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In vitro labelling of muscle type nicotinic receptors using a fluorophore-conjugated pinnatoxin F derivative

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ABSTRACT

Fluorescent molecules are regularly utilised to study ligand–receptor interactions. Many ligands for nicotinic receptors have been conjugated with fluorophores to study receptor kinetics, recycling and ligand binding characteristics. These include small agonist molecules, as well as large peptidic antagonists. However, no small molecule antagonists have been investigated using this method. Pinnatoxin F is a newly discovered non-peptidic muscle type nicotinic receptor antagonist produced by the marine dinoflagellate species *Vulcanodinium rugosum*. This molecule has the potential for conjugation to a fluorophore, allowing subsequent visualisation of interactions with nicotinic receptors. Pinnatoxin F was modified by addition of diaminopolyether spacers, to which a fluorophore (VivoTag® 645) was conjugated. The fluorescent pinnatoxin was then applied to muscle sections from *thy1-YFP-H* transgenic mice, which express YFP in motor nerves, to allow direct visualization of fluorescent binding at the neuromuscular junction. The addition of both the diaminopolyether spacer and the VivoTag® 645 reduced the potency of pinnatoxin F, as evidenced by a reduction in *in vitro* neuromuscular blocking activity and *in vivo* toxicity. Despite this reduced potency, the fluorescent molecule selectively labelled endplate regions in *thy1*-YFP mouse muscle sections and this labelling was inhibited by pre-exposure of muscle sections to native pinnatoxin F or the nicotinic antagonist α-bungarotoxin. This study proves nicotinic receptor binding activity of pinnatoxin F and is the first example of a fluorophore-conjugated small-molecule antagonist for nicotinic receptors. These results indicate the potential for other small-molecule nicotinic receptor antagonists to be fluorescently labelled and used as probes for specific nicotinic receptor subtypes.

1. Introduction

Ligand binding and receptor function can be studied using fluorescent ligands, facilitating direct visualization of binding sites and molecular re-organisation during receptor-gating. Nicotinic receptors (nAChRs) are pentameric ligand-gated ion channels that mediate signal

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transduction at the neuromuscular junction (NMJ) and have been probed using fluorescent ligands for some 40 years. Fluorescent agonists are most commonly used to drive receptors into a desensitized state that can be more easily probed (Auerbach, 2003; Edelstein et al., 1997). Initial attempts involved the creation of fluorescent ACh analogues, through the attachment of fluorophores such as dansyl, pyrene, *N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl) (NBD) and 7-diethylaminocoumarin (DEAC) (Barrantes et al., 1975; Cohen and Changeux, 1973; Jürss et al., 1979; Krieger et al., 2008; Song et al., 2003; Waksman et al., 1976). Fluorescent analogues of the natural nAChR agonists epibatidine and anabaseine have also recently been described and used for more selective nAChR fluorescent studies (Grandl et al., 2007; Talley et al., 2006).

Fluorophore-conjugated natural antagonists have also been utilized, with in many cases the highly potent peptidic snake toxin α-bungarotoxin (α-BgTx) conjugated to a wide range of different fluorophores including rhodamine, fluorescein isothiocyanate (FITC), Alexa dyes or biotin, for use in immunofluorescence studies with streptavidin-linked fluorophores (Anderson and Cohen, 1974; Baier et al., 2010; Bruneau et al., 2005; Qu et al., 1990; Wheeler et al., 1994). Other natural peptidic antagonists used in fluorescent studies include α-cobratoxin and conotoxins (Hone et al., 2009; Johnson et al., 1990; Yang et al., 2011). However, there have been no reports to date of the conjugation of fluorophores to non-peptide (i.e. small-molecule) nAChR antagonists.

Pinnatoxin F (PnTX-F) is a member of the pinnatoxin family, produced by the dinoflagellate species *Vulcanodinium rugosum* (Nézan and Chomérat, 2011; Rhodes et al., 2011). As a member of the cyclic imine group of toxins, PnTX-F contains a 6, 7-linked cyclic imine moiety within its chemical structure (Fig 1). This moiety is thought to be the key structural requirement for the toxicity of this toxin group, which also includes gymnodimines, spirolides, pteriatoxins and prorocentrolides (Molgó et al., 2007). PnTX-F is highly toxic in rodent bioassays, causing death within minutes via respiratory depression (Munday et al., 2012; Selwood et al., 2010). Such activity is believed to be due to the pinnatoxins' ability to bind to and antagonize both muscle-type and neuronal nAChRs. In particular, PnTX-F has demonstrated low nanomolar inhibition of neuromuscular transmission *in vitro* (Araoz et al., 2011; Hellyer et al., 2013; Hess et al., 2013).

The aim of the current study was to conjugate a red fluorescent dye (VivoTag® 645) to PnTX-F, to enable visualization of PnTX-F binding at the mammalian NMJ. The biological and physical properties of the VivoTag® coupled pinnatoxin were examined using *in vitro* and *in vivo* toxicity studies and *in vitro* fluorescent co-localisation studies in muscle sections from *thy1-YFP-H* transgenic mice, whose motor nerves express yellow fluorescent protein (YFP).

2. Materials and methods

2.1. Synthesis of aminated pinnatoxin F derivatives

Pure PnTX-F was isolated from cultured *Vulcanodium rugosum* cells, following previously described procedures (Selwood et al., 2010). All reactions were monitored by LC–MS using a Waters Acquity uPLC (Waters, Milford, MA) coupled to a Waters–Micromass Quattro Premier triple quadrupole mass spectrometer (Manchester, U.K.).

1.4 mmol of 2,2′-(ethylenedioxy)bis(ethylamine) (Sigma–Aldrich, St Louis, MO) was added to 0.52 μmoles of dry PnTX-F, and the mixture was then heated to 50° C. The reaction was complete after 3 h. The product PnTX-F–Sp1 was separated from the excess 2,2′-(ethylenedioxy)bis(ethylamine) over a 200 mg Strata-X SPE cartridge (Phenomenex, Torrance, CA) using a water/methanol stepwise gradient. Fractions containing PnTX-F–Sp1 were combined and dried under a stream of nitrogen. A smaller quantity of PnTX-F–Sp2 was prepared following this protocol using the diaminopolyether 4,7,10-trioxa-1,13-tridecanedimine.

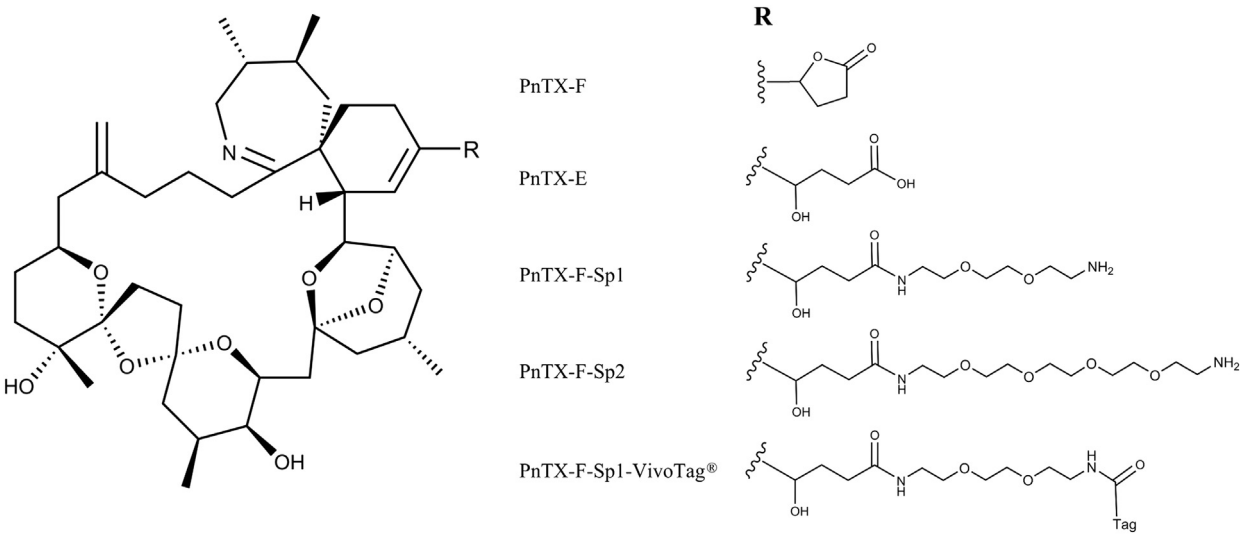

Fig. 1. Chemical structures of pinnatoxin F and derivatives.