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Discovery and characterization of EII_B, a new α-conotoxin from *Conus ermineus* venom by nAChRs affinity capture monitored by MALDI-TOF/TOF mass spectrometry

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ABSTRACT

Animal toxins are peptides that often bind with remarkable affinity and selectivity to membrane receptors such as nicotinic acetylcholine receptors (nAChRs). The latter are, for example, targeted by α-conotoxins, a family of peptide toxins produced by venomous cone snails. nAChRs are implicated in numerous physiological processes explaining why the design of new pharmacological tools and the discovery of potential innovative drugs targeting these receptor channels appear so important. This work describes a methodology developed to discover new ligands of nAChRs from complex mixtures of peptides. The methodology was set up by the incubation of *Torpedo marmorata* electrocyte membranes rich in nAChRs with BSA tryptic digests (>100 peptides) doped by small amounts of known nAChRs ligands (α-conotoxins). Peptides that bind to the receptors were purified and analyzed by MALDI-TOF/TOF mass spectrometry which revealed an enrichment of α-conotoxins in membrane-containing fractions. This result exhibits the binding of α-conotoxins to nAChRs. Negative controls were performed to demonstrate the specificity of the binding. The usefulness and the power of the methodology were also investigated for a discovery issue. The workflow was then applied to the screening of *Conus ermineus* crude venom, aiming at characterizing new nAChRs ligands from this venom, which has not been extensively investigated to date. The methodology validated our experiments by allowing us to bind two α-conotoxins (α-EI and α-EIIA) which have already been described as nAChRs ligands. Moreover, a new conotoxin, never described to date, was also captured, identified and sequenced from this venom. Classical pharmacology tests by radioligand binding using a synthetic homologue of the toxin confirm the activity of the new peptide, called α-EII_B. The K_i value of this peptide for *Torpedo* nicotinic receptors was measured at 2.2 ± 0.7 nM.

1. Introduction

Nicotinic acetylcholine receptors (nAChRs) are implicated in important physiological processes mediating chemical communication in the peripheral and central nervous systems. They have been validated as drug targets for several pathologies such as schizophrenia, drug addiction, Alzheimer's disease and Parkinson's disease (Taly et al., 2009). nAChRs are members of the pentameric Cys-loop ligand-gated ion channel superfamily. In vertebrates, 17 genetically distinct subunits compose nAChR ($\alpha_{1\text{-}10}$, $\beta_{1\text{-}4}$, γ, δ, ε) as homo- or heteropentamers (Molgó et al., 2013; Taly et al., 2009; Yenugonda et al., 2013). This high number of subtypes induces an important diversity of physiological roles. Therefore, specific care must be taken in the elaboration of new drugs to optimize the therapeutic effects against the side effects, the selectivity of the nAChRs probes being then crucial. Standard radio-ligand binding assays and electrophysiology recordings are intensively used to characterize nAChRs ligands (Aronstam and Witkop, 1981). Although, radio-ligand binding is compatible with High-Throughput Screening (HTS), its major limitation is the requirement for a ligand of good affinity for the selected nAChR and to be compatible with radio-labeling. Moreover, α-conotoxin binding analysis through competition with [^{125}I]-α-BgTx is often a hard task as the results are directly depending on the incubation time. These

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radio-assays also implies expensive manufacturing and waste treatments, limiting the use of such assays to specialized laboratories.

During the last decade, intensive efforts have been made to develop high-throughput receptor-binding and electrophysiological assays to screen large libraries of compounds on nAChRs (Molgó et al., 2013). Other physico-chemical techniques have been exploited to develop binding assays. For example, the combination of receptor engineering (solid-phase receptors (Rodríguez et al., 2011), microsphere receptors (Rodríguez et al., 2013)), building of large ligand library (combinatorial chemistry (Yenugonda et al., 2013), virtual screening (Kombo and Bencherif, 2013)) and improvement of detection systems (fluorescence polarization (Fonfría et al., 2010; Vilariño et al., 2009), Surface Plasmon Resonance (Retra et al., 2010), Microplate receptor-binding assays (Aráoz et al., 2012) or calorimetry (Feig, 2007)) can be cited for this purpose. Alternatively, automated electrophysiology measurement devices have been developed and are widely used in an HTS framework (Dunlop et al., 2008). While all these techniques enable the detection of a potential ligand among a mixture of compounds, none of them is capable of identifying directly the molecule that binds to the receptor from a compound mixture. In this context, the group of Kool developed an elegant strategy to solve this problem. It consists in a flow cytometry-based cellular assay, coupled to a LC system with parallel MS detection. Thanks to this innovative combination, FC assay can be applied to complex mixtures of compounds. (Heus et al., 2014, 2013, 2010, Otvos et al., 2016a, 2016b, 2013).

Animal venoms are rich sources of ligands acting on various receptors such as ion channels or GPCRs. The major limitation of venom screening is the final isolation of the active toxin.

Affinity-selection mass spectrometry (AS-MS) is based on an incubation of a purified soluble target with a library of potential ligands. Target–ligand complexes are then purified by size-exclusion chromatography (Wanner and Höfner, 2007; Whitehurst and Annis, 2008). A reverse-phase chromatography step in denaturing conditions dissociates the complex allowing the detection and characterization of the ligand by mass spectrometry. This methodology needs pure and soluble receptors, which are hardly available when the considered receptors are naturally embedded in the cell membrane. In this way, Frontal Affinity Chromatography coupled to Mass Spectrometry (FAC-MS) is based on the continuous infusion of ligands over a protein target, immobilized onto a solid phase. The injection of a mixture of molecules produces variable retention times depending on the affinity of each molecule for the target (Slon-Usakiewicz et al., 2005). This methodology allows the detection of ligands ranging from pM to 100 μM affinities. It has been used to characterize ligand of Human Serum Albumin (de Moraes et al., 2014; Marszałł et al., 2011), of alpha-glucosidase (Tao et al., 2013), of G Protein Coupled Receptors (Temporini et al., 2013, 2009) or of melittin (McFadden et al., 2010). However, all these examples concern the binding of small organic molecules, peptide ligands being more difficult to capture and less described in the literature (Ershov et al., 2012; Fu et al., 2012; Puch et al., 2011). In 2015, we developed a mass spectrometry-based methodology to detect and identify peptides that bind to GPCRs (Cologna et al., 2015). This approach, designed as a proof-of-concept, did not however describe any screening of peptide library and any discovery of new ligand. Based on the observation that cone snail venoms contain α-conotoxins (Lewis et al., 2012), potential ligands of nAChRs, the present work extends the approach to screen *Conus ermineus* venom for new nAChRs ligands. nAChRs coming from *T. marmorata* were chosen not only to mimic muscle-type $\alpha1_2\beta\gamma\delta$ subtype, but also because it has been described as being the structural prototype of all members of the nAChR family (Tsetlin and Kasheverov, 2014). It is important to note that the method is not limited to overexpressed receptors. One could apply the same protocol to classical membranes or even living cells. The sensitivity would substantially decrease, but a concentration step, implemented to the method, would very probably lead to similar results. Experimental workflow was set up by binding known ligands from synthetic mixtures of peptides using *Torpedo* membranes (positive control), membranes over-expressing vasopressin type-2 receptors (negative control) and by performing competition with nicotine, an agonist of nAChRs. The methodology was finally applied to the discovery of nAChRs ligands from the crude venom of *Conus ermineus*. Three nAChRs ligands were finally captured. Two of them having been already described: α-EI (Martinez et al., 1995) and α-EII$_A$ (Quinton et al., 2013). The third one was unknown and was characterized by MS/MS experiments. A synthetic homologuous was produced by solid-phase peptide synthesis. Radio-binding experiments confirmed finally the binding to nAChRs. The new conotoxin was finally named α-EII$_B$.

2. Materials and methods

2.1. Receptors

Torpedo electrocyte membranes rich in Nicotinic Acetylcholine Receptors (nAChRs, subtype $\alpha_2\beta\gamma\delta$, over-expression rate ~40% of membrane protein content) were purified from the electric organ of *Torpedo marmorata* as previously described (Aráoz et al., 2012; Hill et al., 1991; Vilariño et al., 2009). Human vasopressin receptors (V_2Rs) membrane preparations were purchased from PerkinElmer (Waltham, USA) and stored at −80 °C according to the corporation's instruction manual. The product number is No 6110541400UA (PerkinElmer, Waltham, USA). The V_2Rs membranes belong to lot no:1551815, with a Bmax of 7.4 pmol mg^{-1} membrane protein and Kd for [^{3}H]-(Arg8)-vasopressin of 0.38 nmol.L^{-1}, both determined using radioactive saturation binding assay. V_2Rs were over expressed in Chinese-hamster ovary (CHO-K1) cell line.

2.2. Chemicals

Nicotine (racemic), UltraPure™Tris Hydrochloride (Invitrogen™), magnesium chloride, ethylenediaminetetraacetic acid, trifluoroacetic acid (TFA), formic acid (FA), dithiothreitol (DTT), and α-cyano-4-hydroxycinnamic acid (CHCA) and ammonium hydrogenocarbonate (NH_4HCO_3) were all purchased from Sigma–Aldrich® (Steinheim, Germany). Acetonitrile (ACN) was purchased from Biosolve (Valkenswaard, the Netherlands). All chemicals were used without further purification except for water, which was purified using a MilliQ water purification system (Millipore, Billerica, MA, USA). The Fmoc protected amino acids, Pyroglutamic acid and HCTU were purchased at Activotec (Cambridge, UK). The Fmoc-Hyp (tBu)-OH was from Novabiochem [Merck (Darmstadt, Germany)]. The ChemMatrix rinkAmide resin, N-methylmorpholine (NMM) and acetic anhydride (Ac_2O) were obtained from Sigma Aldrich (St Quentin en Fallavier, France). N-methyl-2-pyrrolidone (NMP) was from Merck (Darmstadt, Germany).

2.3. Peptides

Conotoxins described in Table 1 were purchased from Sigma–Aldrich® (Steinheim, Germany). To test the method on a more complex mixture of peptides, chromatographic fractions of *Conus ermineus* snail venom was studied. *Conus* venom was obtained and fractionated as described elsewhere (Quinton et al., 2013).