JOURNAL OF NATURAL PRODUCTS

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Falcarindiol Allosterically Modulates GABAergic Currents in Cultured Rat Hippocampal Neurons

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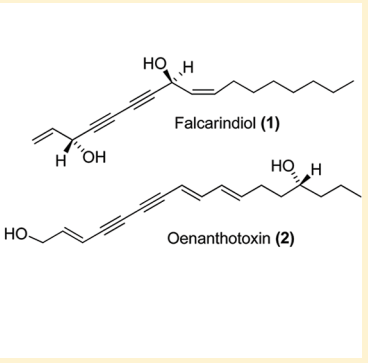
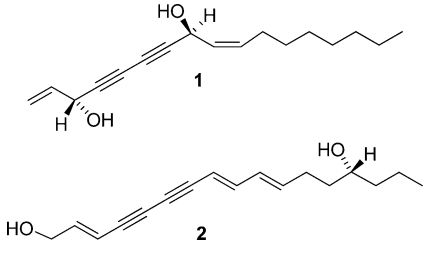
ABSTRACT: Falcarindiol (**1**), a C-17 polyacetylenic diol, shows a pleiotropic profile of bioactivity, but the mechanism(s) underlying its actions are largely unknown. Large amounts of **1** co-occur in water hemlock (*Oenanthe crocata*) along with the convulsant polyacetylenic toxin oenanthotoxin (**2**), a potent $GABA_A$ receptor ($GABA_AR$) inhibitor. Since these compounds are structurally and biogenetically related, it was considered of interest to evaluate whether **1** could affect GABAergic activity, and for this purpose a model of hippocampal cultured neurons was used. Compound **1** significantly increased the amplitude of miniature inhibitory postsynaptic currents, accelerated their onset, and prolonged the decay kinetics. This compound enhanced also the amplitude of currents elicited by 3 μM GABA and accelerated their fading, reducing, however, currents evoked by a saturating (10 mM) GABA concentration. Moreover, kinetic analysis of responses to 10 mM GABA revealed that **1** upregulated the rate and extent of desensitization and slowed the current onset and deactivation. Taken together, these data show that **1** exerts a potent modulatory action on $GABA_AR$s, possibly by modulating agonist binding and desensitization, overall potentially decreasing the toxicity of co-occurring GABA-inhibiting convulsant toxins.



Falcarindiol (**1**), a C-17 polyacetylenic diol, is common in plants from the family Apiaceae, both in edible [carrot (*Daucus carota* L.), celery (*Apium graveolens*), parsley (*Petroselinum crispum* Mill.)] and in poisonous [hemlock (*Conium maculatum* L.) and water dropwort (*Oenanthe crocata* L.)] species.[1] The literature on **1** is rich in biological observations (e.g., antifungal,[2,3] cytotoxic,[4−6] anticancer,[7,8] antioxidant,[9] immunosuppressive, and anticomplement properties[10,11]), but relatively little is known on the molecular mechanism(s) underlying the action of **1** and its cellular targets. Compound **1** has been shown to affect neural function. In particular, apart from protecting neurons from cell death induced by excessive release of nitric oxide,[12,13] this polyacetylene shows also a weak binding affinity for opioid receptors, a moderate affinity for the dopamine D1 receptors, and an affinity for 5-HT7,[14] suggesting overall a potential for interference with pain sensation and drug addiction.[15] These findings indicate that **1** is able to affect the functioning of the CNS by interfering with receptors for key neurotransmitters. The CNS is also the target of the convulsant polyacetylenes. This class of compounds downregulates $GABA_A$ receptors[16,17] and is exemplified by oenanthotoxin (**2**) from water dropwort (*O. crocata*), possibly the most toxic plant of the European flora.[1,16] Falcarindiol (**1**) and oenanthotoxin (**2**) share a diynediol structural motif, but differ in the relative location of the hydroxy groups and the diyne moieties and in the olefinic decoration of the linear chain. Since both compounds occur in the same toxic species, it was considered of interest to evaluate if **1** also can affect GABAergic signaling, playing a potential role, either protective or aggravating, in poisoning from *O. crocata*. Preliminary evidence that **1** downregulates GABA-transaminase and increases the GABA levels in CNS has been presented,[17] providing a further rationale to investigate if **1** also has a direct impact on synaptic and exogenously evoked GABAergic currents. Using hippocampal cultured neurons, we present evidence that **1** shows a potent allosteric modulatory action on $GABA_A$ receptors.



RESULTS AND DISCUSSION

The effect of **1** was examined initially on miniature inhibitory postsynaptic currents (mIPSC). Under control conditions, the

Received: October 21, 2011
Published: March 20, 2012

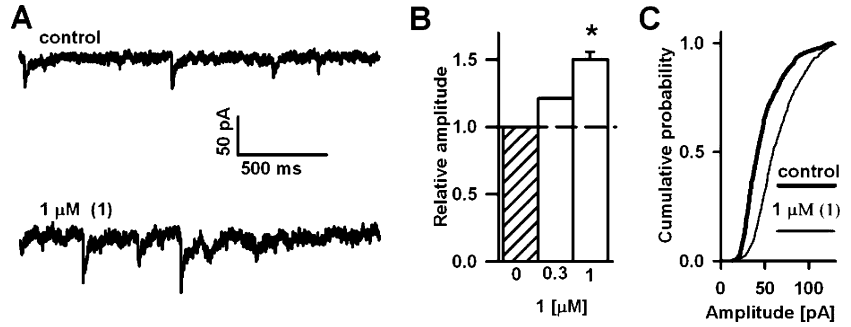


Figure 1. Falcarindiol (**1**) upregulates the amplitude of mIPSCs. (A) Typical trace recorded in control conditions (upper trace) and in the presence of 1 μM **1** (lower trace). (B) Statistics of the effects of **1** on mIPSC amplitudes. (C) Cumulative amplitude histogram (thick line, control; thin line, in the presence of 1 μM **1**). The asterisk indicates a significant ($p < 0.05$) difference with respect to the respective control.

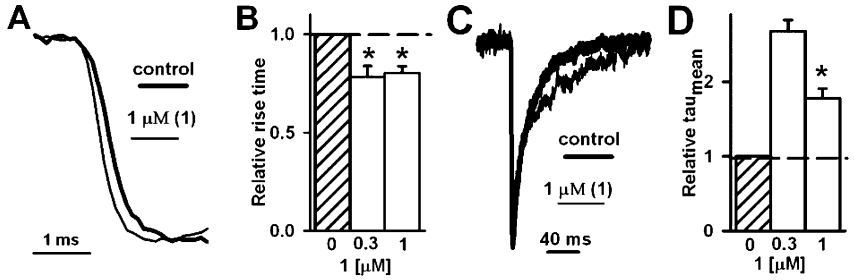


Figure 2. Falcarindiol (**1**) affects the time course of mIPSCs. (A) Typical examples of normalized and superimposed rising phases of mIPSCs in control conditions (thick line) and in the presence of 1 μM **1**. (B) Statistics on the 10–90% rise time. (C) Typical example of normalized and superimposed mIPSCs in control conditions and in the presence of 1 μM **1**, showing a prolonged mIPSC decay in the presence of **1**. (D) Statistics of the effect on the mean deactivation time constant (τ_{mean}). Asterisks indicate significant differences ($p < 0.05$).

mIPSC amplitude measured at −70 mV was −40.4 ± 2.3 pA (n = 8), and the effect of **1** on this parameter was assessed by calculating its activity relative to a control value, measured from the same cell (Figure 1A, B). Falcarindiol (**1**) increased the relative mIPSC amplitude, reaching statistical significance at 1 μM ($p < 0.05$, Figure 1B), and the cumulative histogram constructed for mIPSC amplitudes measured from all neurons included in the statistics showed that the increase in mIPSC amplitude in the presence of 1 μM **1** was characterized by a roughly parallel shift to the right, suggesting a lack of discrimination between mIPSCs characterized by low or high amplitudes.

Under control conditions, the mIPSC frequency was 0.73 ± 0.14 Hz (n = 6), and **1** at 1 μM apparently reduced this parameter (0.52 ± 0.08 Hz, n = 6), but this difference was not statistically significant (p = 0.065). Next, the effect of activity of **1** on the time course of mIPSCs was evaluated. Falcarindiol at 0.3–1 μM concentrations significantly accelerated the onset kinetics of mIPSCs (Figure 2A, B) and also prolonged their decay (Figure 2C, D). Taken together, these data show that **1** at concentrations up to 1 μM exerts a strong modulatory action on GABAergic synaptic currents, enhancing their amplitude, accelerating their onset, and prolonging their decaying phase without affecting cell vitality. Conversely, signs of deterioration of the cell (lower patch stability and a trend to increase the signal run-down), especially upon long-term recordings (>15 min), could be observed at concentrations > 3 μM.

Current responses to exogenous rapid applications of GABA were next investigated in the presence of falcarindiol (**1**). GABAergic synaptic currents represent a very important physiological pattern of rapid inhibitory signaling in the CNS, but the mechanism of their modulation is often difficult to establish, since this potentially may take place both pre- and postsynaptically. Moreover, synaptic signals may result from a release of an a priori unknown amount of neurotransmitter, thus preventing a classic analysis based on dose dependence. Current responses to 3 μM GABA (10 s application time) were recorded at −40 mV in the whole-cell configuration (Figure 3A), and their mean amplitude was −1533 ± 202 pA (n = 17). Similar to mIPSCs, responses measured in the presence of **1** had larger amplitudes than controls, but this effect was significant statistically only at 1 μM (Figure 3A–C). Interestingly, in the presence of **1**, the fading of current responses (decay observed during GABA application) appeared more profound than in control recordings (Figure 3A). This effect was better visualized when normalized control currents were superimposed with those recorded in the presence of **1** (Figure 3C). For currents evoked by 3 μM GABA (10 s application), the extent of fading was assessed as $(I_{Peak} - I_{End})/I_{Peak}$, with I_{End} being the current value at the end of GABA application. As shown in Figure 3 D, **1** significantly enhanced this parameter compared to control values.

It was also evaluated if **1** could exert any effect on the onset and deactivation (current time course after agonist removal) kinetics of responses evoked by 3 μM GABA. Under control conditions, the mean 10–90% rise time was 416 ± 62 ms (n = 14). In the presence of 0.3 μM **1**, there was apparently a trend of acceleration of the onset kinetics (relative 10–90% rise time 0.846 ± 0.062), but this did not reach statistical significance (p = 0.07) and disappeared at a 1 μM concentration (relative value 0.99 ± 0.036, $p > 0.05$). The mean deactivation time constant (τ_{mean}) under control conditions was 535 ± 52 ms (n = 14), and this parameter was not affected in the presence of **1** (relative τ_{mean}: 0.904 ± 0.06 and 1.04 ± 0.05 for 0.3 and 1 μM FL, respectively, $p > 0.05$).

The effect of membrane voltage on the action of **1** was investigated next. Thus, amplitude, onset kinetics, and deactivation time course for responses evoked by 3 μM GABA at the membrane voltage of −40 or +40 mV were

dx.doi.org/10.1021/np2008522 | J. Nat. Prod. 2012, 75, 610–616

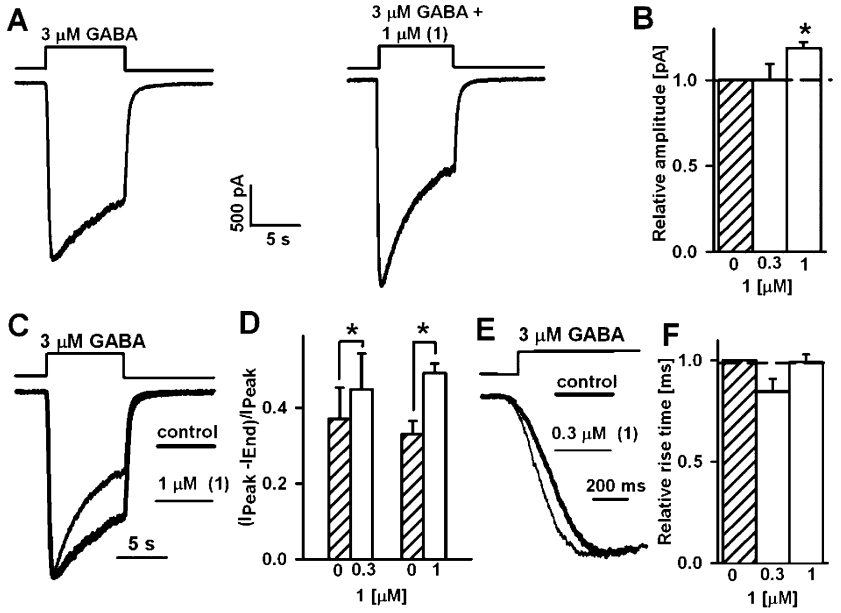


Figure 3. Falcarindiol (**1**) upregulates the amplitude and effects the time course of currents evoked by 3 μM GABA. (A) Typical examples of current responses elicited by 3 μM GABA in control conditions (left) and in the presence of **1** (right). (B) Statistics of the effect on the amplitude of current responses to 3 μM GABA. (C) The same traces as in A but normalized and superimposed, with the increased fading in the presence of **1**. (D) Statistics of fading calculated as $(I_{Peak} - I_{End})/I_{Peak}$. (E) Typical normalized initial phases (onsets) of current responses evoked by 3 μM GABA. (F) Statistics of the 10–90% rise times for currents elicited by 3 μM GABA. Insets above current traces indicate time of agonist applications. Asterisks indicate significant differences ($p < 0.05$).

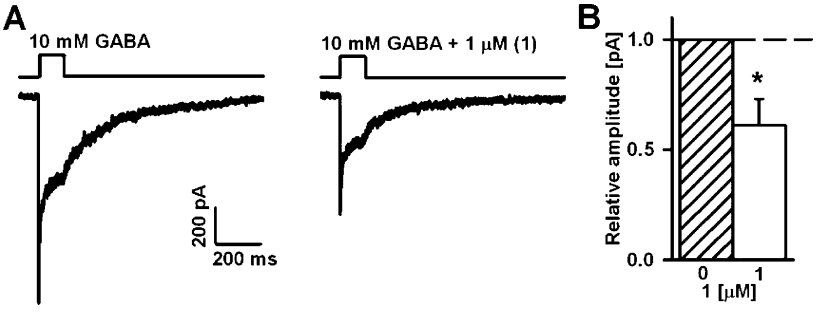


Figure 4. Falcarindiol (**1**) downregulates the amplitude of current responses elicited by a saturating GABA concentration (10 mM). (A) Typical currents evoked by 10 mM GABA in control conditions (left) and in the presence of 1 μM **1** (right). (B) Statistics of the effect on the amplitude of current responses, with a marked reduction of current amplitude induced by **1**. Insets with the above current traces indicate the time of agonist applications. The asterisk indicates a significant difference ($p < 0.05$).

assessed in the presence of **1**, without, however, showing any change in the action of this compound (data not shown). Taken together, the observation of an increase in amplitude of mIPSCs and of responses evoked by 3 μM and the trend to accelerate the rising phase of mIPSCs suggest an upregulation of the binding rate by **1**. To evaluate this possibility, current responses to higher GABA concentrations were recorded. It is expected that the strongest dependence of amplitude and the onset kinetics on the binding step takes place at a GABA concentration close to the EC_{50} value. In the case of GABA-evoked currents in cultured hippocampal neurons, EC_{50} is believed to be close to 30 μM.[21−23] The sensitivity of current responses elicited by 30 μM GABA to **1** was thus checked. Since at this GABA concentration, the application speed of a multibarrel application system (RSC-200, Bio-Logic) becomes comparable to or slower than the current kinetics (especially the rising phase), the ultrafast theta-glass-based perfusion system (Experimental Section) was used in the excised-patch configuration. Using this system, currents evoked by 30 μM GABA at −40 mV had an amplitude of −512 ± 107 pA ($n = 6$) with the rise time 14.6 ± 3.07 ms ($n = 6$) and the deactivation time constant τ_{mean} 203 ± 31 ms ($n = 6$). Falcarindiol (**1**) at 1 μM induced only a slight and not statistically significant increase in the current amplitude (relative amplitude 1.14 ± 0.04, $p > 0.05$). No effect was observed either on the current onset (relative 10–90% rise time: 1.22 ± 0.10, $p > 0.05$) or in the deactivation time constant (relative τ_{mean}: 0.85 ± 0.22, $p > 0.05$).

Although these data may argue against the involvement of **1** in the modulation of the agonist binding, this effect cannot be ruled out. Thus, it is possible that a strong modulatory action on the bound conformational transitions of the receptor could mask effects on the binding step. To address this issue, responses evoked by saturating [GABA] (10 mM) in the presence of **1** were analyzed. At this agonist concentration, the binding step is believed to quickly reach saturation, and conformational transitions between bound states and the unbinding process are expected to become rate limiting.[24−26] Typical current responses to 10 mM GABA applied for 100 ms are shown in Figure 4A, showing a rapid onset, a prominent

dx.doi.org/10.1021/np2008522 | J. Nat. Prod. 2012, 75, 610–616

desensitization (current fading during GABA application), and a slow deactivation. Remarkably, and in contrast to that observed for mIPSCs and for responses to low [GABA], **1** potently reduced the amplitude of currents elicited by saturating GABA concentration (in control conditions 1020 ± 395 pA, $n = 6$, relative amplitude in the presence of 1 μM **1**: 0.61 ± 0.12, $p < 0.05$).

To evaluate whether **1** could affect the kinetics of conformational transitions between bound states, the effect of **1** on the time course of current responses was examined. Under the control conditions, the 10−90% rise time for currents elicited by 10 mM GABA was 0.23 ± 0.012 ms ($n = 5$, excised patch, −70 mV), and 1 μM **1** significantly slowed the current onset (relative rise time 1.51 ± 17, $p < 0.05$, Figure 5A, B). In general, desensitization of $GABA_A$ receptors plays a crucial role in determining the kinetic shape of GABAergic currents.[25] To monitor the desensitization onset, long pulses of saturating [GABA] were therefore applied (Figure 5C). Under control conditions, the time constant of the desensitization onset (τ_{Des}) was 5.45 ± 0.9 ms ($n = 7$), while in the presence of **1** (1 μM) this parameter was significantly shortened (relative τ_{Des}: 0.64 ± 0.08, $p < 0.05$, Figure 5C, D). Interestingly, the acceleration of the desensitization onset was accompanied by an increase in the extent of desensitization, an effect manifested by a reduction in the steady-state to peak ratio (ss/peak in control conditions: 0.66 ± 0.04 and in the presence of **1**: 0.439 ± 0.04, $n = 7$, $p < 0.05$, Figure 5C, F). The analysis of the deactivation phase of currents evoked by 10 mM GABA (Figure 5 F) revealed that **1** clearly slowed the deactivation time course compared to the control (183 ± 18 ms, $n = 6$, relative change in the presence of 1 μM **1**: 1.52 ± 0.22, $p < 0.04$, Figure 5F, G). Overall, currents evoked by saturating [GABA] were therefore affected strongly by **1**, showing in the presence of this compound a decrease in amplitude, an acceleration of the onset, an upregulation of desensitization, and a prolonged deactivation process.

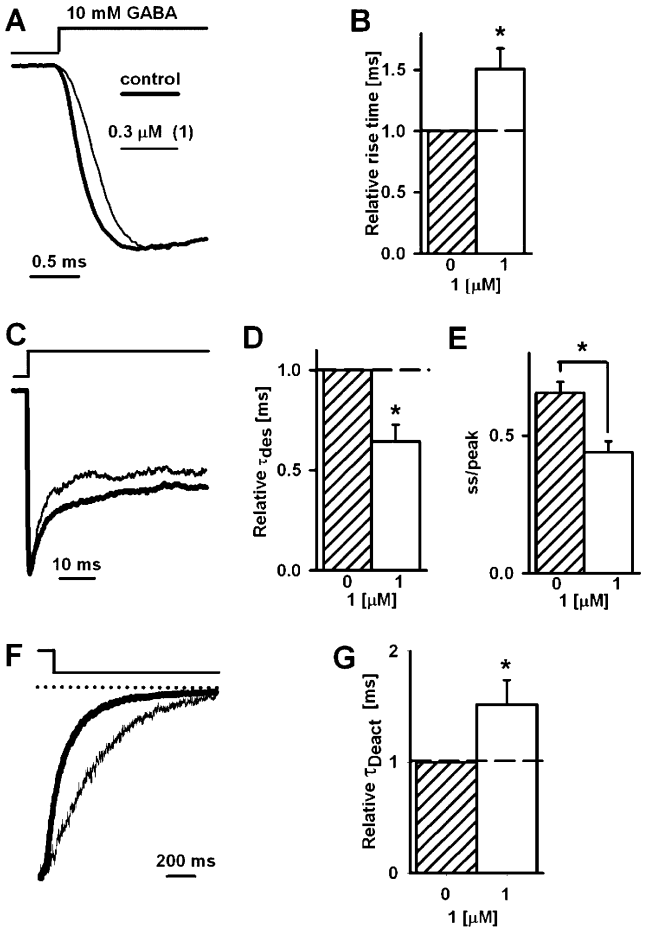


Figure 5. Falcarindiol (**1**) affects the time course of current responses evoked by a saturating GABA concentration (10 mM). (A) Typical examples of normalized and superimposed rising phases of currents elicited by application of 10 mM GABA in control conditions (thick line) and in the presence of 1 μM **1** (thin line). (B) Statistics of the effect on the 10−90% rise time. (C) Typical examples of normalized and superimposed currents elicited by application of 10 mM GABA under control conditions (thick line) and in the presence of 1 μM **1** (thin line). (D and E) Statistics of the effect on the desensitization time constant (τ_{Des}) and on the steady-state to peak parameter, respectively. (F) Typical examples of deactivation phases of currents elicited by application of 10 mM GABA in control conditions (thick line) and in the presence of 1 μM **1** (thin line). Currents were normalized to the current amplitude recorded immediately before agonist removal. (G) Statistics of the effect on the mean deactivation time constant (τ_{mean}). Insets above current traces indicate time of agonist applications. The asterisk indicates a significant difference ($p < 0.05$).

It is known that current responses to exogenous agonist applications recorded from neurons represent the activity of a variety of $GABA_A$ receptors with different kinetic and pharmacological properties.[34,35] Since hippocampal neurons in the long-term culture[36,37] express predominantly the $\alpha_1\beta_2\gamma_2$ $GABA_A$ receptors, it seemed interesting to additionally examine the effect of falcarindiol (**1**) on these receptors under experimental conditions similar to those used for neurons. For this purpose, recombinant $\alpha_1\beta_2\gamma_2$ $GABA_A$ receptors expressed in HEK293 cells were employed. The amplitude of the whole-cell currents, elicited by 3 μM GABA, was increased by a 1 μM concentration of **1** (relative amplitude 1.39 ± 0.15, $n = 5$, $p < 0.05$), and this effect was accompanied by increased fading (from 0.09 ± 0.04 in control conditions to 0.32 ± 0.07, $n = 5$, in the presence of 1 μM of 1, $p < 0.05$). The impact of falcarindiol (**1**) on $\alpha_1\beta_2\gamma_2$ receptors was tested additionally on current responses to saturating (10 mM) GABA. Similar to what is observed for neurons, **1** significantly decreased the amplitude of these currents (relative amplitude 0.59 ± 0.07, $n = 7$, $p < 0.05$) and reduced the ss/peak value (relative value 0.49 ± 0.09, $n = 5$, $p < 0.05$). However, the desensitization time constant τ_{des} was not affected by **1** (data not shown). Thus, the effects of **1** on $\alpha_1\beta_2\gamma_2$ $GABA_A$ receptors were similar qualitatively to those observed in neurons. In particular, the opposite effect of **1** on amplitudes of currents elicited by low (3 μM) or high (10 mM) GABA concentrations were reproduced in the recombinant model.

These observations show that the profile of action of **1** is opposite that of oenanthotoxin (**2**),[16,17] a compound that potently inhibits GABAergic currents.[16,17] From a mechanistic standpoint, the action of **1** on $GABA_A$ receptors is complex, potentially comprising both binding and gating properties. The effects on the amplitude of mIPSC and of the currents evoked by low [GABA] suggest an upregulation of agonist binding. Despite its relatively high peak concentration, synaptically released GABA is commonly believed to be nonsaturating,[23,27,28] due to a particularly short presence within the synaptic cleft. This very short synaptic GABA transient renders the synaptic signals strongly susceptible to modulation by compounds affecting the agonist binding site.[23,29−32] However, upregulation of binding would be expected to accelerate the rate of onset for current responses to nonsaturating [GABA],

dx.doi.org/10.1021/np2008522 | J. Nat. Prod. 2012, 75, 610−616

an effect that has not been observed for the effect of **1** on responses evoked by either 3 or 30 μM GABA. Moreover, for responses to 30 μM GABA (i.e., close to the EC_{50} value) no significant effect was observed on amplitude, while, at saturating GABA concentrations, a significant reduction was observed in the presence of **1**. This pattern of modulation of currents evoked by saturating or subsaturating [GABA] may reflect an upregulation of desensitization. Indeed, compounds upregulating desensitization processes, such as bases and benzodiazepines, or membrane depolarization tends to reduce the amplitude of current responses evoked by high [GABA].[23,30,33] Importantly, benzodiazepines that increase the amplitude of mIPSCs and of currents evoked by low [GABA] have an opposite effect on responses elicited by [GABA] close to EC_{50} or higher,[23] as observed for **1**. In a further analogy to the benzodiazepines, **1** could also prolong the decaying phase of mIPSCs (Figure 2), and it does not seem unreasonable to assume that this compound affects GABAergic currents by upregulating both binding and desensitization of $GABA_A$ receptors. However, while the impact of **1** on desensitization is solidly supported by results obtained for saturating [GABA] applications (Figure 5C–G), the lack of a clear effect of **1** on the onset of currents evoked by nonsaturating [GABA] complicates the interpretation of the effect on the agonist binding. Finally, as revealed by the analysis of current responses to saturating [GABA], **1** slowed the onset of these currents (Figure 5), indicating that it may downregulate the transition rate between bound closed and bound open states. It is thus possible that the acceleration of the current onset related to upregulation of the binding rate is counterbalanced by an opposite action on the conformational transitions of the channel macromolecule in the bound states. It needs to be considered additionally that current responses to exogenous agonist applications recorded from neurons represent the activity of a mixture of $GABA_A$ receptors with different kinetic and pharmacological properties.[34,35] It is thus possible that **1** affects some kinetic features of recorded currents; for example, the rise time could be obscured by a differential effect of this compound on different $GABA_A$ receptor subtypes. However, experiments on recombinant $\alpha1\beta2\gamma2$ receptors have demonstrated that major effects of falcarindiol (**1**) on neuronal GABAergic currents (impact on the amplitude at low or high [GABA] and on the extent of desensitization) were reproduced qualitatively. It should be additionally borne in mind that **1** could exert both a pre- and postsynaptic effect, as suggested by the trend of reduction of mIPSC frequency. Although the present data are insufficient to precisely distinguish between the pre- and postsynaptic component of the action of **1**, a combination of upregulation of binding and desensitization seems most compatible with the observations made.

In principle, the complexity of the action of **1** on $GABA_A$ receptors makes it difficult to assess its overall effect. However, since saturating [GABA] are unlikely to occur under physiological conditions, the enhancement of GABAergic inhibition by **1** should predominate under physiological conditions. The increased GABAergic drive would manifest in an upregulation of both phasic (synaptic currents) and tonic (long lasting and elicited by low [GABA]) inhibition.[35] Moreover, it is possible that effects of **1** on ionotropic GABAergic inhibitory drive could work synergistically with the inhibition of GABA-transaminase,[18] a process that would lead to an increase in the overall GABA level.

In conclusion, we have shown that **1** at micromolar concentrations potently modulates both the amplitude and time course of GABAergic currents. While synaptic currents are upregulated, the effect on responses to exogenous GABA depends on the agonist concentration. Overall, the upregulation of GABAergic drive may have potentially a therapeutic anticonvulsant or tranquilizing effect, moderating the convulsant properties of oenanthotoxin (**2**) and related neurotoxins. Owing to an overall weaker effect of **1** on $GABA_A$ receptors compared to **2**, this action is probably significant only when the concentration of falcarindiol is much higher than that of the convulsant neurotoxins, as in *O. fistulosa*. In *O. crocata*, where an opposite situation is evident, the effect is, most likely, negligible. Interestingly, no cases of poisoning from *O. fistulosa* have been reported to date, despite the wide distribution of this plant in Europe and the presence of oenanthotoxin (**2**) and related compounds.[16]

The isolation of compounds with opposite bioactivity from the same plant source is not unprecedented, both within food plants and within medicinal and poisonous plants. Thus, the sweetness-modifying phenolic cynarin occurs in artichoke leaves with the bitter sesquiterpene lactone cynaropicrin,[38] while *Cannabis sativa* contains both the cannabinoid agonist Δ^9-THC and its lower homologue and antagonist Δ^9-THCV,[39] with the antivitamin ginkgotoxin and its antidote bilobalide cooccurring in ginkgo leaves.[40] Given the diversity of the phytochemical profile of plants and our basic lack of knowledge on their ecological roles, these observations, albeit rare, are not surprising.

EXPERIMENTAL SECTION

General Experimental Procedures. ^{1}H (300 MHz) and ^{13}C (75 MHz) NMR spectra were measured on a JEOL Eclipse 300 spectrometer. Low- and high-resolution ESIMS were obtained on a LTQ OrbitrapXL (Thermo Scientific) mass spectrometer. Silica gel 60 (70–230 mesh) was used for gravity column chromatography.

Plant Material. Falcarindiol (**1**) was isolated from the underground parts of *Conium maculatum* L., a plant more easily available than water hemlock in Northern Italy. *C. maculatum* was collected on the outskirts of Ceva (CN, Italy) in July 2010 and was identified by Dr. Edoardo Luciano. A voucher specimen (DL008) is kept at the Novara Laboratory.

Extraction and Isolation. Dried and powdered roots (430 g) were extracted with acetone (2 × 2 L), affording, after evaporation, 11 g of a brownish oil, which was fractionated directly by gravity column chromatography (200 g of silica gel, petroleum ether–EtOAc, 95:5, as eluant, fractions of 10 mL). Fractions 33–38 afforded 410 mg of falcarindiol (0.10%) as a colorless oil, indentified by comparison of the NMR (^{1}H and ^{13}C NMR) and MS data with those reported in the literature.[19]

Storage of Falcarindiol (1). Falcarindiol was stored in frozen DMSO or frozen benzene to avoid rapid degradation. These frozen solutions were stable for at least six months at 4 °C (^{1}H NMR control) and did not developed a reddish color that signifies the degradation of polyacetylenes. For the electrophysiological experiments, stock solutions of **1** were prepared by diluting the DMSO mother solution to a final concentration of 10 mM. In all experiments, the solvent (DMSO) was present at the same concentration in the controls and in the solutions of **1**.

Neuronal Primary Cell Culture. The neuronal culture was prepared from P2–P4 Wistar rat pups. Animals were killed by decapitation, and efforts were made to minimize the number of sacrificed animals. This procedure is in agreement with the Polish Animal Welfare Act and was approved by the local Ethical Committee (decision number 18/2010). Brains were removed and hippocampi were dissected on ice in dissociation medium (DM) (in mM: 81.8 Na_2SO_4; 30 K_2SO_4; 5.8 $MgCl_2$; 0.25 $CaCl_2$; 1 HEPES; 20 glucose; 1 kynureic acid; 0.001% phenol red). Hippocampi were cut manually into pieces with a blade and incubated twice for 15 min at 37 °C in papain solution (100 U papain in DM, Worthington, NY, USA). The

dx.doi.org/10.1021/np2008522 | J. Nat. Prod. 2012, 75, 610–616

tissue was then rinsed three times in DM and three times in plating medium (MEM, 10% FBS, 1% penicillin/streptomycin). Tissue was mechanically dissociated using Pasteur pipets with decreasing tip diameter in OptiMEM (Invitrogen) and centrifuged at 1000 rpm for 10 min at room temperature. Neurons were plated in the plating medium on 1 mg/mL poly-L-lysine (Sigma) and 2.5 μg/mL laminin (Roche) coated coverslips at a density of ~400−500 cells/mm^2. Three hours later, plating medium was exchanged for growth medium (Neurobasal-A without phenol red, 2% B-27 supplement, 1% penicillin/streptomycin, 0.5 mM glutamine, 12.5 μM glutamate, 25 μM β-mercaptoethanol). Neurons were cultured at 37 °C in a 5% CO_2 incubator. Experiments were performed on neurons cultured for 9−16 days.

Expression of Recombinant Receptors. Human embryonic kidney cells (HEK 293) were cultured in Dulbecco's modified Eagle medium (Life Technologies, Warsaw, Poland) with 10% fetal bovine serum (Life Technologies, Warsaw, Poland) at 37 °C in a humidified incubator with 5% CO_2. For cell transfections, a calcium phosphate precipitation method was used.[41] Cells were co-transfected with rat $GABA_A$R subunits: α_1, β_2, γ_2 introduced in pCMV separate vector and with pCMVCD4 plasmid encoding human CD4 receptor. To identify cells expressing recombinant $GABA_A$ receptors, CD4 binding magnetic beads were used (Dynabeads M-450 CD4, Dynal Biotech ASA, Oslo, Norway). Recordings were performed 48−72 h after transfection.

Electrophysiological Recordings. Currents were recorded in the outside-out or in the whole-cell configuration of the patch-clamp technique using an Axopatch 200B amplifier (Molecular Devices Corporation, Sunnyvale, CA, USA) at −40 mV. The pipet solution contained (in mM) 137 CsCl, 1 $CaCl_2$, 2 $MgCl_2$, 11 BAPTA (tetracesium salt), 2 ATP, and 10 HEPES, pH 7.2 with CsOH. The external solution contained (in mM) 137 NaCl, 5 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 20 glucose, and 10 HEPES, pH 7.2 with NaOH. Miniature IPSCs were recorded in the whole-cell configuration in the gap-free mode (mIPSC recordings), and these signals were low-pass filtered with a Butterworth filter at 3 kHz and sampled at 10 kHz using the analog-to-digital converter Digidata 1322A (Molecular Devices Corporation). Analysis of current responses to rapid GABA applications (especially at high GABA concentrations) required a higher time resolution, and these signals were filtered at 10 kHz and sampled at 50−100 kHz. For acquisition and signal analysis pClamp 10.1 software was used (Molecular Devices Corporation). Miniature GABAergic IPSCs were recorded in the presence of 1 μM tetrodotoxin and kynurenic acid (1 mM) at a holding voltage of −70 mV. Solutions were supplied by gravitation through a glass tube (i.d. 1 mm) directly onto the recording area with a flux of ca. 2 mL/min. This system allowed a reliable control of the surrounding of neurons from which the recordings were made. Cells exhibiting a mIPSC amplitude run-down larger than 20% during the entire recording period were excluded from the statistics. For the whole-cell recordings, pipet resistance (electrodes filled with the internal solution) was 2−3.5 MΩ. Access resistance was monitored and compensated at 30−80%. Cells for which access resistance was larger than 15 MΩ (after compensation) were rejected. Current responses to 3 μM GABA were barely detectable in the excised patch configuration and for this reason were recorded in the whole-cell mode using a multibarrel system (RSC-200, Bio-Logic, Grenoble, France, exchange time ca. 15−50 ms).

Rapid Application Experiments. GABA was applied to excised patches using the rapid perfusion system based on a piezoelectric-driven theta-glass application pipet.[20] The theta-glass tubing was from Hilgenberg (Malsfeld, Germany), and piezoelectric translator was from Physik Instrumente (preloaded HVPZT translator 80 μm, Waldbronn, Germany). The application speed was assessed by open tip recordings of the liquid junction potentials, which revealed 10−90% exchange within 60−120 μs. In experiments in which the effect of falcarindiol (**1**) was addressed, the compound was present at the same concentration in solutions supplied by both channels (wash and GABA-containing) of the theta-glass capillary. Before applying the agonist (in the presence or absence of **1**), the patch was exposed to the washing solution for at least 2 min. In some recordings, a brief pretreatment with **1** (ca. 1 min) was applied, and the effect of this compound was very similar to that observed after 2 min, implying that pretreatment time within the range of minutes was sufficient. In order to avoid excessive accumulation of **1** in the recording chamber (30 mm Petri dish), in all the electrophysiological experiments, the cells were superfused with fresh Ringer solution at the rate of 2 mL/min.

Data Analysis. The onset kinetics of current responses was quantified as 10% to 90% rise time. The kinetics of current deactivation (current time course after agonist removal) or the decaying phase of mIPSCs was fitted with a sum of two exponents: $y(t) = A_1 \exp(-t/\tau_{fast}) + A_2 \exp(-t/\tau_{slow})$, where A_1 and A_2 are the amplitudes of respective components, while τ_{fast} and τ_{slow} are the time constants. For normalized currents, $A_1 + A_2 = 1$. The mean deactivation or decay time constant was calculated as $\tau_{mean} = A_1\tau_{fast} + A_2\tau_{slow}$. The desensitization kinetics were described by a sum of one exponential function and a constant factor representing the steady-state current ($y(t) = A \exp(-t/\tau_{Des}) + C$). The effect of falcarindiol (**1**) on mIPSCs or on current responses was assessed from the comparison between control and test recordings obtained from the same neuron (or excised patch), and therefore the results are presented as relative values normalized to the respective controls. Data are expressed as mean ± SEM, and paired Student's *t*-test was used for data comparison; differences were considered significant when $p < 0.05$. All experiments were performed at room temperature (22−24 °C).

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Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

J.W.M. and P.W. were supported by the Foundation for Polish Science award "Mistrz" (contract no. 7/2008).

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