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Novel Plant Substances Acting as β Subunit Isoform-Selective Positive Allosteric Modulators of $GABA_A$ Receptors

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ABSTRACT

$GABA_A$ receptors are modulated by a large variety of compounds. A common chemical characteristic of most of these modulators is that they contain a cyclic entity. Three linear molecules of a polyacetylene structure were isolated from the East African medicinal plant *Cussonia zimmermannii* Harms and shown to allosterically stimulate $GABA_A$ receptors. Stimulation was not abolished by the absence of the γ_2 subunit, the benzodiazepine antagonist Ro15-1788 (8-fluoro-5,6-dihydro-5-methyl-6-oxo-4*H*-imidazo[1,5-*a*][1,4]benzodiazepine-3-carboxylic acid ethyl ester), or the point mutation β_2N265S that abolishes effects by loreclezole. At a concentration of 30 μM, the substances by themselves elicited only tiny currents. Maximal stimulation at $\alpha_1\beta_2\gamma_2$ amounted to 110 to 450% for the three substances, and half-maximal stimulation was observed at concentrations of 1 to 2 μM. Stimulation was subunit composition-dependent and was for the substance MS-1, $\alpha_1\beta_2\gamma_2 \approx \alpha_1\beta_2 \approx \alpha_3\beta_2\gamma_2 > \alpha_2\beta_2\gamma_2 > \alpha_5\beta_2\gamma_2 \approx \alpha_1\beta_3\gamma_2 \approx \alpha_6\beta_2\gamma_2 > \alpha_1\beta_1\gamma_2$, for MS-2 $\alpha_1\beta_2\gamma_2 \approx \alpha_3\beta_2\gamma_2 \approx \alpha_1\beta_2 > \alpha_2\beta_2\gamma_2 \approx \alpha_6\beta_2\gamma_2 \approx \alpha_5\beta_2\gamma_2 > \alpha_1\beta_1\gamma_2$, and for MS-4, $\alpha_1\beta_2\gamma_2 \approx \alpha_1\beta_2 \approx \alpha_5\beta_2\gamma_2 \approx \alpha_3\beta_2\gamma_2 \approx \alpha_2\beta_2\gamma_2 > \alpha_6\beta_2\gamma_2 \gg \alpha_1\beta_1\gamma_2$. Maximal stimulation by MS-1 was 450% at $\alpha_1\beta_2\gamma_2$, 80% at $\alpha_1\beta_1\gamma_2$, and 150% at $\alpha_1\beta_3\gamma_2$. MS-1 was thus specific for receptors containing the β_2 subunit. The reversal potential was unaffected by 10 μM MS-1, whereas apparent picrotoxin affinity for current inhibition was increased approximately 3-fold. In summary, these positive allosteric modulators of $GABA_A$ receptors of plant origin have a novel unusual chemical structure and act at a site independent of that of benzodiazepines and loreclezole.

GABA is the major inhibitory neurotransmitter in the mammalian central nervous system. It acts at three types of receptors, the G-protein-coupled $GABA_B$ receptor, and the $GABA_A$ and $GABA_C$ receptors, which both constitute ion channels. Two subunits of the $GABA_A$ receptor have initially been purified (Sigel et al., 1983), and their coding DNA has been cloned (Schofield et al., 1987). Numerous subunits have since been cloned (for review, see Macdonald and Olsen, 1994; Rabow et al., 1995; Barnard et al., 1998). These subunits show homology to subunits of the nicotinic acetylcholine receptors, the glycine receptor, and the 5HT3 receptor. The $GABA_A$ receptors are heteromeric protein complexes consisting of five subunits that are arranged around a central Cl^--selective channel (Macdonald and Olsen, 1994). The major receptor isoform of the $GABA_A$ receptor in the brain presumably consists of α_1, β_2, and γ_2 subunits (Laurie et al., 1992; Benke et al., 1994; Macdonald and Olsen, 1994; Rabow et al., 1995; McKernan and Whiting, 1996; Barnard et al., 1998). Different approaches have indicated a 2α:2β:1γ subunit stoichiometry for this receptor (Backus et al., 1993; Chang et al., 1996; Tretter et al., 1997; Farrar et al., 1999; Baumann et al., 2001, 2002).

The $GABA_A$ receptor is the site of action of many modulatory compounds, among them the benzodiazepines (for review, see Sieghart, 1995). Both binding sites, those for the channel agonist GABA and those for benzodiazepines, are thought to be located at subunit interfaces in a homologous position (for review, see Galzi and Changeux, 1994; Sigel and Buhr, 1997). Most of the allosteric modulators contain a ring in their chemical structure.

Cussonia zimmermannii Harms belongs to the genus *Cussonia* of the family Araliaceae. It occurs in Kenya and Tanzania and grows in lowland rain forests, lowland dry evergreen forests, and woodlands at altitudes of 0 to 400 m (Tennant, 1968). The marrow of the stem and branches is eaten to treat epilepsy, and a decoction of the root is taken as a remedy for labor pain (Haerdi, 1964). In addition, an infu-

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ABBREVIATIONS: DMSO, dimethyl sulfoxide; MS-1, 1-ethyl-6-hydroxypentadeca-2,4-dinyl acetate; MS-2, 6-hydroxy-1-vinylpentadeca-2,4-dinyl acetate; MS-4, (6*S*)-16-acetoxy-6-hydroxy-1-vinylhexadeca-2,4-diynyl acetate; EBOB, ethynylbicycloorthobenzoate; DHA, docosahexaenoic acid; Ro 15–1788, 8-fluoro-5,6-dihydro-5-methyl-6-oxo-4*H*-imidazo[1,5-*a*][1,4]benzodiazepine-3-carboxylic acid ethyl ester.

sion of the leaves is used as a wash for people suffering from fever or ague and a decoction of the roots is taken as a remedy for gonorrhea (Kokwaro, 1976).

Herein, we describe three potent positive allosteric modulators of $GABA_A$ receptors with a linear polyacetylene structure isolated from the plant *C. zimmermannii* Harms. They are shown to act at a site independent of both the benzodiazepine and the loreclezole site. These compounds display a most interesting and unprecedented subunit specificity.

Materials and Methods

Substances. MS-1, MS-2, and MS-4 were isolated from the East African medicinal plant *C. zimmermannii* Harms. The methods of isolation and structure determination will be described elsewhere.

[^{3}H]Flunitrazepam Binding. Cortex material derived from rats (stem RORO; RCC Ltd., Basel, Switzerland) was homogenized on ice with 50 mM Tris/HCl, pH 7.4, 120 mM NaCl, and 5 mM KCl using a Polytron homogenizer (Kinematica, Basel, Switzerland). The homogenate was centrifuged at 31,000*g* for 10 min at 4°C. The pellet was resuspended with 50 mM Tris/HCl, pH 7.4, and centrifuged as described above for a total of three washing steps. The membrane fraction was stored at −80°C. For the binding of [^{3}H]flunitrazepam (final concentration, 1 nM; Amersham Biosciences Inc., Piscataway, NJ), 200 mg of membrane protein (BCA protein assay) was used. Nonspecific binding was determined in the presence of 10 μM diazepam. Binding equilibrium was reached within 1 h at room temperature, and binding assays were terminated after this time by rapid filtration using GF/C filters (Whatman, Maidstone, UK). Filters were washed three times with ice-cold Tris/HCl, pH 7.4, buffer. Radioactivity on filters was determined by liquid scintillation counting (Tri-Carb 2100TR; PerkinElmer Life and Analytical Sciences, Boston, MA). Results are given as the mean ± S.E.M. of two to four individual experiments performed in triplicate.

Construction of Receptor Subunits. The cDNAs coding for the α_1, β_2, and γ_2S (γ_2) subunits of the rat $GABA_A$ receptor channel have been described elsewhere (Lolait et al., 1989; Malherbe et al., 1990a,b). For cell transfection, the cDNAs were subcloned into the polylinker of pBC/CMV (Bertocci et al., 1991). This expression vector allows high-level expression of a foreign gene under control of the cytomegalovirus promoter. The cDNAs coding for α_2, α_3, α_5, α_6, β_1, and β_3 were prepared similarly. α Subunits were cloned into the EcoRI, and the β subunits were subcloned into the SmaI site of the polylinker by standard techniques.

Expression in *Xenopus laevis* Oocytes. Capped cRNAs were synthesized (Ambion, Austin, TX) from the linearized pBC/CMV vectors containing wild-type α_1, α_2, α_3, α_5, α_6, β_1, β_2, β_3, and γ_2, respectively. A poly(A) tail of ~400 residues was added to each transcript using yeast poly(A) polymerase (U.S. Biochemical Corp., Cleveland, OH). The concentration of the cRNA was quantified on a formaldehyde gel using Radiant Red stain (Bio-Rad, Hercules, CA) for visualization of the RNA and known concentrations of RNA ladder (Invitrogen, Carlsbad, CA) as standard on the same gel. cRNA combinations in nuclease-free water were stored at −80°C. Isolation of oocytes from the frogs, culturing of the oocytes, injection of cRNA, and defolliculation were performed as described previously (Sigel, 1987; Sigel et al., 1990). Oocytes were injected with 50 nl of the cRNA solution. The combination of wild-type α_1 and β_2 subunits was expressed at 75 and 75 nM, and the combination of wild-type α_x, β_x, and γ_2 subunits was expressed at 10, 10, and 50 nM (Boileau et al., 2002). For control purposes, cRNA coding for a voltage-gated sodium channel (Kuhn and Greeff, 1999) was used at a concentration of 40 nM. The injected oocytes were incubated in modified Barth's solution [10 mM HEPES, pH 7.5, 88 mM NaCl, 1 mM KCl, 2.4 mM $NaHCO_3$, 0.82 mM $MgSO_4$, 0.34 mM $Ca(NO_3)_2$, 0.41 mM $CaCl_2$, 100 U/ml penicillin, and 100 μg/ml streptomycin] at 18°C for at least a day before the measurements.

Two-Electrode Voltage-Clamp Measurements. All measurements were done in medium containing 90 mM NaCl, 1 mM $MgCl_2$, 1 mM KCl, 1 mM $CaCl_2$, and 5 mM HEPES, pH 7.4, at a holding potential of −80 mV. To quantify stimulation by MS compounds, agonist concentrations eliciting ~5% of the maximal current amplitude were applied alone or in combination with increasing concentrations of MS compounds between 0.03 and 30 μM for 20 s, and a washout period of 4 min was allowed to ensure full recovery from desensitization. The stimulation was then calculated as Stimulation = $[(I_{\text{after MS}}/I_{\text{before MS}}) - 1] \times 100\%$. Stimulation was fitted to the Hill equation: $I = I_{max}/1 + (EC_{50}/A)^{n_H}$, where I is the current amplitude at a given concentration of MS compound A, I_{max} is the current amplitude at maximal stimulation, EC_{50} is the concentration of MS compound yielding half-maximal current amplitudes, and n_H is the Hill coefficient. GABA-evoked currents (at ~10% of the maximal current amplitude) were inhibited by varying concentrations of picrotoxin. Inhibition curves for picrotoxin were fitted with the equation $I(c) = I(0)/(1 + (IC_{50}/c)$, where $I(0)$ is the control current in the absence of picrotoxin standardized to 100%, $I(c)$ is the relative current amplitude, c is the concentration of picrotoxin, and IC_{50} is the concentration of picrotoxin causing 50% inhibition of the current. Voltage-dependent sodium currents were determined by a potential jump from a holding potential of −100 to −15 mV.

Data are given as the mean ± S.E.M. (number of experiments for at least two batches of oocytes). The perfusion system was cleaned between drug applications by washing with 100% dimethyl sulfoxide (DMSO) to avoid contamination. The stock solution of MS compounds was 40 mM in DMSO. The final concentration of DMSO in the medium was always adjusted to 0.5%. These concentrations of DMSO did not by themselves significantly affect GABA-elicited currents. Currents were measured using a modified OC-725 amplifier (Warner Instruments, Hamden, CT) in combination with an XY-recorder or digitized using a MacLab/200 (ADInstruments, Oxfordshire, UK).

Results

Three Polyacetylene Compounds Stimulate [^{3}H]Flunitrazepam Binding

The compounds were named MS compounds, and their structures are shown in Fig. 1. All three compounds are linear, polyunsaturated with two triple bonds, and contain an acetate group. These compounds were isolated from the plant *C. zimmermannii* Harms. Elucidation of the chemical structures was performed using UV spectroscopy, infrared spectroscopy, ^{1}H NMR, ^{13}C NMR, heteronuclear multiple quantum correlation, heteronuclear multiple bond correlation, correlated spectroscopy, electron ionization mass spec-

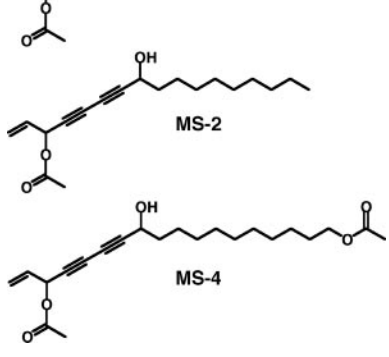

Fig. 1. Structures of the three polyacetylene compounds.

troscopy, fast atom bombardment mass spectrometry, and high-resolution electron ionization mass spectrometry (manuscript in preparation). In traditional local medicine, this plant is used, among many other purposes, for the treatment of epilepsy. Within a screening approach, the plant was shown to contain components able to allosterically interact with the benzodiazepine-binding site on $GABA_A$ receptors. The property to stimulate the binding of [^{3}H]flunitrazepam was then used to isolate three substances. Figure 2 shows the dose-dependent stimulation of [^{3}H]flunitrazepam binding of MS-1, MS-2, and MS-4.

Electrophysiological Studies

Positive Allosteric Modulation. Functional effects of MS compounds were investigated in electrophysiological studies at recombinant $GABA_A$ receptors expressed in *X. laevis* oocytes. Because of solubility, the MS compounds were only used up to a concentration of 30 μM. GABA was always used at concentrations eliciting 2 to 6% of the maximal current amplitude in the corresponding $GABA_A$ receptor type. At $\alpha_1\beta_2\gamma_2$, each MS compound by itself (30 mM) elicited tiny currents amounting to $<0.1\%$ of the maximal current elicited by GABA, but all of the compounds exhibited a potent positive allosteric modulatory effect by enhancing the GABA-stimulated current at $\alpha_1\beta_2\gamma_2$. This concentration-dependent stimulation is documented for MS-1 at $\alpha_1\beta_2\gamma_2$ with a GABA concentration of 7 μM (Fig. 3). Maximum stimulation at $\alpha_1\beta_2\gamma_2$ was achieved with $\sim$10 μM MS-1. We also tested whether 10 μM MS-1 stimulated near-maximal currents elicited by GABA. In the presence of 500 μM GABA, 10 μM MS-1 did not significantly affect the current amplitudes [97.7 $\pm$ 1.5% of the control ($n = 3$)]. Figure 4 shows the effect of 10 μM MS-1 on the GABA concentration dependence of the current. In the absence of MS-1, the K_a for GABA and the Hill coefficients were 24 $\pm$ 5 μM ($n = 4$) and 1.5 $\pm$ 0.1 ($n = 4$), respectively. In the presence of 10 μM MS-1, the concentration response curve was less steep and characterized by K_a for GABA of 21 $\pm$ 3 μM ($n = 4$) and a Hill coefficient of 1.1 $\pm$ 0.1 ($n = 4$). Stimulation by 10 μM MS-1 at low concentrations of GABA amounts to only $\sim$2.3-fold compared with the value of 4-fold expected from the data shown below. The reason for this discrepancy is not known, but it may be due to the repetitive application of 10 μM MS-1 in these experiments.

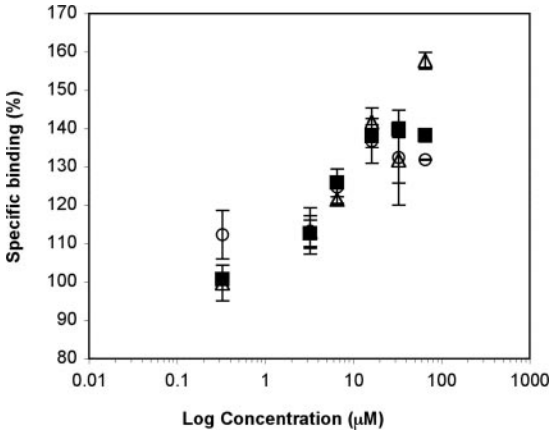

Fig. 2. Stimulation of [^{3}H]flunitrazepam binding by MS-1, MS-2, and MS-4. Specific binding stimulated by MS-1 (■), MS-2 (○), and MS-4 (△) is shown relative to control binding in the absence of drugs. Data are expressed as the mean $\pm$ S.E.M. of two to four individual experiments.

Subunit Specificity of MS-1. Figure 5A shows an averaged concentration response curve of this type of experiment for $\alpha_1\beta_2\gamma_2$. Maximal stimulation is $\sim$450%, and half-maximal stimulation was observed at a concentration (EC_{50}) of $\sim$1.5 mM. Replacement of α_1 in this subunit combination by other α subunit isoforms such as α_2, α_3, α_5, or α_6 had little effect on EC_{50}, which varied between 0.6 and 1.0 μM, but had in some cases a drastic effect on the maximal stimulation (Fig. 5A). The extent of stimulation was $\alpha_1\beta_2\gamma_2 \approx \alpha_3\beta_2\gamma_2 > \alpha_2\beta_2\gamma_2 > \alpha_5\beta_2\gamma_2 \approx \alpha_6\beta_2\gamma_2$. Figure 5B shows the effect of the β subunit isoform and the lack of effect upon omitting γ_2 from $\alpha_1\beta_2\gamma_2$. Replacing β_2 in $\alpha_1\beta_2\gamma_2$ by β_1 or β_3 drastically reduced maximal stimulation from 450% to 80 and 150%, respectively. Introducing the point mutation β_2N265S that is known to strongly reduce stimulatory effects by loreclezole (Wingrove et al., 1994) into $\alpha_1\beta_2\gamma_2$ had only a relatively weak effect in this case, reducing maximal stimulation by approximately one third (Fig. 5B).

Subunit Specificity of MS-2 and MS-4. Figures 6 and 7 show subunit specificities of MS-2 and MS-4, respectively. Again, the replacement of α_1 in $\alpha_1\beta_2\gamma_2$ by other α subunit isoforms such as α_2, α_3, α_5, or α_6 had little effect on EC_{50}, which varied between 0.8 and 1.3 μM for MS-2 and 1.4 and 3.5 μM for MS-4, but had in some cases a drastic effect on the maximal stimulation (Figs. 6 and 7). Maximal stimulation was $\sim$300% for MS-2 and $\sim$110% for MS-4. The following specificity in this respect was observed for MS-2, $\alpha_1\beta_2\gamma_2 \approx \alpha_3\beta_2\gamma_2 \approx \alpha_1\beta_2 > \alpha_2\beta_2\gamma_2 \approx \alpha_6\beta_2\gamma_2 \approx \alpha_5\beta_2\gamma_2 > \alpha_1\beta_1\gamma_2$, and for MS-4, $\alpha_1\beta_2\gamma_2 \approx \alpha_1\beta_2 \approx \alpha_5\beta_2\gamma_2 \approx \alpha_3\beta_2\gamma_2 \approx \alpha_2\beta_2\gamma_2 > \alpha_6\beta_2\gamma_2 \gg \alpha_1\beta_1\gamma_2$.

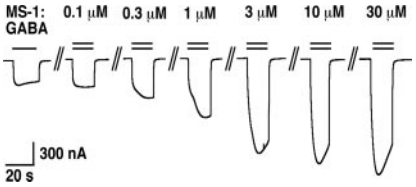

Fig. 3. Concentration dependence of allosteric stimulation by MS-1 at $\alpha_1\beta_2\gamma_2$ $GABA_A$ receptors. Recombinant $\alpha_1\beta_2\gamma_2$ $GABA_A$ receptors were expressed in *X. laevis* oocytes and exposed to either 7 mM GABA alone or in combination with increasing concentrations of MS-1. The experiment was repeated twice on oocytes of two different batches with similar results.

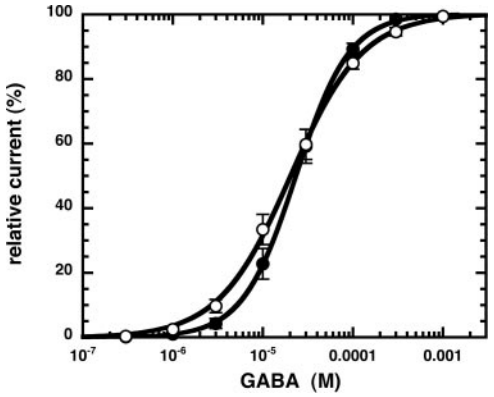

Fig. 4. Effect of MS-1 on the GABA concentration dependence. A, recombinant $\alpha_1\beta_2\gamma_2$ $GABA_A$ receptors were expressed in *X. laevis* oocytes and exposed to increasing GABA concentrations either alone (●) or in combination with 10 μM MS-1 (○). Data are given as the mean $\pm$ S.E.M. (up to four oocytes from two different batches).

Lack of Inhibition by the Benzodiazepine Antagonist Ro15-1788

We tested whether MS-1 would act at a known site on the $GABA_A$ receptor. Currents elicited by 2 to 7 μM GABA were stimulated by 10 μM MS-1 (377 $\pm$ 43%; $n = 7$). If 1 μM benzodiazepine antagonist Ro15-1788 was coapplied, stimulation would not be significantly altered with 391 $\pm$ 90% ($n = 3$) (Fig. 8). In control experiments, the current stimulation by 1 μM diazepam was completely abolished in the presence of 1 μM Ro15-1788 (Fig. 8). In contrast to Ro15-1788, the antagonist of the ROD site, ROD178B (Sigel et al., 2001), at a concentration of 100 μM, significantly reduced stimulation to 134 $\pm$ 9% ($n = 3$).

Costimulation by MS-1 and Diazepam

The above observations indicate independent sites of action of MS-1 and diazepam. Even if this were the case, whether the two drugs act additively cannot be predicted. Currents were elicited by 2 to 7 μM GABA followed by coapplication with 1 μM diazepam and subsequently the combination of 1 μM diazepam and 10 μM MS-1. As shown in Fig. 8, diazepam induced a stimulation of 244 $\pm$ 12% ($n = 8$) and the combination of diazepam and MS-1 induced a stimulation of 871 $\pm$ 152% ($n = 4$), demonstrating an additive effect of the two drugs.

Effect of MS-1 on the Apparent Affinity of Picrotoxin

Currents elicited by 3 or 4 μM GABA were inhibited by increasing concentrations of the channel pore blocker picrotoxin. The half-maximal concentration of picrotoxin for current inhibition was 2.6 $\pm$ 0.4 μM ($n = 3$) in the absence and 1.0 $\pm$ 0.1 μM ($n = 3$) in the presence of 10 μM MS-1. MS-1 increased apparent picrotoxin affinity ~2.6-fold (Fig. 9).

Effect of MS-1 on the Apparent Affinity of Bicuculline

GABA-induced currents amounting to ~20% of the maximal current amplitude were inhibited by increasing concentrations of the competitive GABA antagonist bicuculline. The half-maximal concentration of bicuculline for current inhibition was 1.3 $\pm$ 0.1 μM ($n = 4$) in the absence of 10 μM MS-1 and nonsignificantly increased to 2.3 $\pm$ 0.4 μM ($n = 4$) in the presence of 10 μM MS-1 (data not shown).

Ion Selectivity Is Maintained and Stimulation Is Independent of Potential

The reversal potential and the potential dependence of the current elicited by GABA were both not altered in the presence of 10 μM MS-1. The reversal potential was -29 ± 1 mV ($n = 3$) in the absence of 10 μM MS-1 and was not altered in its presence (data not shown).

Specificity of the MS Compounds

Because the structure of the MS compounds suggests interaction with the lipid bilayer, they could in principle nonspecifically interact with any membrane protein. Even if their specificity for receptors containing the β_2 subunit argued for a specific effect, we were concerned with this possibility. Therefore, we tested the effects of MS-1 on the rat brain voltage-gated sodium channel IIA. We chose concentrations of MS-1 of 1, 5, and 30 μM, which should be compared with the concentrations eliciting half-maximal effects (200% stimulation) at $\alpha_1\beta_2\gamma_2$ $GABA_A$ receptors (0.6–1.5 μM). 1, 5, and 30 μM MS-1 weakly stimulated peak sodium currents elicited by a voltage jump from -100 to -15 mV by 8.8 $\pm$ 2.5% ($n = 3$), 3.8 $\pm$ 2.5% ($n = 4$), and 6.6 $\pm$ 3.6% ($n = 3$),

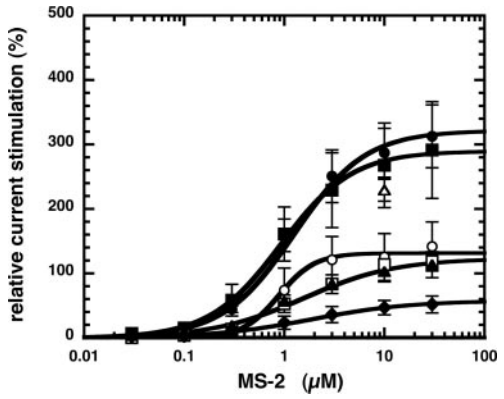

Fig. 6. Subunit isoform specificity of MS-2. Recombinant $\alpha_1\beta_2\gamma_2$ (●), $\alpha_1\beta_2$ (△), $\alpha_2\beta_2\gamma_2$ (○), $\alpha_3\beta_2\gamma_2$ (■), $\alpha_5\beta_2\gamma_2$ (□), $\alpha_6\beta_2\gamma_2$ (▲), and $\alpha_1\beta_1\gamma_2$ (♦) $GABA_A$ receptors were expressed in *X. laevis* oocytes and exposed to either GABA alone or in combination with increasing concentrations of MS-2. Data are given as the mean $\pm$ S.E.M. (up to three oocytes from two different batches).

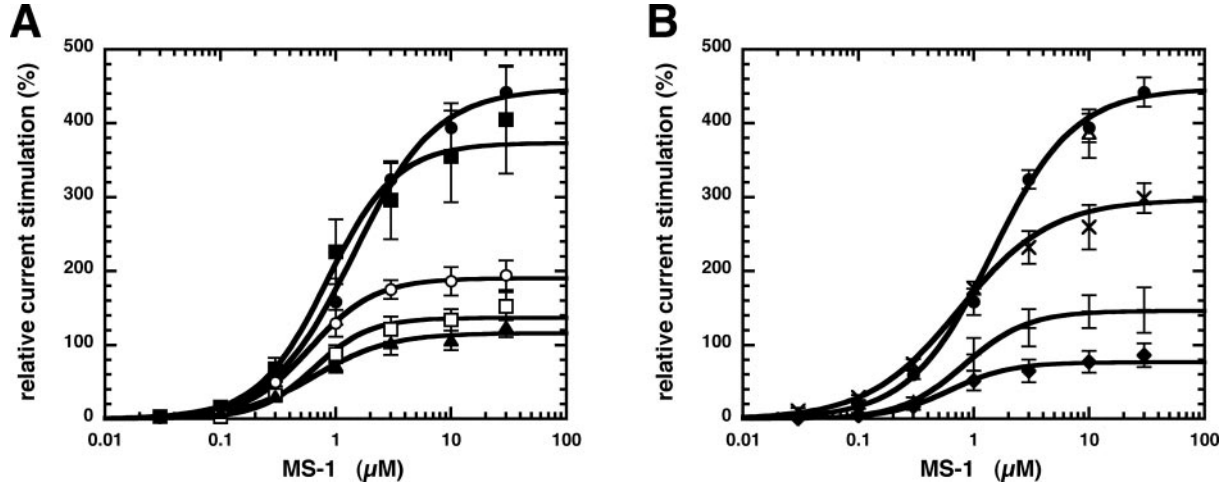

Fig. 5. Subunit isoform specificity of MS-1. Recombinant $\alpha_1\beta_2\gamma_2$ (●), $\alpha_2\beta_2\gamma_2$ (○), $\alpha_3\beta_2\gamma_2$ (■), $\alpha_5\beta_2\gamma_2$ (□), and $\alpha_6\beta_2\gamma_2$ (▲) (A) and $\alpha_1\beta_2\gamma_2$ (●), $\alpha_1\beta_2$ (△), $\alpha_1\beta_2$N265Sγ_2 (×), $\alpha_1\beta_3\gamma_2$ (+), and $\alpha_1\beta_1\gamma_2$ (♦) (B) $GABA_A$ receptors were expressed in *X. laevis* oocytes and exposed to either GABA alone or in combination with increasing concentrations of MS-1. Data are given as the mean $\pm$ S.E.M. (up to three oocytes from two different batches).

respectively (data not shown). In contrast, currents induced by a voltage jump from −60 to −15 mV were inhibited and inhibition amounted to 20.3 ± 3.1% (n = 3), 11.0 ± 2.6% (n = 4), and 15.6 ± 2.6% (n = 3), respectively. With both potential protocols and all three concentrations of MS-1 tested, voltage-dependent inactivation was slowed down to a similar extent. For example, inactivation after a pulse from −100 to −15 mV was fitted with mono-exponential function characterized by the time constant t. This time constant was slightly increased by 1 μM MS-1 from 5.6 ± 0.3 ms (n = 3) in its absence to 9.3 ± 0.4 ms (n = 3) in its presence. In summary, the effects of MS-1 on the voltage-gated sodium channel are small, arguing again against a nonspecific membrane effect.

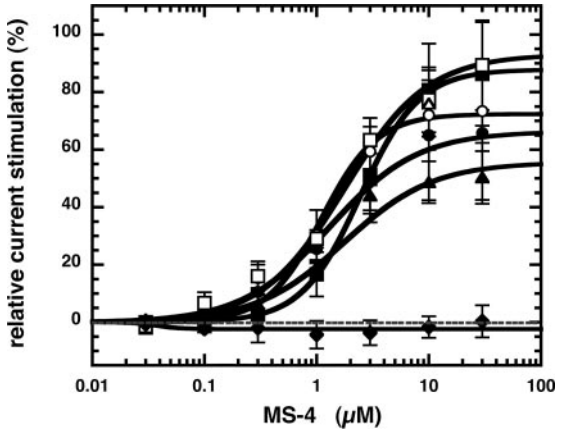

Fig. 7. Subunit isoform specificity of MS-4. Recombinant $\alpha_1\beta_2\gamma_2$ (●), $\alpha_1\beta_2$ (△), $\alpha_2\beta_2\gamma_2$ (○), $\alpha_3\beta_2\gamma_2$ (■), $\alpha_5\beta_2\gamma_2$ (□), $\alpha_6\beta_2\gamma_2$ (▲), and $\alpha_1\beta_1\gamma_2$ (♦) $GABA_A$ receptors were expressed in *X. laevis* oocytes and exposed to either GABA alone or in combination with increasing concentrations of MS-4. Data are given as the mean ± S.E.M. (up to three oocytes from two different batches).

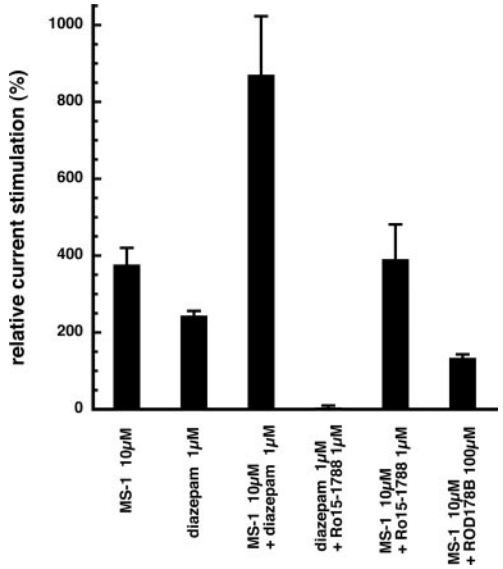

Fig. 8. Lack of inhibition by the benzodiazepine antagonist Ro15-1788 and additive stimulation by diazepam and MS-1. Recombinant $\alpha_1\beta_2\gamma_2$ $GABA_A$ receptors were exposed to one of the following: 1) GABA in combination with 10 μM MS-1 or 1 μM diazepam; 2) GABA in combination with 10 μM MS-1 and 1 μM Ro15-1788, 100 μM ROD178B, or 1 μM diazepam; or 3) GABA in combination with 1 μM diazepam and 1 μM Ro15-1788. Data are given as the mean ± S.E.M. (up to three oocytes from two different batches).

Discussion

In this study, we showed that three substances isolated from *C. zimmermannii* Harms act as potent positive allosteric modulators at $GABA_A$ receptors with a half-maximal stimulation at a concentration of 0.6 to 3.5 μM and a maximal stimulation of 110 to 450%. The threshold of stimulation was below 0.1 μM. The substances did not open the channels themselves, even at high concentrations, and the reversal potential was not affected. This, together with data shown in Fig. 4, suggest an action on channel gating.

All three substances are of a polyacetylene structure (Fig. 1). The only report known to us on a connection between polyacetylenes and the $GABA_A$ receptor deals with cicutoxin isolated from water hemlock and analogs (Uwai et al., 2000). Cicutoxin has been shown to displace the channel blocker [^{3}H]EBOB (PerkinElmer Life and Analytical Sciences) from its binding site with an IC_{50} of 0.5 μM. No functional studies were performed, except it was shown that the compound was able to kill mice with a LD_{50} of ~3 mg/kg. Another compound, virol A, was shown to have approximately half of the potency in both cases. Virol A was later shown to inhibit currents elicited by GABA in acutely dissociated rat hippocampal CA1 neurons with an IC_{50} of ~1 μM (Uwai et al., 2001). Most remarkably, the compounds described here are linear molecules. The majority of positive allosteric modulators of $GABA_A$ receptors contain at least one cyclic entity. Other linear compounds affecting $GABA_A$ receptors are unsaturated fatty acids, including docosahexaenoic acid (DHA) (Nabekura et al., 1998). At low concentrations ≤1 μM, DHA stimulates GABA responses up to 20% in a γ subunit-dependent way. At higher concentrations, DHA inhibits GABA responses in a γ subunit-independent manner.

The three novel compounds showed unique subunit selectivity profiles. Omitting the γ subunit from $\alpha_1\beta_2\gamma_2$ did not affect the extent of stimulation. In contrast, the β subunit present seems to strongly influence stimulation. Replacing β_2 with β_1 reduced maximal stimulation more than 4-fold. Replacing β_2 with β_3 reduced maximal stimulation approximately 3-fold. Thus, MS-1 has specificity for receptors containing the β_2 subunit. Introducing the point mutation into β_2, N265S, which is known to strongly reduce stimulatory effects by loreclezole

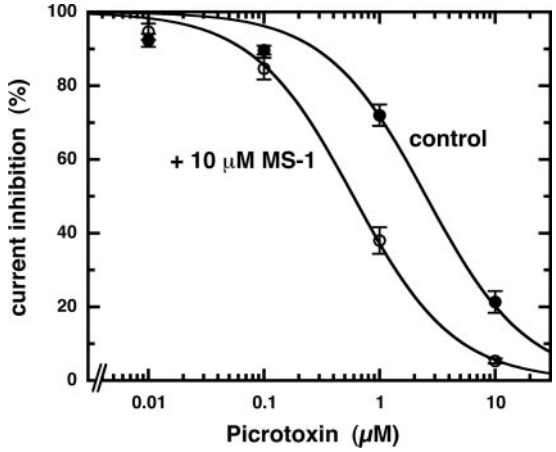

Fig. 9. MS-1 affects inhibition by picrotoxin. Recombinant $\alpha_1\beta_2\gamma_2$ $GABA_A$ receptors were exposed to either GABA alone (●) or in combination with 10 μM MS-1 (○). Currents were inhibited by increasing concentrations of picrotoxin. Data are given as the mean ± S.E.M. (three oocytes from two different batches).

(Wingrove et al., 1994), had only a relatively weak effect, reducing maximal stimulation by ~35%. This indicates that the site for MS compounds is probably different from that for loreclezole. This conclusion is enforced by the fact that loreclezole shows a different β subunit specificity, eliciting large simulation at receptors containing both the β_2 and β_3 subunits and only a small stimulation at receptors containing the β_1 subunit (Wafford et al., 1994). The α subunit isoform present in $\alpha_x\beta_2\gamma_2$ profoundly affected the extent of stimulation. For all three studied compounds, strongest modulation was seen in $\alpha_1\beta_2\gamma_2$, whereas only a weak stimulation was observed in $\alpha_1\beta_1\gamma_2$. The precise sequence for the extent of stimulation differed for the three compounds, $\alpha_1\beta_2\gamma_2 \approx \alpha_1\beta_2 \approx \alpha_3\beta_2\gamma_2 > \alpha_2\beta_2\gamma_2 > \alpha_5\beta_2\gamma_2 \approx \alpha_1\beta_3\gamma_2 \approx \alpha_6\beta_2\gamma_2 > \alpha_1\beta_1\gamma_2$ for MS-1, $\alpha_1\beta_2\gamma_2 \approx \alpha_3\beta_2\gamma_2 \approx \alpha_1\beta_2 > \alpha_2\beta_2\gamma_2 \approx \alpha_6\beta_2\gamma_2 \approx \alpha_5\beta_2\gamma_2 > \alpha_1\beta_1\gamma_2$ for MS-2, and $\alpha_1\beta_2\gamma_2 \approx \alpha_1\beta_2 \approx \alpha_5\beta_2\gamma_2 \approx \alpha_3\beta_2\gamma_2 \approx \alpha_2\beta_2\gamma_2 > \alpha_6\beta_2\gamma_2 \gg \alpha_1\beta_1\gamma_2$ for MS-4. The precise sequence of specificity is different for the three compounds, but for all compounds, action at $\alpha_3\beta_2\gamma_2$ is similar to action at $\alpha_1\beta_2\gamma_2$.

The relative lack of effects on the voltage-gated sodium channel together with the observed $GABA_A$ receptor subunit specificity argues against nonspecific perturbance of the membrane by the present linear hydrophobic molecules. It would be interesting to know where these novel compounds have their site of action in $GABA_A$ receptors. The independence of the stimulation from the presence of the γ subunit and its resistance to the benzodiazepine antagonist Ro15-1788 clearly document a site of action different from the benzodiazepine-binding site. The β subunit specificity and the relative lack of effect of the mutation β_2N265S argues for a site of action different from loreclezole. The α subunit specificity differs from that of classic benzodiazepines, which fail to affect $\alpha_6\beta_2\gamma_2$ receptors.

In summary, we have described novel positive allosteric modulators of $GABA_A$ receptors belonging to the polyacetylenes. The pharmacological characterization is at the in vitro level, and the suitability of the compounds for therapeutic applications needs to be shown. Because chemical synthesis seems feasible, in vivo experiments are within reach.

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