μM GABA-activated Cl^- uptake (0 μM zolpidem), nor did pressure significantly affect basal levels of Cl^- uptake. The findings indicate that the characteristics of the antagonism by pressure of zolpidem are similar to those found for diazepam (Fig. 1) and for flunitrazepam in the present and in previous studies (Davies et al., 2001). Collectively, the findings suggest that "classical" non-selective benzodiazepine receptor agonists and selective Type-1 benzodiazepine receptor agonists have a common, pressure antagonism sensitive mechanism of coupling.

3.2. Negative benzodiazepine receptor ligands

To determine whether pressure antagonism of benzodiazepine receptor ligands extends to negative benzodiazepine receptor ligands, we measured the effects of 12 ATA heliox versus the benzodiazepine receptor partial inverse agonist, Ro15-4513, and the benzodiazepine receptor full inverse agonist, DMCM. As depicted in Fig. 3, Ro15-4513 significantly decreased GABA-activated Cl^- uptake compared to

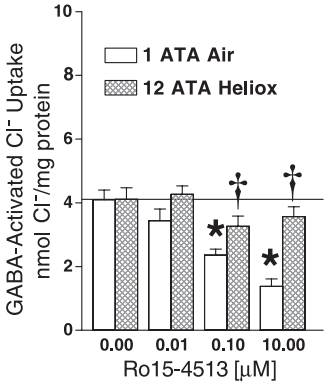

Fig. 3. Antagonism of Ro15-4513 by 12 ATA heliox. Two-way ANOVA revealed a significant main effect of atmospheric condition on GABA-activated Cl^- uptake [$F(1,27)=19.05$, $P<0.001$]. There was also a significant main effect of drug condition [$F(3,27)=12.53$, $P<0.0001$] and a significant interaction between main effects [$F(3,27)=3.83$, $P<0.05$]. Post-hoc analyses revealed that Ro15-4513 (0.1 and 10.0 μM) inhibition of GABA-activated Cl^- uptake measured at 12 ATA heliox was not significantly different than that of Ro15-4513 (0.0 μM) potentiation of GABA-activated Cl^- uptake measured at 1 ATA air (Bonferroni multiple comparison test). Thus exposure to 12 ATA heliox completely antagonized Ro15-4513-induced inhibition of GABA-activated Cl^- uptake. The horizontal line represents the effects of 10 μM GABA when tested at 1 ATA Air. See Fig. 1 legend for other figure parameters (*$P<0.05$, inverse agonist action compared with respective 0 μM Ro15-4513 control, †$P<0.05$, antagonist action compared to respective 1 ATA Air control; $n=6$ per condition).

10 μM GABA alone in a concentration-dependent manner under 1 ATA control conditions. In agreement with its effects on the positive benzodiazepine receptor agonists tested, exposure to 12 ATA completely antagonized inhibition of GABA-activated Cl^- uptake by 0.1 and 10 μM Ro15-4513 without altering the effects of 10 μM GABA or baseline functions of the receptor.

DMCM also significantly decreased GABA-activated Cl^- uptake in a direct, concentration-dependent manner compared to 10 μM GABA alone when tested under 1 ATA air control conditions (Fig. 4). This direct relationship between DMCM concentration and inhibition of GABA-activated Cl^- uptake agrees with prior biochemical studies (Morrow and Paul, 1988), but differs from electrophysiological studies in recombinant $GABA_A$ receptors (Sigel et al., 1990; Im et al., 1995; Stevenson et al., 1995). The latter studies have shown a concentration-dependent switch from inverse agonist activity to agonist activity. These concentration-related differences in the effects of DMCM on $GABA_A$ receptor function most likely reflect the differences in resolution between in vitro biochemical assays, which measure the net effect of the modulator on a spectrum of receptor subtypes, versus electrophysiological studies in recombinant receptors that measure changes in defined receptor populations.

Exposure to 12 ATA heliox significantly antagonized the inhibiting effects of DMCM on GABA-activated Cl^- uptake. The degree of pressure antagonism was inversely related to the concentration of DMCM tested. That is,

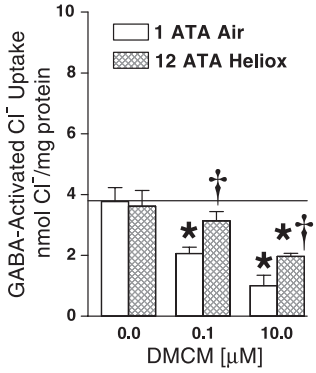

Fig. 4. Antagonism of DMCM by 12 ATA heliox. Two-way ANOVA revealed a significant main effect of atmospheric condition on GABA-activated Cl^- uptake [$F(1,26)=4.69$, $P<0.05$]. There was also a significant main effect of drug condition [$F(2,26)=19.90$, $P<0.0001$]). Post-hoc analysis revealed significant inverse agonist actions by DMCM at 0.1 and 10μM when tested at 1 ATA air, but only at 10 μM when tested at 12 ATA heliox. The horizontal line represents the effects of 10 μM GABA when tested at 1 ATA Air. Other figure details are the same as in Fig. 1 (*$P<0.05$, inverse agonist action compared with respective 0 μM DMCM control, †$P<0.05$, antagonist action compared to respective 1 ATA Air control; $n=6$ per condition).

exposure to 12 ATA heliox completely antagonized 0.1 μM DMCM, but only partially antagonized 10.0 μM DMCM (Fig. 4). The inverse relationship of pressure antagonism and DMCM concentration suggests that pressure antagonism of DMCM is surmountable by increasing DMCM concentration. As in the studies described above, pressure did not affect receptor function or response to

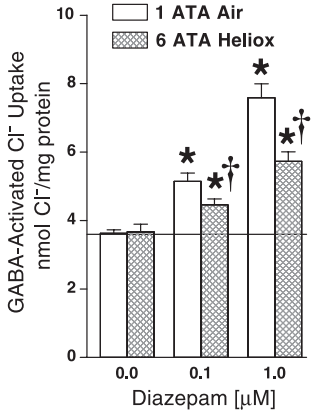

Fig. 5. Antagonism of diazepam by 6 ATA heliox. Two-way ANOVA revealed a significant main effect of atmospheric condition on GABA-activated Cl^- uptake [$F(1,34)=16.07$, $P<0.001$]. There was also a significant main effect of drug condition [$F(2,34)=71.48$, $P<0.0001$]) and a significant interaction between main effects [$F(2,34)=7.12$, $P<0.01$]. Post-hoc analysis revealed that diazepam significantly potentiated GABA-activated Cl^- uptake when tested at 1 ATA air and 6 ATA heliox and that exposure to 6 ATA heliox significantly antagonized diazepam at both concentrations. The horizontal line represents the effects of 10 μM GABA when tested at 1 ATA Air. Other figure details are the same as in Fig. 1 (*$P<0.05$, agonist action compared with respective 0 μM diazepam control, †$P<0.05$, antagonist action compared to respective 1 ATA Air control; $n=6$ per condition).

GABA in the absence of DMCM. Taken together, the findings with DMCM and other benzodiazepine receptor ligands indicate that the characteristics of the antagonism by pressure of DMCM (Fig. 4) differ from those of benzodiazepine receptor agonists (Figs. 1 and 2) and benzodiazepine receptor partial inverse agonists (Fig. 3).

3.3. Pressure-dependent antagonism of allosteric modulation

We tested the effects of a lower pressure (6 ATA heliox) on diazepam enhancement of $GABA_A$ receptor function to determine if the magnitude of pressure antagonism is pressure (concentration)-dependent. As illustrated in Fig. 5, exposure to 6 ATA heliox significantly antagonized diazepam enhancement of GABA-activated Cl^- uptake without significantly affecting $GABA_AR$ function. As with 12 ATA, the degree of antagonism by 6 ATA heliox was greater at the high versus the low concentration of diazepam indicating that the antagonism by pressure is not surmountable. On the other hand, pressure did not completely antagonize diazepam and the magnitude of pressure antagonism by 6 ATA heliox (Fig. 5) was less than the magnitude of antagonism found with 12 ATA heliox (Fig. 1). Taken together, the results at 6 and 12 ATA indicate that the magnitude of pressure antagonism is directly related to the pressure applied.

4. Discussion

The current studies represent the first attempt to use pressure as a tool to dissect benzodiazepine receptor coupling. These findings indicate that pressure antagonized allosteric coupling by a spectrum of benzodiazepine receptor ligands with different functional properties. This ability of pressure to antagonize all benzodiazepine receptor ligands tested suggests that there is a common, pressure antagonism sensitive structural/functional element underlying coupling for benzodiazepine receptor agonists, benzodiazepine receptor partial inverse agonists and benzodiazepine receptor full inverse agonists. The characteristics and pattern of antagonism by 12 ATA heliox among the family of benzodiazepine receptor ligands also suggests similarities and differences in coupling among benzodiazepine receptor ligands.

Exposure to 12 ATA heliox completely antagonized flunitrazepam, diazepam, zolpidem and Ro15-4513 induced potentiation or inhibition of GABA-activated Cl^- uptake, respectively, at the moderate and maximally effective concentrations tested. The ability of pressure to completely antagonize these agents at maximally effective concentrations (Mehta and Ticku, 1988; Harris et al., 1988, 1995; Allan et al., 1992a) indicates that antagonism by 12 ATA of these agents is complete and is not surmountable. This conclusion is supported by prior demonstration that pressure completely antagonizes flunitrazepam at 10 times its maximum effective concentration (Davies et al., 2001). The non-surmountability of pressure antagonism of these ligands fits expectations of antagonists acting in a non-competitive-like manner. This is not meant to suggest that pressure acts via the traditional mechanisms associated with competitive and non-competitive models. The comparison is made to put the present findings in the context of other antagonist–agonist relationships. The similar characteristics in response to 12 ATA heliox of diazepam, flunitrazepam, zolpidem and Ro15-4513 at maximal effective concentrations suggest similar pressure antagonism sensitive coupling for these ligands.

Differences between the effects of 6 ATA and 12 ATA heliox on diazepam provide further insight into the characteristics of pressure antagonism of benzodiazepine receptor agonists. The results are similar to those at 12 ATA with one exception. As with 12 ATA, 6 ATA heliox antagonized diazepam at 0.1 and at 1.0 μM and the degree of antagonism was greater at the high than at the lower concentration of diazepam indicating that the antagonism by 6 ATA is not surmountable. The primary difference was that 6 ATA did not completely antagonize the effects of diazepam at either concentration. This suggests that pressure antagonism of benzodiazepine receptor agonists is not an all or none phenomenon, but appears to vary directly with the magnitude of pressure.

Exposure to 12 ATA heliox also antagonized the effects of the benzodiazepine receptor full inverse agonist DMCM on GABA-activated Cl^- uptake. The antagonism occurred from 0.1 to 10 μM. Prior electrophysiological studies suggest that the action of DMCM can switch from inverse agonist to agonist as the DMCM concentration increases (Sigel et al., 1990; Im et al., 1995; Stevenson et al., 1995), suggesting that such a switch might be interpreted as pressure antagonism of DMCM at the high concentration. However, in agreement with prior biochemical findings (Morrow and Paul, 1988), DMCM inhibited GABA-activated Cl^- uptake in a direct concentration-dependent manner in the current study. Hence, pressure antagonism of DMCM in the present work cannot be attributed to changes in the action of DMCM.

In contrast to the effects of 12 ATA heliox on the other benzodiazepine receptor ligands tested, the degree of DMCM antagonism decreased as the concentration of inverse agonist increased from 0.1 to 10 μM. This DMCM concentration-related decrease in the efficacy of pressure antagonism indicates that pressure antagonism of DMCM is surmountable. The inverse relationship between DMCM concentration and pressure antagonism fits a competitive model for the antagonism. Collectively, these findings suggest that coupling for the full inverse agonist DMCM differs from coupling for benzodiazepine receptor agonists and benzodiazepine receptor partial inverse agonists.

DMCM is a β-carboline that differs structurally from the other tested compounds. Hence, the differences in effects of

pressure may reflect the structural differences between the agents. Future studies focused on investigating the effects of pressure on β-carboline agonists (e.g., abecarnil) can test this hypothesis.

Electrophysiological studies suggest another possible explanation for the lack of complete antagonism by pressure of DMCM. This work indicates that DMCM can interact with $GABA_A$ receptors via at least two separate sites: a high affinity site at nM concentrations and a low affinity site at μM concentrations (Sigel et al., 1990; Im et al., 1995; Stevenson et al., 1995). Therefore, the incomplete antagonism by pressure of DMCM could be explained by inverse agonist action of DMCM on two sites, one of which is pressure antagonism sensitive, and one of which is not.

Interestingly, the results of 6 ATA heliox with diazepam suggest that DMCM might be completely antagonized by pressures greater than 12 ATA. If this were the case, it would bring the characteristics of pressure antagonism of DMCM closer to those of benzodiazepine receptor agonists and Ro15-4513. Testing higher pressure is beyond our capability at the present time. Regardless, even if testing at higher pressure completely antagonized the effects of DMCM, this demonstration would not change the evidence that pressure antagonism is surmountable for DMCM, but is not surmountable for the other ligands.

The molecular mechanism by which hyperbaric heliox antagonizes benzodiazepine receptor ligands is not yet fully understood. Nonetheless, considerable evidence points to a direct mechanism of antagonism targeting coupling (transduction) of the signal initiated by binding of benzodiazepine receptor ligands. First, hyperbaric exposure did not alter high affinity benzodiazepine receptor binding (Davies et al., 2001). Second, several lines of evidence indicate that the mechanism of antagonism of allosteric modulation does not result from pressure-induced changes in the GABA effector including: (1) Responsiveness of $GABA_A$ receptors to GABA; (2) Properties of the $GABA_A$ receptor chloride ion channel and (3) Cl^- levels in the absence of exogenous GABA (basal uptake) (Davies and Alkana, 1998; Davies et al., 1999, 2001). Taken together, these findings support the hypothesis that hyperbaric heliox antagonizes benzodiazepine receptor ligands by uncoupling the benzodiazepine receptor binding site from the $GABA_A$ receptor effector.

Findings from several laboratories indicate that the ability of hyperbaric heliox to uncouple benzodiazepine receptor binding from the $GABA_A$ receptor effector results from the action of pressure per se not from a pharmacological effect of helium or oxygen. This work found that 1 ATA helium does not significantly affect allosteric modulators of $GABA_A$ receptor function (Davies et al., 1996, 1999, 2001; Davies and Alkana, 1998) or other anesthetics (Dodson et al., 1985; Wann and MacDonald, 1988). Moreover, exposure to 4 and 6 ATA 100% oxygen antagonized the behavioral effects of ethanol, another allosteric modulator of $GABA_A$ receptor function (Alkana and Malcolm, 1980). This antagonism by oxygen was not mediated by increased oxygen partial pressure or oxygen-induced changes in ethanol pharmacokinetics (Alkana and Malcolm, 1980, 1982). Therefore, the antagonism by 12 ATA heliox in the latter experiments cannot be due to a specific action of hyperbaric helium, since antagonism by pressure also occurred in the absence of helium. These findings demonstrate pressure antagonism of allosteric modulation of $GABA_A$ receptor function is not related to the presence of helium or to the oxygen partial pressure.

In contrast to traditional pharmacological antagonists, the differences in response to pressure among benzodiazepine receptor allosteric modulators in the present study cannot be based on structure activity relationships between pressure and recognition binding sites since pressure is a physical force that does not have a chemical structure. Rather, these differences must be based on the ability of pressure to block or offset common physical–chemical changes underlying allosteric coupling initiated by ligand binding to recognition sites on $GABA_A$ receptors (Davies et al., 2001; Davies and Alkana, 2001). Physical–chemical changes reflect the underlying molecular structure. Therefore, the similarities and differences in the characteristics of pressure antagonism among benzodiazepine receptor ligands suggest that pressure can be used to identify coupling processes that have similar pressure antagonism sensitive structural/functional elements (Davies et al., 2001).

These findings with pressure compliment and extend recent molecular studies suggesting differences in coupling initiated by benzodiazepine receptor agonist and the benzodiazepine receptor inverse agonist DMCM. This work in other laboratories has begun to switch from a focus on investigating the molecular architecture and function of the benzodiazepine receptor ligand binding region of $GABA_A$ receptors to study the structural and functional bases mediating allosteric coupling. These studies provided insight into the possible structures and mechanisms underlying allosteric coupling processes (Mihic et al., 1994; Buhr et al., 1996; Carlson et al., 2000; Williams and Akabas, 2000; Lynch et al., 1997; Kelly et al., 2002). Further studies, using chimeras and point mutations, found structural determinants in the TM2–TM3 regions of $GABA_A$ receptors that were necessary for coupling of benzodiazepine receptor agonists (Boileau et al., 1998; Boileau and Czajkowski, 1999). In contrast, these regions were not necessary for coupling of the benzodiazepine receptor inverse agonist DMCM, which suggests, in agreement with the present findings, that DMCM is coupled through different structural elements than is coupling for benzodiazepine receptor agonists. Taken together, these molecular and hyperbaric findings suggest that a site of pressure antagonism of benzodiazepine receptor coupling may lie in the TM2–TM3 region of $GABA_A$ receptors.

Surprisingly, the characteristics of pressure antagonism for the benzodiazepine receptor partial inverse agonist Ro15-4513 did not fit expectations based on its pharmacological end point. That is, the pattern of the antagonism

resembled the pattern seen with the full benzodiazepine receptor agonists diazepam and flunitrazepam, but differed from the characteristics of the antagonism of the benzodiazepine receptor full inverse agonist, DMCM. On a pharmacological basis, this is unexpected, since one would predict that the characteristics of the antagonism for coupling of a partial inverse agonist would more closely resemble those of a full inverse agonist as opposed to resembling coupling for benzodiazepine receptor agonists.

Taken together, these findings suggest that there are subtle structural/functional differences underlying benzodiazepine receptor coupling for benzodiazepine receptor ligands that cannot be differentiated on the bases of pharmacological response alone. These differences could reflect action by DMCM on one or more sites with differences in the structural elements that mediate coupling, which are not shared by benzodiazepine receptor agonists and partial inverse agonists. Further studies, which more completely test the effects of pressure versus Ro15-4513 and DMCM, as well as other benzodiazepine receptor partial inverse and full inverse agonists, are necessary before conclusions can be drawn and to determine whether the present finding generalize across the spectrum of benzodiazepine receptor ligands.

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