Alcohol, Vol. 19, No. 3, pp. 197–205, 1999

0741-8329/99/$–see front matter

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PII S0741-8329(99)00040-3

Furosemide Action on Cerebellar $GABA_A$ Receptors in Alcohol-Sensitive ANT Rats

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Received 5 October, 1998; Accepted 4 May, 1999

MÄKELÄ, R., M. UUSI-OUKARI, S. S. OJA, H. ALHO, I. ANGHELESCU, C. KLAWE, H. LÜDDENS AND E. R. KORPI. *Furosemide action on cerebellar $GABA_A$ receptors in alcohol-sensitive ANT rats.* ALCOHOL **19**(3) 197–205, 1999.—Furosemide increases the basal *tert*-[^{35}S]butylbicyclophosphorothionate ([^{35}S]TBPS) binding and reverses the inhibition of the binding by γ-aminobutyric acid (GABA) in the cerebellar $GABA_A$ receptors containing the α6 and β2/β3 subunits. These effects are less pronounced in the alcohol-sensitive (ANT) than in the alcohol-insensitive (AT) rat line. The difference between the rat lines in the increase of basal [^{35}S]TBPS binding was removed after a longer preincubation with ethylendiaminetetraacetic acid (EDTA) containing buffer, but long preincubation did not reduce the GABA content of the incubation fluid or remove the difference in GABA antagonism by furosemide. The GABA sensitivity of the [^{35}S]TBPS binding did not differ between the rat lines. There was no nucleotide sequence difference in the β2 or β3 subunits between the rat lines and similar β2/3 subunit-dependent agonistic actions by methyl-6,7-dimethoxy-4-ethyl-β-carboline-3-carboxylate (DMCM) in the rat lines were detected. The data suggest that there are still unknown structural alterations in the cerebellar $GABA_A$ receptors between the AT and ANT rat lines, possibly associated with differential alcohol sensitivity. © 1999 Elsevier Science Inc. All rights reserved.

$GABA_A$ receptors Furosemide Methyl-6,7-dimethoxy-4-ethyl-β-carboline-3-carboxylate (DMCM) Cerebellum Selected rat lines Autoradiography

γ-AMINOBUTYRIC acid type A ($GABA_A$) receptors are ligand-gated ion channels that mediate most of the fast inhibitory neurotransmission in the mammalian brain. Nineteen subunits (α1–6, β1–4, γ1–3, δ, γ1–3, ϵ and π) are presently known to participate in the formation of the $GABA_A$ receptors in the brain (2,18,28). $GABA_A$ receptors are targets for many pharmacologically and clinically important drugs; e.g., benzodiazepines. The loop diuretic furosemide has recently been found to be the first $GABA_A$ receptor subtype-specific antagonist. It specifically antagonizes α6β2/3γ2 receptor combination that is largely expressed in the granule cell layer of cerebellum (14,15,17,33).

Alcohol-sensitive (ANT) rat line, selectively outbred for high sensitivity to the motor-impairing effects of an acute, moderate dose (2 g/kg) of ethanol (4), shows also increased behavioral sensitivity to benzodiazepines, such as diazepam and lorazepam as compared to alcohol-insensitive (AT) rat line (7,37). Cerebellar $GABA_A$ receptors of female and male ANT rats have been shown to be less sensitive to the actions of furosemide than those of female and male AT rats in increasing the basal *tert*-[^{35}S]butylbicyclophosphorothionate ([^{35}S]TBPS) binding to the ionophore-binding sites and reversing the GABA-induced inhibition of the [^{35}S]TBPS binding (20). In the AT rats, the actions of furosemide were comparable to those in the Wistar rats (14). Cerebellar α6 subunit-containing $GABA_A$ receptors display also an abnormal benzodiazepine pharmacology in the ANT rats. The binding of ^{3}H-labeled ethyl-8-azido-5,6-dihydro-5-methyl-6-oxo-4H-imidazo[1,5-a][1,4]benzodiazepine-3-carboxylate ([^{3}H]Ro 15-4513) to the cerebellar α6 subunit-containing "diazepam-insensitive" (DIS) benzodiazepine site (23) in the ANT rats is about 100-fold more sensitive to benzodiazepine agonists

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than the binding in the AT rat samples (31,32). This difference in the amount of DIS ([^{3}H]Ro 15-4513) binding has been explained by an amino acid exchange from arginine (R) to glutamine (Q) in the position 100 of the $GABA_A$ receptor $\alpha 6$ subunit in ANT rats, caused by a single point mutation (13). However, the point mutation in the putative benzodiazepine binding site has been excluded as a cause for the blunted furosemide sensitivity of ANT rats using recombinant $GABA_A$ receptors (20). Since direct evidence is lacking that this mutation explains anything else than the enhanced benzodiazepine sensitivity of the ANT rats, other $GABA_A$ receptor alterations might be involved in the mechanism of alcohol sensitivity, perhaps being also the reason for diminished furosemide antagonism.

Because furosemide is one of the few compounds displaying highly subtype-selective action on $GABA_A$ receptor, dissolving its mechanisms of action would also be beneficial in explaining the function of a single $GABA_A$ receptor subtype. Therefore, in the present study, we examined in detail possible mechanisms of action of furosemide on $GABA_A$ receptors in cerebellar sections of the AT and ANT rat lines.

METHOD

Animals

Adult female and male ANT and AT rats, developed by selective outbreeding for high and low sensitivity to motor-impairing effects of a moderate dose of ethanol (2 g/kg, intraperitoneally) (4), from the F_{50}, F_{52} and F_{54} generations were maintained in groups of four to six animals in stainless steel wire cages under a 12/12 h light/dark cycle (lights on at 6:00 A.M.) at an ambient temperature of 22 ± 2°C and a relative humidity of 55 ± 5%. The rats had free access to RM1E rodent feed (SDS, Witham, England) and tap water. A total number of 25 female and 33 male ANT and 25 female and 33 male AT rats were used.

Preparation of Cryostat Sections

The rats were killed by decapitation and the whole brains were rapidly dissected out. The brains were frozen as a whole, or the cerebella were dissected out, cut into halves, and frozen on dry ice. From the whole brains and the halves of the cerebella, 14 μm coronal and sagittal cryostat sections, respectively, were cut using a Leitz 1720 cryostat and mounted onto gelatin-coated slides for autoradiography (14) and dried at room temperature.

For most of assays four sections (two ATs and two ANTs), were cut on each object glass. For determining endogenous GABA concentrations, five AT or ANT sections were cut onto each object glass.

Autoradiography

The autoradiographic procedures for regional localization of [^{35}S]TBPS and [^{3}H]Ro 15-4513 binding were as previously described in detail (3,14). Briefly, serial cryostat sections were preincubated in an ice-water bath for 15 min in 50 mM Tris-HCl buffer supplemented with 120 mM NaCl, pH 7.4, (normal preincubation) for [^{35}S]TBPS and [^{3}H]Ro 15-4513 autoradiography assays. In some [^{35}S]TBPS experiments, sections were preincubated in an ice-water bath three times for 10 min in 50 mM Tris-HCl supplemented with 1mM ethylendiaminetetraacetic acid (EDTA), pH 7.4 (long preincubation) (21). Incubations with 6 nM [^{35}S]TBPS (DuPont de Nemours-New England Nuclear, Dreieich, Germany), supplemented with 5.25 nM cold TBPS, were performed for 90 min at room temperature in the 50 mM Tris-HCl, 120 mM NaCl buffer, pH 7.4, using 750 μl liquid bubbles over sections on object glasses in a humid chamber. Incubations with 8 nM [^{3}H]Ro 15-4513 (DuPont de Nemours) were performed for 60 min at 0–4°C in the preincubation buffer. The effects of 1–50 μM bicuculline, 1–50 μM 2′-(3′-carboxy-2′,3′-propyl)-3-amino-6-p-methoxyphenylpyrazinium (SR 95531; Research Biochemicals, Natick, MA), 100 nM–10 μM GABA, 30 μM–1 mM furosemide (Sigma Chemical Co., St Louis, MO), 1 and 30 μM methyl-6,7-dimethoxy-4-ethyl-β-carboline-3-carboxylate (DMCM; Research Biochemicals) were tested on [^{35}S]TBPS binding, whereas effects of 10 μM and 100 μM diazepam (Orion Pharmaceutica, Espoo, Finland) were tested on [^{3}H]Ro 15-4513 binding. Nonspecific binding was determined with 10 μM ethyl-8-fluoro-5,6-dihydro-5-methyl-6-oxo-4H-imidazo[1,5-a][1,4]benzodiazepine-3-carboxylate (flumazenil, Ro 15-1788) (F. Hoffmann-La Roche, Basel, Switzerland) and 10 μM picrotoxinin (Sigma) in [^{3}H]Ro 15-4513 and [^{35}S]TBPS autoradiography, respectively. After the incubation, the sections were washed three times for 15 s in an ice-cold 10 mM Tris-HCl buffer, pH 7.4, dipped into distilled water, air-dried at room temperature, and exposed with plastic ^{3}H- or ^{14}C-standards to Hyperfilm-^{3}H or Hyperfilm-β_{max} autoradiographic films (Amersham, Buckinghamshire, U.K.), respectively, for five days to six weeks.

Thionin Staining

Cerebellar sections of AT and ANT rat brains were stained in 0.1% (w/v) thionin (Sigma) solution for 2 min. Excess of thionin was removed by incubating the sections in ascending concentrations of ethanol. The incubation in Histo-Clear (National Diagnostics, Atlanta, GA) was followed by drying the sections at room temperature and covering them with coverslips.

Sequencing

For sequencing of the β1, β2 and β3 subunits of the ANT and AT rats, total RNA was prepared by the single step guanidinium thiocyanate method (1) from the halves of the AT and ANT cerebella. Poly(A)$^+$-RNA was prepared batchwise from the total RNA embloying oligo(dT) cellulose. Total RNA was heated to 70°C for 10 min and the solution was adjusted to 0.5 M LiCl before adding the buffer-equilibrated oligo(dT) cellulose. After incubation the cellulose was washed by centrifugation and the poly(A)$^+$-RNA eluted with 2 mM EDTA/0.1% sodium dodecylsulfate and reapplied to regenerated oligo(dT) cellulose. The RNA was precipitated, and the quality was controlled on a 1% agarose gel. Heat-denatured poly(A)$^+$-RNA was reversly transcribed for 90 min at 42°C using an oligo(dT) primer and avian myeloblastoma-virus reverse transcriptase. Produced single-strand DNA was purified and precipitated. The first strand was converted to double-standed DNA with *E. Coli* DNA polymerase and *E. Coli* ligase, for 16 h at 14°C, and purified and precipitated. Cycle sequencing with subunit-specific primers covering the whole coding area of the β1, β2 and β3 subunits of the rat lines and most of the published 5′- and 3′-adjacent noncoding areas was performed on a Perkin Elmer 470 sequencer.

Amino Acid Analysis

For GABA assays 200-μl samples of autoradiographic incubation media were mixed with 20 μl of 50% sulphosalicylic acid containing L-2,4-diaminobutyric acid as internal stan-

dard. The mixtures were left for 1 h at room temperature and then centrifuged to precipitate any proteins present. Saturated LiOH (10 μl) were added to 120-μl samples from the supernatants and mixed with 70 μl of lithium citrate buffer (pH 2.2). The solutions were filtrated and 30 μl samples from the filtrates subjected to ion-exchange chromatography using an automatic Pharmacia LKB Alpha Plus amino acid analyzer (Cambridge Medical Diagnostics, U.K.). The assays were based on post-column derivatization with o-phthalaldehyde and fluorescense detection with the highest sensitivity usable.

Data Analyses

Regional labeling intensities were quantitated from the autoradiography films using MCID M4 image analysis devices and programs (Imaging Research, St. Catharines, Canada) as earlier described (14,20). Binding densities for granule cell and molecular layers of each cerebellum were averaged from four to six measurements. The plastic radioactivity standards exposed simultaneously with the brain sections were used as a reference with the resulting binding values converted to radioactivity levels standardized for gray matter areas [nCi/mg (1 nCi/mg = 0.37 pBq/kg) for ^3H and nCi/g (1 nCi/g = 0.37 fBq/kg) for ^{14}C].

The proportional area of the granule cell layer of the whole cerebellar area was measured with the same image analysis system from the thionin stained sagittal sections using area calibration with the MCID reticule and intensity limits for separating the dark staining of the granule cell layer from the lighter staining of the other cerebellar regions.

Statistical significances of the differences between the rat lines were assessed using Student's *t*-test or two-way ANOVA with the Prism 2 program (GraphPAD Software, San Diego, CA), as were the Pearson correlation coefficients.

RESULTS

Furosemide, Bicuculline and SR 95531 Actions on Cerebellar Sections of the AT and ANT Rats

Using ligand binding technique on cerebellar membranes we have shown earlier that the ability of furosemide to enhance basal [^{35}S]TBPS binding and to antagonize GABA-induced inhibition of [^{35}S]TBPS binding is blunted in ANT rat membranes as compared to AT membranes (20). To dissect out the contribution of the cerebellar granule cell and molecular layers to these effects, we now used [^{35}S]TBPS autoradiography on cerebellar sections of AT and ANT rats to study actions of furosemide. [^{35}S]TBPS labeled the granule cell and molecular layers intensively in both AT and ANT cerebellar sections. In contrast to our earlier results with the AT and the ANT cerebellar membranes (20), the basal [^{35}S]TBPS binding was slightly but significantly ($p < 0.01$) lower to the granule cell layer of the ANT rat cerebellar sections than to those of the AT sections (305 ± 29 nCi/g versus 257 ± 17 nCi/g, mean ± SD, n=8, for AT and ANT rats, respectively). The binding to the cerebellar molecular layer did not differ between the rat lines (186 ± 16 nCi/g and 200 ± 16 nCi/g, $n = 8$, for AT and ANT rats, respectively). The difference ($p < 0.001$) between rat lines in the basal [^{35}S]TBPS binding to granule cell layer was confirmed with the cerebellar sections cut from nonhomogenized halves of the same female rat cerebella we used in our previous homogenate binding study (20) (215 ± 28 nCi/g versus 176 ± 21 nCi/g, n = 25, for AT and ANT rats, respectively). Furosemide (30 μM–1 mM) increased the basal [^{35}S]TBPS binding in granule cell layers less effectively, $F(1,56) = 10.91$, $p = 0.0017$ by two-way ANOVA, $n = 8$, in the ANT than in the AT rat line (Fig. 1A). In the molecular layer, there was no difference in the action of furosemide between the rat lines (Fig. 1B).

For comparison, we tested the effects of SR 95531 and bicuculline, two competitive GABA site antagonists (6), on the basal [^{35}S]TBPS binding in the cerebella of the AT and the ANT rats. SR 95531 tended to be $F(1,40) = 5.19$, $p = 0.0281$ by two-way ANOVA, $n = 8$, and bicuculline was clearly $F(1,42) = 8.81$, $p = 0.0049$, $n = 8$, more effective in increasing the basal binding in the AT than the ANT granule cell layer (Fig. 1C, 1E). In contrast, SR 95531 did not affect and bicuculline similarly inhibited the binding to molecular layers of the rat lines (Fig. 1D, 1F).

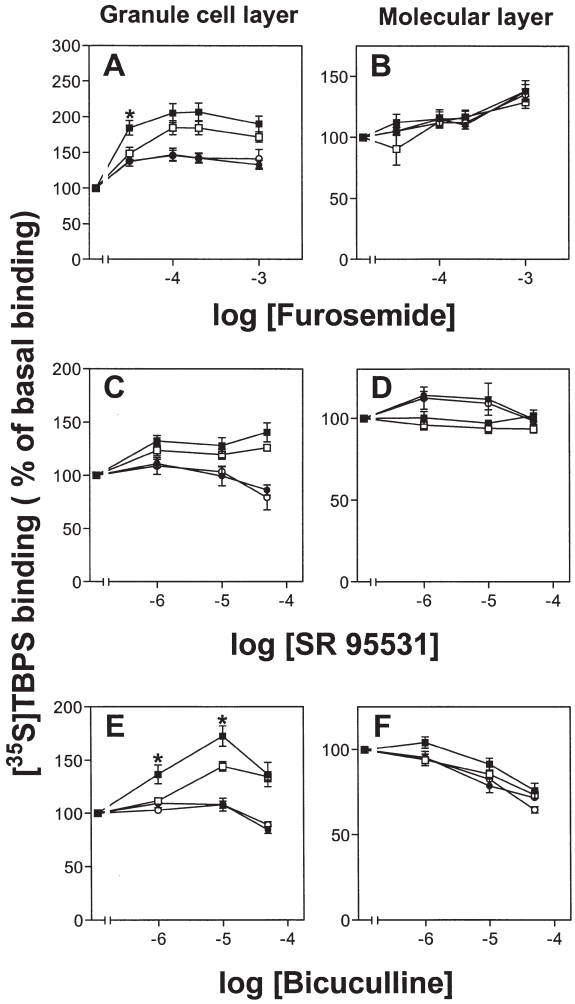

FIG. 1. Effects of furosemide, SR 95531 and bicuculline on the picrotoxinin-sensitive [^{35}S]TBPS binding in cerebellar sections of male AT (■, ●) and ANT (□, ○) rats determined in standard (■, □) and long (●, ○) preincubation conditions. The results are expressed as percentages (mean ± SE of eight animals in each group) of basal binding determined in the absence of added furosemide, SR 95531 or bicuculline (100 %). The results are shown for the granule cell layer (A, C, E) and for the molecular layer (B, D, F). Values to the left of the gaps were obtained in the absence of added drugs. Statistical significance of the difference between the rat lines (Student's *t*-test): $*p < 0.05$.

Effect of Long Preincubation With EDTA-Containing Buffer on Antagonist Actions

As the enhancement of the basal [^{35}S]TBPS binding by furosemide, SR 95531 and bicuculline is most likely due to antagonism of endogenous GABA still present in the sections, we performed an autoradiography after long preincubation with EDTA-containing buffer (21) to remove most of the GABA (3,29). This assay was performed in parallel to the normal assay using adjacent brain sections and the same incubation solutions.

The long preincubation did not affect the difference ($p < 0.01$) in the basal [^{35}S]TBPS binding between the rat lines, but it increased the amount of the binding to the granule cell layer (357 ± 39 nCi/g and 292 ± 40 nCi/g, $n = 8$, for AT and ANT rats, respectively). There was no difference in the basal [^{35}S]TBPS binding to the molecular layer of AT and ANT cerebella (159 ± 9 nCi/g and 163 ± 13 nCi/g for AT and ANT rats, respectively). The rat line difference ($p < 0.001$) in the basal [^{35}S]TBPS binding to granule cell layer was confirmed with another set of cerebellar sections from male AT and ANT rat brains that were also preincubated three times for 10 min with the EDTA-containing buffer (298 ± 44 nCi/g versus 260 ± 24 nCi/g, $n = 25$, for AT and ANT rats, respectively).

The level of increase of the basal [^{35}S]TBPS binding by furosemide decreased from about 180% to 140%. Simultaneously, the difference between rat lines was abolished [$F(1, 56) = 0.13$, $p = 0.7148$ by two-way ANOVA ($n = 8$)] (Fig. 1A). In the molecular layer, there was no difference in the effects of furosemide between the rat lines or the preincubation conditions (Fig. 1B). Both SR 95531 and bicuculline inhibited ($p < 0.001$) the basal [^{35}S]TBPS binding to granule cell layers of both rat lines and there was no difference between rat lines (Fig. 1C, 1E). In the molecular layer of both rat lines, SR 95531 did not affect and bicuculline inhibited the basal [^{35}S]TBPS binding (Fig. 1D, 1F).

We also studied the ability of furosemide to antagonize the inhibition of [^{35}S]TBPS binding by GABA in cerebellar sections treated with long preincubation in EDTA-containing buffer. There was no difference in the inhibition of the basal [^{35}S]TBPS binding by 3 μM GABA either to granule cell or molecular layer between the rat lines (Fig. 2A, 2B) Furosemide (200 μM) antagonized the inhibition of the [^{35}S]TBPS binding by GABA more effectively ($p < 0.0001$) in the granule cell layer of AT than of ANT rats (Fig. 2A). In the molecular layer, furosemide was not able to affect the inhibition of [^{35}S]TBPS binding by GABA (Fig. 2B).

GABA Sensitivity of the AT and ANT Cerebella

The difference in the properties of furosemide, SR 95531 and bicuculline between the rat lines that can be abolished by the long preincubation of the cerebellar sections could be due to an increased sensitivity of AT granule cell receptors as compared to ANT granule cell receptors to GABA or to a differential amount of endogenous GABA. However, the basal [^{35}S]TBPS binding to the ANT granule cell layer was

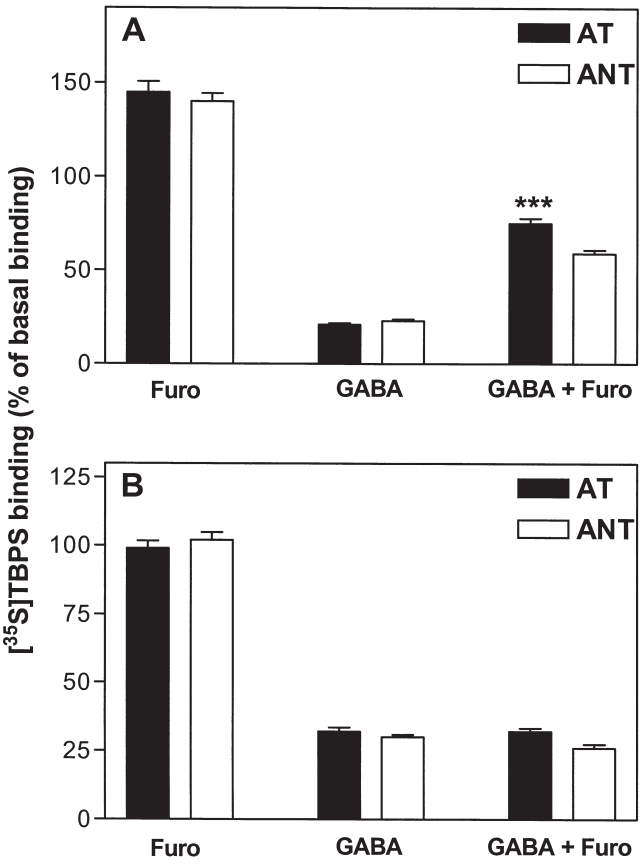

FIG. 2. The ability of furosemide to enhance the basal [^{35}S]TBPS binding and to antagonize the inhibition of [^{35}S]TBPS binding by GABA in the long-preincubated male AT and ANT cerebellar sections. The effects of 200 μM furosemide (Furo), 3 μM GABA and GABA together with furosemide on the [^{35}S]TBPS binding to the granule cell (A) and the molecular (B) layer are expressed as percentages of the basal [^{35}S]TBPS binding. Values shown are means ± SE for 25 cerebella per rat line. Statistical significance of the difference between the rat lines (Student's *t*-test): ***$p < 0.001$.

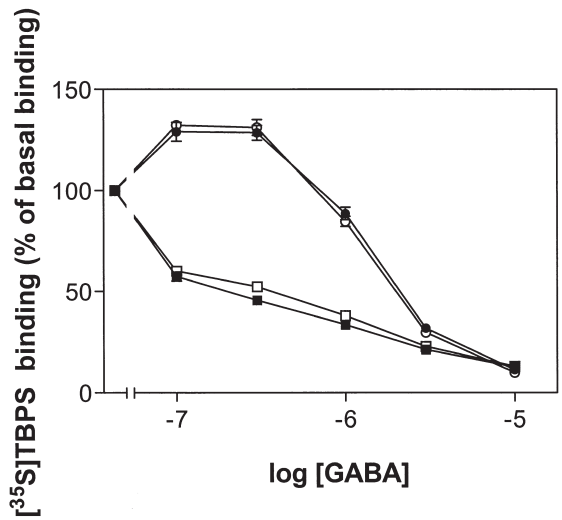

FIG. 3. Effect of GABA on the picrotoxin-sensitive [^{35}S]TBPS binding to the granule cell (■, □) and molecular (●, ○) layers of the long-preincubated male AT (■, ●) and ANT (□, ○) cerebellar sections. The results are expressed as percentages (mean ± SE of 25 animals in each group) of basal binding determined in the absence of added GABA (100%). Values to the left of the gaps were obtained in the absence of GABA. Basal [^{35}S]TBPS binding values were 298 ± 44 nCi/g for granule cell layer and 162 ± 18 nCi/g for molecular layer of AT and 260 ± 24 nCi/g for granule cell layer and 163 ± 16 nCi/g for molecular layer of ANT rats.

similarly sensitive to GABA (100 nM–10 μM) as that of the AT rats after most endogenous GABA had been removed by the long preincubation with EDTA-containing buffer (Fig. 3). The granule cell layer in both rat lines was over 10 times more sensitive to GABA than the molecular layer, previously shown to be due to a 10-fold higher GABA sensitivity of granule cell restricted α6-containing receptors than the more ubiquitous α1 subunit-containing receptors (11).

Further, we examined whether the AT and ANT cerebellar sections differ in the endogenous GABA amount released during incubation. We performed a normal (15 min, 50 mM Tris-HCl + 120 mM NaCl, pH 7.4) and a long preincubation (3 × 10 min, 50 mM Tris-HCl + 1 mM EDTA, pH 7.4) followed by 90 min of incubation with 6 nM cold TBPS on cerebellar sections, collected the incubation fluid and measured the GABA concentrations. Using the normal preincubation GABA concentration was 80 ± 20 nM, mean ± SD, $n = 5$ object glasses (5 sections per glass), for AT and 90 ± 20 nM for ANT rats. After the long preincubation assay, GABA concentrations of 90 ± 30 nM for AT and 100 ± 30 nM for ANT rats were detected; that is, no difference was observed between the rat lines or the preincubation conditions.

Area of the Cerebellar Granule Cell Layer in AT and ANT Rat Sections

We found no difference in the area of the granule cell layer proportional to the whole cerebellar outline between female AT and ANT rat lines (32 ± 5% versus 31 ± 5% of the whole cerebellar area, mean ± SD, $n = 25$, respectively).

Effect of Chloride Ions on the Actions of Furosemide

For [^{35}S]TBPS autoradiography, sections are normally preincubated and incubated in 50 mM Tris-HCl plus 120 mM NaCl buffer, pH 7.4, whereas in [^{35}S]TBPS ligand binding studies, membranes are washed well in 50 mM Tris-citrate, 1 mM EDTA, pH 7.4, and the incubation is performed in 50 mM Tris-citrate buffer supplemented with 200 mM NaCl, pH 7.4. Because we did not detect the difference in the basal [^{35}S]TBPS binding between cerebella of AT and ANT rats using well-washed cerebellar membranes in a previous ligand binding study (20), we studied the effect of different NaCl concentrations in preincubation and the incubation buffers on [^{35}S]TBPS autoradiography (Fig. 4). To determine [^{35}S]TBPS binding in the nominal absence of Cl^-, an autoradiography was performed using 100 mM Na-phosphate buffer, pH 7.4. Little [^{35}S]TBPS binding was detected to the granule cell and molecular layers of the rat lines in the absence of Cl^- (Fig. 4A, 4B). The binding to both cerebellar layers increased with the concentration of Cl^-. There was significantly [$F(1, 70) = 18.16$, $p < 0.0001$ by ANOVA (n = 12)] more binding to the granule cell layer of AT than to the granule cell layer of ANT rats at all Cl^--concentrations, whereas no difference was detected at any Cl^--concentration in the molecular layer.

The ability of furosemide to increase basal [^{35}S]TBPS binding and to reverse the GABA-induced inhibition of the [^{35}S]TBPS binding to the cerebellar granule cell layer was independent of the presence of Cl^- in the incubation buffer, as the effects were detected with the Na-phosphate buffer. However, in Cl^--free buffer no differences in actions of furosemide between the rat lines were detected.

Furosemide enhanced the basal [^{35}S]TBPS binding significantly [$F(1, 70) = 7.443$, $p = 0.0080$], more in AT granule cells than in ANT granule cells in the presence of different Cl^--concentrations (Fig. 4C), whereas the inhibition of the binding by 3 μM GABA was similar between the rat lines in all Cl^--concentrations (Fig. 4E). Furosemide antagonized the inhibition by GABA more effectively in granule cells of AT than ANT rats, $F(1,70) = 45.82$, $p < 0.0001$ (Fig. 4G).

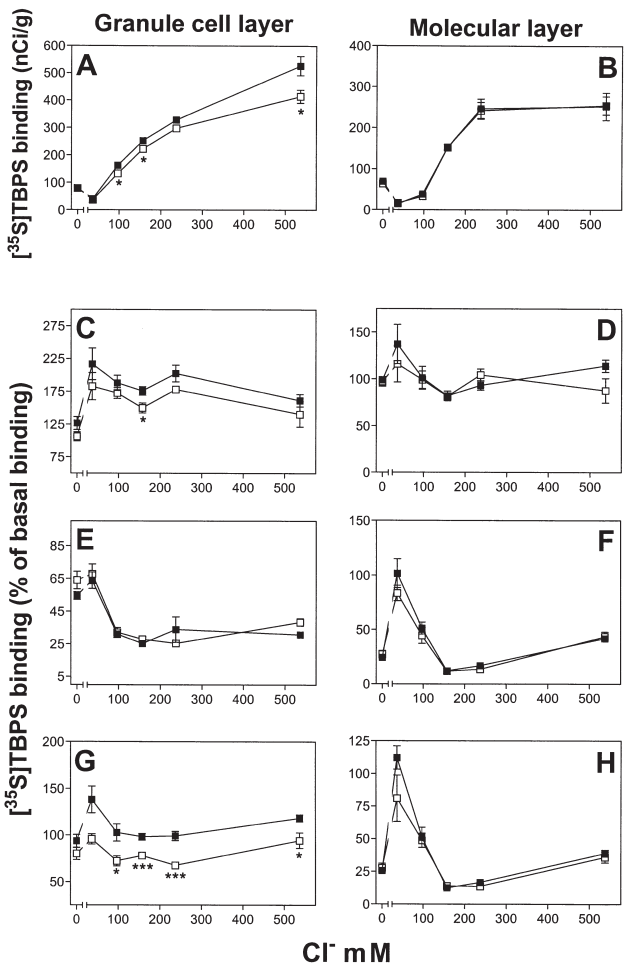

FIG. 4. Effects of different Cl^--concentrations in the buffer on basal [^{35}S]TBPS binding in the absence (A, B) and in the presence of 200 μM furosemide (C, D) and in the presence of 3 μM GABA (E, F) and GABA and furosemide (G, H) to the cerebellar sections of the male AT (■) and the male ANT (□) rats. The results are shown for the granule cell layer (A, C, E, G) and for molecular layer (B, D, F, H). A and B depict the basal binding values at various NaCl concentrations in nCi/g, the rest of the results being expressed as percentages (mean ± SE of 12 animals in each group) of the basal binding at the corresponding NaCl concentration. Values to left of the gaps were obtained in the presence of Cl^--free 100 mM Na-phosphate buffer, pH 7.4. Statistical significance of the difference between the rat lines (Student's t-test): $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

DMCM Actions on Cerebella of the AT and ANT Rats

The benzodiazepine site inverse agonist DMCM paradoxically potentiates $GABA_A$ receptor responses at high micromolar concentrations dependent on the β2 and β3 subunits, but independently of the α subunit variant at a site different from the benzodiazepine receptor (30,34,36). However, the lack of DMCM inhibition via the benzodiazepine site in α6 subunit-containing receptors (30) makes the potentiation more pronounced on these receptors. Because the effect of

furosemide is not only dependent on the presence of the α6 subunit, but also on that of the β2 or the β3 subunit, we compared the effect of DMCM on the [^{35}S]TBPS binding in cerebella of AT and ANT rats to detect possible alterations in the expression of the β subunits or in the assembly of β subunit-containing receptors.

In the long-preincubated cerebellar sections, 30 μM DMCM increased the inhibition by 0.3 μM GABA on [^{35}S]TBPS binding in the granule cell layer of AT and ANT rats ($p < 0.001$) (Fig. 5A). The potentiation was slightly more pronounced in the AT than in the ANT rat line. Potentiation of GABA inhibition, but no rat line difference, was observed in the molecular layer (Fig. 5B). The benzodiazepine antagonist flumazenil (10 μM) did not affect this action of DMCM neither in the granule cell nor molecular layer.

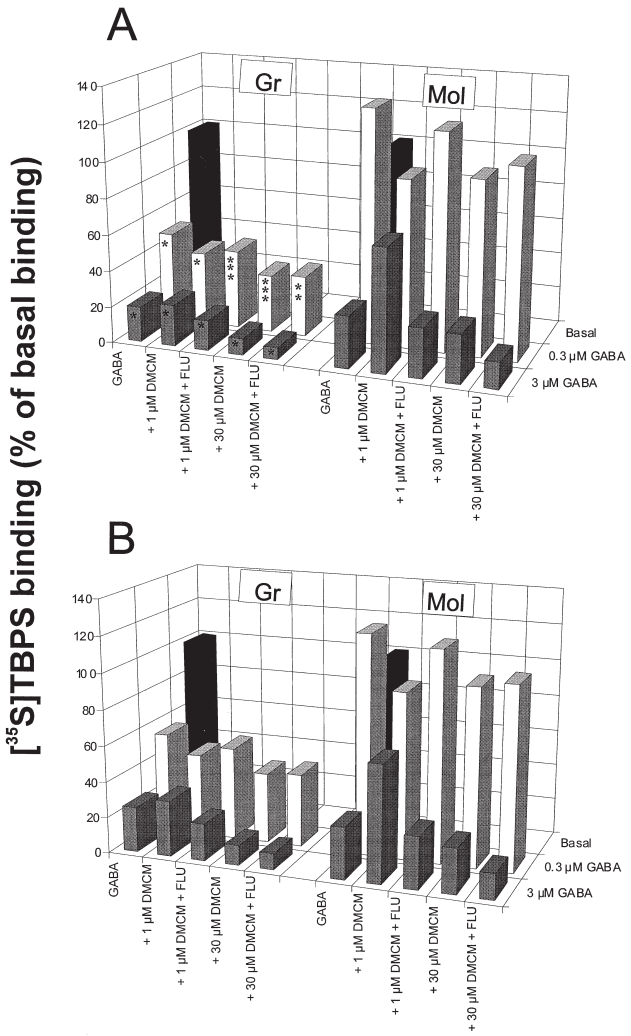

FIG. 5. Effect of 1 and 30 μM DMCM and of 10 μM of the benzodiazepine antagonist flumazenil on DMCM actions in the granule cell (Gr) and molecular layers (Mol) of the AT (A) and ANT (B) rat lines. Data are mean values ($n = 33$ in the presence of 0.3 μM GABA and $n = 8$ in the presence of 3 μM GABA) with standard deviations (not shown) within 30% of the mean values. Statistical significance between the rat lines (Student's t-test): *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

In the molecular layer of both rat lines, 1 μM DMCM acted as an inverse agonist antagonizing the potentiating effect by 0.3 μM GABA and the inhibiting effect of 3 μM GABA on [^{35}S]TBPS binding. Flumazenil was able to antagonize the effect of 1 μM DMCM at the both GABA concentrations (Fig. 5B). In the granule cell layer of the ANT ($p < 0.01$) but not the AT rats ($p > 0.05$), flumazenil antagonized the effect of 1 μM DMCM at low GABA concentration.

$GABA_A$ Receptor β Subunit Sequences of AT and ANT Rats

As the furosemide antagonism has been shown to depend on the presence of the β2 or β3 subunits in the receptor complex in addition to the α6 subunit (14), but no other difference than the previously detected point mutation that causes the arginine to glutamine exchange at position 100 between the sequences of α6 subunit of AT and ANT rat lines has been detected (13), there could be an additional mutation in the β2 or β3 subunits. Such a mutation could hinder the effect of furosemide in ANT rats. However, no differences were detected between the β1, β2 and β3 subunit sequences in the AT and ANT rat lines, respectively. The rat β1, rat β2 and rat β3 coding sequences were verified to be identical to the earlier published sequences of these subunits (27,38,16, respectively).

Benzodiazepine Binding Site

The amount of cerebellar [^{3}H]Ro 15-4513 (8 nM) binding in the presence of 10 and 100 μM diazepam [diazepam-insensitive (DIS) binding] varied between the AT and ANT rat lines and between individual animals of these lines, in agreement with earlier studies (18,19,29). In the granule cell layer of the cerebellum, DIS binding was significantly ($p < 0.001$) lower in the ANT (26 ± 4% and 6.8 ± 4.6% of total binding, mean ± SD, $n = 25$, for the binding in the presence of 10 and 100 μM diazepam, respectively) than in the AT rats (52 ± 17% and 38 ± 21%, $n = 25$). In the molecular layer, diazepam was able to displace most [^{3}H]Ro 15-4513 binding in the AT (7.2 ± 1.7% and 5.7 ± 1.0% of total binding) and the ANT (6.3 ± 1.2% and 4.5 ± 0.8%) samples. A difference between the rat lines was observed in the total [^{3}H]Ro 15-4513 binding only to the granule cell layer (8.9 ± 1.9 versus 11.2 ± 1.0 nCi/mg for AT and ANT rats, respectively), but not to the molecular cell layer (8.2 ± 1.0 versus 8.5 ± 1.2 nCi/mg for AT and ANT rats, respectively), confirming our earlier results (20).

Correlations Between Ligand Binding Properties

The ability of furosemide to antagonize the inhibition of [^{35}S]TBPS binding in the AT and ANT cerebellar membranes correlated with the amount of DIS binding to the granule cell layer of AT and ANT rats (20). In the present study, this was confirmed using cerebellar sections. DIS binding in the granule cell layer of individual rats in the presence of 10 and 100 μM diazepam correlated well with percentages of reversal by furosemide of GABA-induced inhibition of the [^{35}S]TBPS binding and with the basal [^{35}S]TBPS binding to the granule cell layer in the whole rat population (Fig. 6). The correlations between DIS binding and percentages of reversal by furosemide were also clearly seen within the AT rat line, in agreement with earlier results (20). Pearson correlation coefficients for ANT, AT and the combined ANT and AT data were −0.0260, 0.6457***, 0.6409*** and −0.0266, 0.6567***, 0.6451*** for the DIS binding in the presence of 10 and 100 μM diazepam, respectively. There was also a weak correla-

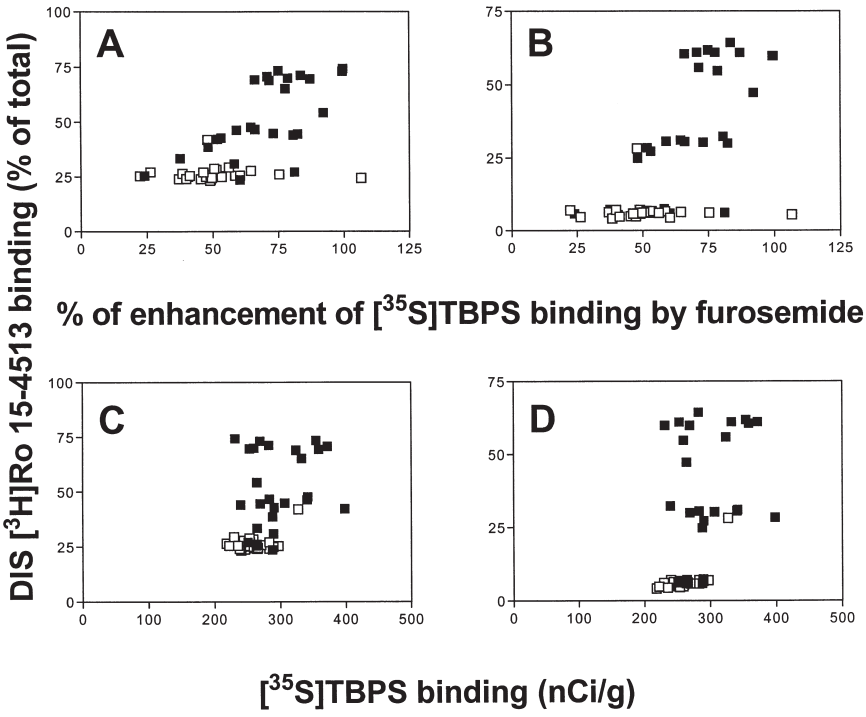

FIG. 6. Correlations between the reversal by furosemide of GABA-induced decrease in [^{35}S]TBPS binding and basal [^{35}S]TBPS binding to diazepam-insensitive (DIS) [^{3}H]Ro 15-4513 binding in the cerebella of individual male AT (■) and ANT (□) rats. Percent enhancement of [^{35}S]TBPS binding by furosemide and % DIS of total [^{3}H]Ro 15-4513 binding in the presence of 10 μM diazepam (A) or 100 μM diazepam (B). Basal [^{35}S]TBPS binding and % DIS of total [^{3}H]Ro 15-4513 binding in the presence of 10 μM diazepam (C) or 100 μM diazepam (D). Percent enhancement of [^{35}S]TBPS binding by furosemide, reflecting furosemide-induced $GABA_A$ receptor antagonism, is expressed as percent reversal by 200 μM furosemide of [^{35}S]TBPS binding inhibited by 3 μM exogenous GABA, full 100% enhancement being defined as recovery of the basal binding. [^{35}S]TBPS binding was performed and effects of furosemide were tested on the long-preincubated cerebellar sections.

tion between basal [^{35}S]TBPS binding and DIS binding in the ANT rat line (Pearson correlation coefficients 0.4696 and 0.6603 for 10 and 100 μM diazepam, respectively), but no correlation was observed in the AT rat line.

Basal [^{35}S]TBPS binding correlated weakly with the total amount of [^{3}H]Ro 15-4513 binding in the whole rat population (−0.4015**) but not within rat lines. There was no correlation between the basal [^{35}S]TBPS binding and the antagonism by furosemide, but the basal [^{35}S]TBPS binding correlated with the percent increase in the basal [^{35}S]TBPS binding by furosemide in the AT rat line (−0.7303***) and in the whole rat population (−0.3684*). Basal [^{35}S]TBPS binding correlated well also with the DIS [^{3}H]Ro 15-4513 binding both in the presence of 10 μM and 100 μM diazepam in the whole rat population (0.4694*** and 0.4957***, respectively) and in the ANT rat line (0.4696* and 0.6603***, respectively).

DISCUSSION

Furosemide enhances [^{35}S]TBPS binding to α6β2/3γ2 $GABA_A$ receptor channels in the absence and presence of GABA by increasing the affinity of binding non-competitively at an allosteric regulatory site (14). This receptor subtype is exclusively expressed in the granule cells of the cerebellum (15,24,25). Furosemide has also been shown to inhibit GABA-induced currents in α4 subunit-containing receptors (10,35), although no antagonism of GABA inhibition of [^{35}S]TBPS binding by furosemide to thalamus or cortex, the brain areas containing most of the α4 subunit, has been detected (11,20). Therefore, furosemide appears to be a useful tool in studying a limited population of cerebellar $GABA_A$ receptors and in developing receptor subtype-specific drugs. Unfortunately, furosemide as a strongly polar compound cannot cross the blood-brain barrier. In order to develop subtype-specific, furosemide-like drugs that penetrate the blood-brain barrier, the exact binding site for furosemide needs to be determined.

We have shown earlier that both of furosemide's actions, enhancement of basal [^{35}S]TBPS binding and the antagonism of GABA-induced inhibition of [^{35}S]TBPS binding, are reduced in cerebellar membranes of ANT rats as compared to those of AT or Wistar rats (14,20). In the present study, these reduced actions of furosemide in ANT rats were confirmed to be a property of cerebellar granule cell layer using autoradiography of cerebellar sections.

In addition to furosemide, the competitive $GABA_A$ receptor antagonists SR 95531 and bicuculline differentiated between ANT and AT granule cell layers in enhancing the basal [^{35}S]TBPS binding in standard autoradiography; that is, shorter preincubation, which could be due to a difference in

the GABA sensitivity of the granule cells between the rat lines, as the enhancement of the basal binding is most likely due to antagonism of endogenous agonists (3,29). Indeed, longer preincubation with EDTA-containing buffer removed the rat line difference in the enhancement of basal [^{35}S]TBPS binding by furosemide, SR 95531 and bicuculline. However, furosemide still enhanced the basal binding, whereas bicuculline did not affect and SR 95531 actually inhibited it. The effect of SR 95531 had been detected on well-washed cerebellar membranes (14).

Interestingly, the treatment with EDTA did not remove the rat line difference in the amount of the basal [^{35}S]TBPS binding, although it increased the binding. The difference in the antagonism by furosemide of GABA-induced inhibition of [^{35}S]TBPS binding between the rat lines also remained, which suggests a structural alteration at the furosemide binding site, since endogenous GABA concentration released into the incubation medium did not differ between the rat lines. However, since our long preincubation failed to reduce the GABA content of the incubation fluid, there may be other endogenous compounds that might differ between the rat lines. This is also suggested by the fact that GABA sensitivity does not differ between the sections of the rat lines.

In order to produce a rapid, reversible block of the $GABA_A$ receptors, furosemide requires a combination of α6 and β2 or β3 subunits, whereas α1 or β1 subunit-containing receptors are insensitive to furosemide (14). The variant of the β subunit is thus important for the action of furosemide. We did not find differences between the AT and ANT β2 or β3 subunit sequences that could explain the furosemide difference, neither could we detect a difference between the rat lines in the β2/3 subunit-dependent agonistic action of DMCM (30).

The difference in the sensitivity to benzodiazepine agonists between AT and ANT rat lines is mediated by a point mutation in ANT α6 subunit (13) and correlates well with the difference in furosemide antagonism in our previous (20) and in the present study. But using recombinant receptors we already excluded the point mutation as a reason for this difference (20). As there are no other differences in genome sequences encoding the α6 (13), or β2 and β3 subunits (present study) between the AT and ANT rats, it could be possible that the point mutation in the α6 subunit hinders proper binding site interaction and/or subunit assembly in the cerebellar $GABA_A$ receptors of ANT rats, in which the δ subunit could play a prominent role. This alternative could explain the reduced muscimol binding in the ANT rats (22), because the high affinity [^{3}H]muscimol binding has been suggested to be a property of α6δ subunit-combination (8,26). Though, we did not detect a difference in cerebellar δ subunit mRNA levels between AT and ANT rats, there still could be different levels of protein expression, comparable to the $\alpha6^{-/-}$ $GABA_A$ subunit-deficient mice, in which the δ protein level is remarkably reduced at the normal expression level of δ subunit mRNA (8). Although the GABA antagonism by furosemide in recombinant receptors is independent of the presence or absence of γ2 and δ subunits, the increase of basal [^{35}S]TBPS binding in the absence of exogenous GABA was detected only in α6β2/3γ2 receptors (12). This leaves room for a role of the δ subunit in the furosemide action.

The main determinant of furosemide inhibition on $GABA_A$ receptors is located close to the first transmembrane domain of the α6 subunit (5,9). It was also shown that there is a low potency-site for furosemide and that it is located at the carboxy-terminal end of the α6, starting with TM2, though the possibility remains that both sites form parts of a single binding pocket. As the difference in furosemide action between the AT and ANT rat lines was not detected when the chloride-free Na-phosphate buffer was used, it could be that the important amino acids for formation of furosemide binding site in chloride ion channel are somehow altered in ANT receptors.

In conclusion, the differential furosemide action appears not to be secondary to any difference in the GABA sensitivity, but rather due to structural, still unknown, modifications via the α6 subunit benzodiazepine site mutation or other, such as posttranslational, alterations. Whether the blunted action of furosemide is associated with alterations in ethanol sensitivity at the cellular and biochemical level still remains to be studied in situ.

ACKNOWLEDGEMENTS

This study was partly supported by the Finnish Foundation of Alcohol Studies (R. M.), the Academy of Finland (E. R. K), the Medical Research Fund of the University of Tampere (H. A.), and the Deutsche Forschungsgemeinschaft (H. L.).

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