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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.

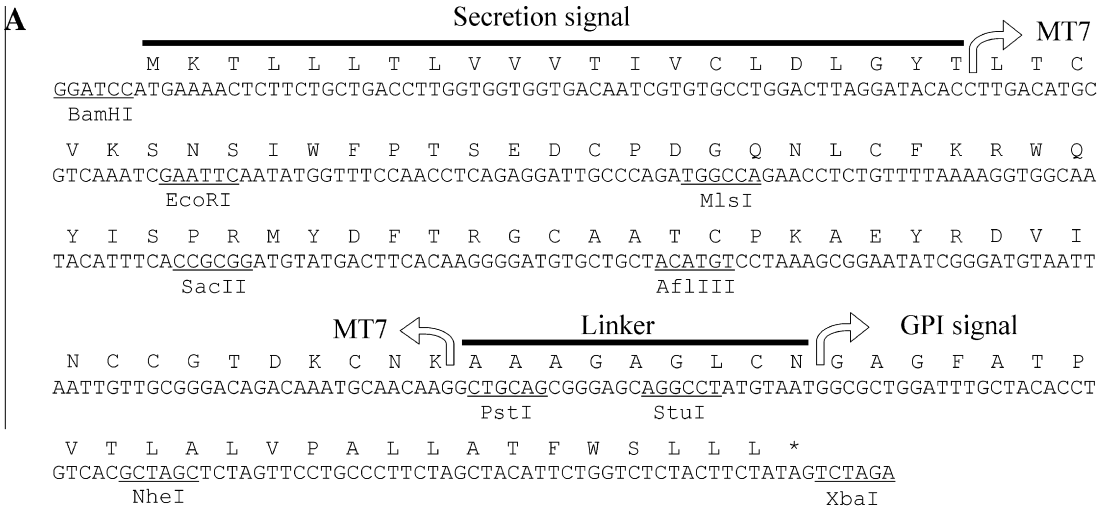

Fig. 1. Schematic drawing of synthesized toxins. (A) DNA and amino acid lettering of the whole MT7–GPI construct. The restriction sites underlined were incorporated in oligonucleotides used in building the DNA sequence and further utilized for the construction of T3L and Tα/1L. In the biosynthetic processing 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.

2.4. Intracellular Ca^{2+} concentration measurement

Cells were detached from plates by gentle pipetting, centrifuged 150*g* for 4 min, resuspended in a smaller volume of growth medium and incubated for 20 min with 4 μM fura-2 acetoxymethyl ester at room temperature. The cells were then diluted with two volumes of growth medium and stored in suspension at room temperature. When the effect of soluble toxin was tested, the toxin was added to an aliquote of cells at this stage and incubation continued for ⩾60 min. Before the experiment, cells were spun down in a microcentrifuge, washed twice in assay buffer and finally resuspended in assay buffer. In the case of soluble toxin testing, the washes and experiment were conducted with toxin in the buffer. The assay buffer was composed of 130 mM NaCl, 5.4 mM KCl, 1 mM $CaCl_2$, 10 mM glucose, 1.2 mM $MgCl_2$, 4.2 mM $NaHCO_3$, 7.3 mM NaH_2PO_4, 63 mM sucrose and 20 mM MES, pH adjusted to 6.3.

Fura-2 fluorescence recordings were monitored in a Hitachi F-2000 fluorescence spectrophotometer with cell suspensions in a thermostatted quartz cuvette with magnetic stirring. Recordings at 340 nm (excitation) and 505 nm (emission) were briefly interrupted for additions of agonist and finally calibrated using 0.04% Triton X-100 for F_{max} and 10 mM EGTA for F_{min}. Intracellular calcium concentrations were calculated according to the equation $[Ca^{2+}] = (F - F_{min})/(F_{max} - F) \times 255$ nM (K_d for fura-2–Ca^{2+} complex at 27 °C).

2.5. Radioligand binding

Infected cells from monolayer culture were harvested in phosphate-buffered saline solution, centrifuged 1500*g* and stored as pellets at −80 °C until use. For binding experiments the cells were resuspended in binding buffer (100 mM NaCl, 20 mM Hepes, 10 mM $MgCl_2$, 1 mM EDTA, pH adjusted to 7.4) and homogenized with an Ultra-Turrax homogenizer (IKA-Werke, Staufen, Germany). The homogenates (50–100 μg of protein) were first incubated with vehicle (control and MT–GPI cells) or soluble toxins (control cells) for 30 min after which 1 nM of [^{3}H]NMS was added and incubation continued for another 60 min. Atropine (10 μM) was used to determine nonspecific binding. Reactions were terminated by rapid filtration through prewashed GF/B filters followed by five washes with cold buffer containing 180 mM NaCl, 25 mM $MgCl_2$, and 20 mM Hepes, pH 7.4, in a microplate harvester. Radioactivity on filters was determined in a TopCount microplate scintillation counter (PerkinElmer, Waltham, MA).

3. Results and discussion

3.1. Construction of GPI-anchored toxins and evaluation of MT7–GPI activity

We have previously reported on the expression of secreted, soluble MT7 in Sf9 insect cells using the baculovirus expression vector system [6]. In this work we modified the synthetic DNA sequence for MT7 by introducing a number of restriction sites to facilitate mutagenesis studies and domain exchanges (Fig. 1A). To examine the GPI anchoring of the toxin, we removed the stop codon from the sequence and added a short linker and the GPI synthesis signal sequence in accordance with a report describing toxin tethering by GPI [18]. Upon expression the GPI signal sequence will be spliced off during processing and the GPI moiety attached to the new carboxy terminal asparagine [21]. The role of the linker is to position the toxin at some distance from the membrane and to confer rotational freedom. There appears not to be strict length requirements for the linker, although an extensive length may reduce the activity of tethered toxins [19]. We used the same nine amino acid length for the linker as was used for α- and κ-neurotoxins previously [18]. We also synthesized an MT3-like (T3L) sequence and an MTα/MT1-like (Tα/1L) sequence (Fig. 1B), which were similarly fused to the same linker and GPI signal sequence.

Coexpression of MT7–GPI and the muscarinic M_1 receptor resulted in complete silencing of the receptor response (Fig. 2A). MT7–GPI did not interfere with M_3 receptor signaling (Fig. 2B). This indicated that the tethered MT7 is able to block the M_1 receptor when expressed in the same cell, and with similar specificity as the soluble MT7. Coexpression of soluble MT7 and the M_1 receptor also resulted in marked inhibition of the receptor response (data not shown). This is likely a result of the secreted toxin binding to cell surface receptors. However, it could also mean that the biosynthetic processing and membrane localization of the receptor is

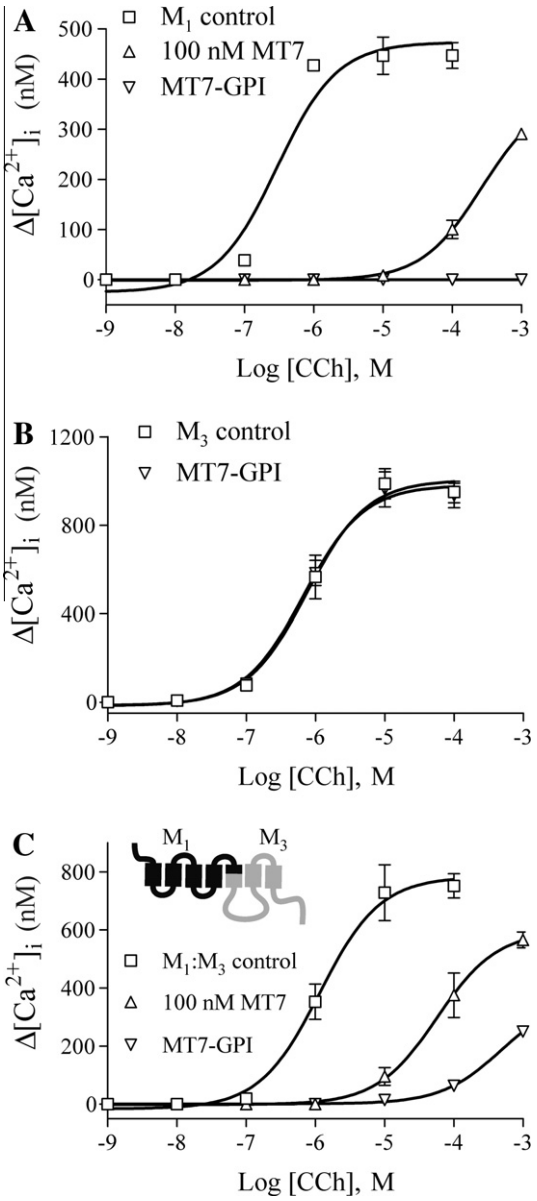

Fig. 2. Effect of MT7–GPI on coexpressed receptors. Sf9 cells expressing receptors alone or coexpressing receptors and MT7–GPI were assayed for carbachol (CCh)-induced Ca^{2+} responses. (A, C) Control cells were additionally tested with soluble MT7 for comparison. (C) The chimeric M_1:M_3 receptor is shown schematically with seven transmembrane helices and extra- and intracellular loops. Data points are means ± SD from three experiments.

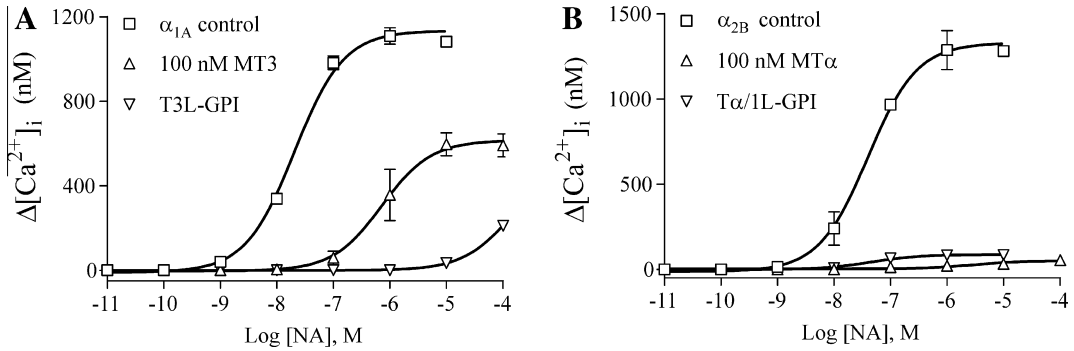

Fig. 3. Effect of GPI-anchored toxin-like molecules on adrenoceptors. Cells expressing human adrenoceptors, α_{1A} (A) α_{2B} (B), alone or together with GPI-anchored toxins were assayed for noradrenaline (NA)-stimulated Ca^{2+} increases. Control cells were also tested with soluble MT3 (A) and MTα (B). Data points are means ± SD from three experiments.

impaired by simultaneous expression of the receptor antagonist. To test various possibilities, we first coexpressed a chimaeric M_1:M_3 receptor that shows about tenfold reduced affinity for MT7 as compared to the M_1 receptor [22] with MT7–GPI (Fig. 2C). With this construct a response to high agonist concentrations was still detectable, demonstrating that the receptor reaches the membrane and signals to G-proteins. To go further, we added eGFP to the M_1 receptor and studied the cellular distribution of expressed protein. There were no detectable alterations in membrane localizations when M_1–eGFP was coexpressed with either soluble MT7 or MT7–GPI as compared to the control without toxin coexpression (Fig. S1). This suggested that both soluble and GPI-anchored MT7 block the cell surface receptors. When expressing soluble MT7, the toxin is secreted into the medium and thus free to bind any M_1 receptor in contact with the medium, whereas MT7–GPI should stay anchored to the cell in which it is expressed. To test this, we infected cells with virus for soluble MT7 and MT7–GPI and collected the media after 28 h. Incubation of M_1-expressing cells with the media resulted in partial inhibition of the M_1 response with soluble MT7 medium, but not with MT7–GPI medium (Fig. S2). Together these results demonstrate that MT7–GPI is expressed as a tethered toxin and blocks the M_1 receptor in a cell-restricted fashion.

3.2. GPI applicability to other toxin molecules

We have recently shown that MT3, which has long been considered an M_4 receptor-specific toxin [7], binds to and antagonizes several adrenoceptors [10]. The GPI-anchored MT3-like toxin (T3L-GPI) inhibited the coexpressed α_{1A}-adrenoceptor considerably more efficiently than 100 nM MT3 (Fig. 3A). Similarly, the MTα/MT1-like toxin (Tα/1L-GPI) almost completely blocked the α_{2B}-adrenoceptor response (Fig. 3B). This indicates that a GPI anchor can probably be added to all three-finger toxins acting on GPCRs without interfering with toxin binding to the receptors. The coexpression experiments do not provide estimates of the affinities of the GPI-linked toxins for the receptors. Earlier studies have shown that MT3 binds to the α_{1A}-adrenoceptor with an EC_{50} of 1.4 nM and MTα has a similar low nanomolar affinity (~3 nM) for the α_{2B}-adrenoceptor [9,10]. The anchoring to the membrane puts the toxin molecules in close vicinity of the receptors and such local concentrations of toxins may be difficult to interpret in terms of affinities. The experiments demonstrated though that the inhibitory effect of anchored toxins is prominent.

3.3. Anchored toxins in radioligand binding

Finally we wanted to test how the tethered toxins affect radioligand binding to homogenized cell preparations. The soluble MTs do not directly compete with orthosteric ligand binding, but they reduce the available binding sites through incompletely understood allosteric interactions [3,9,23]. We expressed the M_1 and M_4 receptors either alone or together with the GPI-anchored toxins and measured the [^{3}H]NMS binding sites (Fig. 4). MT7–GPI almost fully blocked the M_1 receptor with no effect on the M_4 receptor. Conversely, the T3L–GPI blocked the M_4 receptor like 100 nM MT3 and inhibited the radioligand binding to M_1 receptors approximately 50%. This is in line with the MT3 affinity for the M_1 receptor which is about 100-fold lower than for the M_4 receptor [7,8]. MTα does not show binding to the muscarinic receptors [9], whereas MT1 has moderate affinity (pIC_{50} = 6.5–7) for both M_1 and M_4 receptors [10,24–26]. MTα and MT1 differ at four positions: amino acids 31–33 and 57 (Fig. 1B). Based on our Tα/1L construct and the

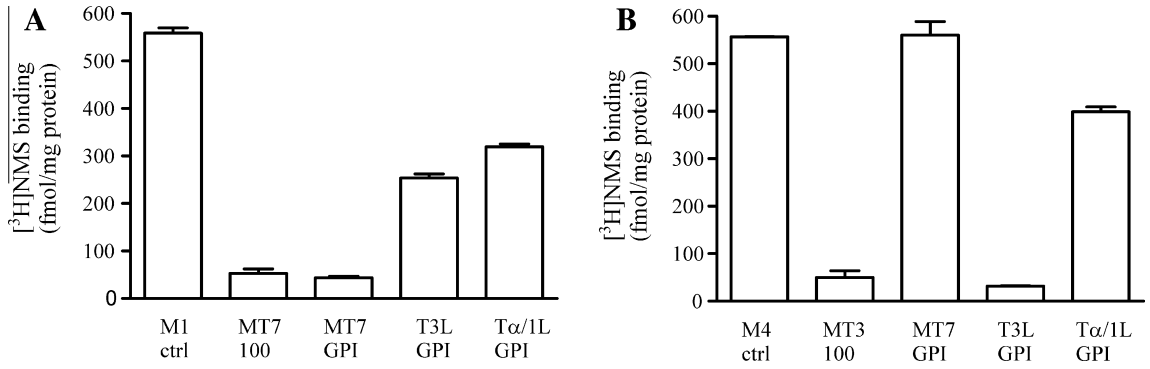

Fig. 4. [^{3}H]NMS binding to muscarinic receptors. Homogenates of cells expressing M_1 receptors (A) or M_4 receptors (B) either alone or together with GPI-anchored toxins were subjected to ligand binding experiments. Soluble MT7 and MT3 at concentrations of 100 nM give saturating inhibitions at M_1 and M_4 receptors, respectively. Data points are means ± SEM from three experiments performed with two different batches of cells.

reduced binding capacity of M_1 and M_4 receptors when coexpressed with the GPI-anchored toxin (Fig. 4), it is tempting to speculate that the proline (P^{33}) in MT1 confers muscarinic receptor affinity to this toxin as compared to MTα. However, this needs to be investigated with soluble toxins before firm conclusions can be drawn. MT1 binds to the α_{2B}-adrenoceptor with similar affinity as MTα [10]. Therefore it is reasonable to suggest that some residues of the toxins are either required for binding to certain receptors or oppositely prohibit or significantly weaken the binding to certain receptors. Such cases open up for technological manipulations of toxins to increase receptor selectivity or other binding properties.

4. Conclusion

The tethered nature of the endogenous nicotinic receptor modulator lynx1 brought about a new discipline in ionic channel research, where snake, spider and cone snail toxins have been used widely for decades [18,27]. In this report we have evaluated the applicability of tethering snake three-finger toxins active on GPCRs by adding a GPI tail. The most well studied GPCR toxin MT7 was found to exhibit strong selectivity and high affinity for the M_1 receptor in its membrane-anchored conformation. We also demonstrated that other toxins or toxin-like molecules with the same folding can be attached to the membrane without detectable alterations in selectivity between receptor subtypes. The complementary data provided here on the tethering technology should encourage future investigations on the specific roles of different receptor subtypes in tissues and organs.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2011.11.062.

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