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Glycosylphosphatidylinositol (GPI)-anchoring of mamba toxins enables cell-restricted receptor silencing

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ABSTRACT

Muscarinic toxins (MTs) are snake venom peptides found to selectively target specific subtypes of G-protein-coupled receptors. In here, we have attached a glycosylphosphatidylinositol (GPI) tail to three different toxin molecules and evaluated their receptor-blocking effects in a heterologous expression system. MT7–GPI remained anchored to the cell surface and selectively inhibited M_1 muscarinic receptor signaling expressed in the same cell. To further demonstrate the utility of the GPI tail, we generated MT3- and MTα-like gene sequences and fused these to the signal sequence for GPI attachment. Functional assessment of these membrane-anchored toxins on coexpressed target receptors indicated a prominent antagonistic effect. In ligand binding experiments the GPI-anchored toxins were found to exhibit similar selection profiles among receptor subtypes as the soluble toxins. The results indicate that GPI attachment of MTs and related receptor toxins could be used to assess the role of receptor subtypes in specific organs or even cells *in vivo* by transgenic approaches.

1. Introduction

Peptide neurotoxins isolated from venoms of the elapid branch of snakes have a high content of disulfides and usually adopt a distinctive three-finger fold [1,2]. Best known are the α-neurotoxins, acting on the ionotropic nicotinic acetylcholine receptors, and the muscarinic toxins (MTs) and related peptides, acting on G-protein-coupled receptors (GPCRs) [3,4]. MT7, or m1-toxin, from the green mamba is highly selective for the M_1 muscarinic receptor, to which it binds in a nearly irreversible manner and blocks the receptor activity [5,6]. MT3, although highly similar to MT7 at the peptide sequence level, binds only weakly to the M_1 receptor, but shows high affinity binding to the M_4 receptor [7,8]. Recently we have demonstrated that several toxins classified as MTs exhibit high affinity binding to receptors of the adrenergic system. MT1 and MTα are most potent at the α_{2B}-adrenoceptor, and MT3 also binds with high affinity to both α_1- and α_2-adrenoceptors [9,10]. In addition, two newly discovered three-finger toxins from the green mamba show selective binding to adrenoceptors [11,12].

The three-finger folded venom peptides have evolutionary kinship with Ly-6 antigens of the immune system [13,14]. The precise function for most of the Ly-6 antigens are still unknown, but this superfamily of proteins also contains molecules that modulate the nicotinic receptors [15–17]. For example, lynx1 (Ly-6/neurotoxin) was discovered as a cholinergic modulator with neurotoxin folding [15]. Lynx1 and several other members of the Ly-6 superfamily differ from snake neurotoxins in their mode of distribution. A glycosylphosphatidylinositol (GPI) tail added to the C-terminus anchors the molecule to the surface of cells in which they are expressed. This feature has been utilized in basic research to anchor venom toxins to cell surfaces by adding a signaling sequence for GPI synthesis to the toxin sequences [18,19]. Using a transgenic approach on zebrafish, it was shown that tethered α-bungarotoxin expressed in muscle cells *in vivo* efficiently blocked the nicotinic receptor currents, while cells with no tethered toxin expression remained normal [18]. Besides from snake three-finger toxins, a number of cone snail and spider toxins acting on various ion channels have also been subjected to GPI anchoring or membrane tethering through fusion with a single transmembrane helix [19]. With the discovery of new selective toxins for GPCRs, we wanted to evaluate if the GPI anchoring can be applied to toxins targeting these receptors. In here we describe the tethering of three toxins or toxin-like molecules and their effect on coexpressed receptors.

2. Materials and methods

2.1. Materials

[^{3}H]*N*-methylscopolamine ([^{3}H]NMS) (76 Ci/mmol) was purchased from Amersham Biosciences (Buckinghamshire, UK). Fura-2

Abbreviations: GPCR, G-protein-coupled receptor; GPI, glycosylphosphatidylinositol; eGFP, enhanced green fluorescent protein; MT, muscarinic toxin; NA, noradrenaline; CCh, carbachol, carbamoylcholine chloride; NMS, *N*-methylscopolamine; $\Delta[Ca^{2+}]_i$, change in intracellular free Ca^{2+} concentration.

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acetoxymethyl ester was from Molecular Probes (Eugene, OR). Atropine hemisulfate, carbamoylcholine chloride (carbachol) and noradrenaline bitartrate were from Sigma–Aldrich (Helsinki, Finland). Synthetic MT3 and MTα were from Peptide Institute (Osaka, Japan). Recombinantly expressed MT7 was purified from insect cell medium as described previously [6] and the concentration was determined spectrophotometrically (A_M = 15,900 M^{-1} cm^{-1}). Other chemicals used were of analytical grade quality.

2.2. DNA construction of toxins

We have previously described the construction of a synthetic DNA sequence for MT7 with an N-terminal secretion signal [6]. We first used this construct to amplify by PCR the secretion signal sequence and a short part of the MT7 sequence, and to introduce an EcoRI site (Fig. 1A). After this, complementary oligonucleotides were used to elongate and introduce restriction sites to the full length MT7 sequence. To add a linker region and a GPI signal sequence we used additional complementary oligonucleotides without the stop codon at the end of the MT7 sequence. The MT3-like toxin T3L and the MTα/MT1-like toxin Tα/1L were built in a similar way. The utilization of the EcoRI (T3L, Tα/1L) and SacII (Tα/1L) sites made the constructs slightly differ from MT3 and MTα, which is the reason for the different naming of our constructs (Fig 1B). A lysine residue at position 59 was changed to a glycine in T3L because a soluble version of T3L with K^{59} was expressed very poorly. Oligonucleotides were purchased TAG Copenhagen (Copenhagen, Denmark). Subcloning was performed using the pBluescript KS vector (Stratagene, La Jolla, CA) or the pFastBac1 vector (Invitrogen, Paisley, UK) and the correct sequences verified by automated DNA sequencing (Turku Centre for Biotechnology, Turku, Finland).

2.3. Baculovirus, cell culture and expression

The toxin constructs in pFastBac1 were used to generate recombinant baculovirus using the Bac-to-Bac expression system (Invitrogen). Baculoviruses for untagged receptor expression have been described earlier [6,9,20]. The enhanced Green Fluorescent Protein (eGFP)-tagged M_1 receptor was generated by fusion of the M_1 receptor cDNA to eGFP in pEGFP-N1 (BD Biosciences Clontech, Palo Alto, CA) after removal of the M_1 stop codon. The fused construct was then transferred to pFastBac1 for baculovirus generation.

Sf9 cells were grown in suspension at 27 °C in Grace's insect medium (Invitrogen) supplemented with 8% heat-inactivated fetal bovine serum (Invitrogen), 50 μg/ml streptomycin (Invitrogen), 50 U/ml penicillin (Invitrogen) and 0.02% Pluronic F68 (Sigma–Aldrich, Helsinki, Finland). For recombinant protein expression, Sf9 cells were plated on tissue culture dishes and allowed to attach. Coinfections were done by removing the medium and bathing the cells in a high-titer virus stock with either MT–GPI virus or an "empty" (no recombinant protein expression) virus in case of control for 30 min. After this, the virus stock was exchanged for respective receptor virus stock and incubation continued for 30 min. Fresh medium was then added to infected cultures and the infections were allowed to proceed for 24–26 h for the functional assays, and cultures used for radioligand binding were harvested after 48 h.

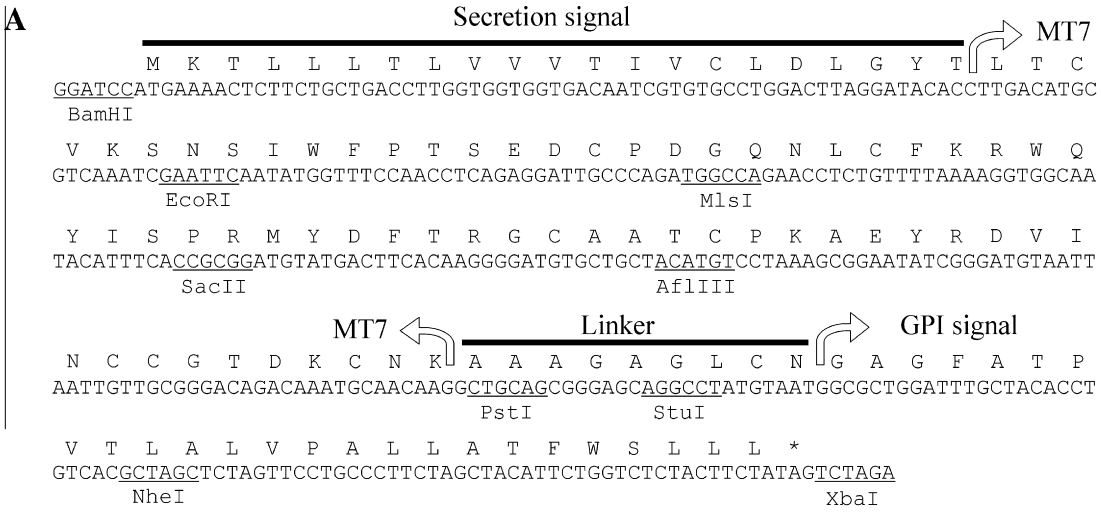

Fig. 1. Schematic drawing of synthesized toxins. (A) DNA and amino acid lettering of the whole MT7–GPI construct. The restriction sites underlined were incorporated in oligonucleotides used in building the DNA sequence and further utilized for the construction of T3L and Tα/1L. In the biosynthetic processing the secretion signal and the GPI signal are cleaved off and the GPI moiety attached to the new C-terminal asparagine. (B) Aligned sequences of selected toxins. The MT3-like construct used here, T3L, differ from MT3 at positions indicated in boldface. The MTα/MT1-like construct Tα/1L differ from MTα at positions indicated in boldface.