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A cembranoid from tobacco prevents the expression of nicotine-induced withdrawal behavior in planarian worms

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Abstract

Using an adaptation of published behavioral protocols, we determined that acute exposure to the cholinergic compounds nicotine and carbamylcholine decreased planarian motility in a concentration-dependent manner. A tobacco cembranoid (1S,2E,4R,6R,7E,11E)-cembra-2,7,11-triene-4,6-diol (4R-cembranoid), also decreased planarian motility. Experiments in the presence of 1 μ M 4R-cembranoid did increase the IC₅₀ for nicotine- but not carbamylcholine-induced decrease in planarian motility. When planarians were exposed for 24 h to either nicotine or carbamylcholine at concentrations near their respective IC₅₀ values and then transferred to plain media, nicotine-exposed, but not carbamylcholine- or cembranoid-exposed worms displayed withdrawal-like distress behaviors. In experiments where planarians were pre-exposed to 100 μ M nicotine for 24 h in the presence of 1 μ M 4R-cembranoid, the withdrawal-like effects were significantly reduced. These results indicate that the 4R-cembranoid might have valuable applications for tobacco abuse research. This experimental approach using planarians is useful for the initial screening of compounds relevant to drug abuse and dependence.

Keywords

Planaria; Nicotine; Cembranoid; Carbamylcholine

1. Introduction

During the course of evolution, many types of organisms have developed substances used for predation or defense. These substances include small organic toxins (Brenner et al., 2003; Mebs, 2001). This is an aspect of the so-called “evolutionary arms race” (Dawkins and Krebs, 1979), where these toxins are structurally optimized for their interaction with molecular targets, usually macromolecules such as receptors, transporters, and enzymes. These macromolecules, conversely, can evolve into toxin-resistant forms (Geffeney et al., 2002, 2005; Hanifin et al., 2008). One of the best-known examples of defensive molecules is nicotine (Soloway, 1976). Nicotine, found primarily in tobacco plants, plays an important role in the resistance of these plants against arthropods (Steppuhn et al., 2004). Nicotine’s main molecular targets are

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Statement of potential conflict of interest

V.A.E. and O.R.P. are co-discoverers on two cembranoid-related patents: US 6,204,289.

B1 — “Cembranoid Inhibitors of Nicotinic Acetylcholine Receptors” and US 6,489,357 B1 — “Tobacco Cembranoids Block the Expression of Behavioral Sensitization to Nicotine and Inhibit Neuronal Acetylcholine Receptors”.

nicotinic acetylcholine receptors (nAChR), which are the best-known members of the ligand-gated ion channel superfamily (Dani and Bertrand, 2007).

The cembranoids are cyclic diterpenoids which display a 14-carbon cembrane ring (Hann et al., 1998; Wahlberg and Eklund, 1992; Fig. 1). Marine invertebrates are the richest cembranoid source, with more than one hundred described examples (Wahlberg and Eklund, 1992).

Cembranoids are present in a variety of organisms, including conifers and tobacco (Dauben et al., 1965; Wahlberg and Eklund, 1992), ants and termites (Edwards and Chambers, 1984; Prestwich, 1984) and alligators (Mattern et al., 1997).

Few biological effects of the cembranoids have been reported. Some cembranoids act as antitumor compounds (Rodríguez and Martínez, 1993); others inhibit cyclic AMP phosphodiesterase (Roengsumran et al., 2002), and prostaglandin synthesis (Olsson et al., 1993). Many cembranoids are ligands of muscle-type and neuronal-type nicotinic acetylcholine receptors (Eaton et al., 2004; Eterović et al., 1993; Ferchmin et al., 2001; Hann et al., 1998; Pagán et al., 2001). A specific cembranoid, eupalmerin acetate, potentiates the function of another type of ligand-gated ion channel, the GABA-A receptor (Li et al., 2008).

Planarian worms (*Platyhelminthes*, *Turbellaria*, *Tricladida*; Ruppert et al. (2004)) are used widely in developmental biology and regeneration research (Newmark and Sanchez-Alvarado, 2002). This group of organisms shows promise in neuropharmacology research, as they have a well-developed nervous system that displays a rudimentary bilateral brain containing every neurotransmitter system found in vertebrates (Cebrià et al., 2002; Okamoto et al., 2005; Sarnat and Netsky, 1985, 2002; Villar and Schaeffer, 1993). In contrast to most invertebrates, the planarian nervous system shares many structural similarities with vertebrate nervous systems, including multipolar neurons and dendritic spines (Sarnat and Netsky, 1985, 2002). Planarians are being rediscovered as a very useful animal model to study abused drugs. These worms display specific responses to psychoactive substances, including behaviors resembling withdrawal syndromes in response to compounds such as cocaine, cannabinoids, amphetamines and opiates (Buttarelli et al., 2002; Kusayama and Watanabe, 2000; Pagán et al., 2008; Palladini et al., 1996; Raffa and Desai, 2005; Raffa and Martley, 2005; Raffa et al., 2006; Raffa and Valdez, 2001; Rawls et al., 2006; Rowlands and Pagán, 2008). In this work, we report the effect of a tobacco cembranoid on nicotine- or carbamylcholine-induced behavior in planarians.

2. Materials and methods

2.1. Materials

Planarian worms (*Dugesia dorotocephala*) were obtained through Ward's (Rochester, NY). General laboratory materials were purchased from Fisher Scientific (Suwanee, GA). The compounds used in this work are shown in Fig. 1. Nicotine ditartrate was purchased from Sigma-Aldrich (St. Louis, MO) and carbamylcholine hydrochloride was purchased from Tocris (Ellisville, MO). The tobacco cembranoid (4R-cebranoid) was purchased from Dr. Khalid A. El Sayed (University of Louisiana at Monroe).

2.2. General methods

All the experiments were done at room temperature using artificial pond water (APW; NaCl, 6 mM; NaHCO₃, 0.1 mM; CaCl₂, 0.6 mM; pH 6.9), containing 0.1% dimethylsulfoxide (DMSO) as a solubility-aiding agent. At this concentration, DMSO does not show any evident behavioral or toxic effects in planaria (Pagán et al., 2006). Planarians were transferred to APW upon receipt and allowed to acclimate to the laboratory conditions for at least 24 h before the experiments. The worms were used within 2 weeks of arrival, and the APW was changed daily.

All graphs and statistical procedures were done using the GraphPad 5/Instat software packages (GraphPad Software, San Diego, CA). Each of the described experiments was performed by two or more independent observers.

2.3. Motility measurements

To measure planarian motility, we used a modification of a published behavioral protocol (Raffa et al., 2001), as modified in Pagán et al. (2006, 2008). This is a simple, yet useful assay that can be used to study the effects of experimental compounds on planarian locomotor behavior. A worm was transferred to a previously APW-rinsed 6 cm polystyrene dish and set on a grid (1 cm² squares, Fig. 2A), followed by adding 5 ml of APW/0.1% DMSO in the absence (control) or in the presence of the experimental compounds. Unless otherwise indicated, motility measurements were recorded after an incubation period of 15 min; it has been reported that nicotine-induced behaviors in planarians display a latency of 10–15 min (Buttarelli et al., 2000). Planarian motility was measured by counting how many times the worm crossed a square per min, over a period of 8 min. Each worm was used only once. The data was graphed as cumulative crosses *vs.* time, and fit to a linear equation (Fig. 2B). In experiments where the worms were exposed to increasing concentrations of the experimental compounds, the slopes obtained by the linear equation fit were normalized to the control slopes, plotted as the fraction of control *vs.* the experimental compound concentration and fit to an empirical Hill-type equation (Eq. (1)):

$$F = IC_{50}^n / (IC_{50}^n + [\text{compound}]^n) \quad (1)$$

where F is the fraction of control, $[\text{compound}]$ is the experimental compound concentration in μM , IC_{50} is the compound concentration that decreased planarian motility by 50% and n is the Hill coefficient.

The commercially available nicotine and carbamylcholine used in this study were in the form of ditartrate and hydrochloride salts respectively. To determine if these ions affected planarian motility on their own, they were tested at the concentrations that corresponded to the IC_{50} of nicotine or CCh in the absence and in the presence of 0.1% DMSO.

2.4. Withdrawal-like behavior measurements

The procedure used to observe and measure withdrawal-like behaviors was adapted from Raffa and Desai (2005) as modified in Rowlands and Pagán (2008). Briefly, planarians were placed into separate 1.5 ml microcentrifuge tubes containing nicotine (100 μM) or carbamylcholine (CCh, 150 μM). Two sets of control worms were also observed, using either planarians pre-exposed to plain APW or to APW/0.1% DMSO. After an overnight incubation period (22–27 h), the worms were individually transferred to glass dishes containing APW in the absence of any experimental compounds and observed with a stereomicroscope during three time periods: 0–5, 30–35 and 60–65 min. The withdrawal-like behaviors observed were based on the work described in Raffa and Desai (2005). These behaviors were named “HeadBop” (“nodding”-like movements while gliding at the bottom of the dish), “HeadSwing” (head rotation in the absence of gliding while the tail is fixed to the bottom of the dish), “TailTwist” (bending of the tail tip) and “Corkscrew” (spiral rotation while floating/swimming). Two other described movements: “Squirming” (shaking) and “Clinging” (scrunching), tended to appear concurrently, therefore, we decided to count these movements together. The data was graphed as the number of events as a function of time.

3. Results

3.1. Effects of the tested compounds on planarian motility

Fig. 2B shows the cembranoid-induced motility decrease in planarians. Similar plots were obtained for nicotine and CCh (data not shown). Based on this data, concentration–response curves for motility decrease induced by the cembranoid, nicotine and CCh were constructed as described in the “Materials and methods” section (Fig. 3).

To determine the effect of tartaric acid and hydrochloric acid on planarian motility, they were tested at the concentrations that corresponded to the IC_{50} of nicotine or CCh. Since tartaric acid is associated with nicotine in a 2:1 ratio and CCh is associated with HCl at a 1:1 ratio, tartaric acid and HCl were tested at a concentration of 200 and 100 μ M respectively, in the absence and in the presence of 0.1% DMSO. None of these compounds affected planarian motility at the tested concentrations (data not shown).

3.2. The presence of tobacco cembranoid significantly decreased the motility inhibition of nicotine, but not carbamylcholine

Fig. 4 shows the effect of 1 μ M cembranoid on the concentration–response motility curves of nicotine or carbamylcholine, as indicated. The tobacco cembranoid induced a significant increase in the nicotine, but not the CCh IC_{50} .

3.3. Nicotine, but not carbamylcholine nor the 4R-cembranoid induces withdrawal-like behaviors

For the first four behaviors (“HeadBop”, “HeadSwing”, “TailTwist” and “Corkscrew”), data were fit to a linear equation and the statistical difference between the slopes obtained for the controls and experimental groups was calculated using the *F*-test (Fig. 5). Neither the APW controls nor the APW/0.1% DMSO controls displayed any stereotypic behaviors upon transfer to APW. Nicotine-exposed, but not carbamylcholine-exposed, showed these withdrawal-like behaviors. For nicotine, the number of observed instances of these behaviors decreased in a linear fashion down to baseline levels over time (Fig. 5). When the planarians were incubated with 1 μ M 4R-cembranoid and then transferred to APW, the worms did not display these withdrawal-like behaviors (data not shown).

A specific behavior, “scrunching/squirming”, did not follow a linear pattern (Fig. 6). In the first measured time period (0–5 min), both nicotine- and CCh-exposed worms displayed this behavior, with an average number of events close to two (Fig. 6). After this time period, the carbamylcholine-exposed worms returned to baseline levels. At the second measured time (30–35 min) nicotine induced a significantly higher number of events when compared to CCh. Finally, at the last measured time (60–65 min), the worms exposed to both compounds were at baseline level.

3.4. The 4R-cembranoid tobacco cembranoid prevents the nicotine-induced, withdrawal-like behaviors

Fig. 7 shows a series of experiments to test the effect of 1 μ M 4R-cembranoid on nicotine-induced withdrawal behaviors. The presence of the cembranoid significantly decreased the induction of these behaviors by nicotine. As in the previous set of experiments, the “scrunching/squirming” behavior induced by nicotine displayed a non-linear nature, being relatively low at 5 min, highest at 30 min and back to baseline at 60 min (Fig. 8). In this case, the cembranoid also abolished these responses.

4. Discussion

To our knowledge, this is the first report about the effect of cembranoids on planarian worms. Previous studies using cholinergic agonists such as acetylcholine and nicotine have demonstrated that these compounds induce behavioral responses in planarians (Best and Morita, 1991; Buttarelli et al., 2000). Acetylcholine is a ubiquitous molecule in biological systems; its presence in vertebrates is well established, but it is also present in microorganisms and plants (Wessler et al., 1999). Acetylcholine receptors are traditionally classified as nicotinic (ionotropic) or muscarinic (metabotropic), based on their specific activation by the naturally-occurring products, nicotine and muscarine, respectively (Daly, 2005). A series of studies have provided evidence for the presence of cholinergic receptors in planarians using microarrays and expression sequence tags (Cebrià et al., 2002; Mineta et al., 2003; Nakazawa et al., 2003). Additionally, a planarian genome project, using the planarian *Schmidtea mediterranea*, is underway (Robb et al., 2008). This database is posted at <http://smedgd.neuro.utah.edu/>. Using this resource, we have found several candidates of nicotinic and muscarinic cholinergic receptors.

Carbamylcholine is a cholinesterase-resistant analog of acetylcholine (Kester et al., 2007). The use of carbamylcholine allowed us to avoid using cholinesterase inhibitors, which can induce behavioral changes in planarians by themselves (Buttarelli et al., 2000; Lenicque and Feral, 1976). Stimulation of the planarian cholinergic system by the cholinesterase inhibitor physostigmine (eserine; Buttarelli et al., 2000; Lenicque and Feral, 1976) induced hypokinesia. We have also found protein candidates consistent with cholinesterases using the aforementioned *S. mediterranea* database (Robb et al., 2008).

In previous studies, the cholinergic agonist nicotine at a concentration of about 300 μM induced hypokinesia, in agreement with our work (Buttarelli et al., 2000). Additionally, nicotine has been used as an anesthetic agent for planaria (Pedersen, 1958). In contrast, known inhibitors of nicotinic–cholinergic transmission such as gallamine, tubocurare and the depolarizing neuromuscular blocking agent succinylcholine, failed to induce any apparent behavioral effects in planarians (Best and Morita, 1991). Interestingly, the inhibition of muscarinic cholinergic responses by mM doses of atropine caused hyperkinesia (Buttarelli et al., 2000). Taken together, these results suggest that stimulation of the nicotinic–cholinergic system reduce planarian motility, while the suppression of muscarinic cholinergic activity increases motility. The hypokinesia induced by 20 μM 4R-cembranoid could be explained by cholinergic activation, since under certain conditions 4R-cembranoid acts as a positive modulator of nicotinic receptors (Ulrich et al., 2008).

Our observations that nicotine and CCh induce hypokinesia in planarians agree with the literature reports discussed above. This is consistent with our results showing nicotine-induced motility decrease. However, it is to be noted that the route of drug administration in rats and planarians is rather different, namely the injection and transport through a circulatory system in rats vs. the addition of the compounds to the water in planarians (which lack a circulatory system). Planarian membranes are permeable to low molecular weight compounds (Palladini et al., 1983), which should allow nicotine and cembranoids to reach their molecular targets easily. Cembranoids can modulate a CNS-regulated behavior (motility) in rats, indicating that these compounds can cross the blood–brain barrier (Ferchmin et al., 2001). This is supported by experiments showing that rats injected intravenously with [^3H]-4R-cembranoid, displayed detectable radioactivity levels in their brains (Eterović et al., unpublished results). Further studies using rodent models are needed.

Our results show that 1 μM 4R-cembranoid decreased the apparent potency of nicotine but not carbamylcholine to induce motility decrease in our experimental system (Fig. 4). Our results

also parallel a series of studies done using *Xenopus* oocytes expressing a specific nAChR subtype from rats (Eaton et al., 2004), where the authors found that tobacco cembranoids inhibit nicotine-, but not acetylcholine-induced currents. That said, it is important to point out that the carbamylcholine curves display a much larger experimental error than the nicotine curves; the non-significant *p*-value obtained may be an artifact of this data spread.

The most significant result in this work is that the 4R tobacco cembranoid prevents the expression of nicotine-induced withdrawal-like behaviors in planarians (Figs. 7 and 8).

One of these nicotine-induced withdrawal-like behaviors, “Scrunching/Squirming”, did not follow a linear decrease as a function of time (Figs. 6 and 8). Two possible explanations for the transient increase in movements at *t*=30 min are, that upon transfer to nicotine-free APW nicotine would diffuse out of the worm differentially, meaning that whatever nicotine accumulated in nervous tissue would remain there longer than in other tissues, triggering the peak responses after 30 min. Alternatively, this may be an effect induced by nicotine metabolites, which are known to be bioactive by themselves in vertebrates (Crooks and Dwoskin, 1997). More studies are clearly needed, since the pharmacokinetics and metabolism of nicotine in planarians is currently unknown.

Our results are consistent with other studies showing that 4R-cembranoid blocks behavioral sensitization to nicotine in rats (Ferchmin et al., 2001). Additionally, transient hypoactivity is also observed in rats exposed for the first time to nicotine (Stolerman et al., 1995; Ferchmin et al., 2001). Taken together, Eaton et al. (2004), Ferchmin et al. (2001) and our results provide evidence for the evolutionary conservation cholinergic responses to specific ligands and other drugs, and demonstrate the potential of cembranoids as compounds with possible applications in tobacco abuse research.

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Fig. 1.

Compounds used in this work. 4R cembranoid (1S,2E,4R,6R,7E,11E-cembra-2,7,11-triene-4,6-diol); (-) nicotine (3-(1-methylpyrrolidin-2-yl)pyridine) and carbamylcholine (2-carbamoyloxyethyl-trimethylammonium).

Fig. 2.

A. Motility assay experimental setup (square size=1 cm², see text). B. Effect of 4R-cembranoid in planarian motility. The data points were fit to a linear equation to generate the plots. Each line represents the average of experiments done with 4–10 worms. Similar plots were obtained for nicotine and carbamylcholine (data not shown). Error bars represent the standard error of the mean.

Fig. 3. Dose–response curves showing the effect of the experimental compounds on planarian motility, based on linear plots like Fig. 2B. Each data point represents the average of 4–10 worms. The lines and the IC_{50} values ($\mu\text{M} \pm \text{SEM}$) were generated by fitting the data to Eq. (1). Error bars represent the standard error of the mean.

Fig. 4.

The presence of 1 μM 4R-cembranoid significantly increases the IC_{50} of nicotine, but not of carbamylcholine to induce motility decrease in planarians. A. Nicotine. B. Carbamylcholine. The lines and the IC_{50} s ($\mu\text{M} \pm \text{SEM}$) were generated using Eq. (1). The p -values were obtained through an F -test. Each data point represents the average of 3–10 worms. Error bars represent the standard error of the mean.

Fig. 5. Nicotine (100 μM, closed symbols), but not carbamylcholine (150 μM, open symbols), induce withdrawal-like behaviors in planarians, as indicated (see methods). Each symbol is the average of three experiments. Error bars are the standard error of the mean. *p*-values (*F*-test) are indicated in the figure.

Fig. 6.

The “Scrunching/Squirming” behavior does not follow a linear pattern (see text). Nicotine (100 μM, closed symbols), carbamylcholine (150 μM, open symbols). The two data sets are significantly different from each other ($p < 0.05$; two-way ANOVA). Each symbol is the average of three experiments. Error bars are the standard error of the mean.

Fig. 7.

Nicotine (100 μM, closed symbols), but not nicotine+4R-cembranoid (100 and 1 μM respectively, open symbols), induce withdrawal-like behaviors in planarians, as indicated (see methods). Each symbol is the average of three experiments. Error bars are the standard error of the mean. *p*-values (*F*-test) are indicated in the figure.

Fig. 8. Nicotine (100 μ M, closed symbols), but not nicotine+4R-cembranoid (100 and 1 μ M respectively, open symbols), induce the “Scrunching/Squirming” behavior (see methods). The two data sets are significantly different from each other ($p < 0.001$; two-way ANOVA). Each symbol is the average of three experiments. Error bars are the standard error of the mean.