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Novel plant substances acting as β subunit isoform selective positive allosteric modulators of $GABA_A$ receptors

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Running title: β subunit isoform selective modulators of $GABA_A$ receptors

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Abbreviations: GABA: γ-aminobutyric acid; $GABA_A$ receptor: γ-aminobutyric acid type A receptor; MS-1: 1-ethyl-6-hydroxypentadeca-2,4-dinyl acetate; MS-2: 6-hydroxy-1-vinylpentadeca-2,4-dinyl acetate, MS-4: (6S)-16-Acetoxy-6-hydroxy-1-vinylhexadeca-2,4-diynyl acetate.

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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 $\gamma 2$ subunit, by the benzodiazepine antagonist Ro15-1788, or the point mutation β_2N265S, that abolishes effects by loreclezole. At a concentration of 30 μM, the substances elicited by themselves only tiny currents. Maximal stimulation at $\alpha_1\beta_2\gamma_2$ amounted to 110 – 450% for the three substances and half maximal stimulation was observed at concentrations of 1 – 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 at $\alpha_1\beta_2\gamma_2$ was 450%, at $\alpha_1\beta_1\gamma_2$ 80%, and at $\alpha_1\beta_3\gamma_2$ 150%. MS-1 was thus specific for receptors containing the β_2 subunit. The reversal potential was unaffected by 10 μM MS-1, while apparent picrotoxin affinity for current inhibition was increased about three-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.

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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 the $GABA_C$ receptors, that 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 been cloned since (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 $5HT_3$ receptor. The $GABA_A$ receptors are heteromeric protein complexes consisting of five subunits, which 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, $\beta_2$and $\gamma_2$subunits (Macdonald and Olsen, 1994; Rabow et al., 1995; Barnard et al., 1998; Laurie et al., 1992; Benke et al., 1994; McKernan and Whiting, 1996). 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 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 of 0 - 400 m (Tennant, 1968). The marrow of the

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stem and branches are eaten to treat epilepsy and a decoction of the root is taken as a remedy for labour pain (Haerdi, 1964). In addition, an infusion 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 gonorrhoea (Kokwaro, 1976).

We describe here three potent positive allosteric modulators of $GABA_A$ receptors with a linear polyacetylene structure isolated from the plant *Cussonia zimmermannii* Harms. They are shown to act at a site independent of both the benzodiazepine and the loreclezole site. Most interestingly, these compounds display an unprecedented subunit specificity.

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Methods

Substances. MS1, MS-2 and MS-4 were isolated from the East African medicinal plant *Cussonia zimmermannii* Harms. Methods of isolation and structure determination will be described elsewhere.

[^{3}H]Flunitrazepam binding. Cortex material derived from rats (stem RORO, RCC Ltd., Switzerland) was homogenized on ice with 50 mM Tris/HCl pH 7.4, 120 mM NaCl, 5 mM KCl using a Polytron homogenizer. The homogenate was centrifuged at 31‘000g 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 (Amersham Pharmacia, 1 nM final concentration) 200 µg of membrane protein (BCA protein assay) was used. Non-specific binding was determined in the presence of 10 µM diazepam. Binding equilibrium was reached within 1 h at RT and binding assays were terminated after this time by rapid filtration using GF/C filters. Filters were washed three times with cold Tris/HCl pH 7.4 buffer. Radioactivity on filters was determined by liquid scintillation counting (TRI-CARB 2100TR, Packard). Results are given as mean ± SEM of two to four individual experiments performed in triplicates.

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

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were cloned into the *Eco*RI and the β subunits were subcloned into the *Sma*I site of the polylinker by standard techniques.

Expression in Xenopus Oocytes. Capped cRNAs were synthesized (Ambion, Austin, Texas) from the linearized pCMV 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 about 400 residues was added to each transcript using yeast poly-A polymerase (USB, Cleveland, Ohio). The concentration of the cRNA was quantified on a formaldehyde gel using Radiant Red stain (Biorad) for visualisation of the RNA and known concentrations of RNA ladder (GIBCO BRL) 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 done as described earlier (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 nM : 75 nM and the combination of wild type α_x, β_x and γ_2 subunits was expressed at 10 nM : 10 nM : 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, 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 about 5% of the maximal current amplitude

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were applied alone or in combination with increasing concentrations of MS-compounds between 0.03 - 30 µM for 20 s and a wash out period of 4 min was allowed to ensure full recovery from desensitization. The stimulation was then calculated as Stimulation = $((I_{after\ NCS}/I_{before\ NCS})-1)$ x 100%. Stimulation was fitted to the Hill equation: $I = I_{max}/1+(EC_{50}/A)^n$ 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 is the Hill coefficient. GABA-evoked currents (at about 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} 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 mean ± standard error of mean (SEM) (number of experiments for at least 2 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 affect significantly GABA elicited currents. Currents were measured using a modified OC-725 amplifier (Warner Instruments Corp.) in combination with an XY-recorder or digitized using a MacLab/200 (AD Instruments).

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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 *Cussonia zimmermanni* Harms. Elucidation of the chemical structures were performed using UV (Ultraviolet Spectroscopy), IR (Infrared Spectroscopy), ^{1}H-NMR (Proton Nuclear Magnetic Resonance), ^{13}C-NMR (Carbon Nuclear Magnetic Resonance), HMQC (Heteronuclear Multiple Quantum Correlation), HMBC (Heteronuclear Multiple Bond Correlation), COSY (Correlated Spectroscopy), EI-MS, FAB-MS (Fast Atom Bombardment Mass Spectrometry) and HR-EI-MS (High Resolution Electron Ionization Mass Spectrometry) (to be published elsewhere). In traditional, local medicine this plant is used among many other purposes against 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 binding of [^{3}H]flunitrazepam was then used to isolate three substances. Fig. 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 *Xenopus laevis* oocytes. For reasons of solubility the MS-compounds were only used up to a concentration of 30 μM. GABA was always used at concentrations eliciting 2-6% of the maximal current amplitude in the corresponding $GABA_A$ receptor type. 30 μM of each MS-compound elicited at $\alpha_1\beta_2\gamma_2$ by itself tiny currents amounting to < 0.1% of the maximal current elicited by GABA.

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But all 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 about 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 ± 1.5% of the control (n = 3)). Fig. 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 ± 5 μM (n = 4) and 1.5 ± 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 ± 3 μM (n = 4) and a Hill coefficient of 1.1 ± 0.1 (n = 4). Stimulation by 10 μM MS-1 at low concentrations of GABA amounts to only about 2.3-fold as compared to the value of 4-fold expected from the data shown below. The reason for this discrepancy is not known, but may be due to the repetitive application of 10 μM MS-1 in these experiments.

Electrophysiological studies: Subunit specificity of MS-1. Fig. 5A shows an averaged concentration response curve of this type of experiment for $\alpha_1\beta_2\gamma_2$. Maximal stimulation is about 450% and half-maximal stimulation was observed at a concentration (EC_{50}) of about 1.5 μM. Replacement of α_1 in this subunit combination by other α subunit isoforms α_2, α_3, α_5, or α_6 resulted in little effect on EC_{50}, that varied between 0.6 and 1.0 μM, but had in some cases a drastic effect on the maximal stimulation (Fig. 5A). Extent of stimulation was $\alpha_1\beta_2\gamma_2 \approx \alpha_3\beta_2\gamma_2 > \alpha_2\beta_2\gamma_2 > \alpha_5\beta_2\gamma_2 \approx \alpha_6\beta_2\gamma_2$. Fig. 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%

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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 to about 65% (Fig. 5B).

Electrophysiological studies: Subunit specificity of MS-2 and MS-4. Figs. 6 and 7 show subunit specificities of MS-2 and MS-4, respectively. Again replacement of α_1 in $\alpha_1\beta_2\gamma_2$ by other α subunit isoforms α_2, α_3, α_5, or α_6 resulted in little effect on EC_{50}, that 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 (Fig. 6 and 7). Maximal stimulation was about 300% for MS-2 and about 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 $\alpha_1\beta_2\gamma_2 \approx \alpha_1\beta_2 \approx \alpha_5\beta_2\gamma_2 \approx \alpha_3\beta_2\gamma_2 \approx \alpha_2\beta_2\gamma_2 > \alpha_6\beta_2\gamma_2 \gg \alpha_1\beta_1\gamma_2$.

Lack of inhibition by the benzodiazepine antagonist Ro15-1788. It was tested if MS-1 would act at a known site on the $GABA_A$ receptor. Currents elicited by 2-7 μM GABA were stimulated by 10 μM MS-1 377 ± 43% (n = 7). If 1 μM of the benzodiazepine antagonist Ro15-1788 was co-applied stimulation was not significantly altered with 391 ± 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 (Sigel et al., 2001) ROD178B at a concentration of 100 μM significantly reduced stimulation to 134 ± 9 % (n = 3).

Co-stimulation by MS-1 and diazepam. The above observations indicate independent sites of action of MS-1 and diazepam. Even if this is the case it can not be predicted if the two drugs act additively or not. Currents were elicited by 2-7 μM GABA, followed

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by co-application 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 ± 12 % (n = 8) and the combination of diazepam and MS-1 a stimulation of 871 ± 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 ± 0.4 μM (n = 3) in the absence and 1.0 ± 0.1 μM (n = 3) in the presence of 10 μM MS-1. MS-1 increased apparent picrotoxin affinity about 2.6-fold (Fig. 9).

Effect of MS-1 on the apparent affinity of bicuculline. GABA induced currents amounting to about 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 ± 0.1 μM (n = 4) in the absence and non-significantly increased to 2.3 ± 0.4 μM (n = 4) in the presence of 10 μM MS-1 (not shown).

Ion selectivity is maintained and stimulation is potential independent. 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 (not shown).

Specificity of the MS-compounds. As the structure of the MS-compounds suggests interaction with the lipid bilayer, they could in principle non-specifically interact with any membrane protein. Even if their specificity for receptors containing the β_2 subunit argues 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

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concentrations of MS-1 of 1, 5 μM 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 μM – 1.5 μM). 1, 5 and 30 μM MS-1 weakly stimulated peak sodium currents elicited by a voltage jump from –100 mV to –15 mV by 8.8 ± 2.5 % (n = 3), 3.8 ± 2.5 % (n = 4) and 6.6 ± 3.6 % (n = 3), respectively (not shown). For a voltage jump from –60 mV to –15 mV, in contrast 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 following a pulse from –100 mV to –15 mV was fitted with mono-exponential function characterized by the time constant τ. 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, effects of MS-1 on the voltage gated sodium channel are small, arguing again against a non-specific membrane effect.

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Discussion

We show here that three substances isolated from Cussonia *zimmermannii* Harms act as potent positive allosteric modulators at $GABA_A$ receptors with a half maximal stimulation at a concentration of 0.6 – 3.5 μM and a maximal stimulation of 110 – 450%. Threshold of stimulation was below 0.1 μM. The fact that the substances did not open the channels themselves even at high concentrations and that the reversal potential is not affected probably indicates that these substances act by increasing the apparent affinity for channel opening.

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 from its binding site with an IC_{50} of 0.5 μM. No functional studies were performed except it was shown that the compound is able to kill mice with a LD_{50} of about 3 mg/kg. Another compound, virol A was shown to have about half 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 about 1 μM (Uwai et al., 2001). Most remarkably, the compounds described here are linear molecules. Most known 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.

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The three novel compounds showed unique subunit selectivity profiles. Omitting the γ subunit from $\alpha_1\beta_2\gamma_2$ did not affect extent of stimulation. In contrast, the β subunit present seems to strongly influence stimulation. Replacing β_2 by β_1 reduced maximal stimulation more than 4-fold. Replacing β_2 by β_3 reduced maximal stimulation about 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 (Wingrove et al., 1994) had only a relatively weak effect, reducing maximal stimulation by about 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 subunit 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 $\alpha_1\beta_2\gamma_2$ while 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, being $\alpha_1\beta_2\gamma_2 \approx \alpha_1\beta_2 \approx \alpha_3\beta_2\gamma_2 > \alpha_2\beta_2\gamma_2 > \alpha_5\beta_2\gamma_2 \approx \alpha_1\beta_3\gamma_2 \approx \alpha_6\beta_2\gamma_2 > \alpha_1\beta_1\gamma_2$ 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 argues together with the observed $GABA_A$ receptor subunit specificity against non-specific perturbance of the membrane by the present linear hydrophobic molecules. It is interesting to know

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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 classical benzodiazepine that do not affect at all $\alpha_6\beta_2\gamma_2$ receptors.

In summary, we describe here 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. As chemical synthesis seems feasible, in vivo experiments are within reach.

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(Footnotes)

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(Legends)

Fig. 1. Structures of the three polyacetylene compounds.

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 mean ± SEM of two to four individual experiments.

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 *Xenopus leavis* oocytes and exposed to either 7 μM 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 *Xenopus leavis* oocytes and exposed to increasing GABA concentrations either alone (●) or in combination with 10 μM MS-1 (❍). Data are given as mean ± SEM (≤ 4 oocytes from two different batches).

Fig. 5. Subunit isoform specificity of MS-1. (A) 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 $\alpha_6\beta_2\gamma_2$ (▲) and (B) $\alpha_1\beta_2\gamma_2$ (●), $\alpha_1\beta_2$ (△), $\alpha_1\beta_2$N265Sγ_2 (x), $\alpha_1\beta_3\gamma_2$ (+), and $\alpha_1\beta_1\gamma_2$ (◆) $GABA_A$ receptors were expressed in *Xenopus leavis* oocytes and exposed to either GABA alone or in combination with increasing concentrations of MS-1. Data are given as mean ± SEM (≤3 oocytes from two different batches).

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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 *Xenopus leavis* oocytes and exposed to either GABA alone or in combination with increasing concentrations of MS-2. Data are given as mean ± SEM (≥ 3 oocytes from two different batches).

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 *Xenopus leavis* oocytes and exposed to either GABA alone or in combination with increasing concentrations of MS-4. Data are given as mean ± SEM (≥ 3 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 either GABA in combination with 10 μM MS-1 or 1 μM diazepam, or to GABA in combination with 10 μM MS-1 and 1 μM Ro15-1788, 100 μM ROD178B or 1 μM diazepam, or to GABA in combination with 1 μM diazepam and 1 μM Ro15-1788. Data are given as mean ± SEM (≥ 3 oocytes from two different batches).

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 (○).

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Currents were inhibited by increasing concentrations of picrotoxin. Data are given as mean ± SEM (3 oocytes from two different batches).

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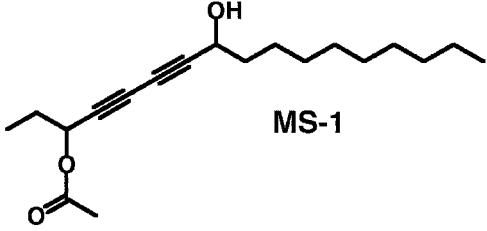
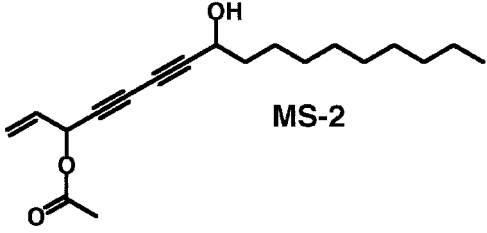
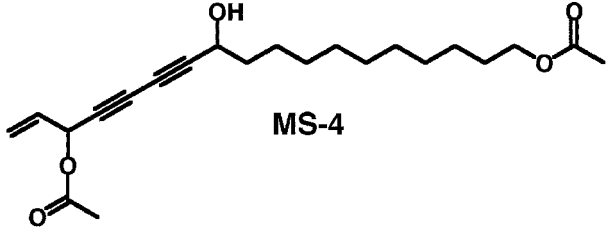


MS-1



MS-2



MS-4

Figure 1

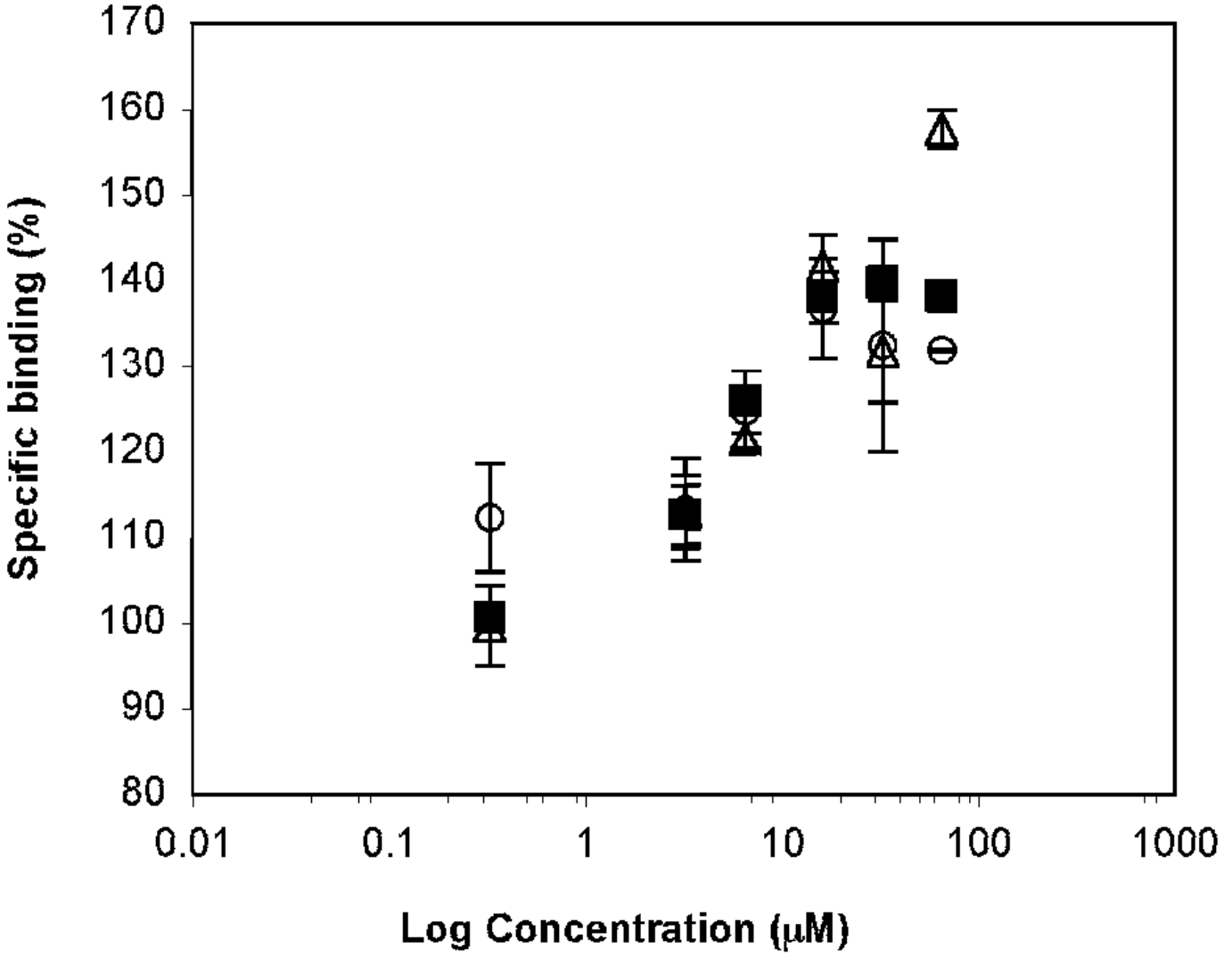

170
160
150
140
130
120
110
100
90
80
Specific binding (%)
0.01
0.1
1
10
100
1000
Log Concentration (μM)

Figure 2

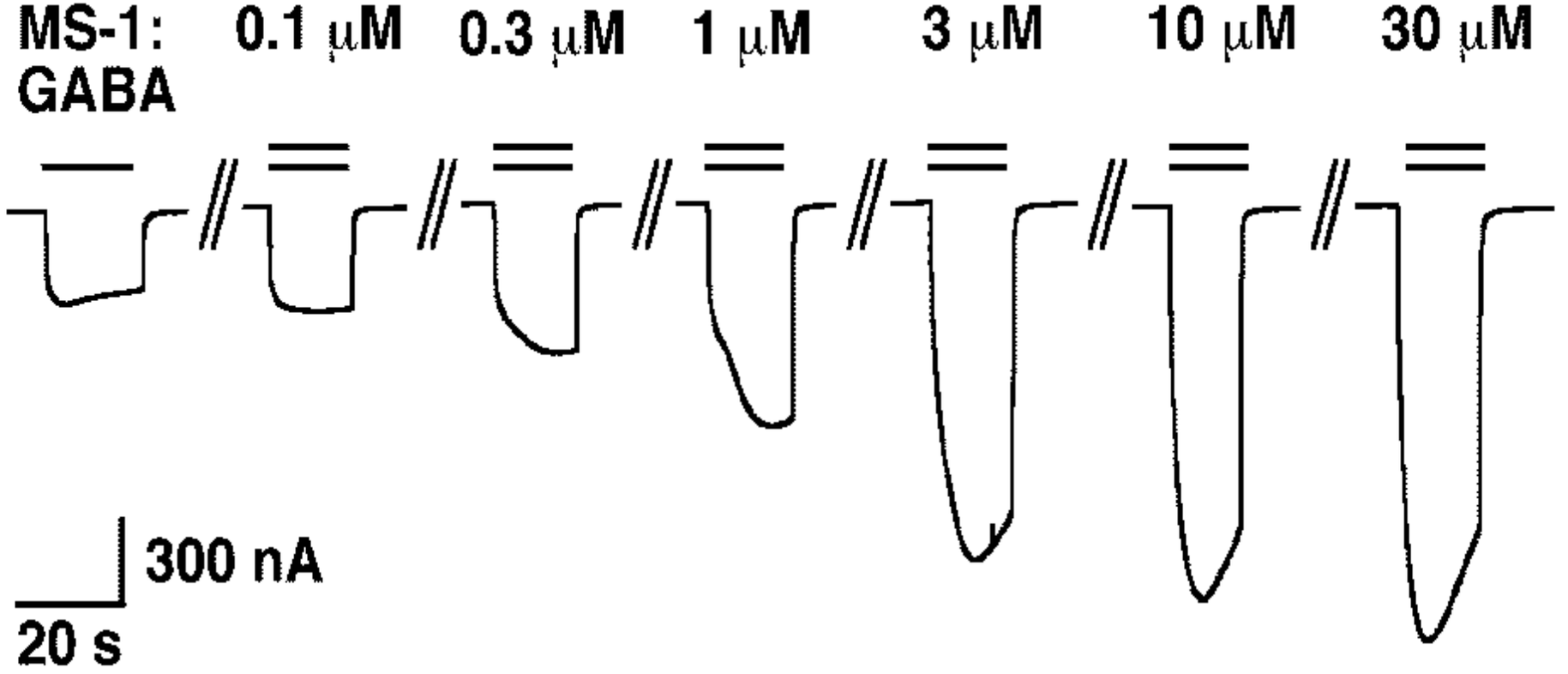


Figure 3

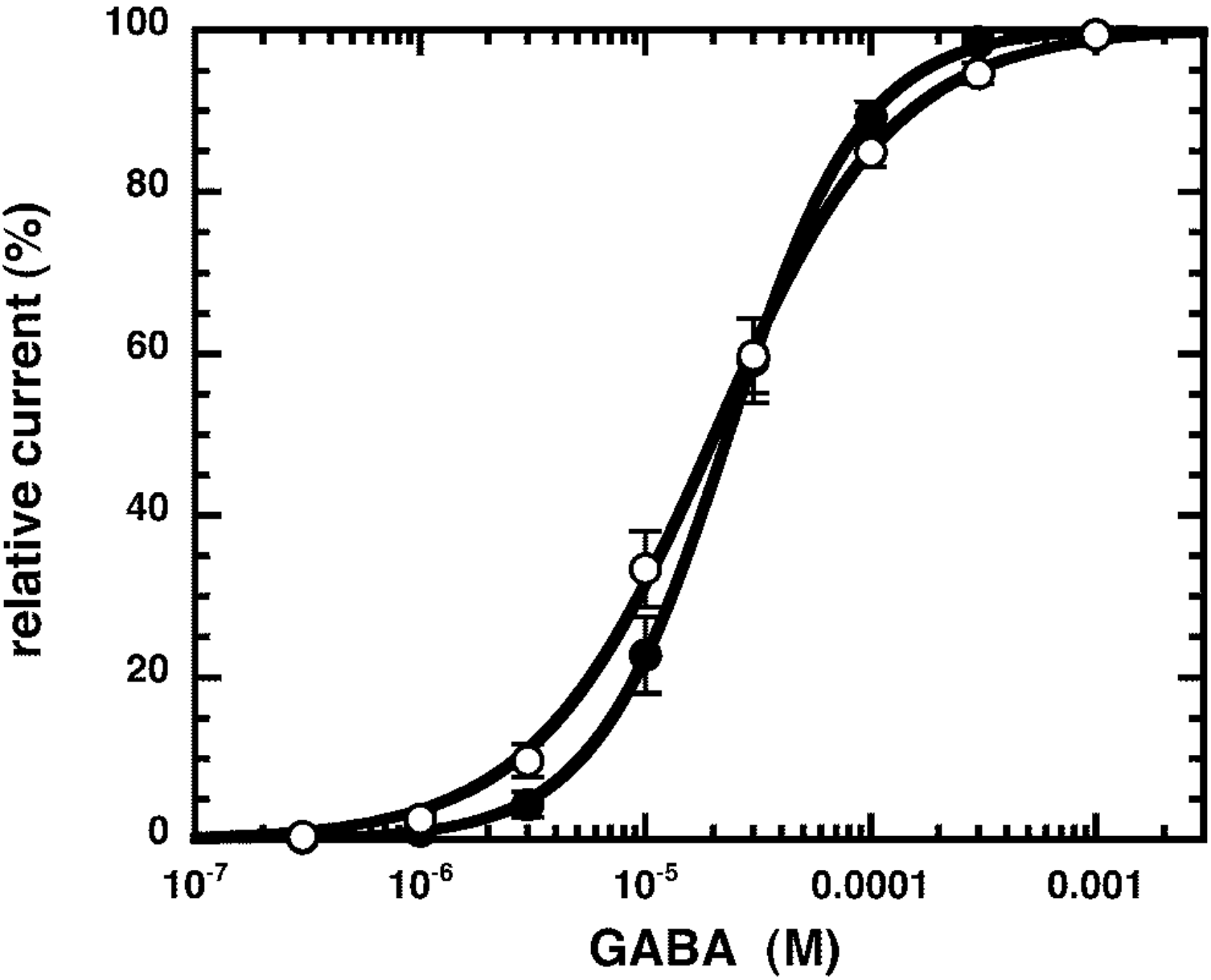

100
80
60
40
20
0
relative current (%)
10-7
10-6
10-5
0.0001
0.001
GABA (M)

Figure 4

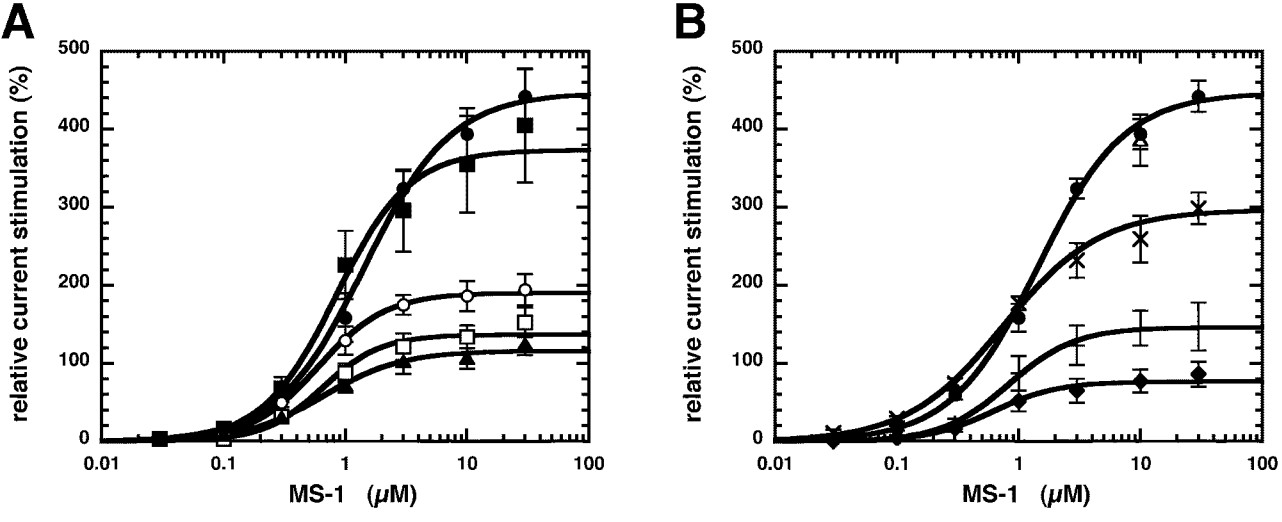

A
B
relative current stimulation (%)
500
400
300
200
100
0
0.01
0.1
1
10
100
MS-1 (μM)

Figure 5

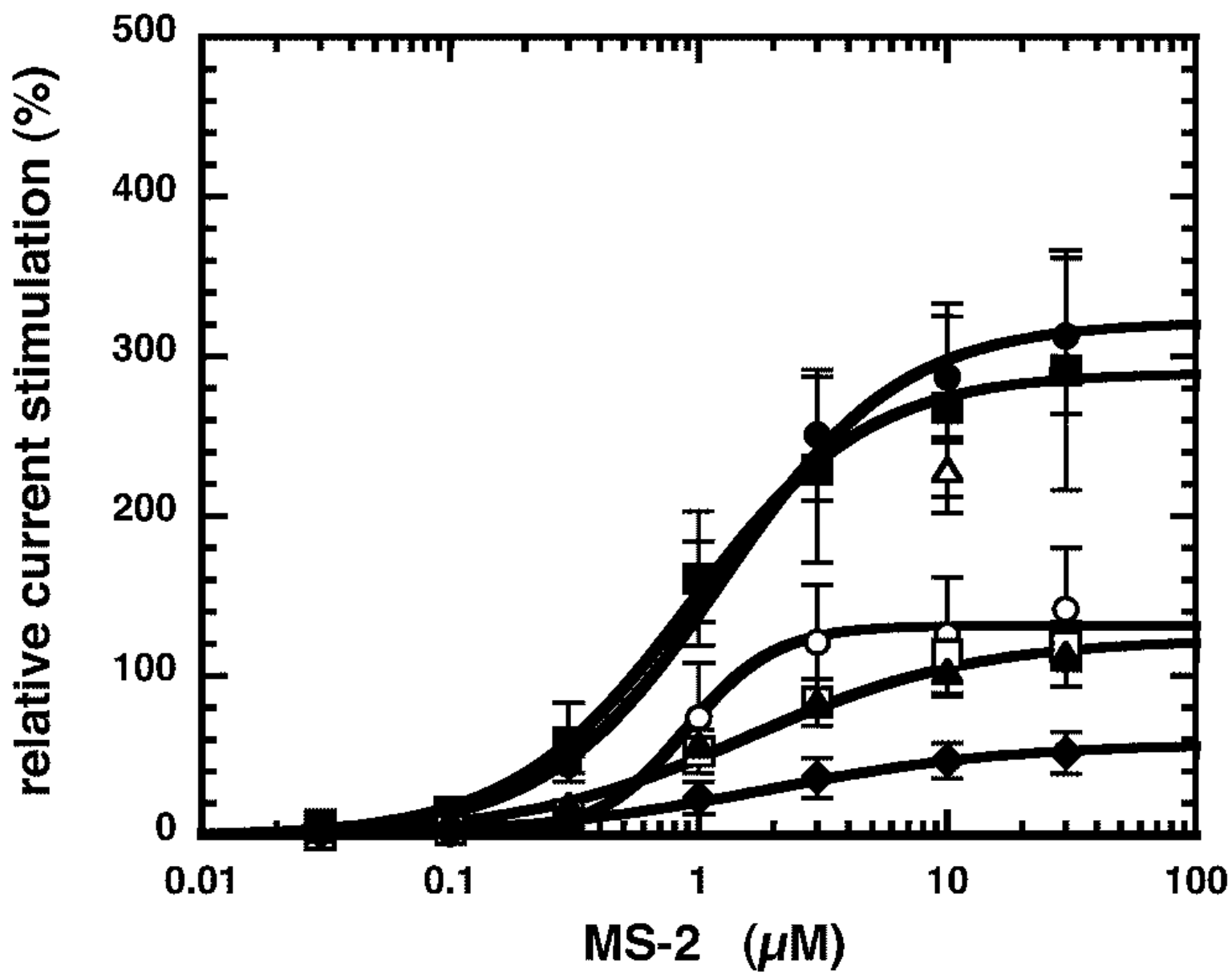

relative current stimulation (%)
500
400
300
200
100
0
0.01
0.1
1
10
100
MS-2 (μM)

Figure 6

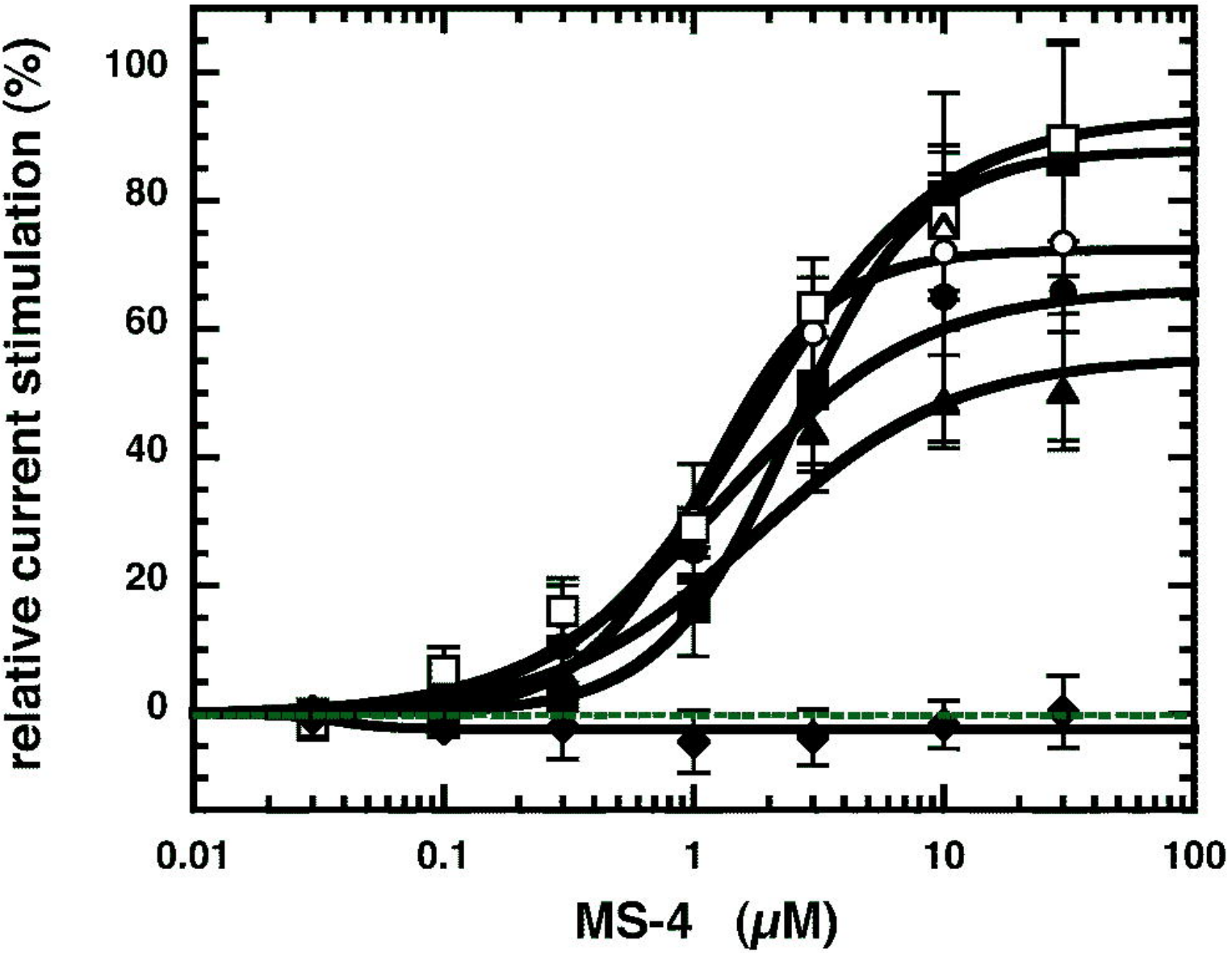

relative current stimulation (%)
100
80
60
40
20
0
0.01
0.1
1
10
100
MS-4 (μM)

Figure 7

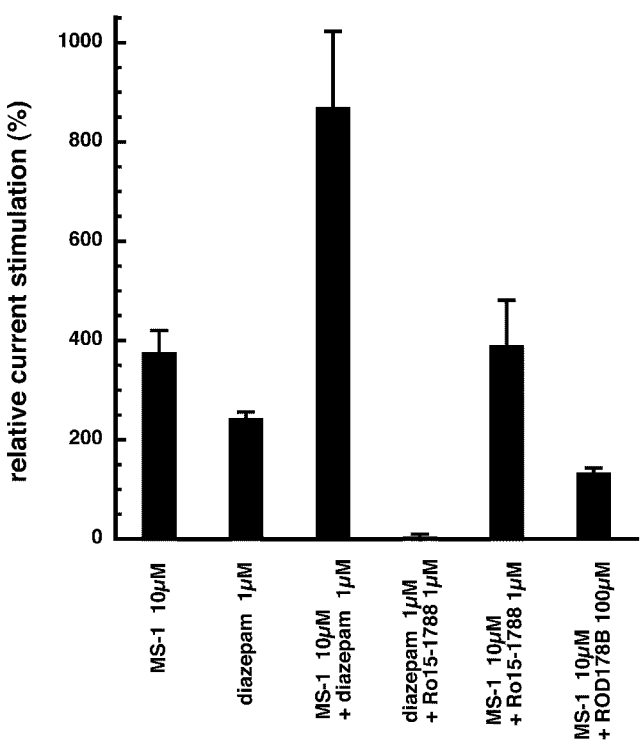

relative current stimulation (%)
1000
800
600
400
200
0
MS-1 10μM
diazepam 1μM
MS-1 10μM + diazepam 1μM
diazepam 1μM + Ro15-1788 1μM
MS-1 10μM + Ro15-1788 1μM
MS-1 10μM + ROD178B 100μM

Figure 8

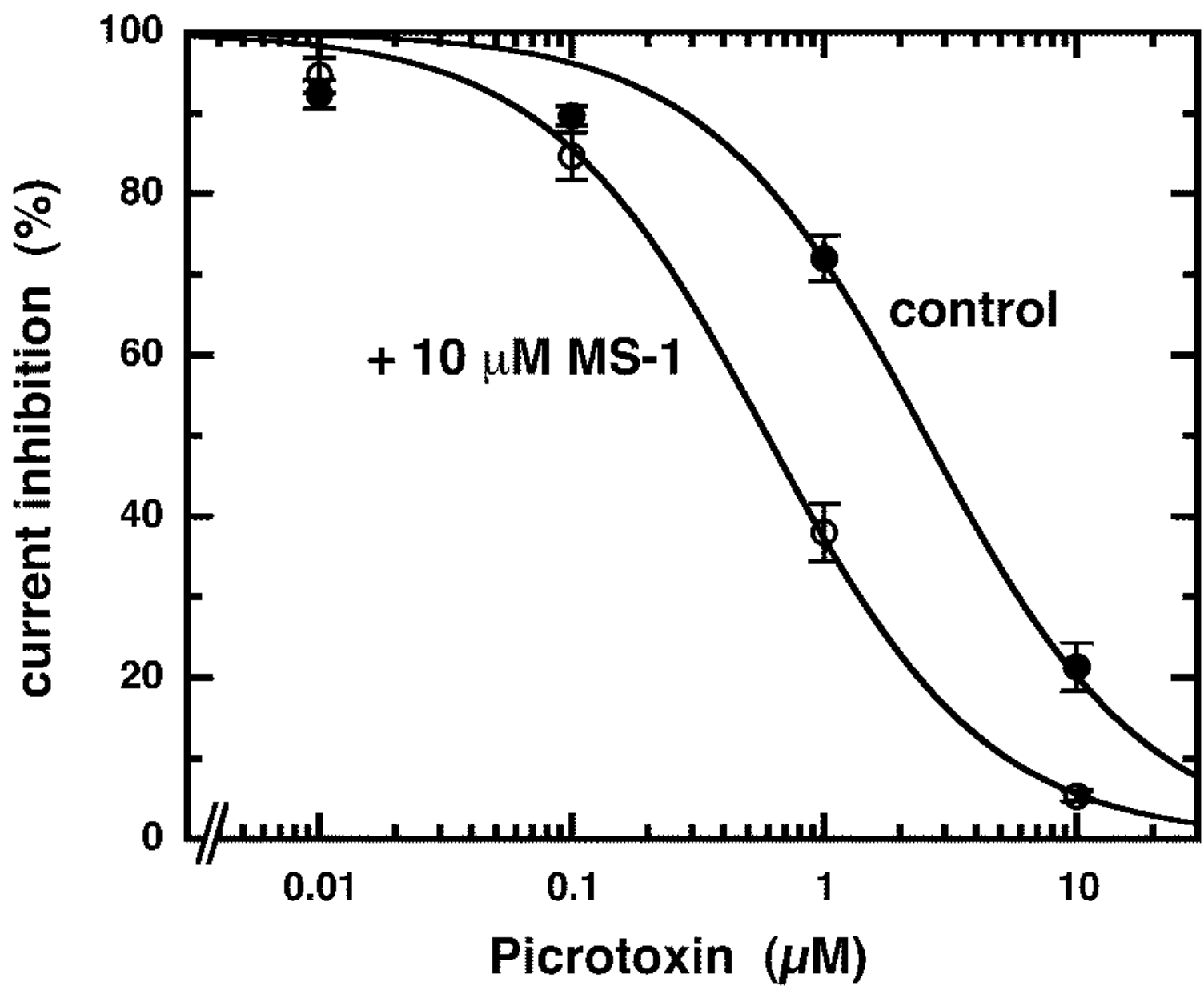

100
80
60
40
20
0
current inhibition (%)
control
+ 10 μM MS-1
0.01
0.1
1
10
Picrotoxin (μM)

Figure 9