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The Neurotoxic Effect of 13,19-Didesmethyl and 13-Desmethyl Spirolide C Phycotoxins Is Mainly Mediated by Nicotinic Rather Than Muscarinic Acetylcholine Receptors

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ABSTRACT

Spirolides are a large family of lipophilic marine toxins produced by dinoflagellates that have been detected in contaminated shellfish. Among them, 13,19-didesmethyl and 13-desmethyl spirolide C phycotoxins are widely distributed and their mode of action needs to be clearly defined. In order to further characterize the pharmacological profiles of these phycotoxins on various nicotinic acetylcholine receptor (nAChR) subtypes and to examine whether they act on muscarinic receptors (mAChRs), functional electrophysiological studies and competition binding experiments have been performed. While 13-desmethyl spirolide C interacted efficiently with sub-nanomolar affinities and low selectivity with muscular and neuronal nAChRs, 13,19-didesmethyl spirolide C was more selective of muscular and homopentameric $\alpha 7$ receptors and recognized only weakly neuronal heteropentameric receptors, especially the $\alpha 4\beta 2$ subtype. Thus, the presence of an additional methyl group on the tetrahydropyran ring significantly modified the pharmacological profile of 13-desmethyl spirolide C by notably increasing its affinity on certain neuronal nAChRs. Structural explanations of this selectivity difference are proposed, based on molecular docking experiments modeling different spirolide-receptor complexes. In addition, the 2 spirolides interacted only with low micromolar affinities with the 5 mAChRs, highlighting that the toxicity of the spirolide C analogs is mainly due to their high inhibition potency on various peripheral and central nAChRs and not to their low ability to interact with mAChR subtypes.

Key words: spirolides; marine phycotoxins; nicotinic acetylcholine receptors; muscarinic acetylcholine receptors; neuromuscular junction; *Xenopus* oocytes; *Xenopus* skeletal myocytes, acetylcholine

ABBREVIATIONS:

nAChR, nicotinic acetylcholine receptor;
mAChR, muscarinic acetylcholine receptor;

BSA, bovine serum albumin;
 α -BTX, α -bungarotoxin;
PBS, phosphate buffered saline;

SPX,	spirolide;
NMS,	N-methyl scopolamine;
CHO,	Chinese hamster ovary;
LD ₅₀ ,	dose producing the death of 50% of animals;
EDL,	<i>Extensor digitorum longus</i> ;
MEPP,	miniature endplate potential;
EPP,	endplate potential;
AChBP,	acetylcholine binding protein.

The spirolides are an emerging family of lipophilic marine toxins belonging to a heterogeneous group of macrocyclic compounds named cyclic imine toxins [for reviews see (Guéret and Brimble, 2010; Molgó et al., 2007, 2014; Stivala et al., 2015)]. These toxins were first detected in extracts of the digestive glands of bivalve mollusks (*Mytilus edulis* mussels and *Placopecten magellanicus* scallops) collected from the Atlantic coast of Nova Scotia (Canada) in the early 1990s (Hu et al., 1995). More recently, spirolides have been detected in shellfish and phytoplankton extracts from European (Aasen et al., 2005; Amzil et al., 2007; Ciminiello et al., 2006; MacKinnon et al., 2006; Villar Gonzalez et al., 2006), North American (Gribble et al., 2005) and South American coasts (Alvarez et al., 2010). The distinguishing chemical feature of these compounds is the presence of a cyclic imine moiety in their structure (Hu et al., 1995, 1996b, 2001), which has been found elsewhere in marine toxins known as gymnodimines A–C (Kharrat et al., 2008; Miles et al., 2000, 2003; Seki et al., 1995), pinnatoxins (Chou et al., 1996; Uemura et al., 1995), pteriatoxins (Takada et al., 2001), prorocentrolides (Hu et al., 1996), and spiro-procentrimine (Lu et al., 2001). The spirolide group of toxins has grown over the years to include 13-desmethyl spirolide C (13-SPX-C) (Hu et al., 2001; Sleno et al., 2004), and new compounds with slightly modified structures like 13,19-didesmethyl spirolide C (13,19-SPX-C), 27-hydroxy-13,19-SPX-C, spirolide G (Aasen et al., 2005; MacKinnon et al., 2006), and more recently, spirolides H and I (Roach et al., 2009) (Fig. 1).

The spirolides are referred to act as fast-acting phycotoxins, because they induce rapid onset of neurotoxic signs similar, to some extent, to those reported for the acute toxicity of paralytic shellfish poisoning toxins, when injected intraperitoneally (ip) or administered orally; followed by rapid death (within tens of minutes after ip administration) of laboratory mice (Gill et al., 2003; Hu et al., 1996; Richard et al., 2001). The toxicity of the various spirolide derivatives, when ip administered to mice, varies drastically. Hence, according to the group of Munday, 13-SPX-C and 20-methyl spirolide G were reported to be highly toxic, with LD₅₀ between 0.007 and 0.008 mg/kg mouse, while spirolides A and B have approximately 5 and 13 times lower toxicity

(Munday et al., 2012). However, higher LD₅₀ values, ie, 0.028 and more than 0.063 mg/kg mouse, were determined by the group of Botana and coworkers for 13-SPX-C and 20-methyl spirolide G, respectively (Otero et al., 2012), showing lower levels of toxicity for these 2 spirolides than those reported by the group of Munday. Interestingly, it was shown that the loss of the methyl group at C-19 in 13-SPX-C, giving 13,19-SPX-C, resulted in a between 1.2 and 5-fold lower toxicity (MacKinnon et al., 2006; Otero et al., 2012). The toxicity of all other known spirolide derivatives (including 27-hydroxy-13-desmethyl spirolide C, 27-oxo-13,19-SPX-C, as well as spirolides E and F) was reported to be greatly decreased and even null (Ciminiello et al., 2010; Hu et al., 1996b; Roach et al., 2009). After ip injection, widespread neuronal damage has been detected in mouse brains, and a clear relationship was observed between the doses of 13-SPX-C and the severity of the induced lesions (Gill et al., 2003).

It has been initially reported that spirolides may affect voltage-gated Ca²⁺ channels (Hu et al., 1995), but functional binding and crystallographic studies revealed recently that 13-SPX-C targets different nicotinic acetylcholine receptor (nAChR) subtypes with affinities ranging from subnanomolar to submicromolar (Aráoz et al., 2009; Bourne et al., 2010; Hauser et al., 2012; Vilariño et al., 2009). Furthermore, both nicotinic and muscarinic acetylcholine receptor (mAChR) gene expressions were reported to be upregulated after spirolide treatment (Gill et al., 2003). The potential interaction of 13-SPX-C on mAChRs was recently investigated. Thus, studies using rat cortical membranes and/or neuroblastoma cells known to express various mAChR subtypes revealed either a reduction of muscarinic function and a decrease of specific muscarinic binding by this toxin at 100 nM (Wandscheer et al., 2010) or, on the opposite, no appreciable effect of the spiroimine toxin at 100 μM (Hauser et al., 2012).

Therefore, this study was designed to define the mode of action and molecular target(s) of both 13,19-SPX-C and 13-SPX-C using *in vitro* electrophysiological experiments, and binding studies on clonal cell lines expressing various nAChR and mAChR subtypes. The choice to study these 2 spirolide C analogs was supported by their global distribution as well as by their capacity to cross the intestinal barrier (Espiña et al., 2011) and reach the central nervous system (Alonso et al., 2013). Moreover, our study aims examining the potential role of an additional methyl group at C-19 of the tetrahydropyran ring B of the bis-spiroacetal ring of 13-SPX-C on the pharmacological profile of this compound. Our results show that 13,19-SPX-C is more potent than 13-SPX-C in blocking muscular nAChR with subnanomolar affinities, while the loss of a methyl group is deleterious on its affinity toward heteropentameric neuronal

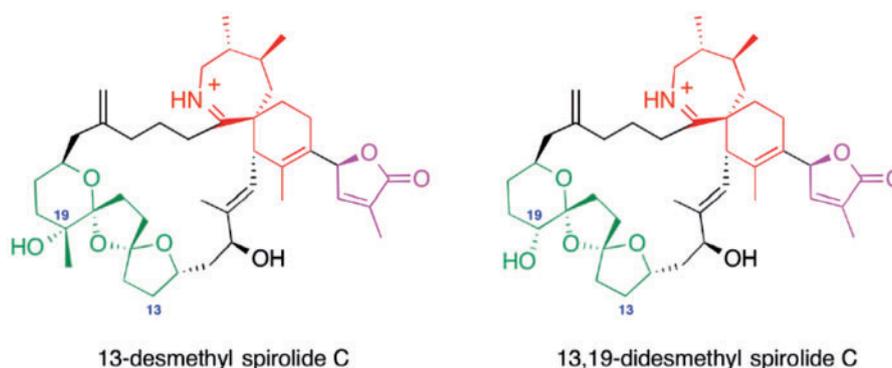


FIG. 1. Chemical structures of 13-desmethyl spirolide C (13-SPX-C) and 13,19-didesmethyl spirolide C (13,19-SPX-C).

subtypes. These functional properties were supported by molecular docking experiments, unveiling common and variable molecular interactions within the different toxin-receptor complexes. Finally, the 2 spiroles interact only with low micromolar affinities with the 5 mAChR subtypes, in contrast to their sub-nanomolar to nanomolar affinity on nAChRs, highlighting the nicotinic basis of the neurotoxicity of 13-SPX-C and 13,19-SPX-C to mammals.

MATERIALS AND METHODS

Phycotoxins and radioligands. The 13,19-SPX-C and 13-SPX-C extracted from laboratory cultures of the dinoflagellate *Alexandrium ostenfeldii* were purchased ($\geq 96\%$ purity) from Cifga S.A. (Lugo, Spain) and from the NRC Canada, Institute for Marine Bioscience (Halifax, Nova Scotia, Canada), respectively. [^{125}I]- α -Bungarotoxin ([^{125}I]- α -BTX) (210–250 Ci/mmol), (\pm)-[^3H]-epibatidine (55 Ci/mmol), and N-methyl-[^3H]-scopolamine methyl chloride ([^3H]-NMS) (78 Ci/mmol) were purchased from PerkinElmer (Courtaboeuf, France). All other chemicals were obtained from Sigma-Aldrich (Saint Quentin Fallavier, France) or other standard sources.

Animals and biological materials. Adult female *Xenopus laevis* frogs were obtained from the Centre de Ressources Biologiques Xénopes – CNRS (Université de Rennes 1, France). Live *Torpedo marmorata* fishes were purchased from the Service Modèles Biologiques of the Station Biologique de Roscoff (France), and adult female Swiss mice were obtained from the CNRS animal house. Experiments were performed in accordance with European Community guidelines for laboratory animal handling and with the official edict presented by the French Ministry of Agriculture and the recommendations of the Helsinki Declaration. The cDNAs coding for chick chimeric $\alpha 7$ -5HT $_3$ and human $\alpha 4$ and $\beta 2$ subunits were kindly provided by Dr P.J. Corringer (Pasteur Institute, Paris, France) and by Prof. O. Steinlein (Institute of Human Genetics, Bonn, Germany). The human $\alpha 3$ cDNA was purchased from Clinisciences (Nanterre, France). The human $\alpha 7$ cDNA (Maskell et al., 2003) was a generous gift from Prof. I. Bermudez (Oxford Brookes University, Oxford, UK).

Nerve-evoked and directly elicited mouse muscle isometric contractions. Mice were anesthetized with isoflurane (Aerrane, Baxter S.A., Lessines, Belgium) inhalation before being euthanized by dislocation of the cervical vertebrae. For isometric twitch tension measurements, the mouse *Extensor digitorum longus* (EDL) muscles with its attached nerve was carefully removed and mounted in a silicone-lined bath filled with Krebs-Ringer solution of the following composition (in mM): NaCl 140, KCl 5, CaCl $_2$ 2, MgCl $_2$ 1, glucose 11, and HEPES 5 (pH 7.4), continuously perfused with pure O $_2$ throughout the experiment. One of the EDL tendons was securely anchored onto the silicone-coated bath and the other tendon was attached via an adjustable stainless-steel hook to an FT03 isometric force transducer (Grass Instruments, West Warwick, USA). Muscle twitches and tetanic contractions were evoked either by stimulating the motor nerve via a suction microelectrode (adapted to the diameter of the nerve), with supramaximal current pulses of 0.15 ms duration delivered by an S-44 stimulator (Grass Instruments) at frequencies indicated in the text, or by direct electrical stimulation through an electrode assembly, build up in the silicone-coated bath, and placed at short distance along the length of the

muscle. The resting tension adjusted for each preparation investigated, with a mobile micrometer stage, allowed incremental adjustments of muscle length to obtain maximal contractile responses. Signals from the isometric transducer were amplified, collected, and digitized with the aid of a computer equipped with a Digidata-1322A A/D interface board (Axon Instruments, Union City, California). Data acquisition and analysis were performed with the WinWCP V3.9.6 software program, kindly provided by Dr. John Dempster (University of Strathclyde, Scotland). Experiments were performed at 20°C–22°C.

Intracellular recordings from the mouse neuromuscular junction. Membrane and synaptic potentials were recorded from end-plate regions with intracellular microelectrodes (filled with 3 M KCl solution, 8–12 M Ω resistance) and an Axoclamp-2A system (Axon Instruments), as previously reported (Minic et al., 2003). The motor nerve was stimulated via a suction electrode with current pulses of 0.1 ms duration and supramaximal voltage (typically 3–8 V).

Primary cell culture of *Xenopus* myocytes. Myotomal tissues from 1-day-old *Xenopus laevis* embryos (stage 22–24) were mechanically dissociated using a Ca $^{2+}$ -Mg $^{2+}$ -free medium containing (in mM): NaCl 115, KCl 2.6, Ethylenediaminetetraacetic acid (EDTA) 0.5, and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) 5 (pH = 7.6) as previously detailed (Kharrat et al., 2008).

Patch-clamp recordings from the *Xenopus* skeletal myocytes. Perforated patch-clamp recordings were performed at 20°C–22°C. Pipettes had resistances of 2–3 M Ω when filled with internal solution. Membrane currents and potential were recorded using an Axopatch 200B patch-clamp amplifier (Axon Instruments). Access resistances were compensated at 80%. Myocytes were voltage-clamped through an amphotericin-perforated membrane patch, using the following intrapipette composition (in mM): NaCl 1, KCl 20, K-gluconate 125, MgCl $_2$ 1, HEPES 10 (pH = 7.2), and amphotericin-B (300 $\mu\text{g}/\text{ml}$). The external solution had the following composition (in mM): NaCl 140, KCl 3, MgCl $_2$ 1, CaCl $_2$ 2, HEPES 10 (pH = 7.4). For ionophoretic ACh application, high-resistance glass pipettes (100–150 M Ω) were filled with 1 M AChCl. ACh efflux was induced by cationic-current pulses (1 ms duration) with a constant-current generator, allowing the use of breaking currents to prevent spontaneous ACh efflux and to avoid nAChR desensitization.

Expression of nAChRs in human embryonic kidney cells. A chimeric cDNA of the neuronal type $\alpha 7$ -5HT $_3$ nAChR was transfected into human embryonic kidney (HEK)-293 cells by calcium phosphate precipitation, as previously described (Eisele et al., 1993; Servent et al., 1997). Briefly, the chick cDNA (15 μg of $\alpha 7$ -5HT $_3$) was transfected by calcium precipitation with a careful control of the pH (6.95). The cells were placed at 37°C under 5% CO $_2$ and 2 days after transfection, were harvested in a phosphate buffered saline (PBS) with 5 mM EDTA, and resuspended in 3 ml/plate of this buffer for binding experiments. For human $\alpha 4\beta 2$ and $\alpha 3\beta 2$ receptor subtypes, 24 h after the calcium phosphate transfection with neuronal nAChRs cDNA, the cells were placed for 2 days at 30°C with 5% CO $_2$ before being collected for binding assays, using a cell density adjusted to specifically bind $\leq 10\%$ of radioligand.

Expression of mAChRs in Chinese hamster ovary cells and membrane preparation. Chinese hamster ovary (CHO) cells stably expressing the different cloned human muscarinic receptors were kindly

provided by Prof. P. O. Couraud (Institut Cochin de Génétique Moléculaire, Paris, France). The cells were grown, harvested, and the membrane prepared as previously described (Aráoz et al., 2011).

Binding assays. The affinity of spiroptides for the chimeric $\alpha 7$ -5HT₃ nAChR was determined as previously described (Kharrat et al., 2008; Servent et al., 1997). This chimeric receptor was used due to its ability to be functionally expressed in cell lines such as CHO or HEK cells, that is not the case for the wild-type $\alpha 7$ receptor. Competition experiments with *Torpedo* nAChRs were performed at equilibrium by incubating for at least 4 h, 0.05 μ g *Torpedo* membrane with different concentrations of 13,19-SPX-C, 13-SPX-C, and [¹²⁵I] α -BTX (0.25–0.45 nM). In these assays, nonspecific binding was determined in the presence of 1 μ M cobratoxin. The filters were washed with cold PBS and counted on a gamma counter (LKB-Multigamma 1261, Uppsala, Sweden). Equilibrium binding experiments on $\alpha 4\beta 2$ and $\alpha 3\beta 2$ subtypes used [³H]epibatidine as radioactive tracer. Cells expressing these receptor subtypes were incubated with 0.5–1 nM [³H]epibatidine and various concentrations of the spiroimine toxins for 4 h. After filtration, GF/C filters were dried and 6 ml of scintillation solution (Ultima Gold F, PerkinElmer) were added before counting the filters on a Liquid Scintillation Analyzers (Tri-Carb 2300 TR, PerkinElmer). In these assays, nonspecific binding was determined in the presence of 100 nM epibatidine.

In equilibrium competition experiments, IC₅₀ values were determined by fitting the competition data by the empirical Hill's equation and converted to K_i constants using the following equation: $K_i = IC_{50} / (1 + L^*/K_d)$ (Cheng and Prusoff, 1973), with a K_d for α -BTX on muscle-type receptor of 50 pM, and a K_d for epibatidine on human $\alpha 3\beta 2$ and $\alpha 4\beta 2$ equal to 35 and 20 pM, respectively. All experiments were done at least 3 times in duplicate.

The effect of spiroptides on the equilibrium binding of [³H]-NMS on the 5 human nAChRs subtypes was determined in inhibition experiments on 96-well plates. With [³H]-NMS as tracer, membrane protein concentrations was adjusted so that no more than 10% of added radioligand was specifically bound (around 1500–2500 cpm) and were incubated in PBS-BSA at 25°C for 120 min with high micromolar concentration of ligands and [³H]-NMS (0.5 nM) in a final assay volume of 100 μ l. Nonspecific binding was determined in the presence of 50 μ M atropine. The reaction was stopped by filtration of the 96-well simultaneously through a GF/C plate presoaked in 0.5% polyethylenimine, using a FilterMate harvester (PerkinElmer, France). The filters were washed twice with ice-cold buffer (PBS), dried, and the bound radioactivity was counted after the addition of 25 μ l of MicroScint 0 per well, by scintillation spectrometry on a TopCount beta counter (PerkinElmer). Each experiment was done 2 times in duplicate.

Expression of human $\alpha 7$ and $\alpha 4\beta 2$ nAChRs in *Xenopus* oocytes. Oocytes were surgically removed from mature female *Xenopus laevis* frogs under anesthesia using ethyl-3-amino benzoate methanesulfonate salt (Sigma-Aldrich, Saint Quentin Fallavier, France) solution (1 g/l) and were recovered in calcium-free medium containing (in mM): NaCl 88, KCl 2.5, MgCl₂ 1, and HEPES 5 (pH 7.6). Following an extensive washing with this solution, the oocytes were transferred to Barth's solution containing (in mM): NaCl 88, KCl 1, MgSO₄ 0.33, CaCl₂ 0.41, MgSO₄ 0.82, Ca(NO₃)₂ 0.33, NaHCO₃ 2.4, and HEPES 10 (pH 7.2) supplemented with kanamycine 0.1 mg/ml. Stage V–VI oocytes were manually defolliculated and microinjected with 50 nl human $\alpha 7$ mRNA (0.1 μ g/ μ l), using a Nanoliter 2000 Micro 4 Controller (World

Precision Instruments, Inc, UK). Oocytes were as well injected with 50 nl of a mixture of plasmids carrying the cDNA of $\alpha 4$ and $\beta 2$ (0.3 μ g/ μ l, each). Oocytes were incubated at 18°C in Barth's solution. Recordings were performed between 3 and 4 days after injection.

***Torpedo* membrane preparation and nAChR microtransplantation to *Xenopus* oocytes.** *Torpedo marmorata* specimens kept in conventional artificial seawater were anaesthetized with 0.03% tricaine (Sigma-Aldrich) in seawater before surgical excision of electric organs. The $\alpha 1_2\beta 1\gamma\delta$ nAChR-rich membranes were prepared at 4°C from freshly dissected and sliced *Torpedo* electric organs using procedures previously described (Hill et al., 1991; Krieger et al., 2008), resuspended in 5 mM glycine, and aliquots stored at –80°C until use. Microtransplantation of *Torpedo* nAChR (Miledi et al., 2006) consisted of a microinjection into the oocyte cytoplasm of a membrane suspension (50 nl at 3.5 mg/ml protein) using a Nanoliter2000 Micro4 Controller mounted on a microscope.

Voltage-clamp recording on oocytes. ACh-evoked currents were recorded with a standard 2-microelectrode voltage-clamp amplifier (OC-725B, Warner Instrument Corp, Hamden, Connecticut) at a holding potential of –60 mV. Voltage and current microelectrodes had 0.5–1.5 M Ω tip resistance. Data were acquired with a pCLAMP-9/Digidata-1322A system (Molecular Devices, Union City, California). The recording chamber (capacity 300 μ l) was superfused (8 ml/min, 20°C) with a modified Ringer's solution containing (in mM): NaCl 100, KCl 2.8, BaCl₂ 0.3 and HEPES 5 (pH 7.4), where BaCl₂ substitution to CaCl₂ prevents secondary activation of Ca²⁺-dependent Cl[–] current (Sands et al., 1993). ACh and the cyclic imine toxins were superfused using a computer-controlled solution exchange system (VC-6, Warner Instruments). Perfusion profile was as follows: a clamped oocyte was perfused twice with ACh (3 s for oocytes expressing human $\alpha 7$ nAChR, or 15 s for oocytes expressing human $\alpha 4\beta 2$ and for oocytes having incorporated *Torpedo* $\alpha 1_2\beta 1\gamma\delta$ nAChR onto their membrane), with an interval of 3 min between successive ACh applications to ensure receptor recovery from desensitization. After 3 min washing, the same oocyte was perfused for 45 s with a given concentration of 13,19-SPX-C or 13-SPX-C followed immediately by the perfusion of a mixture of ACh containing the same concentration of a given spiroptide. Concentration-inhibition curves were analyzed as previously detailed (Bourne et al., 2010). Each oocyte was tested only against 1 toxin concentration. Several xenope female donors were used to obtain the dose-response curves.

Molecular modeling. The molecular modeling protocol followed in this study is similar to that previously described for the interaction of pinnatoxin A with nAChRs (Aráoz et al., 2011). In brief, homology models for the extracellular domain of the human $\alpha 7$, human $\alpha 4\beta 2$ and *Torpedo* $\alpha 1_2\beta 1\gamma\delta$ nAChRs subtypes were generated using the *Aplysia*-AChBP crystal structure (Protein Data Bank code 2WZY) (Bourne et al., 2010) as template. As no docking software is currently able to deal natively with the macrocycle flexibility, the docking procedure was carried out in 2 steps: (1) conformational search of the ligands using MacroModel (Schrödinger) ensembles of 61 (13-SPX-C) and 90 (13,19-SPX-C) conformers incorporating macrocycle flexibility; (2) molecular docking using Gold (Cambridge Crystallographic Data Centre) of the 2 ligands at the subunit interfaces of the $\alpha 7$, $\alpha 4\beta 2$ and $\alpha 1_2\beta 1\gamma\delta$ homology models, which takes care of the flexibility of macrocycle substituents. Images

of the receptor-ligand complexes were generated using PyMol (Schrödinger).

RESULTS

Block of Nerve-Evoked Muscle Contractions and Neuromuscular Transmission in Mouse EDL Nerve-Muscle Preparations

To determine whether 13,19-SPX-C and 13-SPX-C had an action on the mature muscle-type nAChR, isolated mouse EDL nerve-muscle preparations expressing the $\alpha 1_2\beta\epsilon\delta$ nAChR subtypes were used. The 2 spirolides caused a time- and concentration-dependent block of isometric twitch responses evoked by motor nerve stimulation, as shown in typical experiments (Fig. 2). The spirolide-induced neuromuscular block was persistent and not reversed by continuous washout of spirolides for 30–45 min with a toxin-free medium. Furthermore, once 13,19-DMS had completely blocked nerve-evoked twitch (Fig. 2A), perfusion of 4-aminopyridine (10–20 μ M), a well-known K^+ channel blocker (Molgó *et al.*, 1977) for 20–30 min was unable to reverse the neuromuscular block produced by the toxin. As shown in Figure 2,

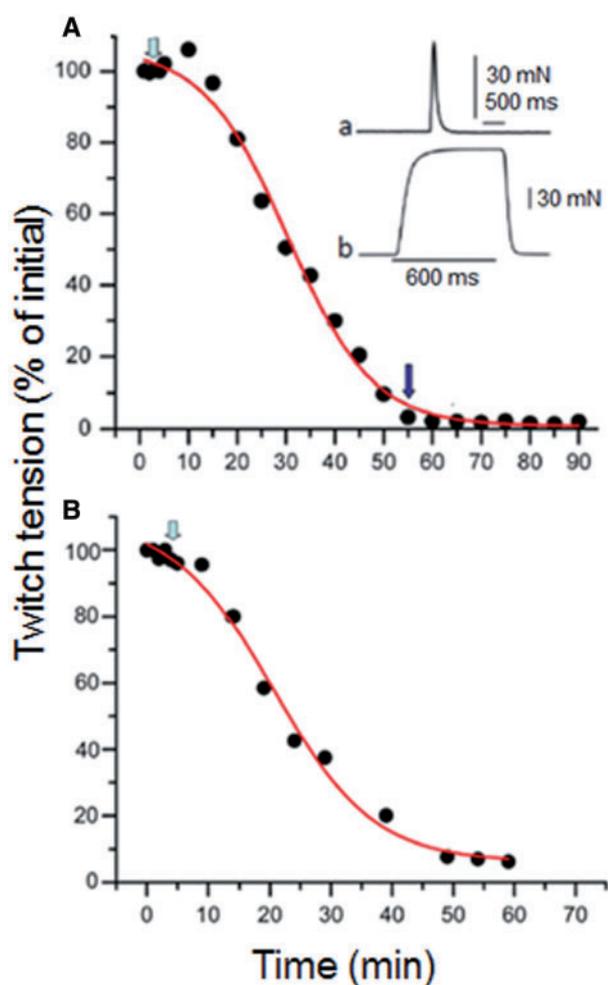


FIG. 2. Block of nerve-evoked isometric twitch tension in isolated mouse EDL nerve-muscle preparations by 2.6 nM 13,19-SPX-C (A), and 6 nM 13-SPX-C (B). Blue arrows indicate the addition of the spirolides to the medium. The black arrow indicates the perfusion of 20 μ M 4-aminopyridine, which was unable to reverse the neuromuscular block produced by 13,19-SPX-C. Inset in (A) shows that after complete block of nerve-evoked twitch-tension, twitch (a) and tetanic contraction (b, 40 Hz) can still be evoked upon direct muscle electrical stimulation.

at concentrations that completely blocked nerve-evoked twitch responses, twitch and tetanic contractions could still be elicited by direct electrical stimulation of the EDL muscle. These results suggest that the 2 spirolides affect endplate nAChRs.

Electrophysiological recordings were performed to determine whether the spirolides affected the membrane potential of muscle fibers, the amplitude of miniature endplate potentials (MEPPs), or other neurotransmission parameters. For this, intracellular recordings were performed from the junctional area of muscle fibers in which MEPPs and endplate potentials (EPPs) were detectable. 13,19-SPX-C (1.5 nM) and 13-SPX-C (3 nM) applied independently to mouse EDL preparation produced a complete block of MEPP amplitudes within 60 min (4 junctions sampled from 4 different EDL muscles under each condition). Typical recordings of MEPPs before and after the action of 13,19-SPX-C are shown in Figure 3A. At the time MEPPs were completely blocked by each of the spirolides, nerve stimulation still evoked muscle contractions in most of the fibers preventing intracellular recordings. When higher concentration of either 13,19-SPX-C or 13-SPX-C was used (2 and 5 nM, respectively), nerve-stimulation evoked subthreshold EPPs could be recorded in all the junctions tested ($n = 14$ from 3 different EDL muscles under each condition) (Fig. 3B). Furthermore, no significant action was detected on the membrane potential of muscle fibers with each of the spirolide C analogs studied. Thus, the mean resting membrane potential under control conditions was -70.2 ± 1.2 mV ($n = 24$ from 4 different EDL muscles), while in the presence of 2 nM 13,19-SPX-C or 5 nM 13-SPX-C, it was -71.2 ± 0.9 mV and 69.8 ± 1.9 mV ($n = 24$ from 4 different EDL muscles for each condition), respectively. Overall, our results strongly suggest that the 2 spirolide C analogs block endplate nAChRs, without affecting the resting membrane potential of skeletal muscle fibers.

Block of Nicotinic Currents in Embryonic *Xenopus* Skeletal Myocytes

The nAChRs at the skeletal neuromuscular junction have been extensively characterized and are known to form heteropentamers consisting of 2 $\alpha 1$ -subunits, single $\beta 1$ - and δ -subunits, and either a γ or a ϵ subunit in the embryonic or mature receptor-types, respectively (Corringer *et al.*, 2000). *Xenopus* myocytes, derived from *Xenopus* embryos, express nAChRs on their membrane surface, and patch-clamp recordings and iontophoretic application of ACh can be used to detect ACh-induced nicotinic currents. In myocytes voltage-clamped at -80 mV, an ACh-filled micropipette was positioned as close as possible to the cell membrane and brief iontophoretic ACh pulses were applied to their surface. Under control conditions, iontophoretic ACh pulses of constant intensity and duration elicited typical inward nicotinic currents caused by the binding of ACh to embryonic-type nAChRs (Fig. 4A, a). The perfusion of an external solution containing 2.5 or 10 nM 13-SPX-C revealed no agonist effect on nAChRs, but a time-dependent reduction of nicotinic currents, followed by complete blockade, as shown in Figure 4A (b, c). The almost complete block of nAChRs by the spirolide was not reversible during 20–30 min washout with a toxin-free solution ($n = 3$). To test whether the spirolide-induced blockade of nAChR depends on the membrane potential, current-voltage relationships were performed with iontophoretic ACh applications on patch-clamped myocytes. As shown in Figure 4B, a linear relationship was observed between the ACh-evoked current peaks and the holding membrane potential (-80 to -20 mV range) under control conditions and after 2.5 nM 13-SPX-C-treatment that blocked approximately 50%–60% of nicotinic peak current amplitudes ($n = 3$). These results indicate that

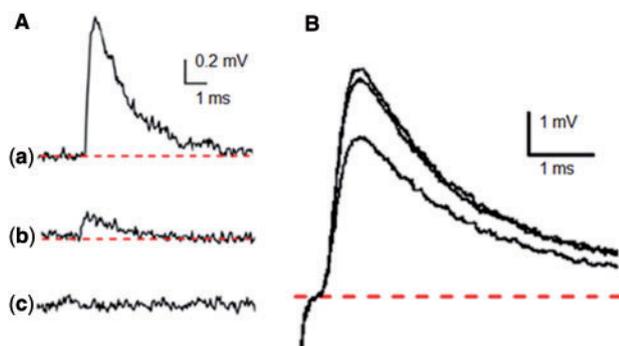


FIG. 3. Block of MEPP and EPP amplitudes by 13,19-SPX-C on EDL junctions. A, MEPPs recorded on the same mouse EDL junction before (a) and after the addition of 1.5 nM 13,19-SPX-C to the medium (b, c). Each trace represents the average of 8 spontaneous MEPPs. Note the reduction in amplitude (b) and the complete blockade of MEPPs (c), 10 and 30 min, respectively, after addition of the spiroside to the medium. Resting membrane potential during MEPP recordings was -68.6 ± 0.8 mV. B, Superimposed subthreshold EPPs recorded in a mouse EDL junction treated for 30 min with 2 nM 13,19-SPX-C. Resting membrane potential during measurements -69.2 ± 1.3 mV.

13-SPX-C induced blockade of ACh-evoked current through the embryonic muscle-type nAChR was not affected by the holding membrane potential. Similar results were obtained with 13,19-SPX-C, but complete blockade of ACh-induced currents occurred at much lower concentrations (2–3 nM) (data not shown).

Block of nAChRs Incorporated or Expressed in *Xenopus* Oocytes

Functional analysis of the effect of 13,19-SPX-C and 13-SPX-C was performed using voltage-clamp recordings from *Xenopus* oocytes, either microtransplanted with *Torpedo* $\alpha_1\beta_1\gamma\delta$ -rich membranes or transfected with human α_7 or $\alpha_4\beta_2$ -encoding cDNAs. In oocytes voltage-clamped at -60 mV, the 2 spiroside C analogs in a concentration range from 10 pM to 500 nM did not evoke inward currents, indicating that they have no direct agonist effect on oocytes having incorporated onto their membrane $\alpha_1\beta_1\gamma\delta$ or expressing the human α_7 and $\alpha_4\beta_2$ nAChR subtypes ($n \geq 50$ oocytes for each nAChR subtype). A good incorporation of the $\alpha_1\beta_1\gamma\delta$ muscle-type nAChR onto the membrane of oocytes was obtained 2–3 days after the microinjection, as judged by the peak amplitude of currents elicited by 25 μ M ACh (Fig. 5) (corresponding to the EC_{50} for ACh determined experimentally, data not shown). 13,19-SPX-C and 13-SPX-C in a concentration-dependent manner blocked the ACh-evoked nicotinic currents in muscle-type nAChR (Fig. 5). The blocking effect of the 2 spiroside C analogs on ACh-elicited currents was not abolished even after 30–40 min washout of the phycotoxin from the medium.

In oocytes expressing the human α_7 nAChR, the perfusion of 350 μ M ACh (corresponding to its EC_{50} determined experimentally on this receptor subtype; data not shown), elicited peak currents amplitudes that varied between oocytes and ranged from 1 to 6 μ A at a -60 mV holding membrane potential ($n = 60$ oocytes tested from 6 different *Xenopus*). The ACh-evoked currents were markedly reduced in amplitude with pico- to nanomolar concentrations of both spiroptides, as revealed by the concentration-inhibition curve shown in Figure 5. The 2 spiroptides, 13,19-SPX-C and 13-SPX-C also blocked the human $\alpha_4\beta_2$ nAChR expressed in *Xenopus* oocytes (Fig. 5). However, higher concentrations were needed to block the current evoked by 150 μ M ACh (EC_{50} ACh for this receptor subtype), indicating that both spiroptides were less potent toward this neuronal receptor subtype when compared with α_7 nAChR (Fig. 5 and Table 1).

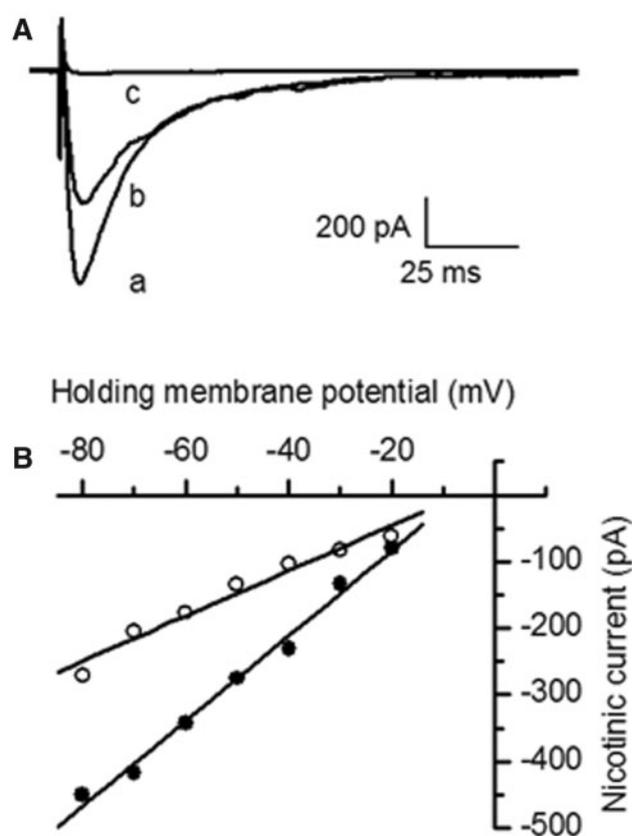


FIG. 4. Block of ACh-evoked nicotinic currents in *Xenopus* skeletal myocytes by 13-SPX-C. A, Nicotinic currents triggered by iontophoretic ACh pulse applications and recorded under patch-clamp conditions at a holding potential of -80 mV before (trace a), and after 13-SPX-C (10 nM) application (trace c). Trace b represents an intermediate blockade obtained after 90 s perfusion of the spiroside. Each current trace represents the average of 6 constant iontophoretic ACh-pulses. B, Representative current-voltage relationship obtained with iontophoretic ACh applications, in control conditions (filled circles), and after addition of a nonsaturating spiroside concentration (2.5 nM) (empty circles). Linear fitting (black lines) of the data points reveals the absence of correlation between the degree of blockade and the membrane potential.

Binding Competition Experiments Between Spiroptides and Radiolabeled nAChR Ligands

To determine the pharmacological selectivity profile of the 2 spiroptides for various nAChRs, their binding affinities and antagonist potencies toward membrane-embedded *Torpedo* muscle-type $\alpha_1\beta_1\gamma\delta$ nAChR (Sine, 2002; Unwin, 2005), neuronal chick chimeric α_7 -5HT₃, and human $\alpha_3\beta_2$ and $\alpha_4\beta_2$ nAChRs (Gotti et al., 2006) were examined through competition binding assays. 13,19-SPX-C and 13-SPX-C, in a concentration-dependent manner, totally displaced [¹²⁵I]α-BTX from *Torpedo* membranes expressing the muscle-type nAChR, and from HEK-293 cells expressing the chimeric α_7 -5HT₃ neuronal nAChR (Fig. 6A). The affinity constants calculated from these competition binding curves are reported in Table 2 and indicate that 13,19-SPX-C interacts with *Torpedo* and α_7 -5HT₃ receptors with high affinities equal to 17 and 220 pM, respectively, ie, 2.5 to 4-fold lower than 13-SPX-C. Interestingly, if the demethylation of the tetrahydropyran ring on the spiroside scaffold seems to favorize its interaction with these receptor subtypes, the opposite was observed on neuronal $\alpha_3\beta_2$ and $\alpha_4\beta_2$ subtypes (Fig. 6B). Indeed, a 25- and 90-fold decrease of affinity was measured for 13,19-SPX-C on $\alpha_3\beta_2$ and $\alpha_4\beta_2$ receptors, when compared with 13-SPX-C (Table 2).

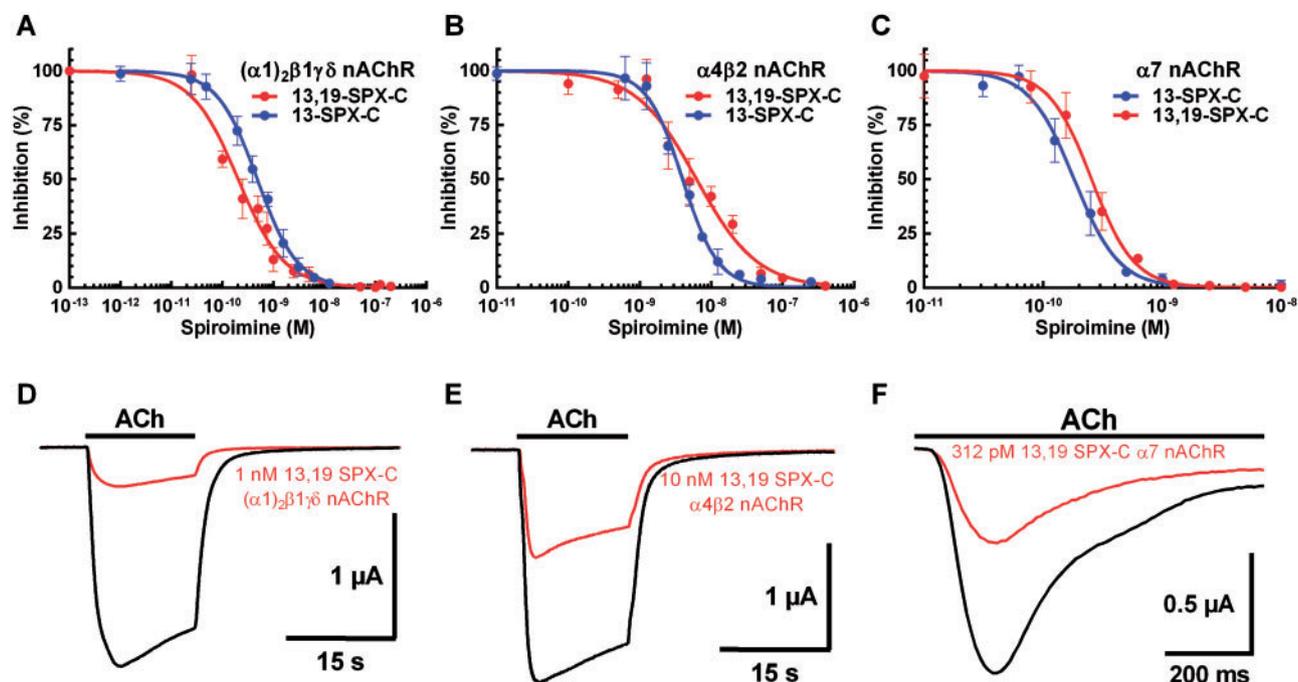


FIG. 5. Comparative inhibition of ACh-evoked currents by 13-SPX-C and 13,19-SPX-C on *Xenopus laevis* oocytes having incorporated the *Torpedo* muscle-type $\alpha_1\beta_1\gamma\delta$ nAChR (A), or expressing the human neuronal $\alpha_4\beta_2$ nAChR (B), and the human neuronal α_7 nAChR (C). The peak amplitude of the ACh current recorded at a holding membrane potential of -60 mV in the presence of a given cyclic imine toxin and at a given concentration was normalized to previously obtained control ACh evoked currents using the same oocyte. In the curves, each point represents the mean \pm SEM of data obtained from 5 different oocytes and fitted to the Hill's equation. D, Typical inward current obtained after 15 s perfusion of $25 \mu\text{M}$ ACh to an oocyte having incorporated the *Torpedo* muscle-type ($\alpha_1\beta_1\gamma\delta$) nAChR (black tracing), and after the antagonistic action of 1 nM 13,19-SPX-C (red tracing). E, Typical inward current obtained after 15 s perfusion of $150 \mu\text{M}$ ACh to an oocyte expressing human neuronal $\alpha_4\beta_2$ nAChR (black tracing), and after the antagonistic action of 10 nM 13,19-SPX-C (red tracing). F, Typical inward current obtained after 3 s perfusion of $350 \mu\text{M}$ ACh on an oocyte expressing human neuronal α_7 nAChR (black tracing), and after the antagonistic action of 312 pM 13,19-SPX-C (red tracing). The ACh concentration used corresponded to the EC_{50} that was experimentally determined for each nAChR subtype studied here.

TABLE 1. Inhibition Constants (IC_{50} , nM)* for 13,19-SPX-C and 13-SPX-C on ACh-Evoked Nicotinic Currents in Oocytes Microtransplanted With the Muscle-Type $\alpha_1\beta_1\gamma\delta$ nAChR or Expressing Neuronal α_7 and $\alpha_4\beta_2$ nAChR Subtypes

Ligand	$\alpha_1\beta_1\gamma\delta$ (<i>Torpedo</i>)	$\alpha_4\beta_2$ (Human)	α_7 (Human)
IC_{50} (nM)			
13,19-SPX-C	0.204 (0.158–0.263)*	6.26 (4.7–8.3)	0.255 (0.239–0.272)
13-SPX-C	0.51 (0.4–0.6)	3.9 (2.9–5.1)	0.18 (0.16–0.21)

*Mean values (95% confidence intervals) from concentration-response curves recorded on 40–50 oocytes for each condition.

Thus, on the receptor subtypes studied, the order of potency of 13-SPX-C was $\alpha_3\beta_2 > \textit{Torpedo} > \alpha_7\text{-5HT}_3 = \alpha_4\beta_2$, while 13,19-SPX-C was clearly more selective of the muscular-type receptor and interact weakly on central neuronal receptor subtypes, especially the $\alpha_4\beta_2$ subtype. The order of potency of its interaction was as follows: *Torpedo* $>$ $\alpha_7\text{-5HT}_3 > \alpha_3\beta_2 > \alpha_4\beta_2$.

Competition Binding Experiments Between Spirolicides and Radiolabeled mAChR Ligands

On the basis of controversial results on the potential interaction of 13-SPX-C with muscarinic receptors (Hauser et al., 2012; Wandscheer et al., 2010), we studied the inhibitory property of 13,19-SPX-C and 13-SPX-C on the 5 human mAChR subtypes stably expressed in the CHO cell line. At the maximal concentration tested ($3 \mu\text{M}$), the 2 toxins induce only a partial reduction of the [^3H]-NMS binding on the 5 mAChR subtypes, 13,19-SPX-C being from 2- to 5-times less potent than 13-SPX-C (Fig. 7).

Affinity constants in the low micromolar range were estimated from these competition binding curves (Table 3), highlighting the very low affinity of these toxins on muscarinic receptors.

DISCUSSION

Receptors for ACh were first divided into nAChRs and mAChRs by Sir Henry Dale, following the observation that ACh action can be mimicked by nicotine (from the tobacco plant *Nicotiana tabacum*) and muscarine (from the fly agaric *Amanita muscaria*), respectively (Dale, 1914). Furthermore, other natural toxins can be used to identify and distinguish these 2 types of receptors, as for example the nicotinic antagonist d-tubocurarine and the muscarinic antagonist atropine. Even if the nicotinic action of marine toxins belonging to the cyclic imine phycotoxin family was recently demonstrated (Bourme et al., 2010; Hauser et al., 2012; Kharat et al., 2008), their potential effect on mAChRs was still under debate due to controversial results reported in the literature (Hauser et al., 2012; Wandscheer et al., 2010). Thus, the pharmacological profiles of 2 molecules produced by the dinoflagellate *Alexandrium ostenfeldii*, the 13-SPX-C and 13,19-SPX-C phycotoxins, were characterized using electrophysiological and binding experiments on the 2 types of cholinergic targets.

First, on isolated mouse EDL nerve-muscle preparations, the 2 spirolicide analogs block endplate nAChRs as evidenced by the block of MEPP amplitudes and the recording of subthreshold EPPs, without any effect on the resting membrane potential of skeletal muscle fibers. Second, studying the effect of both compounds on the nicotinic currents in embryonic *Xenopus* skeletal myocytes reveals no agonist effect on nAChRs, but a complete,

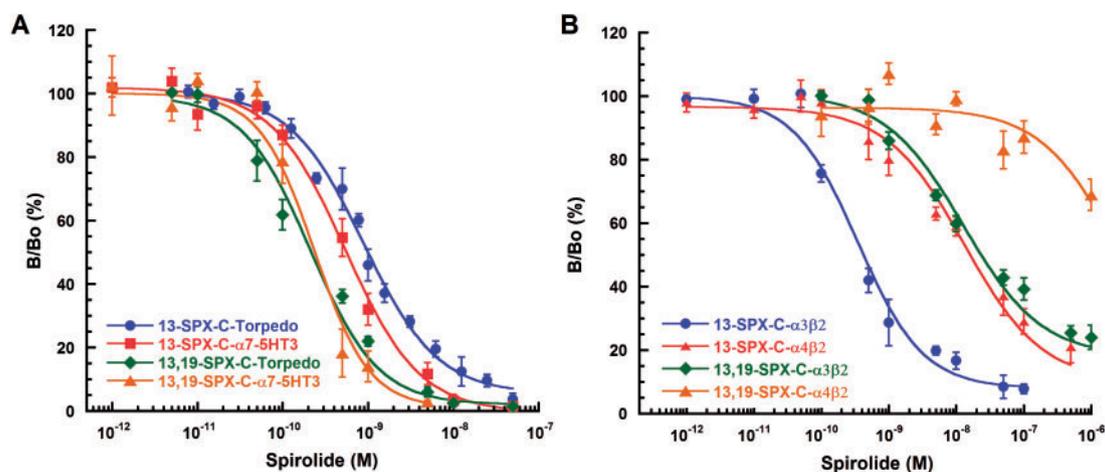


FIG. 6. Inhibition of specific [125 I] α -bungarotoxin (A) and [3 H]epibatidine binding (B) on nAChRs by spiroimine toxins. Binding inhibition by increasing the concentrations of 13,19-SPX-C or 13-SPX-C on *Torpedo* membranes or HEK-293 cells expressing the chimeric neuronal α 7-5-HT $_3$ nAChR (A), and neuronal-type α 4 β 2 and α 3 β 2 nAChRs, respectively. The results are expressed as the ratio of the specific tracer binding measured with (B), or without the spiroimide C analogs (Bo) expressed in percentage. Curve fitting was based on a nonlinear regression analysis using the Hill's equation. Data are mean values \pm SEM of 3 distinct inhibition experiments made in duplicate.

TABLE 2. Affinity Constants of 13,19-SPX-C and 13-SPX-C on Muscular and Neuronal Nicotinic Receptor Subtypes Determined by Equilibrium Competition Binding Experiments

$K_i \pm$ SEM (nM)	$\alpha_{12}\beta_1\gamma\delta$ (<i>Torpedo</i>)	α 7-5HT3 (Chick)	α 3 β 2 (Human)	α 4 β 2 (Human)
13,19-SPX-C	0.017 \pm 0.003	0.22 \pm 0.06	0.51 \pm 0.14	53 \pm 25
13-SPX-C	0.080 \pm 0.002 ^a	0.53 \pm 0.08	0.021 \pm 0.005 ^a	0.58 \pm 0.07 ^a

^aCalculated from IC $_{50}$ values reported in Bourne et al. (2010).

almost irreversible and voltage-independent blockade of nicotinic currents. The apparent irreversible neuromuscular block produced by spiroimides on skeletal myocytes is in agreement with the unsurmountable effect observed for gymnodimine and 13-SPX-C in the inhibition of the nicotine-evoked responses on various neuronal nAChRs (Hauser et al., 2012). In addition, this mode of action looks like those produced by some α -neurotoxins from snake venoms (α -bungarotoxin, erabutoxin-b, and α -cobratoxin), which bind with high affinity to skeletal muscle nAChRs, producing flaccid paralysis (Katz and Miledi, 1973).

Functional analysis of the effect of 13-SPX-C and 13,19-SPX-C was also performed using voltage-clamp recordings on *Xenopus* oocytes, either microtransplanted with *Torpedo* $\alpha_{12}\beta_1\gamma\delta$ -rich membranes or expressing the human α 7 or α 4 β 2 nAChRs. The 2 phycotoxins blocked similarly, with higher affinity and in a concentration-dependent manner, the ACh-evoked nicotinic currents through muscular and α 7 subtypes when compared with α 4 β 2 subtype (Fig. 5 and Table 1).

Competition binding assays performed on muscular-type (*Torpedo* membrane) and neuronal (homopentameric α 7-5HT $_3$ and heteropentameric α 3 β 2, α 4 β 2) receptors allowed to determine the affinity constants of the spiroimides studied (Table 2). Thus, 13-SPX-C appears as a highly potent and relatively nonselective ligand of nAChRs, while 13,19-SPX-C is more selective of the muscular-type receptor and interacts more weakly with neuronal receptor, especially the α 4 β 2 subtype. Even if the antagonist potencies determined in functional assays (IC $_{50}$ values) and affinity constants (K_i) measured in competition binding may differ, due to differences in equilibrium conditions of these

experiments, the higher and lower affinities of both phycotoxins, respectively, on *Torpedo* and α 4 β 2 nAChRs, were observed with the 2 approaches. Furthermore, the weak increase in affinity (2- to 4-fold) of the 13,19-SPX-C when compared with the 13-SPX-C analog for the muscular and α 7 subtypes is also commonly observed. Nevertheless, the large affinity decrease measured on α 4 β 2 with binding assays was only partially observed in electrophysiological measurements. This difference could be due to the presence in cell binding preparations of intracellular immature receptors that may differ functionally to mature receptors expressed at the oocyte plasma membrane. Another explanation could be related to variation in subunit stoichiometry in the expressed receptor used in these different experiments, variation which is known to affect the pharmacological property of the α 4 β 2 receptor (Nelson et al., 2003). Supporting this hypothesis, Hauser et al. (2012) recently reported a variation of the 13-SPX-C affinity on α 4 β 2 subtype ranging from 96 nM in binding experiments to 0.7 nM in functional assays.

The structural determinants involved in the interaction of 13-SPX-C and gymnodimine A with a functional surrogate of the ligand binding domain of nAChR, the *Aplysia* acetylcholine binding protein (AChBP), were recently identified with the 2.4 Å resolution of the complex between these 2 toxins and the *Aplysia*-AChBP (Bourne et al., 2010). The subnanomolar affinity of the 13-SPX-C for various nAChRs subtypes was explained by the remarkable surface complementarity between this toxin and the ligand binding pocket, as shown by the deeply buried location of the toxin at the binding interface. In this complex, the bulky and rigid bis-spiroacetal ring system that emerges on the apical side of the interface tightly interacts with the tip of

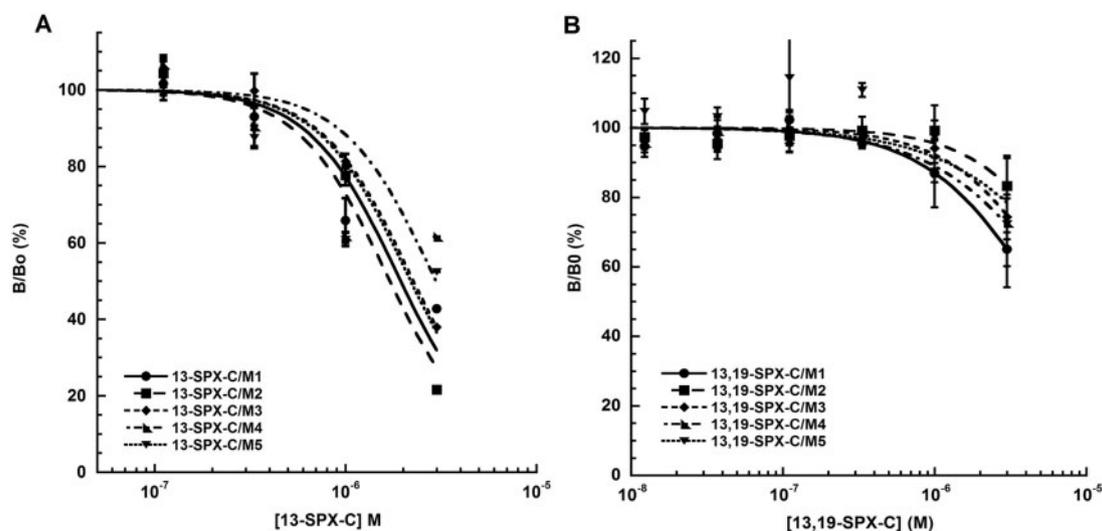


FIG. 7. Inhibition of specific [^3H]-NMS binding by spiroimine toxins. Inhibition of binding by increasing concentrations of 13-SPX-C (A) or 13,19-SPX-C (B) on CHO cells expressing the 5 human mAChR subtypes. The results are expressed as the ratio of the specific tracer binding measured with (B), or without the spiroimide C analogs (Bo) expressed in percentage. Curve fitting was based on a nonlinear regression analysis using the Hill's equation. Data are mean values \pm SEM of 2 distinct inhibition experiments made in duplicate.

TABLE 3. Affinity Constants of 13,19-SPX-C and 13-SPX-C on 5 Human mAChR Subtypes Determined by Equilibrium Competition Binding Experiments Using [^3H]-NMS

mAChR	$K_i \pm \text{SEM}$ (μM)				
	M_1	M_2	M_3	M_4	M_5
13,19-SPX-C	$1.10 \pm 0.20^*$	5.10 ± 3.60	3.10 ± 1.40	0.60 ± 0.10	5.40 ± 2.50
13-SPX-C	0.45 ± 0.07	1.09 ± 0.42	1.22 ± 0.26	0.33 ± 0.14	1.62 ± 0.23

*Data are mean values \pm SEM of 2 distinct inhibition experiments made in duplicate.

loop C and loop E, respectively, located on the principal and complementary interaction faces. In particular, it was found that the tetrahydropyran ring B interacts with Val108 (loop E) from the (-) face, suggesting that the hydroxyl and methyl substitutions on ring B are ideally positioned to hydrogen bond with Tyr195 (Bourne *et al.*, 2010). In this study, the interactions of 13-SPX-C and 13,19-SPX-C with the extracellular domain of 3 nAChRs subtypes (human $\alpha 7$, human $\alpha 4\beta 2$, and *Torpedo* $\alpha 1_2\beta 1\gamma\delta$) were evaluated using molecular docking. The resulting receptor-ligand complexes are represented in Figure 8, showing binding modes similar with those observed in the crystal structure of 13-SPX-C in complex with *Aplysia*-AChBP (Bourne *et al.*, 2010) and in the docking complexes between pinnatoxin A and various nAChRs subtypes (Ar oz *et al.*, 2011). In all complexes generated in this study, we observed a conserved hydrogen bond interaction between the spiroimine NH group and the backbone C=O from Trp147. The butyrolactone ring of both 13-SPX-C and 13,19-SPX-C ligands interacts through hydrogen bonds with the side chain of Arg186 from the $\alpha 7$ and $\alpha 4\beta 2$ receptors, whereas the *Torpedo* $\alpha 1_2\beta 1\gamma\delta$ subtype has a Val residue in this position, and an alternative hydrogen bond is established with the side chain of Lys143 (Fig. 8). Additionally, the nature of the amino acid residues present in the positions 77 and 108 has an important influence on the positioning of the spiroketal ring system in the binding site and therefore on the subtype selectivity that was experimentally determined (Tables 1 and 2). The subtypes $\alpha 7$ and $\alpha 1_2\beta 1\gamma\delta$ have relatively small residues in the position 77 (Thr and Ile, respectively), which can accommodate the spiroketal fragment, and bulky hydrophobic residues in the position 108 (Leu in both cases) which can stabilize the ligand

through nonpolar interactions. In contrast, the $\alpha 4\beta 2$ nAChR subtype presents a Lys residue in the position 77, whose size encumbers the accommodation of the spiroketal ring system in this region of the binding site. Moreover, the smaller Val residue in the position 108 is unable to provide the stabilizing nonpolar interactions that were evidenced for the other 2 subtypes. As shown in Figure 8, the overall consequence is the repositioning of the spiroketal fragment in the binding site of $\alpha 4\beta 2$ nAChRs, which also hampers the hydrogen bond interactions that were observed between this fragment and the side chain of Tyr195.

Finally, it was previously suggested that the impairment of the cholinergic neurotransmission associated with the macrocyclic imine phycotoxins might be due to their action on muscarinic receptors. For example, transcriptional analyses revealed an upregulation of various mAChR gene expression after 13-SPX-C treatment (Gill *et al.*, 2003). In addition, this toxin was shown either to reduce the binding of muscarinic ligands to neuroblastoma cells (Wandscheer *et al.*, 2010) or to produce no appreciable effect on rat cortical or TE671 cell membranes (Hauser *et al.*, 2012). Thus, to directly study the potential effect of 13-SPX-C and 13,19-SPX-C on mAChRs, their ability to interact with these targets was evaluated with competition binding experiments on membrane of cells expressing 5 human muscarinic receptor subtypes. Both toxins interacted only with very low affinity with the 5 mAChR subtypes (micromolar range), 3 to 4 orders of magnitude lower when compared with nAChR subtypes. In conclusion, the toxicological profile on rodent bioassays of both 13-SPX-C and 13,19-SPX-C, which are recognized as "fast-acting toxins", can be explained by their specific high

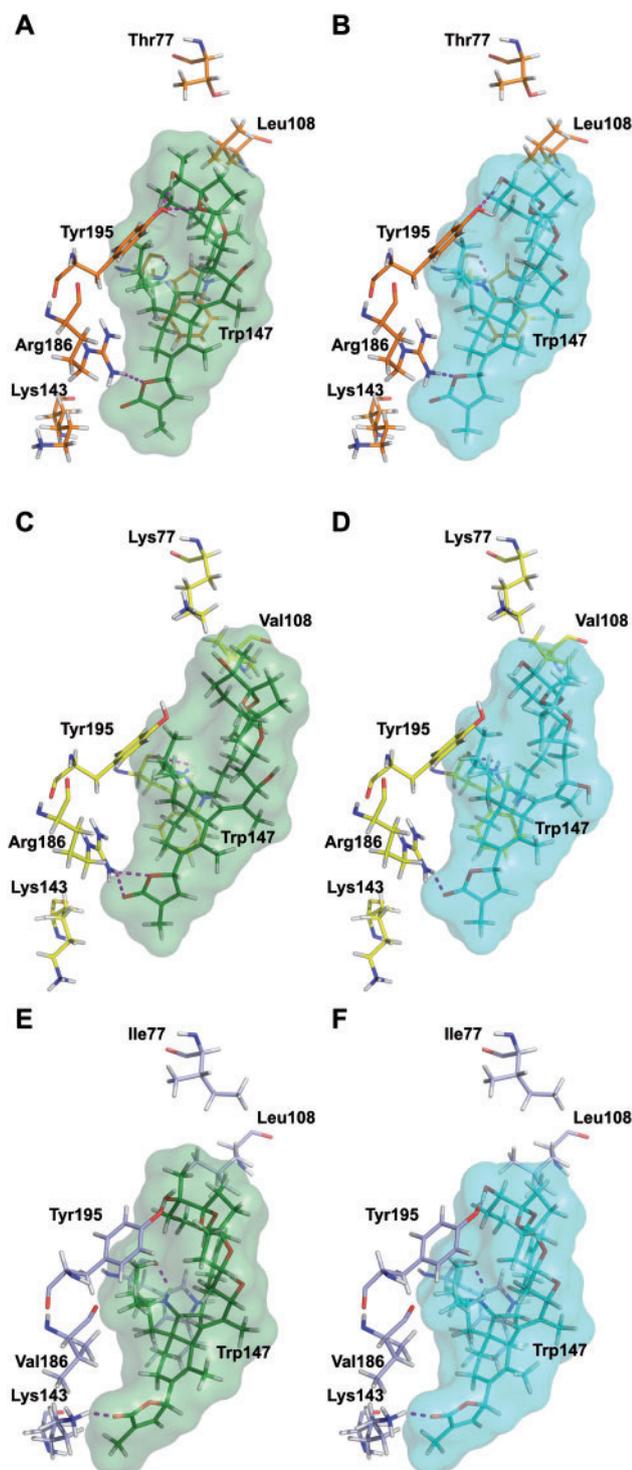


FIG. 8. Representative protein-ligand interactions in the docking complexes of 13-SPX-C (A, C, and E), and 13,19-SPX-C (B, D, and F) with 3 nAChRs subtypes : human $\alpha 7$ (A and B, $\alpha 7$ - $\alpha 7$ interface), human $\alpha 4\beta 2$ (C and D, $\alpha 4$ - $\beta 2$ interface), and *Torpedo* $\alpha 1_2\beta 1\gamma\delta$ (E and F, $\alpha 1$ - δ interface). Only amino acids interacting through hydrogen bonds with the ligand or involved in subtype selectivity as well as the residues from equivalent positions in the sequence alignment are shown. Residue numbering is the same as in Bourne et al. (2010).

affinity interaction with muscle-type nAChRs existing at the neuromuscular junction of skeletal muscle and with the major neuronal nAChRs present in the peripheral and central nervous system.

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