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Selective Antagonist for the Cerebellar Granule Cell-Specific γ-Aminobutyric Acid Type A Receptor

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SUMMARY

Numerous ligands affect inhibitory γ-aminobutyric acid $(GABA)_A$ receptors, none of them showing strict receptor subtype specificity. We report here that a cerebellar $GABA_A$ receptor subtype can be uniquely modulated by furosemide but not by bumetanide, another Cl^-/cation transport blocker. Furosemide specifically reversed the inhibition by GABA of *t*-[^{35}S]butylbicyclophosphorothionate ([^{35}S]TBPS) binding in the cerebellar granule cell layer, as detected by autoradiography of rat brain sections. With recombinant receptors expressed in *Xenopus* oocytes, furosemide antagonized potently (IC_{50}, about 10 μM), rapidly, and reversibly GABA-evoked currents of cerebellar granule cell-specific α6β2γ2 receptors but not α1β2γ2 receptors (IC_{50}, >3 mM). Furosemide reversed GABA inhibition of [^{35}S]TBPS binding and elevated basal [^{35}S]TBPS binding only with α6β2γ2 and α6β3γ2 receptors and not with α6β1γ2 or α1β1/2/3γ2 receptors. It appeared to interact with the receptor complex via a novel recognition site that allosterically regulates the Cl^- ionophore. Furosemide is the first subtype-selective $GABA_A$ receptor (α6β2/3γ2) antagonist and should facilitate studies on cerebellar physiology. It might serve as a prototypic structure for the development of additional subtype-selective $GABA_A$ ligands.

GABA is the major inhibitory neurotransmitter in the mammalian brain. Its postsynaptic action is largely mediated by GABA A-type receptors, which are integral membrane proteins forming an anion-selective channel (for review, see Ref. 1). Under physiological conditions, the activation of $GABA_A$ receptors increases Cl^- conductance, leading to hyperpolarization of the postsynaptic cell membrane and reduction of the probability of action potentials.

$GABA_A$ receptors are widely distributed in neuronal and glial cell populations in the brain, a fact that has made this receptor system an important target for drug design. Each receptor is believed to be assembled from five subunits derived from the α (α1–6), β (β1–3) and γ (γ1–3) subunit classes (2, 3). GABAergic activity can be enhanced by, for example, benzodiazepines, barbiturates, and anesthetics, whereas numerous other compounds, such as bicuculline and picrotoxinin, can act as receptor blockers (4).

Ligand binding studies on the $GABA_A$ receptor have revealed different pharmacological profiles in various brain regions. These profiles can be simulated *in vitro* using recombinant receptors composed of three different subunits. Several $GABA_A$ receptor subtypes differ with respect to benzodiazepine ligand pharmacology (5). For example, zolpidem has been suggested to be an α1 subunit-preferring ligand (6, 7), but it has since been observed that zolpidem has no affinity for α1βxγ3 receptors (8). The cerebellar granule cell-specific, α6 subunit-containing receptors differ from $GABA_A$ receptors elsewhere in the brain in their [^{3}H]Ro 15-4513 binding site (9–13). However, no known ligand affects only α6 subunit-containing receptors. Recently, Wingrove *et al.* (14) reported that loreclezole, a compound not acting at the benzodiazepine site, recognizes only $GABA_A$ receptors that contain β2 or β3 subunits. These examples illustrate the complexity of the structural features that build up a certain ligand binding site, and they indicate that $GABA_A$ receptor classification cannot be based solely on the contribution of single subunits. Attempts to identify ligands specific for $GABA_A$ receptor subtypes, e.g., α1β2γ2 or α6β2γ2 receptors, have so far failed. The receptor antagonists have proved to be the least subtype selective.

We have now focused on the effects of a loop diuretic, furosemide (15), which appears to reduce GABA responses in various neuronal cell populations. For example, furosemide blocks the $GABA_A$ responses in the cingulate cortex after prolonged perfusion by interfering with the recovery of Cl^- gradients via inhibition of Cl^-/cation co-transporters (16). In

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ABBREVIATIONS: GABA, γ-aminobutyric acid; HEK, human embryonic kidney; SR 95531, 2′-(3′-carboxy-2′,3′-propyl)-3-amino-6-*p*-methoxyphenylpyrazinium bromide; TBPS, *t*-butylbicyclophosphorothionate; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

hippocampal neurons, Misgeld *et al.* (17) found no blockade of GABA responses by furosemide, whereas Zhang *et al.* (18) observed a blockade by 0.5–1.5 mM furosemide in neurons at an early postnatal period (days 2–5) but not at more mature stages (days 15–20). More recently, Pearce (19) reported two components in the $GABA_A$ receptor response in hippocampal slices from adult rats, with only one showing sensitivity to furosemide (500 μM). These data suggest that furosemide might act on selected populations of central $GABA_A$ receptors. Therefore, we tested the effects of furosemide on the binding properties of $GABA_A$ receptors in membranes from different brain regions. We observed a specific interaction with cerebellar receptors at low micromolar concentrations. The present report summarizes the experiments that identify furosemide as the first $GABA_A$ receptor subtype-specific antagonist.

Experimental Procedures

Preparation of brain membranes. Adult male Wistar rats (Department of Laboratory Animals, University of Helsinki, Finland) were decapitated, and the cerebral cortex, hippocampus, and cerebellum were dissected and frozen. For each membrane preparation, the cerebral cortical, hippocampal, and cerebellar tissues were pooled from two, four, and four rats, respectively. Tissues were homogenized with a Polytron homogenizer in 50 volumes of ice-cold 50 mM Tris-citrate buffer, pH 7.4, supplemented with 1 mM EDTA and were centrifuged at 20,000 × *g* for 20 min. Pellets were resuspended in the same buffer and recentrifuged five times. The final suspension was prepared in 50 mM Tris-citrate buffer, pH 7.4, divided into aliquots, and stored frozen at −80°.

Production of recombinant receptors. HEK 293 cells were transfected (20) with rat cDNAs encoding α1, α1(Arg-101), α6, α6(His-100), β1, β2, β3, and γ2S subunits, subcloned individually into eukaryotic expression vectors (11, 21–24). Quantitative ratios of the cDNAs for the α, β, and γ subunits were as described earlier (8, 25). Briefly, cells plated on dishes 15-cm in diameter (Becton Dickinson Labware, Lincoln Park, NJ) were transfected 2–3 days later. About 20 hr after transfection the medium was changed, and 48 hr after transfection the cells were washed and harvested in phosphate-buffered saline. Cell pellets were homogenized with a Polytron homogenizer in 50 mM Tris-citrate buffer, pH 7.4, centrifuged, resuspended, and stored frozen at −80°.

Ligand binding assays. Frozen membranes were thawed, resuspended, and centrifuged once before final resuspension in 50 mM Tris-citrate buffer to a protein concentration of 100–240 μg/ml (Bio-Rad protein assay kit), in a total volume of 0.5 ml/assay tube (25). After defined incubation times with duplicate samples, bound and free ligands were separated by rapid filtration of the membranes onto Schleicher & Schuell no. 52 or Whatman GF/B glass fiber filters. The samples were rinsed twice with 5 ml of ice-cold 10 mM Tris·HCl, pH 7.4. Air-dried filters were immersed in 4 ml of scintillation fluid (Wallac Optiphase HiSafe 2 or Packard Ultima Gold), and radioactivity was determined in a Wallac or Beckman scintillation counter.

[^{35}S]TBPS (DuPont de Nemours-New England Nuclear) binding was determined during a 90-min incubation at 22° in 50 mM Tris-citrate buffer supplemented with 200 mM NaCl. Nonspecific binding was determined with 20 μM picrotoxinin (Sigma Chemical Co., St. Louis, MO). [^{35}S]TBPS was used at 2–6 nM concentrations and was diluted with unlabeled TBPS in saturation experiments to cover a range from 6 to 200 nM. In some experiments, furosemide (Sigma), bumetanide (Leo Pharmaceutical Products, Ballerup, Copenhagen, Denmark), or SR 95531 (Research Biochemicals, Natick, MA) was included, with or without GABA (Serva, Heidelberg, Germany).

[^{3}H]Muscimol and [^{3}H]Ro 15-4513 (both from DuPont de Nemours) binding (at 6 nM each) was determined in 50 mM Tris-citrate buffer after 60 min at 0°. The nonspecific binding of the radioligands was determined with 100 μM GABA and 10 μM flumazenil, respectively.

Autoradiographic localization. The procedure used was modified from the work of Olsen *et al.* (26). Briefly, 14-μm horizontal cryostat sections from adult male Wistar rats ($n = 7$) were preincubated in an ice-water bath for 15 min, in 50 mM Tris·HCl, pH 7.4, supplemented with 120 mM NaCl. Incubation with [^{35}S]TBPS (200 dpm/μl, adjusted to 6 nM with unlabeled TBPS) for 90 min at room temperature (22°) was performed in the same buffer, using 600-μl liquid bubbles over sections on object glasses. Effects of GABA, furosemide, bumetanide, and SR 95531 were tested. After incubation, the sections were washed three times for 15 sec in ice-cold incubation buffer, dipped into distilled water, air-dried at room temperature, and exposed to Hyperfilm-βmax (Amersham) for 3–5 days. Picrotoxinin (20 μM) reduced the signal to background levels (data not shown). Autoradiographs were photographed as positive images.

Electrophysiological methods. *Xenopus laevis* oocytes were injected with cRNA (2α/1β/1γ) transcribed from linearized α1, α6, β2, and γ2S expression vectors using SP6 polymerase (80 units/μl; Promega). Handling of oocytes and electrophysiological recordings were carried out as described (27). Recordings were performed in frog Ringer solution (115 mM NaCl, 2.5 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES, adjusted to pH 7.5 with NaOH). Oocytes were clamped at −70 mV membrane potential and different concentrations of furosemide (0.3–3000 μM) were administered for 1 min in the presence of GABA (1 or 10 μM). GABA sensitivities were studied using 0.1–100 μM GABA with and without 20 μM furosemide. Applications were separated by 5-min washes. Voltage ramps (+50 mV to −130 mV in 2 sec) were applied 60 sec after application of GABA was started. Control ramps recorded immediately before the application of GABA were subtracted from the agonist-induced ramps to obtain corrected voltage ramp current-voltage curves. The same protocol was applied using GABA together with furosemide.

Data analyses. The Inplot program (GraphPAD Software, San Diego, CA) was used to calculate the best-fitting values for the parameters of saturation isotherms (K_d and B_{max}) and displacement curves. The IC_{50} values and Hill coefficients for the inhibition of GABA responses by furosemide and the EC_{50} values for GABA in *Xenopus* oocytes were calculated from the peak currents. Statistical significance of the difference from the control mean was assessed using analysis of variance and Student's *t* test, with the GraphPAD Instat program.

Results

Furosemide interaction with cerebellar $GABA_A$ receptors through a novel recognition site. The picrotoxin-sensitive convulsant site labeled by [^{35}S]TBPS was differently affected by furosemide in forebrain and cerebellar membranes (Fig. 1). In cerebrocortical and hippocampal membranes, furosemide at concentrations up to 1 mM did not influence [^{35}S]-TBPS binding. However, furosemide, but not bumetanide, another loop diuretic and chloride transport blocker (15), greatly enhanced the binding in the cerebellar membranes at micromolar concentrations. Qualitatively similar effects were obtained when [^{35}S]TBPS binding was determined in the presence of exogenous GABA (1 and 5 μM). Furosemide in a concentration-dependent manner fully reversed the inhibitory effect of 1 μM GABA, and partially reversed that of 5 μM GABA, on the [^{35}S]TBPS binding to cerebellar receptors. In contrast, bumetanide potentiated the GABA-induced decrease of the binding. To a lesser degree, this action was also observed with higher concentrations of furosemide in hippocampal and cerebrocortical membranes when exogenous GABA was added (Fig. 1), indicating a nonselective action of furosemide at these high concentrations in noncerebellar tissues.

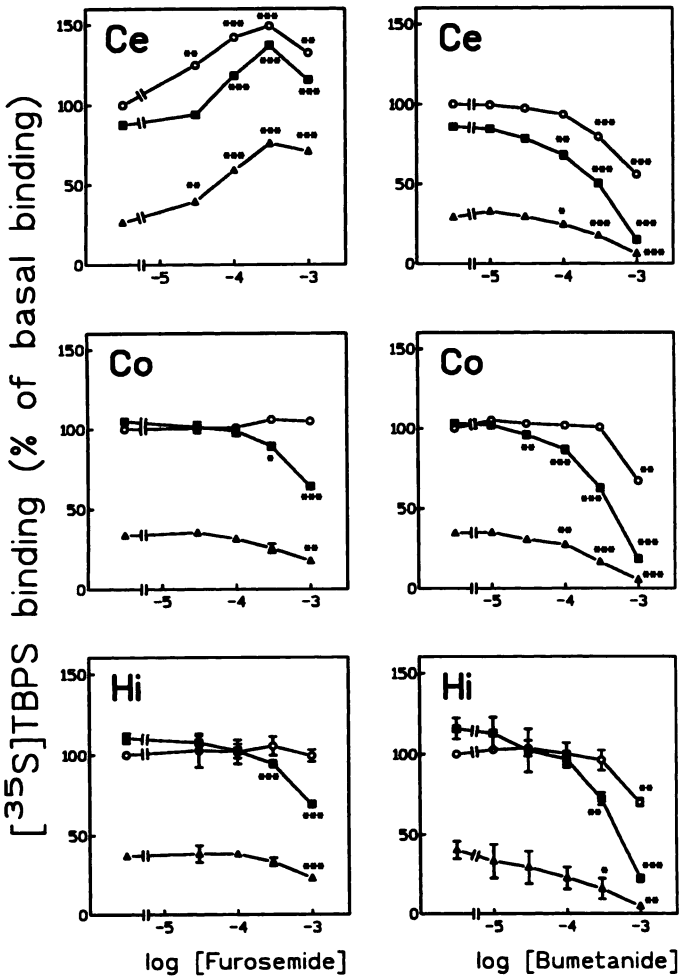

Fig. 1. Effects of furosemide (*left*) and bumetanide (*right*) on picrotoxin-in-sensitive [^{35}S]TBPS binding in cerebellar (*Ce*), cerebrocortical (*Co*), and hippocampal (*Hi*) membranes. [^{35}S]TBPS binding was determined in the absence (○) and presence (■, 1 μM; ▲, 5 μM) of exogenous GABA. The results are expressed as percentages (mean ± standard error of three determinations) of basal binding, determined in the absence of added GABA (100%). *Values to the left of the gaps*, values obtained in the absence of furosemide or bumetanide. Significance of the difference from the corresponding control values (Student's *t* test) is as follows: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

TABLE 1

Effects of furosemide on parameters of [^{35}S]TBPS binding in cerebellar and cerebrocortical membranes

Picrotoxinin-sensitive [^{35}S]TBPS binding was determined in the presence and absence of furosemide at 6 nM radioligand concentration, diluted with unlabeled TBPS to cover a concentration range from 6 to 200 nM. Values are means ± standard errors (three experiments).

Tissue	Furosemide concentration	K_d	B_{max}
	mM	nM	pmol/mg of protein
Cerebellum	0 (control)	117 ± 6	6.1 ± 0.1
	0.3	73 ± 4[a]	6.3 ± 0.2
Cerebral cortex	0 (control)	74 ± 4	8.2 ± 0.3
	0.3	96 ± 8	7.9 ± 0.2

[a] Significance of the difference from the corresponding control value (paired *t* test), $p < 0.05$.

Furosemide (300 μM) increased the affinity, i.e., decreased the K_d value, of [^{35}S]TBPS for cerebellar but not cerebrocortical receptors (Table 1). There was no effect on the density of binding sites (B_{max}), suggesting that the effect of furosemide reflects conformational alteration(s) in the receptor. This excludes the possibility that furosemide competes with [^{35}S]TBPS for its binding site.

To characterize the recognition site of furosemide on GABA$_A$ receptors, we determined the binding of [^{3}H]muscimol and [^{3}H]Ro 15-4513 in the presence of various concentrations of furosemide and bumetanide. Cerebellar [^{3}H]muscimol binding was unaffected by these ligands (Fig. 2A), indicating that the antagonism by furosemide of GABA-induced decreases in [^{35}S]TBPS binding is not due to blockade of GABA binding sites. Total binding of [^{3}H]Ro 15-4513 (diazepam-sensitive plus diazepam-insensitive components) was affected equally by high concentrations of furosemide and bumetanide in cerebellar and cerebrocortical membranes (Fig. 2B). This suggests that the benzodiazepine recognition site on the GABA$_A$ receptor is not involved in the differential actions of these ligands on the convulsant site. Interestingly, neither furosemide nor bumetanide had any significant effect on the cerebellar "diazepam-insensitive" [^{3}H]Ro 15-4513 binding, a hallmark of α6 subunit-containing GABA$_A$ receptors (11). At 10 μM, flumazenil (Ro 15-1788), an antagonist at all benzodiazepine sites (28), did not affect the modulation of [^{35}S]TBPS binding by furosemide (data not shown). These data exclude the GABA and benzodiazepine binding sites as sites of action of furosemide and suggest the existence of a novel effector site on the receptor complex.

Localization of furosemide-sensitive GABA$_A$ recep-

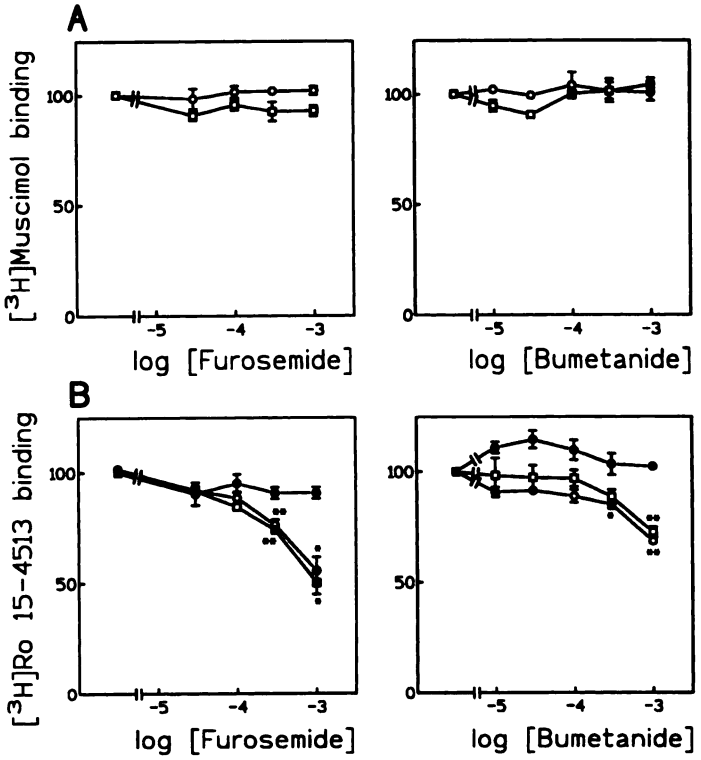

Fig. 2. Effects of furosemide on [^{3}H]muscimol (A) and [^{3}H]Ro 15-4513 (B) binding in cerebellar (●, ○) and cerebrocortical (□) membranes. With cerebellar membranes, both total flumazenil-sensitive [^{3}H]Ro 15-4513 binding (○) and the diazepam-insensitive, GABA$_A$ receptor α6 subunit-specific component of [^{3}H]Ro 15-4513 binding (●) were determined. The latter binding component was determined in the presence of 10 μM diazepam. The results are expressed as percentages (mean ± standard error of three determinations) of basal binding. Significance of the difference from the corresponding control values (Student's *t* test) is as follows: *, $p < 0.05$; **, $p < 0.01$.

tors. The localization of the furosemide-induced increase in the binding of the convulsant [^{35}S]TBPS within the cerebellum was investigated using ligand autoradiography on rat brain sections. Furosemide (1 mM) enhanced the binding only in the cerebellar granule cell layer (data not shown). SR 95531 (50 μM), a specific $GABA_A$ receptor antagonist (29), also enhanced [^{35}S]TBPS binding in the cerebellar granule cell layer, in agreement with our previous observation that [^{35}S]TBPS binding in this layer can be revealed only after blockade of endogenous GABA (25, 30). The furosemide effect on the cerebellar granule cell layer was most pronounced in the presence of 5 μM exogenous GABA, which itself inhibited the binding in all brain regions (Fig. 3). SR 95531 reversed the GABA inhibition of [^{35}S]TBPS binding in the forebrain and in the cerebellar granule cell and molecular layers, whereas furosemide antagonized GABA only in the cerebellar granule cell layer. In all other brain regions, furosemide appeared to slightly potentiate the GABA-induced decrease in [^{35}S]TBPS binding. Bumetanide (1 mM) did not enhance [^{35}S]TBPS binding in any brain region but, rather, potentiated the GABA-induced decrease (Fig. 3). These data document a selective antagonistic action of furosemide on cerebellar granule cell $GABA_A$ receptors.

Furosemide interaction with recombinant α6 subunit-containing $GABA_A$ receptors. We investigated the structural basis for the furosemide sensitivity by using recombinant $GABA_A$ receptors. Furosemide greatly enhanced the [^{35}S]TBPS binding to $\alpha6\beta2\gamma2$ receptors but not to $\alpha1\beta2\gamma2$ receptors (Fig. 4A). Similarly, furosemide, in a concentration-dependent manner, reversed the GABA inhibition only with $\alpha6\beta2\gamma2$ receptors and left $\alpha1\beta2\gamma2$ receptors unaffected. SR 95531 fully reversed the GABA inhibition of [^{35}S]TBPS binding to both receptor types but had no effects on basal binding (Fig. 4B). Similar effects were also seen with 10 μM GABA (Fig. 4C). These results indicate that the furosemide effect on [^{35}S]TBPS binding depends on the presence of the α6 subunit.

A single amino acid residue (arginine at position 100 in the α6 subunit) (11) determines the benzodiazepine agonist insensitivity of α6-containing receptors (23). We excluded the role of this residue in furosemide action by observing wild-type receptor-like furosemide antagonism of GABA inhibition of [^{35}S]TBPS binding with α6(His-100)$\beta2\gamma2$ receptors and no antagonism with α1(Arg-101)$\beta2\gamma2$ receptors, similarly to the wild-type α1-containing receptors (data not shown).

Furosemide sensitivity in recombinant receptors with different β subunit variants. When substituting the β variants in $\alpha6\beta x\gamma2$ receptors, we observed that the furosemide interaction was identical with $\alpha6\beta2\gamma2$ and $\alpha6\beta3\gamma2$ receptors, whereas β1-containing receptors were insensitive to furosemide (Fig. 5, A and B). However, $\alpha6\beta1\gamma2$ receptors exhibited inhibition of [^{35}S]TBPS binding by micromolar concentrations of GABA. Therefore, the structures mediating the furosemide sensitivity are determined by both α6 and β2 or β3 subunits. The specific localization of the furosemide-sensitive sites in the brain reflects the restricted expression of the α6 subunit, because all β subunits are widely expressed in the central nervous system (31–33).

$\alpha1\beta1/2/3\gamma2$ receptors showed small increases in [^{35}S]TBPS binding with 600 μM furosemide in the absence of GABA (Fig. 5C) but failed to show any GABA antagonism when exogenous GABA was added (Fig. 5D), further emphasizing that only the unique interplay between the α6 subunit and β2/3 subunits results in a proper target for the action of furosemide.

Furosemide blockade of $\alpha6\beta2\gamma2$ receptor responses to GABA. We used a *Xenopus* oocyte expression system to compare furosemide actions on GABA-induced currents of recombinant $\alpha6\beta2\gamma2$ and $\alpha1\beta2\gamma2$ receptors. Initial experiments confirmed the higher GABA sensitivity of $\alpha6\beta2\gamma2$ receptors (EC_{50} = 2.3 ± 0.8 μM, mean ± standard error of five oocytes), compared with $\alpha1\beta2\gamma2$ receptors (EC_{50} = 19.9 ± 3.3 μM), as observed earlier for native cerebellar granule cell receptors and receptors expressed in HEK cells (25). Furosemide inhibited GABA-induced currents of $\alpha6\beta2\gamma2$ receptors in a concentration-dependent manner, with an IC_{50} of 10.9 ± 1.6 μM (Hill coefficient, 0.99 ± 0.06; mean ± standard error of three batches of two or three oocytes each) (Fig. 6A). Furosemide at 20 μM did not change the GABA sensitivity of these receptors (EC_{50} = 2.4 ± 0.4 μM, mean ± standard error of five oocytes), as expected for noncompetitive inhibition. Furosemide at 3 mM resulted in only 46 ± 8% inhibition of GABA-induced currents for $\alpha1\beta2\gamma2$ receptors (Fig. 6B). Furosemide alone did not induce any currents in $\alpha6\beta2\gamma2$ or $\alpha1\beta2\gamma2$ receptor-expressing oocytes (data not shown). The action of furosemide was voltage independent with $\alpha6\beta2\gamma2$ receptors (Fig. 6C). With $\alpha1\beta2\gamma2$ receptors, outward currents were blocked more potently than inward currents (Fig. 6D), and at 3 mM furosemide a slightly voltage-dependent blockade of inward currents was observed (Fig. 6D). The reversal potential (−22 mV) was close to the E_{Cl^-} of the oocyte membrane (−25 mV) (34) for both $\alpha6\beta2\gamma2$ and $\alpha1\beta2\gamma2$ receptor-mediated currents under all conditions. Fig. 6E demonstrates rapid onset and decay of the blockade by furosemide

Fig. 3. Localization of furosemide action on [^{35}S]TBPS binding in rat brain horizontal sections. The autoradiographs of picrotoxinin-sensitive [^{35}S]TBPS binding demonstrate widespread basal binding. GABA (5 μM) greatly decreased the binding in all brain regions. Furosemide (1 mM) reversed the GABA-induced decrease of binding in the cerebellar granule cell layer, whereas bumetanide (1 mM) did not. The specific $GABA_A$ receptor antagonist SR 95531 (50 μM) reversed the GABA inhibition in forebrain and in cerebellar molecular and granule cell layers. In the absence of exogenous GABA, furosemide and SR 95531 produced similar enhancement in granule cell layer binding, whereas bumetanide was inactive (data not shown). This protocol was repeated seven times, using independent brain sections, with identical results. *Ce*, cerebellum; *CPu*, caudate-putamen; *Cx*, cerebral cortex; *Hi*, hippocampus; *OB*, olfactory bulb; *Th*, thalamus; *Gr*, cerebellar granule cell layer.

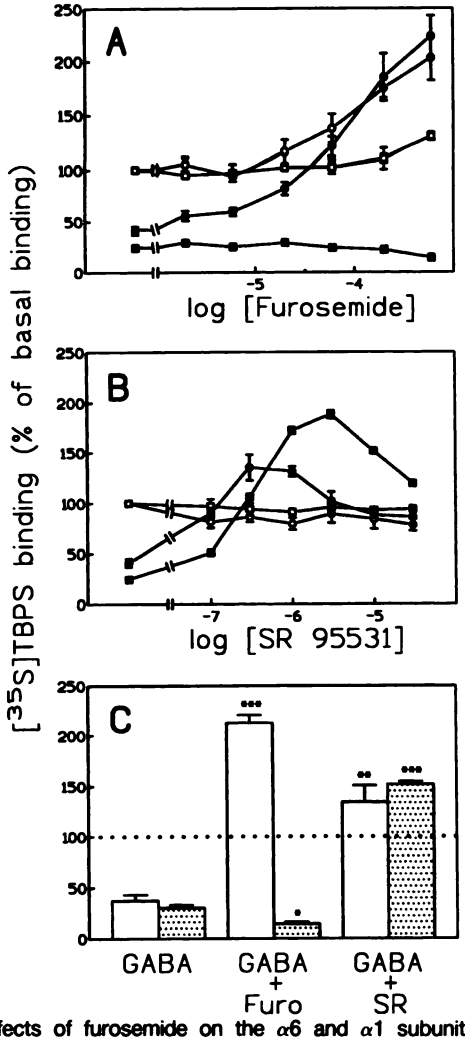

Fig. 4. Effects of furosemide on the $\alpha 6$ and $\alpha 1$ subunit-containing recombinant GABA$_A$ receptors produced by transient transfection of HEK 293 cells. A, [^{35}S]TBPS binding, as affected by furosemide, with $\alpha 6\beta 2\gamma 2$ receptors (*circles*) and $\alpha 1\beta 2\gamma 2$ receptors (*squares*) in the absence (*open symbols*) and presence (*closed symbols*) of exogenous GABA. GABA concentrations were 1 and 10 μM in the experiments with $\alpha 6\beta 2\gamma 2$ and $\alpha 1\beta 2\gamma 2$ receptors, respectively, because $\alpha 6\beta 2\gamma 2$ receptors are at least 10-fold more sensitive to GABA than are $\alpha 1\beta 2\gamma 2$ receptors (25). B, Effects of SR 95531 on [^{35}S]TBPS binding to $\alpha 6\beta 2\gamma 2$ and $\alpha 1\beta 2\gamma 2$ receptors. Symbols are as in A. C, Selective reversal by furosemide (*Furo*) (200 μM) of inhibition by 10 μM GABA with $\alpha 6\beta 2\gamma 2$ receptors (□); SR 95531 (*SR*) (10 μM) was nonselective, being active also with $\alpha 1\beta 2\gamma 2$ receptors (□). *Dotted line*, basal level of [^{35}S]TBPS binding. Results are expressed as percentages (mean ± standard error of three determinations) of the basal binding of each receptor. Significance of the difference from the corresponding control values (Student's *t* test) is as follows: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

in $\alpha 6\beta 2\gamma 2$ receptors. Thus, furosemide blocked the GABA-induced Cl^- flux through $\alpha 6\beta 2\gamma 2$ receptor channels but not $\alpha 1\beta 2\gamma 2$ channels. These data functionally substantiate our results derived from [^{35}S]TBPS binding, although furosemide concentrations needed to affect the binding were about 10 times higher (Fig. 4) than the IC_{50} in oocytes.

Discussion

Electrophysiological studies on the effect of furosemide on GABA-induced currents have been carried out on frog spinal neurons (35), cat (36) and frog (37) dorsal root ganglion cells, and rat sensory ganglion cells (38). In those studies, millimolar

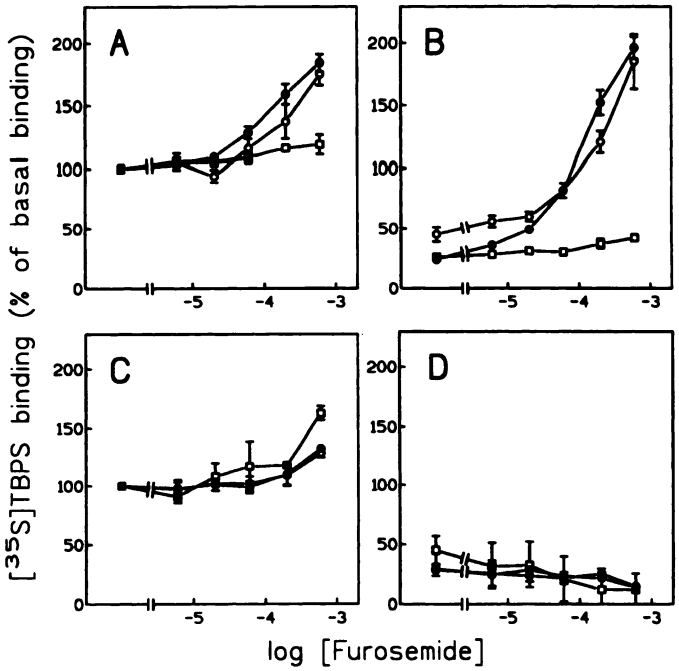

Fig. 5. Effects of β subunit variants on furosemide enhancement of [^{35}S]TBPS binding with $\alpha 6\beta x\gamma 2$ (A and B) and $\alpha 1\beta x\gamma 2$ (C and D) receptors. A and B, [^{35}S]TBPS binding to $\alpha 6\beta 1\gamma 2$ (□), $\alpha 6\beta 2\gamma 2$ (○), and $\alpha 6\beta 3\gamma 2$ (●) receptors in the absence (A) and presence (B) of added GABA (1 μM) was measured. C and D, [^{35}S]TBPS binding to $\alpha 1\beta 1\gamma 2$ (□), $\alpha 1\beta 2\gamma 2$ (○), and $\alpha 1\beta 3\gamma 2$ (●) receptors in the absence (C) and presence (D) of added GABA (10 μM) was measured. Results are given as percentages of basal binding (mean ± standard error of three determinations).

concentrations of furosemide diminished GABA currents. A perfusion time of several minutes was needed to reach the equilibrium effect (36, 39). In contrast, our results indicate that furosemide inhibits a GABA$_A$ receptor subtype specific to cerebellar granule cells. The inhibition was rapid and reversible and occurred at low micromolar concentrations ($IC_{50} \cong 10$ μM). The speed and low concentration distinguish the action of furosemide on $\alpha 6$ subunit-containing GABA$_A$ receptors from that described for spinal and ganglion neurons.

Earlier studies (18, 19) have suggested that furosemide-sensitive GABA$_A$ receptors occur in hippocampal neurons. Our autoradiographic data exclude the presence of any major neuronal group in the rat forebrain possessing furosemide-sensitive GABA$_A$ receptor channels.

Furosemide and bumetanide both inhibit Cl^-/cation cotransporters at low micromolar concentrations, consistent with their renal diuretic actions (15). At higher concentrations bumetanide and furosemide appeared to potentiate the GABA-induced decrease in [^{35}S]TBPS binding, with the exception of the cerebellar granule cell layer. Because this effect did not show any brain region heterogeneity, it may be due to a general action on chloride channels. The fact that bumetanide did not share the action of furosemide on cerebellar granule cell GABA$_A$ receptors strongly suggests that furosemide directly interacts with $\alpha 6$ subunit-containing GABA$_A$ receptors to block channel function.

The lack of specific interference of furosemide with the [^{3}H]muscimol binding sites indicates a noncompetitive interaction with GABA and excludes a competitive mode of inhibition of GABA-induced chloride currents in $\alpha 6\beta 2\gamma 2$ receptor-expressing oocytes. Furosemide also failed to specifically affect

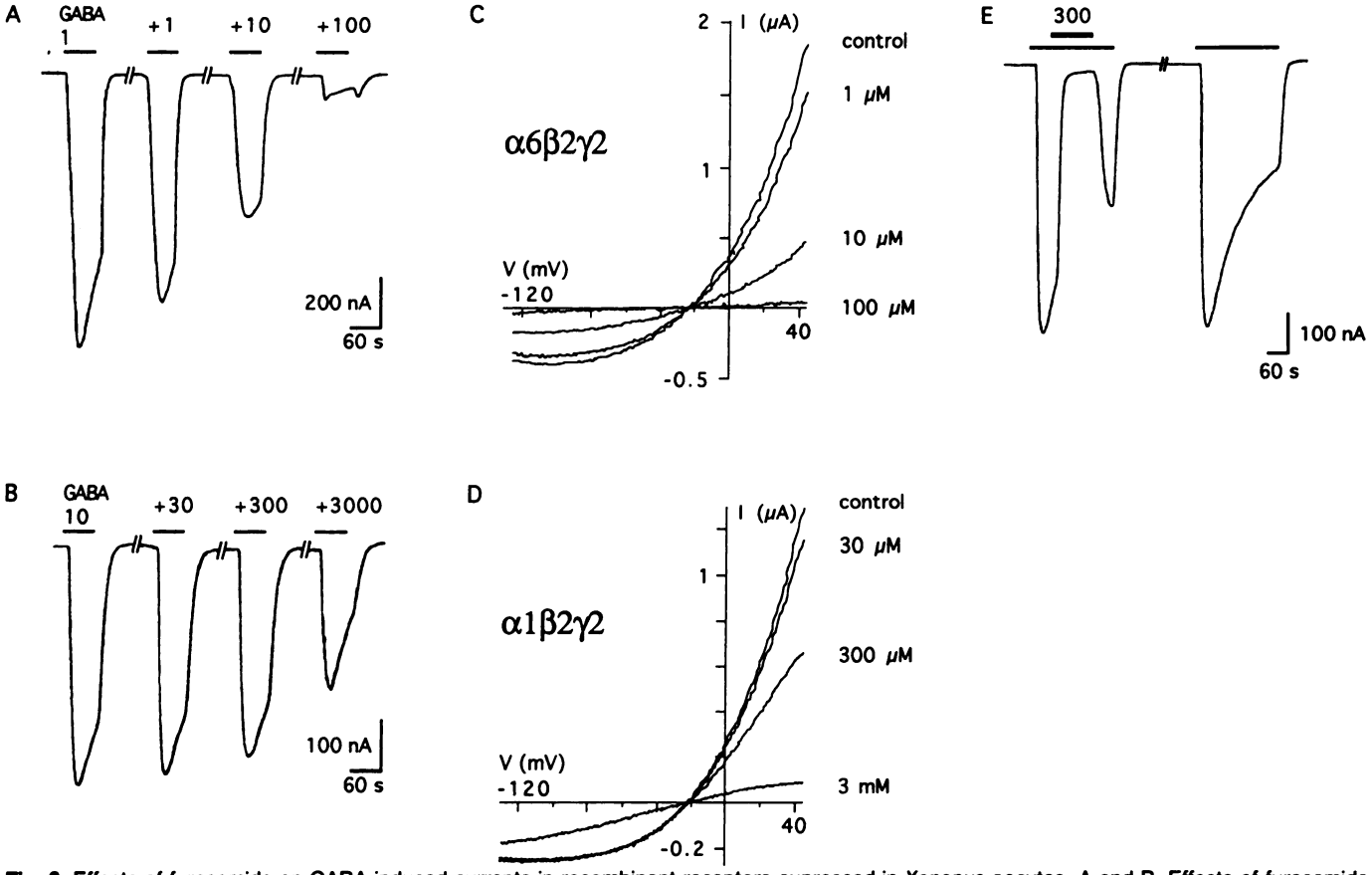

Fig. 6. Effects of furosemide on GABA-induced currents in recombinant receptors expressed in *Xenopus* oocytes. A and B, Effects of furosemide (concentrations in μM) on current responses to 1 μM GABA with $\alpha6\beta2\gamma2$ receptors (A) and to 10 μM GABA with $\alpha1\beta2\gamma2$ receptors (B), at a membrane potential of −70 mV. GABA and furosemide were simultaneously applied by bath perfusion. C and D, Voltage ramps applied in the presence of 1 μM GABA with $\alpha6\beta2\gamma2$ receptors (C) and 10 μM GABA with $\alpha1\beta2\gamma2$ receptors (D), with or without different concentrations of furosemide. E, Reversibility of furosemide blockade of GABA responses of $\alpha6\beta2\gamma2$ receptors. GABA (1 μM) was continuously perfused for 4 min with (*left*) or without (*right*) a 2-min pulse of GABA plus furosemide (300 μM). After discontinuation of the furosemide pulse, the amplitude recovered immediately to about that found in the desensitizing control trace. *Gaps*, 5-min washes between applications in representative traces.

cerebellar [^{3}H]Ro 15-4513 binding, indicating that furosemide action is apparently unaffected by the benzodiazepine recognition site. Furthermore, furosemide increased [^{35}S]TBPS binding and therefore cannot recognize the convulsant site itself. Numerous other binding sites exist on $GABA_A$ receptors, e.g., those for neurosteroids, barbiturates, and loreclezole (14, 40). However, furosemide interacted with the cerebellar convulsant site labeled by [^{35}S]TBPS in a distinct manner, i.e., it enhanced the binding in the absence and presence of GABA by increasing the affinity. This suggests that furosemide acts on a novel allosteric site.

Furosemide can be used to define the structural domains important for $GABA_A$ receptor channel function. Using recombinant receptors, we excluded the involvement of the arginine/histidine residue (position 100 in α6 cDNA) (11) in the extracellular domain. The residue in this position determines the differential benzodiazepine agonist affinities of α6 and α1 subunit-containing recombinant and naturally occurring receptor mutants (21, 23). The structural correlates for furosemide antagonism appear to be more complex, however, because furosemide action also depended on the presence of β2 or β3 variants, with the β1 variant being inactive. Our data further indicate that the majority of native α6 subunit-containing receptors are likely to contain either β2 or β3 subunits, a notion supported by localization of the β variant mRNAs (31, 32). Cerebellar granule cells also express putative $GABA_A$ receptor δ subunits (31), which may assemble in a portion of α6 subunit-containing receptors (41). Their role in $GABA_A$ receptor function and in furosemide-sensitive receptors remains to be studied.

The roles of the $GABA_A$ receptor subtypes in cerebellar granule cells are unknown. Puia *et al.* (42), using the whole-cell voltage-clamp mode, electrophysiologically characterized the components of the granule cell spontaneous inhibitory postsynaptic potentials with fast and slow decays. Similar components were observed by rapid application of GABA to nucleated outside-out patches of granule cells. The structural basis of these findings remains to be established. Because furosemide (500 μM) failed to alter the decay times of the spontaneous inhibitory postsynaptic potentials, we may now exclude the participation of the $\alpha6\beta2/3\gamma2$ receptors. Because the α6 subunit is detected only in Golgi cell/granule cell synapses, whereas the α1 subunit protein is found both in synaptic membranes and in nonsynaptic somatic membranes (43), it is possible that Puia *et al.* (42) detected only the non-α6 receptor responses. Further studies might use the specific $\alpha6\beta2/3\gamma2$ receptor antagonism of furosemide to investigate the physiology of this receptor subtype in preparations with intact cerebellar circuitry.

In summary, we have described the first subtype-specific antagonist for any $GABA_A$ receptor, which could prove useful for research on neurobiology and receptor structure. Although usage of furosemide *in vivo* is complicated by its potent diuretic effects due to blockade of the $Na^+/2\ Cl^-/K^+$ co-transporter (44) at concentrations similar to those needed to block the cerebellar $\alpha6\beta2/3\gamma2$ $GABA_A$ receptor, furosemide may serve as a lead molecule for the design of novel compounds selectively acting on different $GABA_A$ receptor subtypes.

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