Marine Alkaloids (–)-Pictamine and (–)-Lepadine B Block Neuronal Nicotinic Acetylcholine Receptors

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Received October 12, 2004; accepted January 18, 2005; published online January 21, 2005

Ascidians (sea squirts) contain a wealth of alkaloids, but their influence over neuronal nicotinic acetylcholine receptors (nAChRs) has not been evaluated. In this study, we examined the effects of two synthetic compounds, (–)-pictamine, a quinolizidine alkaloid from *Clavelina picta*, and (–)-lepadine B, a decahydroquinoline alkaloid from *Clavelina lepadiformis*, on major types of neuronal nicotinic receptors ($\alpha 4\beta 2$ and $\alpha 7$) expressed in *Xenopus* oocytes. We found that these alkaloids are potent blockers at these receptors: acetylcholine-elicited currents through $\alpha 4\beta 2$ and $\alpha 7$ receptors were blocked by (–)-pictamine with IC_{50} values of 1.5 μM and 1.3 μM , respectively, and by (–)-lepadine B with IC_{50} values of 0.9 μM and 0.7 μM , respectively. Interestingly, no recovery was observed after the removal of (–)-pictamine in oocytes expressing $\alpha 4\beta 2$ receptors, whereas the inhibited $\alpha 7$ currents quickly recovered after the removal of (–)-pictamine. Since there are few compounds that elicit irreversible blocks of $\alpha 4\beta 2$ receptors, (–)-pictamine will be a novel, valuable tool to remove the $\alpha 4\beta 2$ -nAChR action from neuronal activities mediated by these two major types of nAChRs.

Key words nicotinic receptor; ascidians alkaloid; pictamine; *Xenopus* oocyte

Neuronal nicotinic acetylcholine receptors (nAChRs) are ligand-gated ion channels that mediate and/or modulate synaptic transmission in the nervous system.^{1–3} In mammalian brain, neuronal nAChR subunits are identified as $\alpha 2$ – $\alpha 7$, $\alpha 9$, $\alpha 10$, and $\beta 2$ – $\beta 4$.^{4,5} Ligand-binding studies indicate that the most abundant native nAChRs in the central nervous system are $\alpha 4\beta 2$ heteromeric receptors and $\alpha 7$ homomeric receptors.⁶ Although nAChRs have been implicated in several neurological disorders including nicotine addiction, epilepsy, Parkinson's disease and Alzheimer's disease,^{7–9} nicotinic ligands and nicotinic-based therapies have only slowly and minimally become available.^{10–13}

A variety of alkaloids were isolated from amphibian skins, including indolizidines, quinolizidines, decahydroquinolines and piperidines, some of which have been identified as non-competitive blockers of nAChRs at neuromuscular junction and in ganglionic PC12 cells¹⁴ and recombinant neuronal nAChRs expressed in *Xenopus* oocytes.¹⁵ To date, a wide range of toxins, including different types of decahydroquinolines and quinolizidines, have been isolated from ascidians (tunicates, subphylum Urochordata, class Ascidiacea) commonly known as sea squirts.¹⁶ Nevertheless, none of the ascidian alkaloids has been tested in pharmacological research to develop therapeutically useful nicotinic drugs. Previously, we have redefined the absolute configuration of two ascidian alkaloids by enantioselective total synthesis: (–)-pictamine, a quinolizidine alkaloid from *Clavelina picta*, and (–)-lepadine B, a decahydroquinoline alkaloid from *Clavelina lepadiformis*. In the present study, we investigated the effects of (–)-pictamine and (–)-lepadine B on the major types of nicotinic receptors ($\alpha 4\beta 2$ and $\alpha 7$) expressed in *Xenopus* oocytes.

MATERIALS AND METHODS

Materials All alkaloids used in this study were synthesized and are shown in Fig. 1. The synthesis of (–)-picta-

mine and (–)-lepadine B was performed as reported previously.^{17,18} The 3-piperidinol alkaloid (+)-prosafrinine, originally isolated from the leaves of *Prosopis africana*, has also been synthesized as described previously.¹⁹ The structures of these alkaloids are identical to those of the natural products. The other reagents were purchased from Sigma (St. Louis, MO, U.S.A.), unless indicated otherwise.

Expression in *Xenopus* Oocytes To assess the nAChR activity, we measured current through the receptor-channel following the techniques described in detail by Tsuneki *et al.*¹⁵ In brief, oocytes were isolated from *Xenopus laevis* frogs and were microinjected with 20 ng cDNAs in the nucleus. The mixture of cDNAs of mouse $\alpha 4$ and mouse $\beta 2$ subunits was injected in a ratio of 1 : 1, whereas mouse $\alpha 7$ cDNA was injected alone. Oocytes were used in electrophysiological recordings 3 to 8 d after the injection. All experiments were carried out in accordance with guidelines approved by the Toyama Medical and Pharmaceutical Univer-

Fig. 1. Structures of (–)-Pictamine, (–)-Lepadine B and (+)-Prosafrinine. Absolute stereochemistry is as follows, (–)-pictamine: 3R, 4S, 6S, 9aS; (–)-lepadine B: 2S, 3S, 4aS, 5S, 8aR; (+)-prosafrinine: 2S, 3S, 6R.

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sity Animal Research Committee.

Electrophysiological Recording An oocyte was placed in a 300- μ l tube-like chamber in which a low- Ca^{2+} Ringer's solution (82.5 mM NaCl, 2.5 mM KCl, 0.5 mM CaCl_2 , 2 mM MgCl_2 , and 5 mM HEPES, pH 7.4) containing 1 μ M atropine to block endogenous muscarinic receptors was perfused by gravity (15 ml/min). The low- Ca^{2+} Ringer's solution is used to prevent strong activation of the endogenous calcium-dependent chloride conductance.²⁰ Current responses were recorded under two-electrode voltage-clamp at a holding potential of -60 mV using a GeneClamp 500 amplifier and pClamp7 software (Axon Instruments, Union City, CA, U.S.A.). The sampling rate was 20 Hz. Electrodes contained 3 M KCl and had resistances of <1 M Ω . The rapid solution exchange was performed by a computer-controlled three-way Teflon solenoid valve (Parker Hannifin Corp., Division of General Valve, NJ, U.S.A.). Curve fittings to the concentration-inhibition curves for alkaloids (antagonists) and to the ACh concentration-response curves were performed as described previously.¹⁵

RESULTS

Concentrations near the 30% effective doses (EC_{30}) of ACh were used to elicit the currents, that is, 1 μ M for $\alpha 4\beta 2$ receptors and 100 μ M for $\alpha 7$ receptors. The $\alpha 4\beta 2$ currents were inhibited by dihydro- β -erythroidine (the best characterized antagonist of $\alpha 4\beta 2$ receptors, 5 μ M) and $\alpha 7$ currents were inhibited by methyllycaconitine (a selective antagonist of $\alpha 7$ receptors, 10 nM) as reported previously.¹⁵ When the oocytes expressing $\alpha 4\beta 2$ or $\alpha 7$ receptors were preincubated with (–)-pictamine (3 μ M) for 3 min, the peak amplitude of currents elicited by ACh (1 μ M for $\alpha 4\beta 2$ receptors, 100 μ M for $\alpha 7$ receptors) was markedly decreased (Fig. 2A). Interestingly, no recovery was observed after the removal of (–)-pictamine in oocytes expressing $\alpha 4\beta 2$ receptors, whereas the inhibited $\alpha 7$ currents completely recovered 12 min after the removal of (–)-pictamine. To quantitatively assess this profile, the oocytes expressing $\alpha 4\beta 2$ receptors were stimulated with ACh pulses (1 μ M, 5 s) at indicated times (Fig. 2B). In the absence of the alkaloid, repeatable, constant currents were obtained. (–)-Pictamine (3 μ M) caused a time-dependent blockade of the $\alpha 4\beta 2$ currents, and the degree of blockade was maintained at least 48 min after the washout of (–)-pictamine (Fig. 2B). These results suggest that the rate of dissociation of (–)-pictamine is extremely different between $\alpha 4\beta 2$ and $\alpha 7$ receptors. To compare the potency of (–)-pictamine on $\alpha 4\beta 2$ and $\alpha 7$ currents, the peak amplitude of the currents was measured after 3 min of the alkaloid incubation. We observed that (–)-pictamine blocked the currents in a concentration-dependent manner, with IC_{50} values of 1.5 μ M for $\alpha 4\beta 2$ currents and 1.3 μ M for $\alpha 7$ currents (Figs. 2C, D, Table 1). Thus, (–)-pictamine equally blocks $\alpha 4\beta 2$ - and $\alpha 7$ -nAChRs. As shown in Fig. 2E, the concentration-response relationship for ACh on $\alpha 7$ receptors was fit by a single Hill equation (EC_{50} = 158 μ M, Hill coefficient nH = 1.31). The ACh concentration-response curve shifted downward in the presence of (–)-pictamine (3 μ M) (Fig. 2E), indicating that (–)-pictamine is not a competitive blocker of $\alpha 7$ receptors.

When the oocytes expressing $\alpha 4\beta 2$ receptors were prein-

Fig. 2. Irreversible Inhibitory Effects of (–)-Pictamine on ACh-Induced Currents in *Xenopus* Oocytes Expressing $\alpha 4\beta 2$ Nicotinic Receptors

Currents were recorded in voltage-clamp mode at -60 mV. (A) Typical traces showing inhibition by (–)-pictamine (3 μ M) on $\alpha 4\beta 2$ currents elicited by 1 μ M ACh and $\alpha 7$ currents elicited by 100 μ M ACh. Horizontal bars indicate the period of perfusion with ACh for 5 s. Vertical scale bars represent 300 nA for $\alpha 4\beta 2$ currents, and 150 nA for $\alpha 7$ currents. (B) Time course of blockade of $\alpha 4\beta 2$ receptors by (–)-pictamine (3 μ M). Each point represents the mean $\pm$ S.E.M. of five consecutive ACh responses that were normalized to the initial current evoked by 1 μ M ACh alone recorded in the same oocyte. The horizontal bar indicates the time of pictamine superfusion. Concentration-response curves for (–)-pictamine on $\alpha 4\beta 2$ (C) and $\alpha 7$ receptors (D). ACh-elicited currents were measured before and 3 min after the (–)-pictamine treatment. The current responses to ACh in the presence of (–)-pictamine in each oocyte were normalized to the ACh responses (control responses) recorded in the same oocytes. Values represent the mean $\pm$ S.E.M. for five to six separate experiments. (E) Concentration-response curves for ACh on $\alpha 7$ nAChRs in the absence (○) and presence of alkaloids. Oocytes were preincubated with either (–)-pictamine (3 μ M, ▲) or (–)-lepadin B (1 μ M, ●) for 3 min, and then exposed to ACh (10 μ M to 10 mM) in the presence of alkaloids. Each current response to ACh in the presence of the alkaloids was normalized to the maximal current evoked by 1 mM ACh alone recorded in the same oocyte. Values represent the mean $\pm$ S.E.M. for five to eight separate experiments.

Table 1. Potency of Blocking Effects of Alkaloids on *Xenopus* Oocytes Expressing Recombinant Nicotinic Receptors

	IC_{50} (95% confidence intervals), μ M		<i>n</i>
	$\alpha 4\beta 2$ 1 μ M ACh	$\alpha 7$ 100 μ M ACh	
(–)-Pictamine	1.5 (1.3, 1.8)	1.3 (1.1, 1.6)	5–6
(–)-Lepadin B	0.9 (0.7, 1.2)	0.7 (0.5, 0.9)	5–8
(+)-Prosafrinine	2.9 (2.2, 3.8)	1.1 (0.7, 1.9)	3

cubated with (–)-lepadin B (3 μ M) for 3 min, the peak amplitude of currents elicited by ACh (1 μ M) was greatly decreased (Fig. 3A). This blocking effect was reversible within 9 min. Similar reversible blockade was observed in ACh (100 μ M)-elicited currents in oocytes expressing $\alpha 7$ receptors. In fact, these currents were blocked by this alkaloid in a

sea squirts for chemical defense.²⁶⁾ It has been reported that lepadin B elicits cytotoxic effects with IC_{50} values of $44\ \mu\text{M}$ for human breast cancer MCF7, $26\ \mu\text{M}$ for glioblastoma U373, and $38\ \mu\text{M}$ for ovarian carcinoma HEY cells.²⁷⁾ However, these toxic concentrations are 30- to 60-fold higher than the IC_{50} values of (–)-lepadin B for blockade of the nAChRs expressed in oocytes (Table 1). In fact, the blocking effect of (–)-lepadin B was reversible and reproducible in the same oocytes (data not shown). Moreover, no changes in the holding current were observed during the application of (–)-lepadin B (data not shown). Therefore, it is not likely that (–)-lepadin B damages oocytes in the present condition.

Recent genetic studies demonstrate that autosomal-dominant nocturnal frontal lobe epilepsy (ADNFLE) is associated with a mutation in the $\alpha 4$ or the $\beta 2$ subunit of nAChRs.⁹⁾ The mutant nAChR expressed in *Xenopus* oocytes exhibits an increased sensitivity to ACh. A promising therapeutic strategy suggested for ADNFLE is to reduce the enhanced responses of the mutant $\alpha 4\beta 2$ -nAChRs by noncompetitive nicotinic antagonists (open channel blockers). That type of blocker could selectively target the receptors displaying a gain of function by blocking them in a use-dependent manner.⁹⁾ Although (–)-pictamine does not appear to be an use-dependent blocker, its longer-term inhibition suggests that further studies of ascidian alkaloids could reveal beneficial developments for nicotinic-based treatment of neurological diseases.

Acknowledgments The present work was supported in part by Smoking Research Foundation (Japan) (to I.K.) and by Grant-in-Aid (16590435) for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology, Japan (to H.T.). J.A.D. was supported by grants from the NINDS and NIDA of the National Institutes of Health (U.S.A.). We are grateful to Dr. Jerry A. Stitzel (University of Colorado) for providing us with plasmid DNA. We would also like to thank Dr. Hideki Sakai (Toyama Medical and Pharmaceutical University) for the continuous support on data acquisition.

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