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Receptor Subtype-Dependent Positive and Negative Modulation of $GABA_A$ Receptor Function by Niflumic Acid, a Nonsteroidal Anti-Inflammatory Drug

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ABSTRACT

In addition to blocking cyclooxygenases, members of the fenamate group of nonsteroidal anti-inflammatory drugs have been proposed to affect brain $GABA_A$ receptors. Using quantitative autoradiography with $GABA_A$ receptor-associated ionophore ligand [^{35}S]*t*-butylbicyclophosphorothionate (TBPS) on rat brain sections, one of the fenamates, niflumate, at micromolar concentration was found to potentiate GABA actions in most brain areas, whereas being in the cerebellar granule cell layer an efficient antagonist similar to furosemide. With recombinant $GABA_A$ receptors expressed in *Xenopus laevis* oocytes, we found that niflumate potentiated 3 μM GABA responses up to 160% and shifted the GABA concentration-response curve to the left in $\alpha1\beta2\gamma2$ receptors, the predominant $GABA_A$ receptor subtype in the brain. This effect needed the $\gamma2$ subunit, because on $\alpha1\beta2$ receptors, niflumate exhibited solely an antagonistic effect at high concentrations. The potentiation was not abolished by the specific benzodiazepine site antagonist flumazenil. Niflumate acted as a potent antagonist of $\alpha6\beta2$ receptors (with or without $\gamma2$ subunit) and of $\alpha X\beta2\gamma2$ receptors containing a chimeric $\alpha1$ to $\alpha6$ subunit, which suggests that niflumate antagonism is dependent on the same transmembrane domain 1- and 2-including fragment of the $\alpha6$ subunit as furosemide antagonism. This antagonism was noncompetitive because the maximal GABA response, but not the potency, was reduced by niflumate. These data show receptor subtype-dependent positive and negative modulatory actions of niflumate on $GABA_A$ receptors at clinically relevant concentrations, and they suggest the existence of a novel positive modulatory site on $\alpha1\beta2\gamma2$ receptors that is dependent on the $\gamma2$ subunit but not associated with the benzodiazepine binding site.

The structure and function of ligand-gated ion channels and their integral receptors is not well understood; therefore, it is important to establish new tools to probe various receptor subunit interactions, which can lead to novel ideas for the development of receptor subtype-selective drugs. GABA is the major inhibitory neurotransmitter in the mammalian brain. Its fast actions are mediated through ligand-gated anion channels, GABA type A ($GABA_A$) receptors, which are distributed throughout the brain. Receptor activation at cell membrane alters the conformation of the receptor normally leading to opening of the ionophore and anion flux, hyperpolarization of the cell, and inhibition of neuronal excitability. The $GABA_A$ receptor subtypes are formed by temporal and spatial regulation of subunit [$\alpha1$–6, $\beta1$–3, $\gamma1$–3, δ, ϵ, θ, $\rho1$–3, and π; Barnard et al., 1998; Bonnert et al., 1999; Sinkkonen et al., 2000) expression in brain regions and/or by cellular regulation of assembly to pentameric receptor complexes. $GABA_A$ergic drugs, such as benzodiazepines, barbiturates, and volatile anesthetics, enhance the actions of GABA (Sieghart, 1995) and are used to treat, e.g., anxiety, insomnia, and epilepsy, and in general anesthesia. Many drug effects on $GABA_A$ receptor function have been shown to depend on subunit combinations (subtypes) and even on critical amino acids in specific subunits (Korpi et al., 2002).

The $GABA_A$ receptor convulsant [^{35}S]*t*-butylbicyclophosphorothionate (TBPS) specifically binds to a picrotoxinin-sensitive site associated with the $GABA_A$ receptor ionophore (Squires et al., 1983). It has been a useful neurochemical tool in $GABA_A$ receptor research, because [^{35}S]TBPS binding can be studied on native receptor populations in brain sections and homogenates. Positive modulators, such as GABA and barbiturates, decrease [^{35}S]TBPS binding (Squires et al., 1983; Maksay and Ticku, 1985), whereas negative modulators, such as bicuculline, block this effect of GABA (Squires

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ABBREVIATIONS: TBPS, *t*-butylbicyclophosphorothionate; EBOB, 4′-ethynyl-4-*n*[2,3-H_2]propyl-bicycloorthobenzoate; NSAID, nonsteroidal anti-inflammatory drug; TM, transmembrane; NFR, normal frog Ringer; ANOVA, analysis of variance.

and Saederup, 1987). Furosemide, presently the most subtype-selective antagonist, noncompetitively increases the binding in the cerebellar granule cell layer and α6 subunit-containing recombinant receptors (Korpi et al., 1995). Modulation of [^{35}S]TBPS binding thus reveals also allosteric interactions within the receptor complex. Importantly, dissociation of [^{35}S]TBPS binding by GABA and other positive modulators has been shown to reflect receptor function itself as measured, for example, by $^{36}Cl^-$ flux assay (Im and Blakeman, 1991). Thus, [^{35}S]TBPS autoradiography on brain sections offers the advantage that pharmacological heterogeneity of the $GABA_A$ receptor subtypes can be visualized in various brain regions and preliminarily correlated with function.

Niflumate (Fig. 1) belongs to the fenamate group of nonsteroidal anti-inflammatory drugs (NSAIDs). It is available for clinical use in several European countries. Its mechanism of action is believed to be based on inhibition of cycloxygenases (Cushman and Cheung, 1976) that results in antipyretic, analgesic, and anti-inflammatory effects (Vane and Botting, 1998). In addition to these effects on prostaglandin synthesis, it has been shown to interact with central $GABA_A$ receptors in vitro. Niflumate decreases the enhancing effect of permeable anions on [^{35}S]TBPS binding (Evoniuk and Skolnick, 1988). It also decreases the binding of another convulsant, 4′-ethynyl-4-*n*[2,3-$^3H_2$]propyl-bicycloorthobenzoate ([^{3}H]EBOB), to the $GABA_A$ receptor picrotoxinin-sensitive site on rat brain membranes and enhances the inhibitory effect of GABA on the binding (Maksay et al., 1998). Niflumate has GABA-enhancing actions on both cortical and cerebellar membranes, whereas furosemide (Fig. 1) antagonizes GABA in cerebellar membranes (Maksay et al., 1998). Niflumate inhibits GABA-induced currents in *Xenopus laevis* oocytes injected with rat brain total RNA (White and Aylwin, 1990), but it has been also reported to have bimodal effects on GABA-induced currents in *X. laevis* oocytes injected with rat cortical poly(A)$^+$ RNA (Woodward et al., 1994), because 10 μM niflumate potentiates responses to 10 μM GABA and inhibits responses to maximal (3 mM) GABA with IC_{50} value of 7 μM. As is evident from the above-mentioned findings, the mechanisms of positive and negative modulatory actions of niflumate on $GABA_A$ receptor function remain to be clarified. Because NSAIDs permeate the blood-brain barrier (Bannwarth et al., 1989), there is a possibility that niflumate affects $GABA_A$ receptors in vivo. In fact, another fenamate, mefenamic acid, has been shown to have severe central effects, including convulsions and coma in human overdose (Smolinske et al., 1990). We have now studied the mode of action of niflumate on $GABA_A$ receptor subtypes by using 1) [^{35}S]TBPS autoradiography on rat brain sections to reveal heterogeneity in niflumate actions on native $GABA_A$ receptors, and 2) two-electrode voltage-clamp recording of *X. laevis* oocytes expressing recombinant $GABA_A$ receptor to assess the receptor subunit dependence of niflumate actions at functional level. We revealed subunit dependence for both positive and negative modulatory actions of niflumate.

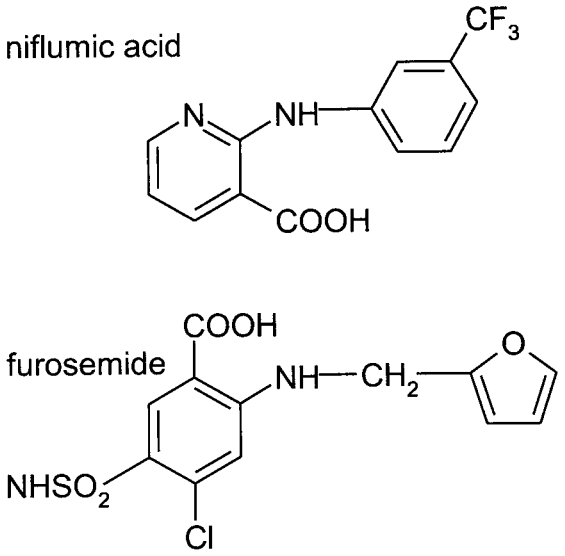

Fig. 1. Structures of niflumic acid and furosemide.

Materials and Methods

All experimental procedures were done with the permission from the Western Finland Provincial Government and the Institutional Animal Use and Care committee of the University of Turku (Turku, Finland).

Ligand Autoradiography. Male Wistar rats (n = 4; Central Animal Laboratory, University of Turku) were maintained in a stainless steel wire-mesh cage with pellet food (Special Diet Service; Witham, Essex, England) and tap water available ad libitum. Rats were decapitated at the age of 4 months, and whole brains were carefully dissected out, rinsed in ice-cold saline, and frozen on dry ice. The frozen brains were wrapped in plastic, and stored at −80°C. [^{35}S]TBPS autoradiography was modified from the standard assay (Sinkkonen et al., 2001). In brief, 14-μm horizontal rat brain sections were cut in a Microm HM 500 OM cryostat (Microm International GmbH, Walldorf, Germany). The sections were thaw-mounted onto gelatin-coated object glasses and stored frozen under desiccant at −20°C. For autoradiography, sections were preincubated in an ice-cold 50 mM Tris-HCl supplemented with 120 mM NaCl, pH 7.4, for 15 min. Final incubation in the preincubation buffer was performed with 3 nM (421 cpm/μl) [^{35}S]TBPS (PerkinElmer Life Sciences, Boston, MA) at room temperature (22°C) for 90 min. Nonspecific binding was determined with 100 μM picrotoxinin (Sigma-Aldrich, St. Louis, MO). Displacement of [^{35}S]TBPS binding was studied in the absence and presence of 3 μM or 1 mM GABA (Sigma-Aldrich). The effects of 10, 30, 100, 300, and 1000 μM niflumate (Sigma-Aldrich, dissolved in 0.1 N NaOH) and 100 μM furosemide (Sigma-Aldrich, dissolved in 0.1 N NaOH) on [^{35}S]TBPS binding were tested with or without 3 μM or 1 mM GABA. Niflumate or furosemide did not affect the pH of the incubation buffer. After incubation, the sections were washed three times for 30 min in ice-cold 10 mM Tris-HCl buffer, pH 7.4. Sections were then dipped into distilled water, air-dried at room temperature, and exposed with a plastic ^{14}C standard to BioMax MR films (Eastman Kodak, Rochester, NY), for 3 days (basal [^{35}S]TBPS binding) or 2 weeks (other binding conditions). Regional labeling intensities of the sections were quantified from the films by using AIS image analysis devices and programs (Imaging Research, St. Catherine's, ON, Canada) the binding values given as radioactivity levels estimated for gray matter areas (in nanocuries per gram).

Recombinant Receptor Expression in *X. laevis* Oocytes. Capped cRNAs coding for rat $GABA_A$ receptor subunits α1, α6, β2, and γ2S (Lüddens et al., 1990; Shivers et al., 1989; Ymer et al., 1989), and α1-16 chimera [where amino acids of the α6 subunit including the first two transmembrane (TM) domains replace the corresponding amino acids in α1 subunit frame to gain furosemide sensitivity; Jackel et al., 1998], were transcribed in vitro from pRK5 plasmids using mMessage mMachine kit (Ambion, Austin, TX) according to manufacturer's instructions. Oocytes were dissected from adult *X. laevis* female frogs (Horst Kähler, Hamburg, Germany) anesthetized with 0.2% tricaine methanesulfonate (Sigma-Aldrich). Isolated oo-

cytes were stored in normal frog Ringer (NFR): 115 mM NaCl, 2.5 mM KCl, 18 mM $CaCl_2$, and 10 mM HEPES, pH 7.5. Oocytes were then defolliculated manually and injected with 46 nl of a solution containing mixtures of subunit cRNAs (0.1–2.5 $\mu g/\mu l$) or pure H_2O with Drummond Nanoject injector (Drummond Scientific Co., Broomall, PA) via a glass micropipette with i.d. of 20 to 40 μm. The oocytes were incubated at 19°C in incubation solution [88 mM NaCl, 1 mM KCl, 2.4 mM $NaHCO_3$, 10 mM HEPES, 0.82 mM $MgSO_4$, 0.33 mM $Ca(NO_3)_2$, 0.91 mM $CaCl_2$, 0.5 mM theophylline, 2 mM sodium pyruvate, 10 U/ml penicillin and 10 μg/ml streptomycin, pH 7.5]. After injection (2 h–1 day) oocytes were digested for 30 min in Ca^{2+}-free medium (82.5 mM NaCl, 2.5 mM KCl, 1 mM $MgCl_2$, 1 mM Na_2HPO_4, and 5 mM HEPES, pH 7.5) containing 0.3 U/ml collagenase type IA (Sigma-Aldrich). Thereafter, the oocytes were incubated in incubation solution until recordings.

Electrophysiological Recordings. For each experiment, oocytes from at least two different frogs were used. Electrophysiological recordings were made 1 to 3 days after cRNA injection. Oocytes were perfused with NFR ± drugs at a flow rate of 1 ml/min at room temperature (22°C) using Ismatec pump (Ismatec, Glattbrugg-Zürich, Switzerland) and 17 channel perfusion system with pinch valves. Drug combinations were mixed before experiments. Oocytes were impaled with two microelectrodes (1.0–2.5 MΩ) filled with 3 M KCl plus 10 mM EGTA, and voltage clamped at −50 mV with Turbo TEC-05 two electrode voltage-clamp amplifier (NPI Electronic GmbH, Tamm, Germany). Experiments were controlled by EggWorks experimental control and data acquisition software program version 3.0.2 (NPI Electronic GmbH). GABA was dissolved in NFR. Niflumate and furosemide were dissolved in 0.1 M NaOH, stocks were diluted in NFR to a concentration of 10 mM, and pH was adjusted to 7.5. Drugs were applied for 10 s unless otherwise stated, and 180- to 600-s washout period was used, depending on drug concentrations. In the GABA concentration-response experiments, any given GABA concentration was first applied alone and thereafter in the presence of niflumate. All different GABA concentrations were tested in every oocyte.

Data Analysis and Statistics. Data analyses were performed using EggWorks Reader version 3.0.2 (NPI Electronic GmbH) and GraphPad Prism version 3.0 (GraphPad Software Inc., San Diego, CA) programs. For autoradiography, the specific [^{35}S]TBPS binding values were determined by subtracting the nonspecific binding values from the corresponding total binding values under each incubation condition. To assess the statistical significance of the niflumate effects on [^{35}S]TBPS binding, one-way analysis of variance (ANOVA) and Dunnett's post hoc test were used. Student's t test was used for 100 μM furosemide effect. For electrophysiological recordings, the amplitudes of peak currents induced by GABA + drug applications were determined from recorded traces, normalized to the corresponding GABA-induced peak currents estimated linearly between the GABA peak currents closest before and after the applications of GABA with the drugs, and presented as a percentage of the control GABA current. The peak currents induced by various GABA concentrations for each oocyte were normalized by setting the maximal GABA current without niflumate to 100%, and the GABA concentration-response curves were generated using nonlinear regression fit. The statistical significance of the niflumate modulation of the GABA response was assessed with one-way ANOVA and Dunnett's post hoc test. Furosemide and niflumate effects at 1,000 μM without additional GABA were assessed using Student's t test.

Results

Niflumate Effects on Native $GABA_A$ Receptors in Brain Sections. [^{35}S]TBPS binding to picrotoxinin-sensitive convulsant site in $GABA_A$ receptors was observed throughout the rat brain in ligand autoradiography (Fig. 2). Niflumate decreased the basal [^{35}S]TBPS binding in all brain regions at 300 and 1,000 μM concentrations, the latter leaving less than 10% of the basal binding (Fig. 2; Table 1). This was in accordance with previous results, because niflumate has been shown to decrease both [^{35}S]TBPS and [^{3}H]EBOB bindings to

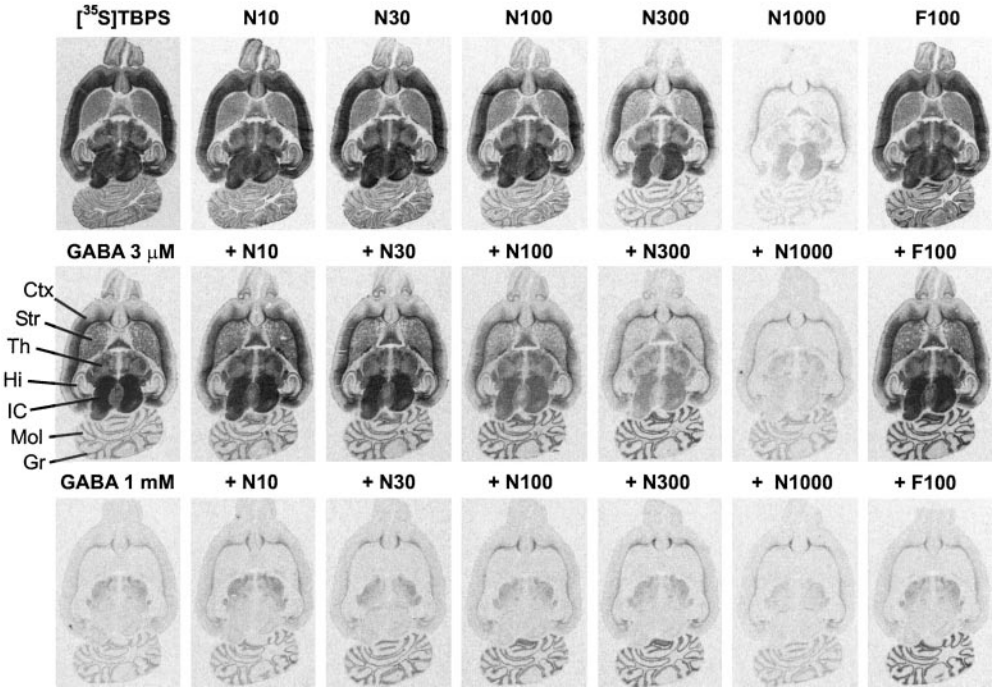

Fig. 2. Regional [^{35}S]TBPS binding to rat brain sections and its modulation by niflumate (N, micromolar concentration) or furosemide (F, 100 μM) in the absence and presence of 3 μM and 1 mM GABA. Ctx, cortex; Gr, cerebellar granule cell layer; Hi, hippocampus; IC, inferior colliculus; Mol, cerebellar molecular layer; Str, striatum; Th, thalamus. Images in the first top row are from a film exposed for 3 days; others are from a film exposed for 2 weeks.

rat brain homogenates without additional GABA (Evoniuk and Skolnick, 1988; Maksay et al., 1998). In contrast to niflumate, 100 μM furosemide increased the binding in the cerebellar granule cell layer but was inactive in the forebrain, in keeping with previous results (Korpi et al., 1995).

Adding 3 μM GABA clearly inhibited the basal [^{35}S]TBPS binding in all brain areas (Fig. 2; Table 2). Regional diversity of niflumate actions was revealed: whereas GABA-inhibited [^{35}S]TBPS binding was further decreased in a concentration-dependent manner in the forebrain, the cerebellar granule cell layer binding component was increased maximally to 200 ± 14% (mean ± S.E., n = 4) by 100 μM niflumate. This latter effect seemed to be biphasic, because 1,000 μM niflumate decreased the binding below control values. Furosemide at 100 μM similarly increased the GABA-inhibited [^{35}S]TBPS binding to maximally 332 ± 36% in the cerebellar granule cell layer. In a previous study, niflumate enhanced the inhibitory effect of 2 μM GABA on [^{3}H]EBOB binding both in the cerebrocortical and cerebellar membranes, whereas furosemide was clearly antagonistic in the cerebellum (Maksay et al., 1998). In the present experiments using brain sections, we were able to differentiate the cerebellar effects of niflumate to an antagonism in the granule cell layer and to an agonism in the molecular layer; the net effect (i.e., when the cerebellum was analyzed as a whole) was negligible up to 300 μM (Table 2). Thus, it seems that in brain homogenate studies (Maksay et al., 1998) the clear GABA antagonism by niflumate in the cerebellar granule cell layer seen now in autoradiography is masked by the agonism on the quantitatively larger receptor population of the molecular layer.

When 1 mM GABA was added to reveal the GABA-insensitive [^{35}S]TBPS binding component (Sinkkonen et al., 2001), [^{35}S]TBPS was displaced to background level in most brain regions, whereas many thalamic nuclei and cerebellar granule cell layer were still labeled (Fig. 2). Adding niflumate (300 μM) in this condition enhanced [^{35}S]TBPS binding in the granule cell layer maximally up to 414 ± 22% from the 1 mM GABA level. The thalamic component of the GABA-insensitive [^{35}S]TBPS binding was also increased about 50% by low niflumate concentrations, whereas it was decreased by 1,000 μM (Table 3). Furosemide efficiently blocked the 1 mM GABA action in the cerebellar granule cell layer and had a weak GABA antagonist effect also in the thalamus.

Positive and Negative Modulation of $\alpha1\beta2\gamma2$ and $\alpha6\beta2\gamma2$ GABA$_A$ Receptor Function by Niflumate. To search for structural determinants for the regional heterogeneity of niflumate effects on GABA-modulated [^{35}S]TBPS binding, we applied two-electrode voltage clamp on *X. laevis* oocytes expressing recombinant GABA$_A$ receptors. Because niflumate possesses some structural similarities to furosemide (Fig. 1), and inhibition of the GABA effect on [^{35}S]TBPS binding by these two compounds was regionally similar, we hypothesized that the antagonism by niflumate is similar

TABLE 1
Regional [^{35}S]TBPS binding to rat brain sections and its modulation by niflumate or furosemide in the absence of GABA as revealed by quantitative autoradiography
Values in the presence of niflumate or furosemide are percentages of the corresponding basal [^{35}S]TBPS binding value. Values are mean ± S.E. (n = 4).

Brain Region	[^{35}S]TBPS	+ Niflumate					+ Furosemide
		10 μM	30 μM	100 μM	300 μM	1,000 μM	100 μM
	nCi/g	%					
Cerebellum	172 ± 8	111 ± 13	106 ± 8	78 ± 12	39 ± 1**	7 ± 1**	160 ± 7***
Granule cell layer	147 ± 4	231 ± 20**	252 ± 10**	220 ± 17**	135 ± 7	34 ± 2**	388 ± 16***
Molecular layer	178 ± 8	58 ± 6**	60 ± 8**	37 ± 9**	14 ± 1**	1 ± 0**	93 ± 5
Cortex	515 ± 26	114 ± 5	102 ± 2	94 ± 6	52 ± 2**	7 ± 1**	97 ± 3
Striatum	253 ± 22	97 ± 6	94 ± 5	85 ± 7	50 ± 2**	4 ± 0**	99 ± 2
Thalamus	428 ± 24	97 ± 5	92 ± 3	87 ± 6	53 ± 2**	7 ± 0**	90 ± 7
Hippocampus	241 ± 9	105 ± 4	92 ± 2	69 ± 6**	31 ± 2**	3 ± 0**	88 ± 5
Inferior colliculus	574 ± 33	100 ± 6	101 ± 5	94 ± 3	71 ± 2**	16 ± 1**	99 ± 5

**, $P < 0.01$; ***, $P < 0.001$, significantly different from the corresponding control values (one-way ANOVA followed by Dunnett's post hoc test for niflumate, Student's t test for furosemide).

TABLE 2
Regional [^{35}S]TBPS binding to rat brain sections and its modulation by niflumate or furosemide in the presence of 3 μM GABA as revealed by quantitative autoradiography
Values in the presence of niflumate or furosemide are percentages of the corresponding GABA-modulated [^{35}S]TBPS binding value. Values are mean ± S.E. (n = 4).

Brain Region	3 μM GABA	% of Basal	+ Niflumate					+ Furosemide
			10 μM	30 μM	100 μM	300 μM	1,000 μM	100 μM
	nCi/g	%	%					
Cerebellum	19 ± 2	11 ± 1	104 ± 12	117 ± 11	135 ± 17	114 ± 18	19 ± 3**	334 ± 45**
Granule cell layer	46 ± 2	31 ± 2	144 ± 8**	160 ± 4**	200 ± 14**	189 ± 8**	54 ± 2**	332 ± 36***
Molecular layer	6 ± 2	3 ± 1	58 ± 31	49 ± 22	9 ± 6*	1 ± 1*	0 ± 0*	124 ± 27
Cortex	124 ± 18	24 ± 3	85 ± 11	68 ± 10	34 ± 3**	12 ± 1**	1 ± 1**	95 ± 7
Striatum	79 ± 10	31 ± 3	90 ± 7	72 ± 5*	39 ± 2**	10 ± 0**	0 ± 0**	106 ± 8
Thalamus	125 ± 9	29 ± 1	86 ± 6	68 ± 5**	39 ± 2**	20 ± 1**	2 ± 0**	108 ± 8
Hippocampus	35 ± 2	14 ± 1	114 ± 10	104 ± 9	54 ± 2**	17 ± 2**	0 ± 0**	131 ± 12*
Inferior colliculus	319 ± 14	56 ± 2	63 ± 3**	50 ± 3**	25 ± 1**	10 ± 0**	0 ± 0**	87 ± 7

*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$, significantly different from the corresponding control values (one-way ANOVA followed by Dunnett's post hoc test for niflumate, Student's t test for furosemide).

to that by furosemide. Furosemide antagonizes GABA noncompetitively in recombinant receptors containing $\alpha 6$ and $\beta 2/3$ subunits (Korpi et al., 1995), which are abundantly expressed in the cerebellar granule cell layer (Wisden et al., 1992). Most native $GABA_A$ receptors contain $\gamma 2$ subunit, which holds true also in the cerebellar granule cell layer (Whiting et al., 1995). For these reasons, recombinant $\alpha 6\beta 2\gamma 2$ receptors were chosen for antagonism studies. Because the GABA-potentiating action of niflumate was seen throughout the brain in the [^{35}S]TBPS autoradiography, subunit combination of $\alpha 1\beta 2\gamma 2$, as the most abundant $GABA_A$ receptor subtype in the native brain (Whiting et al., 1995), was selected for studies on positive modulation.

Different GABA concentrations were applied to oocytes to determine the GABA sensitivities of $\alpha 1\beta 2\gamma 2$ and $\alpha 6\beta 2\gamma 2$ receptors (Fig. 3b, inset). Nonlinear regression fit of normalized GABA currents yielded concentration-response curves with EC_{50} values of 8.0 and 1.8 μM for $\alpha 1\beta 2\gamma 2$ and $\alpha 6\beta 2\gamma 2$ receptors, respectively. In autoradiography, niflumate and furosemide effects were tested in the presence of 3 μM GABA, which concentration induced about 30 and 60% of the maximal response in $\alpha 1\beta 2\gamma 2$, and in $\alpha 6\beta 2\gamma 2$ receptors, respectively. These were optimal responses for studies on positive and negative modulations, respectively. When niflumate was applied to oocytes expressing $\alpha 1\beta 2\gamma 2$ receptors, it potentiated the 3 μM GABA-evoked currents in a concentration-dependent manner (EC_{50} of 31 ± 3 μM, E_{max} of 162 ± 4% of the basal response), with potentiation of 61 ± 7% at the highest 1,000 μM concentration used (Fig. 3). When niflumate was applied alone, it elicited a small inward current at 1,000 μM concentration, lower concentrations having no effect (data not shown). Furosemide at 100 μM tended to enhance GABA-induced currents in $\alpha 1\beta 2\gamma 2$ receptors, but the effect was not statistically significant. Niflumate antagonized the 3 μM GABA-evoked currents in oocytes expressing $\alpha 6\beta 2\gamma 2$ receptors in a concentration-dependent manner (Fig. 3). Inhibition by 1000 μM niflumate was 74 ± 4% of the GABA-induced current. When 1,000 μM niflumate was applied alone, it elicited a small outward current, lower concentrations being ineffective (data not shown). Furosemide at 100 μM inhibited 3 μM GABA responses similarly to high niflumate concentrations. The traces shown in Fig. 3a suggest some "run-up" of the GABA response during experiments. This was variable between oocytes from different frogs and was not caused by repeated niflumate applications. For example, there was no run-up in the GABA concentration-response experiments (data not shown), when each GABA concentration was applied twice, first without and then with niflumate, in random order of GABA concentrations (see below).

To study the mechanisms of different niflumate effects in oocytes expressing $\alpha 1\beta 2\gamma 2$ and $\alpha 6\beta 2\gamma 2$ receptors, the GABA concentration-response curves were determined in the presence and absence of niflumate. Niflumate concentration of 100 μM was chosen for these experiments, because it resulted in clear positive and negative actions on 3 μM GABA response in $\alpha 1\beta 2\gamma 2$ and $\alpha 6\beta 2\gamma 2$ receptors, respectively (Fig. 3b). In $\alpha 1\beta 2\gamma 2$ receptors, niflumate shifted the GABA concentration-response curve to the left (EC_{50} of 12.5 and 6.3 μM in the absence or presence of niflumate, respectively), without notable change in the efficacy (maximal response 93 ± 5% of the response without niflumate; Fig. 4). In $\alpha 6\beta 2\gamma 2$ receptors, niflumate reduced robustly the efficacy (E_{max} 58 ± 3% of the maximal GABA response without niflumate), whereas affinity to GABA was unaltered (EC_{50} of 2.0 and 2.5 μM in the absence or presence of niflumate, respectively). These results suggest that niflumate acts as a positive allosteric modulator in $\alpha 1\beta 2\gamma 2$ receptors, and as a noncompetitive negative modulator in $\alpha 6\beta 2\gamma 2$ receptors.

To study whether niflumate effects on $\alpha 1\beta 2\gamma 2$ and $\alpha 6\beta 2\gamma 2$ receptors were reversible, we applied short niflumate pulses during long GABA applications. In both cases, niflumate elicited fast and reversible effects on GABA currents (Fig. 5).

Niflumate has been shown to block Ca^{2+}-activated Cl^- channels endogenously expressed in *X. laevis* oocytes (White and Aylwin, 1990). To assess the possible interference of this effect in our experiments, 1,000 μM niflumate was applied to oocytes injected with distilled H_2O. Only a minute outward current was detected (8.4 ± 3.5 nA, $n = 8$). This was negligible compared with niflumate effects on GABA-induced currents in oocytes expressing $GABA_A$ receptors. Furthermore, our assay being sensitive to positive and negative modulations of niflumate should not involve this current at all, because both $\alpha 6\beta 2(\gamma 2)$ and $\alpha 1\beta 1\gamma 2$ pass anions and not allow Ca^{2+} to activate the additional current.

A Segment of $\alpha 6$ Subunit Is Sufficient to Induce Niflumate Antagonism in $\alpha 1\beta 2\gamma 2$ Receptors. Furosemide antagonism on $\alpha 6$ receptors has been extensively studied using $\alpha 1/\alpha 6$ subunit chimeras and point mutations. It was first shown with chimeric constructs that the main determinant for furosemide action is located in the N-terminal part of the TM1 of $\alpha 6$ subunit (Fisher et al., 1997; Jackel et al., 1998), where isoleucine at position 228 was later pin-pointed as the crucial amino acid (Thompson et al., 1999). Substitution of a 258-base pair fragment, including the TM1 and TM2 domains of the $\alpha 1$ subunit with that of $\alpha 6$ subunit gene ($\alpha 1$-16 chimera), is thus enough to confer furosemide antagonism (Jackel et al., 1998). To study whether the same structural requirements apply for niflumate antagonism, we coexpressed chimeric $\alpha 1$-16 subunit together with wild-type $\beta 2$

TABLE 3

Regional [^{35}S]TBPS binding to rat brain sections and its modulation by niflumate or furosemide in the presence of 1 mM GABA as revealed by quantitative autoradiography

Values in the presence of niflumate or furosemide are percentages of the corresponding GABA-modulated [^{35}S]TBPS binding value. Values are mean ± S.E. ($n = 4$).

Brain Region	1 mM GABA	% of Basal	+ Niflumate					+ Furosemide
			10 μM	30 μM	100 μM	300 μM	1,000 μM	100 μM
	nCi/g	%	%					
Granule cell layer	15.9 ± 1.2	10.8 ± 1.0	192 ± 8**	263 ± 12**	381 ± 30**	414 ± 22**	106 ± 11	680 ± 61***
Thalamus	9.8 ± 0.7	2.3 ± 0.1	165 ± 6*	157 ± 16*	144 ± 19	106 ± 16	31 ± 12**	129 ± 8*

*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$, significantly different from the corresponding control values (one-way ANOVA followed by Dunnett's post hoc test for niflumate, Student's t test for furosemide).

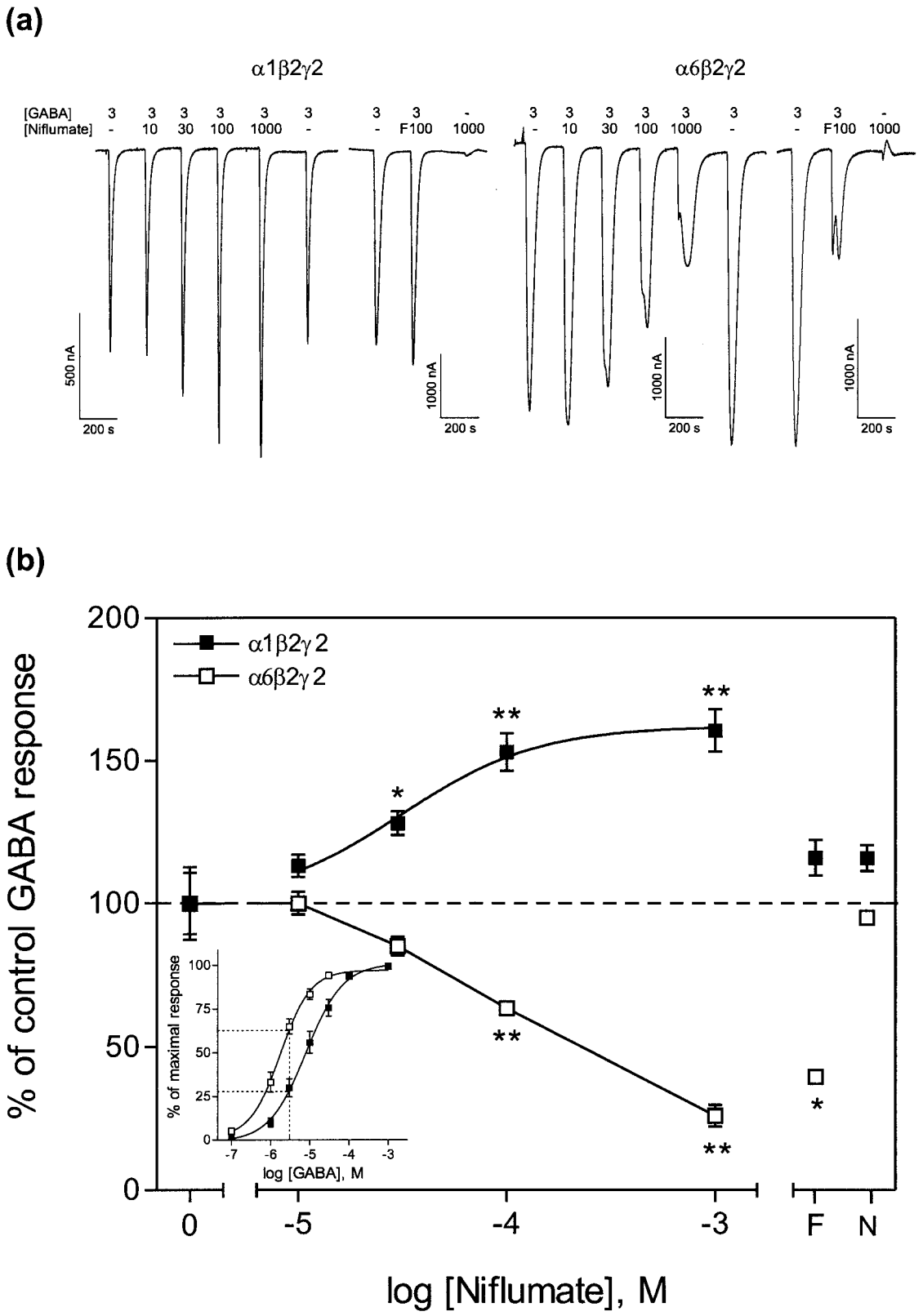

Fig. 3. Modulation of GABA-induced currents by niflumate and furosemide in recombinant $\alpha1\beta2\gamma2$ and $\alpha6\beta2\gamma2$ $GABA_A$ receptors expressed in *X. laevis* oocytes. a, representative current traces for the receptor subtypes as affected by 3 μM GABA, various niflumate concentrations, and furosemide (F100, 100 μM). b, values are presented as mean ± S.E. (n = 20 and 7 for $\alpha1\beta2\gamma2$ and $\alpha6\beta2\gamma2$ combinations, respectively), the 3 μM GABA control response being set to 100%. F, 100 μM furosemide in the presence of 3 μM GABA; N, 1000 μM niflumate alone. Significance of the difference from the corresponding control value (one-way ANOVA followed by Dunnett's post hoc test for niflumate in the presence of GABA, Student's t test for furosemide or niflumate effects alone) as follows: *, $p < 0.05$; **, $p < 0.01$. Inset, the GABA concentration-response curves for both receptor subtypes. Dashed line indicates the efficacy of 3 μM GABA. Maximal current peaks elicited by GABA were 3.7 ± 0.6 (n = 14) and 2.3 ± 0.4 μA (n = 6) for $\alpha1\beta2\gamma2$ and $\alpha6\beta2\gamma2$ combinations, respectively.

and γ2 subunits in *X. laevis* oocytes. The GABA affinity of the chimeric receptor was between α1β2γ2 and α6β2γ2 receptors, EC_{50} value being 5.1 μM (Fig. 6b, inset). When tested in the presence of 3 μM GABA, instead of potentiation seen in α1β2γ2 receptors (Fig. 3), niflumate tended to decrease current amplitudes at all concentrations, but only at 1,000 μM the effect was statistically significant (Fig. 6). Furosemide at 100 μM antagonized GABA currents similarly to the highest niflumate concentration used (1000 μM). When applied alone, 1,000 μM niflumate elicited a small outward current.

Niflumate Potentiation Is Dependent on Receptor γ2 Subunit. The α6β2/3 receptors are furosemide-sensitive irrespective of the presence of γ2 subunit (Korpi et al., 1995; Korpi and Lüddens, 1997). To test the role of γ2 subunit on niflumate actions, α1β2 and α6β2 receptors were expressed in *X. laevis* oocytes. Incorporation of γ2 subunit in the receptor complex decreases GABA affinity, which was also seen in our GABA concentration-response studies, as EC_{50} values for GABA were 1.7 and 0.46 μM for α1β2 and α6β2 receptors, respectively (Fig. 7b, inset). Because niflumate was hypothesized to have GABA-enhancing effects in α1β2 receptors, 1 μM GABA resulting in about 35% of maximal response was used. When niflumate was applied together with 1 μM GABA to α1β2 receptors, it was only active at the 1,000 μM concentration, which inhibited the GABA response by 29 ± 5% (Fig. 7). When applied alone, the same concentration of niflumate was inactive, as was furosemide. GABA at 3 μM induced about 90% of maximal response in α6β2 receptors and was suitable for antagonist studies. When niflumate was applied with 3 μM GABA on α6β2 receptors, concentration-dependent potent antagonism was evident (Fig. 7). Furosemide at 100 μM inhibited the GABA currents, and 1,000 μM niflumate alone elicited a small outward current.

Flumazenil Does Not Affect Niflumate Potentiation of α1β2γ2 Receptors. Benzodiazepine potentiation of $GABA_A$ receptors is dependent on the presence of a suitable α subunit and the γ2 subunit in the receptor complex (Pritchett et al., 1989). Because positive modulation by niflumate was present in α1 and γ2 subunit-containing receptors, it was of interest to study, whether it would be mediated by the benzodiazepine binding site. We applied the benzodiazepine site positive modulator zolpidem at 1 μM concentration on α1β2γ2 receptors together with 3 μM GABA. This resulted in 39 ± 6% potentiation of the GABA-induced current, which was totally blocked by 1 μM flumazenil, a benzodiazepine site antagonist (Fig. 8). When flumazenil was applied together with 100 μM niflumate and 3 μM GABA, it failed to affect the niflumate potentiation of the GABA-induced current (38 ± 4 versus 36 ± 6%).

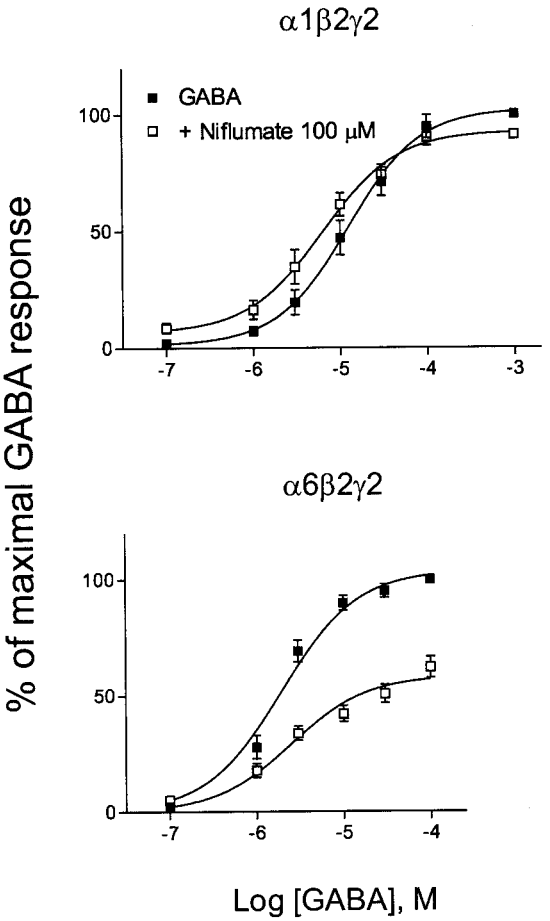

Fig. 4. Effect of 100 μM niflumate on GABA concentration-response curves in recombinant α1β2γ2 and α6β2γ2 $GABA_A$ receptors expressed in *X. laevis* oocytes. The values are presented as mean ± S.E. (n = 7 for both receptor subtypes), the maximal GABA-induced currents without niflumate being set to 100%. They were 3.3 ± 0.3 and 2.4 ± 0.2 μA for α1β2γ2 and α6β2γ2 receptors, respectively.

Discussion

$GABA_A$ receptor is well known for its many binding and effector sites for various chemical classes of ligands

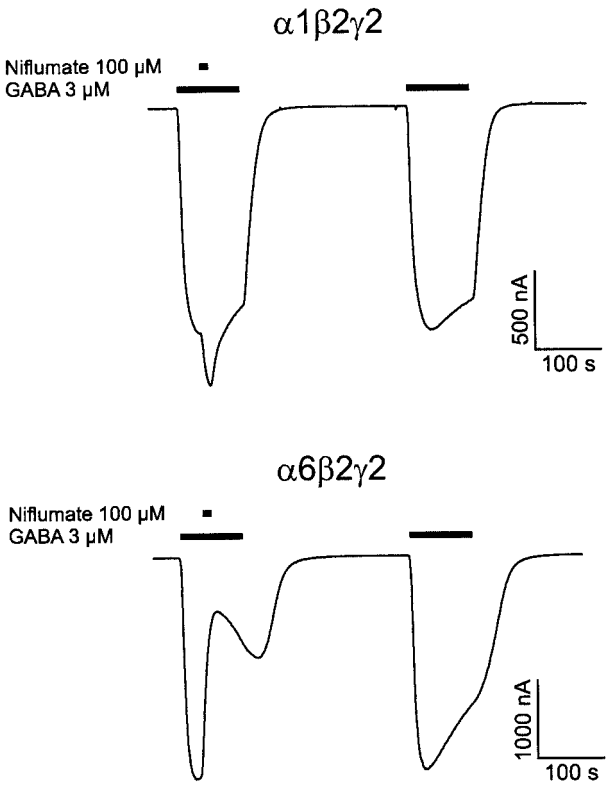

Fig. 5. Reversibility of the niflumate effects on GABA-elicited currents in α1β2γ2 and α6β2γ2 $GABA_A$ receptors expressed in *X. laevis* oocytes. In the traces on the left, 3 μM GABA was applied for 90 s, and 100 μM niflumate for 10 s in the middle of GABA application, as indicated by horizontal bars. The traces on the right show the GABA-induced current without niflumate.

(Sieghart, 1995; Korpi et al., 2002). Many ligands may have more than one binding/effector site on the receptor complex, mediating, for example, GABA-potentiating and direct "GABA site-independent" agonistic effects on the $GABA_A$ receptor. Good examples are intravenous anesthetics, such as propofol and pentobarbital (Inomata et al., 1988; Hales and Lambert, 1991; Korpi et al., 2002). Fewer compounds have been demonstrated to have distinct binding/effector sites mediating attenuation and potentiation of GABA responses. The β-carboline negative allosteric modulator methyl-6,7-dimethoxy-4-ethyl-β-carboline-3-carboxylate has a potent inhibitory action via the γ2 subunit-dependent benzodiazepine site and at higher concentrations a stimulatory "positive allosteric" effect via the coupling mechanism dependent on β2 and β3 subunits (Stevenson et al., 1995). The latter site is also involved in the antagonism of the GABA responses by furosemide (Thompson et al., 1999), this effect being also dependent on the α6 and α4 subunits (Korpi et al., 1995; Knoflach et al., 1996; Wafford et al., 1996). The present study reports on a fenamate NSAID, niflumate, that is a positive or negative modulator of the GABA responses, depending on the receptor subunit combination in both native and recombinant receptors.

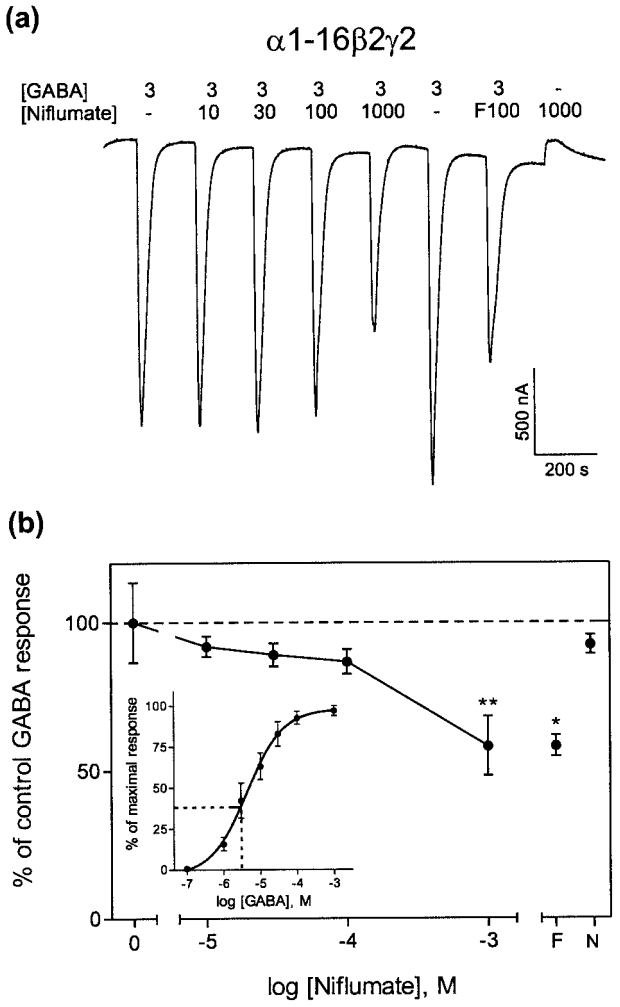

Fig. 6. Modulation of GABA-induced currents by niflumate and furosemide in chimeric α1-16β2γ2 $GABA_A$ receptors expressed in *X. laevis* oocytes. a, representative current trace as affected by 3 μM GABA, various niflumate concentrations, and furosemide (F100, 100 μM). b, values are presented as mean ± S.E. (n = 7), the 3 μM GABA control response being set to 100%. F, 100 μM furosemide in the presence of 3 μM GABA; N, 1000 μM niflumate alone. Significance of the difference from the corresponding control value (one-way ANOVA followed by Dunnett's post hoc test for niflumate in the presence of GABA; Student's t test for furosemide or niflumate effects alone) as follows: *, $p < 0.05$; **, $p < 0.01$. Inset, the GABA concentration-response curve, dashed line indicates the efficacy of 3 μM GABA. Maximal current elicited by GABA was 2.8 ± 0.2 μA (n = 5).

The native receptors were studied using autoradiography of rat brain sections with picrotoxinin-sensitive [^{35}S]TBPS binding to the $GABA_A$ receptor convulsant binding site. Without added GABA, niflumate decreased [^{35}S]TBPS binding in all brain areas (Fig. 2; Table 1), in keeping with previous binding studies (Evoniuk and Skolnick, 1988; Maksay et al., 1998). However, even without added GABA, brain sections containing endogenous GABA affecting [^{35}S]TBPS binding, which can be antagonized by GABA site antagonists (Korpi et al., 1992). It has been estimated that the endogenous GABA concentration reaches 1 μM in synaptosomal membranes (Im and Blakeman, 1991), and it is unlikely that the 15-min preincubation used here removes it completely. Thus, these niflumate effects might be interpreted as positive modulation of GABA effects rather than as independent "direct" action. This is in line with the negligible action of niflumate on recombinant receptors expressed in oocytes in the absence of exogenous GABA. These conclusions would imply that the niflumate potentiation of GABA action is a pure allosteric mechanism.

In search for structural correlates of the positive modulation by niflumate, we studied recombinant $GABA_A$ receptors composed of α1, β2, and γ2 subunits, the main receptor subtype in the brain (Whiting et al., 1995). The EC_{50} for GABA α1β2γ2 receptors was reduced by about 50%, indicating an allosteric potentiation by niflumate (Fig. 3). Furosemide did not consistently potentiate GABA, in keeping with Korpi et al. (1995). However, the α1β2 receptors were insensitive to niflumate up to 100 μM, and at 1,000 μM niflumate inhibited the GABA response by 30% in contrast to the potentiation of α1β2γ2 receptors (Fig. 7). This suggests that niflumate potentiation is dependent on the presence of γ2 subunit together with α1. The same subunit requirement is obligatory for the benzodiazepine binding site modulators, such as zolpidem (Pritchett et al., 1989). However, zolpidem and niflumate effects were differentially sensitive to the benzodiazepine binding site antagonist flumazenil in α1β2γ2 receptors, because only the GABA potentiation by zolpidem was blocked by 1 μM flumazenil (Fig. 8), a concentration that saturates the benzodiazepine binding sites ($K_D \approx$ 1 nM in α1βxγ2 receptors; Pritchett and Seeburg, 1990). These data suggest that niflumate potentiation is mediated via a novel site of the $GABA_A$ receptors dependent on α, β, and γ subunits.

Another fenamate group NSAID, mefenamic acid, was recently shown to potentiate GABA on α1 and β2/3 subunit-containing receptors, but to be inactive or inhibitory in β1 subunit-containing receptors (Halliwell et al., 1999). The GABA potentiating action was dependent on the asparagine residue of TM2 in β2 and β3 subunits (Halliwell et al., 1999), which is the critical residue for furosemide and methyl-6,7-dimethoxy-4-ethyl-β-carboline-3-carboxylate modulations as well (Stevenson et al., 1995; Thompson et al., 1999). Whether niflumate shows similar selectivity for the β subunits is still open, but as its potentiating effect needs the γ2 subunits, the mode of action is clearly different from that of the ligands

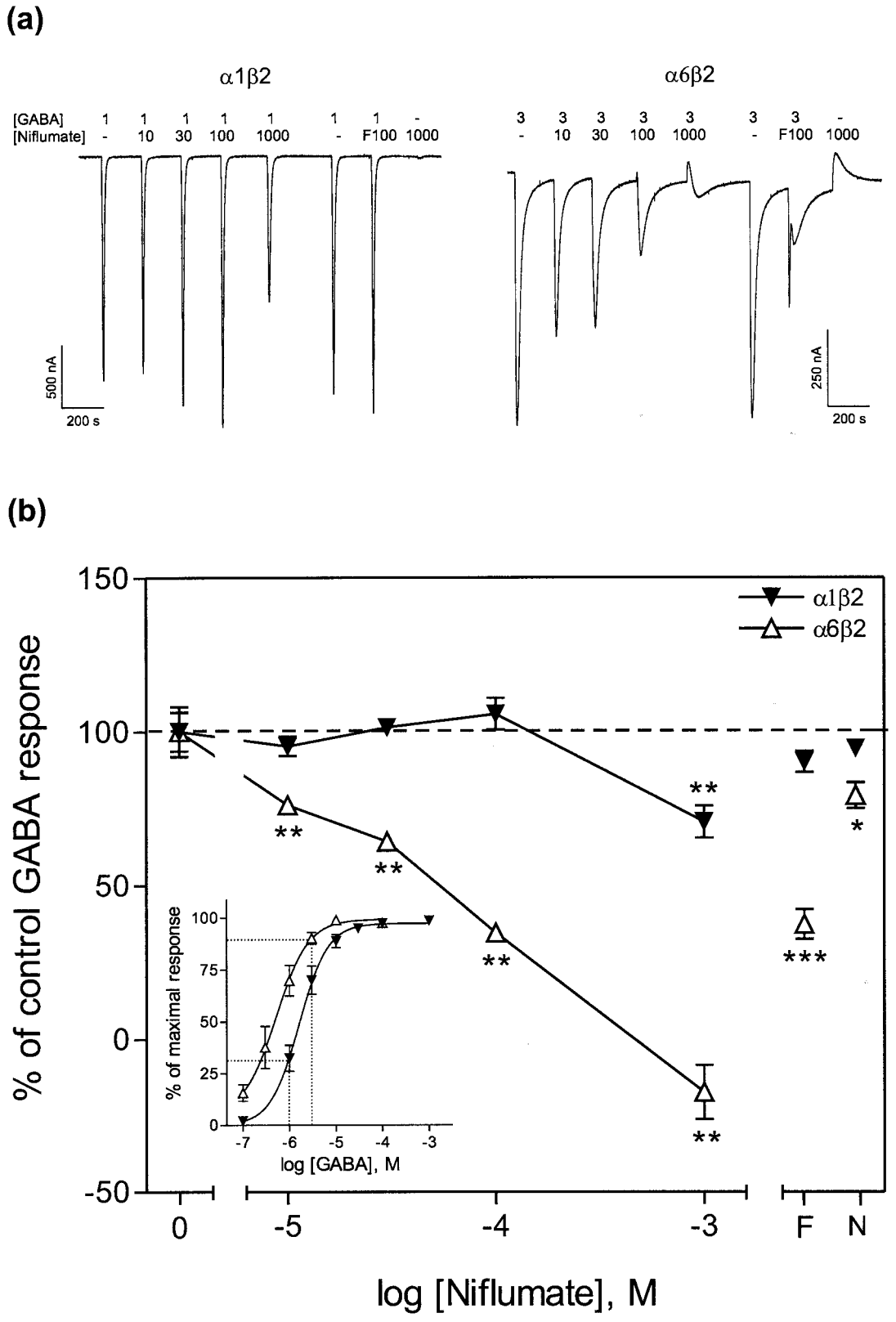

Fig. 7. Modulation of GABA-induced currents by niflumate and furosemide in $\alpha1\beta2$ and $\alpha6\beta2$ recombinant GABA$_A$ receptors expressed in *X. laevis* oocytes. a, representative current traces for different receptor subtypes as affected by GABA, various niflumate concentrations, and furosemide (F100, 100 μM). b, values are presented as mean ± S.E. (n = 13 and 8 for $\alpha1\beta2$ and $\alpha6\beta2$ combinations, respectively), the GABA control response being set to 100%. F, 100 μM furosemide in the presence of GABA; N, 1000 μM niflumate alone. Significance of the difference from the corresponding control value (one-way ANOVA followed by Dunnett's post hoc test for niflumate in the presence of GABA; Student's t test for furosemide or niflumate effects alone) as follows: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. Inset, the GABA concentration-response curves for both receptor subtypes. Dashed line indicates the efficacy of GABA concentrations, which were used in the following experiments. Maximal current peaks elicited by GABA were 3.4 ± 0.4 (n = 9) and 1.1 ± 0.2 μA (n = 8) for $\alpha1\beta2$ and $\alpha6\beta2$ combinations, respectively.

solely needing α subunits interacting with the β2 or β3 subunits and requiring specific feature in the TM2 domain.

Niflumate modulated the inhibition of [^{35}S]TBPS binding by 3 μM GABA depending on the brain region (Fig. 2; Table 2). Whereas positive modulation predominated in most brain areas, the negative modulation (antagonism) was evident in the cerebellar granule cell layer. When the cerebellum was analyzed as whole, the profound enhancement of [^{35}S]TBPS binding in the granule cell layer (GABA antagonism) was masked by reduced binding in the molecular layer (GABA potentiation), even with 3 μM GABA that inhibited the binding in the whole cerebellum already to 11% of basal binding. Our results on the whole cerebellum agree with the previous results on cerebellar membranes (Maksay et al., 1998), but they demonstrate the advantage gained by the spatial resolution when working with brain sections in comparison with brain homogenates.

GABA at 1 mM reveals an atypical GABA-insensitive [^{35}S]TBPS binding component in brain sections (Fig. 2), constituting a minor fraction of basal binding in selected brain regions (Sinkkonen et al., 2001). Niflumate greatly antagonized 1 mM GABA in the cerebellar granule cell layer and to a lesser degree in the thalamus (Table 3), whereas hardly altering anything in other regions up to 1,000 μM niflumate, which reduced the binding. Furosemide had similar effects, but it was more efficient in the cerebellar granule cell layer than thalamus. Furosemide antagonism in the cerebellar granule cell layer is related to the granule cell-restricted α6 subunit (Korpi et al., 1995). Using recombinant receptors, furosemide antagonism has also been observed in thalamus-enriched α4 subunit-containing receptors (Wisden et al., 1992; Knoflach et al., 1996; Wafford et al., 1996). A smaller furosemide antagonism was observed there now, although it was not detected in the thalamus previously, when a less sensitive [^{35}S]TBPS binding autoradiography was performed (Korpi and Lüddens, 1997), possibly because α4 subunit-containing receptors constitute only 20 to 27% of the thalamic receptors (Sur et al., 1999). These results suggest that niflumate antagonism is dependent on the presence of α6 or α4 subunits.

Niflumate antagonized 3-μM GABA-elicited currents in a concentration-dependent manner in α6β2 and α6β2γ2 combinations (Figs. 3 and 7). In α6β2γ2 receptors, niflumate failed to alter the EC_{50} value for GABA, but reduced the maximal GABA currents by about 40%. This is consistent with noncompetitive antagonism, which has been shown also for furosemide (Korpi et al., 1995). To study the site of niflumate antagonism more precisely, we coexpressed the α1-16 chimera, which has been shown to be furosemide-sensitive (Jackel et al., 1998), with β2 and γ2 subunits. These receptors were also inhibited by niflumate (Fig. 6), supporting the idea that furosemide and niflumate share a binding/effector site on α6 subunit. Interestingly, the negative modulatory actions were not saturable, but fully blocked the GABA effects. Indeed, especially in α6β2 receptors high niflumate concentrations produced small outward, as opposed to GABA-induced inward, currents even in the absence of GABA. We believe this outward current is a small leakage current produced by spontaneously open channels, which has been suggested for homomeric β1 and β3 receptors (Sigel et al., 1989; Wooltorton et al., 1997). This property might have obscured the kinetics of drug action and prevented us from calculating IC_{50} values. However, as can be seen from the present data of both ligand binding and electrophysiology experiments, niflumate concentrations needed for positive and negative effects were similar and already low micromolar ones were significantly effective.

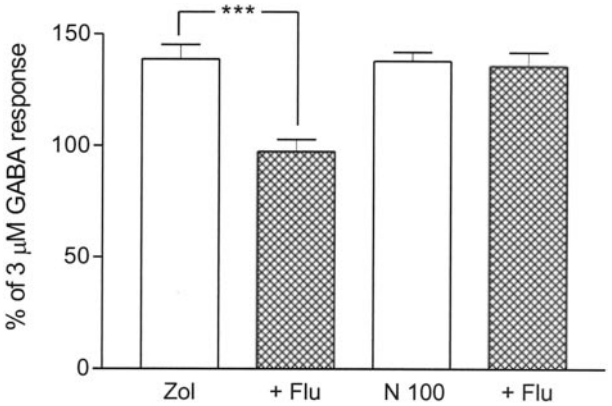

Fig. 8. Potentiation of 3 μM GABA-induced currents by zolpidem (Zol, 1 μM) and niflumate (N, 100 μM), and their modulation by flumazenil (Flu, 1 μM) in α1β2γ2 recombinant $GABA_A$ receptors expressed in *X. laevis* oocytes. Values are presented as mean ± S.E. (n = 8 and 13 for zolpidem and niflumate effects, respectively). ***, $p < 0.001$ for the statistical significance of the flumazenil effect on the zolpidem potentiation (paired t test).

Niflumate is clinically used in Europe. The usual clinical oral dosage of 250 mg of three times daily results in steady-state plasma concentrations of 20 to 70 μM (Houin et al., 1983). Although most of the drug is bound to plasma proteins, free niflumate concentrations in plasma reach the micromolar range. Because fenamates pass the blood-brain barrier efficiently (Bannwarth et al., 1989), niflumate can be estimated to reach micromolar concentrations in the brain. NSAID overdoses have been shown to result in severe central effects, such as convulsion and coma (Smolinske et al., 1990). Thus, some adverse effects of niflumate and other fenamates could be mediated by alterations of $GABA_A$ receptor function.

In conclusion, niflumate has positive-negative modulatory profile on both native and recombinant $GABA_A$ receptors depending on receptor subtype at concentrations that may be clinically or toxicologically relevant. Niflumate acts as a positive allosteric modulator on α1β2γ2 and as a negative modulator on α6β2 and α6β2γ2 (and α1β2) $GABA_A$ receptors. The noncompetitive antagonistic action of niflumate is mediated by the same site as the furosemide action, whereas the site for the positive allosteric modulator action depends on the presence of the γ2 subunit, but is different from the benzodiazepine binding site and remains to be characterized in detail. The present findings add to the diverse list of structural requirements of $GABA_A$ receptor ligands and might offer a novel lead for subtype-selective drug development with a potential of having forebrain-preferring GABA potentiation without cerebellum-related motor impairment.

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