Tethering Naturally Occurring Neurotechnique Peptide Toxins for Cell-Autonomous Modulation of Ion Channels and Receptors In Vivo

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Summary

The physiologies of cells depend on electrochemical signals carried by ion channels and receptors. Venomous animals produce an enormous variety of peptide toxins with high affinity for specific ion channels and receptors. The mammalian prototoxin lyn1 shares with α-bungarotoxin the ability to bind and modulate nicotinic receptors (nAChRs); however, lyn1 is tethered to the membrane via a GPI anchor. We show here that several classes of neurotoxins, including bungarotoxins and cobratoxins, retain their selective antagonistic properties when tethered to the membrane. Targeted elimination of nAChR function in zebrafish can be achieved with tethered α-bungarotoxin, silencing synaptic transmission without perturbing synapse formation. These studies harness the pharmacological properties of peptide toxins for use in genetic experiments. When combined with specific methods of cell and temporal expression, the extension of this approach to hundreds of naturally occurring peptide toxins opens a new landscape for cell-autonomous regulation of cellular physiology in vivo.

Introduction

Our current understanding of the development and function of the mammalian brain rests in large part on conclusions from experimental or accidental lesions that perturb CNS function. These studies have involved a wide variety of techniques, including the analysis of behavioral deficits in people suffering from disease or acute brain injury (Manns et al., 2003), sensory deprivation during development of experimental animals (Shatz and Stryker, 1978), pharmacologic manipulations of CNS receptors and ion channels (Bauer et al., 2002; Shatz and Stryker, 1988), and genetic ablations of specific CNS cell types in experimental animals (Champtiaux and Changeux, 2004; Kofuji et al., 2000; Nakazawa et al., 2004). This rich history and the advances in genetic techniques that enable in vivo manipulations of molecules, cells, and circuits (KOs, BACs, siRNA) provide a powerful incentive for the development of genetic approaches that extend our ability to alter the physiologic properties of specific neurons in vivo. For example, the ability to block presynaptic vesicle fusion using botulinum neurotoxins has been introduced as an effective approach to genetically interfere with neurotransmission in specific CNS cell types (Saint-Amant and Drappeau, 2001; Steinhardt et al., 1994). Likewise, targeted overexpression of K⁺ channels has been shown to be highly effective at silencing neuronal activity both in mammalian and Drosophila excitable cells (Johns et al., 1999; Nadeau et al., 2000; Nitabach et al., 2002; Paradis et al., 2001), although in mammalian cells this approach can induce apoptotic cell death in neurons (Nadeau et al., 2000).

Based on the knowledge that the electrical properties of neurons are controlled by a remarkable variety of ionic currents carried by many specific ion channels and receptors (Catterall, 1999; Champtiaux and Changeux, 2004; Coetzee et al., 1999) and the fact that small specifically acting soluble peptide neurotoxins have evolved to block many of these currents (Adams and Olivera, 1994; McIntosh et al., 1999; Olivera et al., 1994; Terlau and Olivera, 2004; Tsutlin and Hucho, 2004), we sought to harness the potential of peptide toxins as a means to genetically manipulate the functional properties of CNS neurons. To do this, we generated fusion proteins based on the lyn1 prototoxin (Ibañez-Tallon et al., 2002; Miwa et al., 1999) but carrying functional domains from naturally occurring peptide neurotoxins. These chimeric toxins are tethered to the cell surface via a GPI anchor yet retain their ability to specifically block ligand and voltage-gated receptors and ion channels cell autonomously in Xenopus oocytes and in vivo in zebrafish muscle fibers.

Results

Generation of Chimeric Tethered Toxins

Tethered toxin constructs containing functional domains from several classes of neurotoxins were expressed on the cell surface using an N-terminal signal peptide, a flexible linker, and sequences controlling the GPI anchor (Table 1). To test the generality of this approach, tethered toxins specific for different receptors and ion channels were prepared (Table 1). To target nAChRs, we have employed domains from the α- and κ-bungarotoxins and the α-conotoxins MII and PnIB (Servent et al., 1997; Terlau and Olivera, 2004; Tsutlin and Hucho, 2004). These were chosen because they have distinct specificities for nAChR subtypes, which would therefore allow manipulations of specific classes of these receptors in vivo. A tethered toxin construct carrying the broad spectrum Na⁺ channel inhibitor MrVIA was also constructed, since this toxin has recently been shown to block rat skeletal muscle and neuronal voltage-gated Na⁺ channels in Xenopus oocytes and in hippocampal pyramidal neurons (Terlau and Olivera, 2004; Terlau et al., 1996). The activity of this toxin, therefore, could be used to block Na⁺ currents that are required for action potential generation and propagation. Finally, to target
Figure 1. Construction of Tethered Toxins for Cell-Autonomous Inactivation of Ligand and Voltage-Gated Channels

(A) Model representing the binding of tethered α-bungarotoxin to the ACh binding site of the nAChR.

(B) Model representing the binding of tethered conotoxins to the channel pore of voltage-gated sodium and calcium channels.

(C) Amino acid alignments and disulfide bridges of lynx1 and toxins. Tethered toxin constructs include mature toxin sequences (green), synthetic polypeptide linkers of different lengths and composition (yellow), lynx1 hydrophobic C-terminal sequence for addition of the GPI anchor (orange), secretion signal sequences (gray), and epitope tag (blue).

voltage-gated Ca\textsuperscript{2+} channels, we have generated a tethered form of the ω-conotoxin MVIIA (Olivera et al., 1994). This toxin can differentiate N-type from P/Q-type, L-type, and T-type Ca\textsuperscript{2+} channels (Olivera et al., 1994). Specific blockades of Ca\textsuperscript{2+} currents can be used to examine a variety of crucial cellular functions, including mechanisms of presynaptic neurotransmitter release, signal transduction, and hormone secretion (Olivera et al., 1994; Tsien, 1983).

Specific Inactivation of nAChR Subtypes with Tethered Bungarotoxins and Conotoxins

Each of the tethered toxins was coinjected into Xenopus oocytes with its appropriate target(s) or controls and was assayed by two-electrode voltage clamp recordings. Given the strong structural homology of the bungarotoxins and lynx1 (Miwa et al., 1999), our previous demonstration that lynx1 modulates nAChRs when GPI anchored to the cell surface (Ibañez-Tallon et al., 2002), and the mode of binding of bungarotoxins to their target receptors (Figure 1A), we first tested whether these molecules could retain their activity and specificity when tethered to the cell surface. Recordings of nicotinic receptor activities in response to these tethered toxins confirmed this expectation (Figure 2). Thus, coexpression of tethered α-bungarotoxin (t-αBgtx) with muscle α1β1γδ or neuronal α7 nAChRs on the surface of oocytes completely blocks current flow in response to acetylcholine, but does not block α4β2 nAChR function. In contrast, tethered κ-bungarotoxin (t-κBgtx) does not block α1β1γδ nAChR function when coexpressed in the oocyte system. Rather, it specifically blocks the neuronal nAChR composed of α7 subunits and partially blocks α4β2 receptors, consistent with the relative affinities described in previous studies using soluble toxins (Table 1). The fact that the tethered bungarotoxins retained

Table 1. Toxins Used in This Study

<table>
<thead>
<tr>
<th>Toxin</th>
<th>Receptor/Ion Channel</th>
<th>Affinity</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-bungarotoxin</td>
<td>α1β1γδ nAChR</td>
<td>0.4 nM</td>
<td>1</td>
</tr>
<tr>
<td>α-bungarotoxin</td>
<td>α7 nAChR</td>
<td>0.95 nM</td>
<td>2</td>
</tr>
<tr>
<td>α-bungarotoxin</td>
<td>α6β2 nAChR</td>
<td>100 μM</td>
<td>3</td>
</tr>
<tr>
<td>κ-bungarotoxin</td>
<td>α1β1γδ nAChR</td>
<td>1 μM</td>
<td>4</td>
</tr>
<tr>
<td>κ-bungarotoxin</td>
<td>α7 nAChR</td>
<td>1–12 nM</td>
<td>2</td>
</tr>
<tr>
<td>κ-bungarotoxin</td>
<td>α4β2 nAChR</td>
<td>&gt;100 nM</td>
<td>5</td>
</tr>
<tr>
<td>α-conotoxin PhIB</td>
<td>α7 nAChR</td>
<td>61.3 nM</td>
<td>6</td>
</tr>
<tr>
<td>α-conotoxin MII</td>
<td>α3β2 nAChR</td>
<td>0.5 nM</td>
<td>7</td>
</tr>
<tr>
<td>μO-conotoxin MrVIA</td>
<td>Na, 1.2</td>
<td>200 nM</td>
<td>8</td>
</tr>
<tr>
<td>α2-conotoxin MVIIA</td>
<td>Ca, 2.2</td>
<td>1 nM</td>
<td>9</td>
</tr>
</tbody>
</table>

References: 1, Lukas et al., 1981; 2, Servent et al., 1997; 3, Grutter et al., 2003; 4, Loring et al., 1986; 5, Grant et al., 1998; 6, Luo et al., 1999; 7, Cartier et al., 1996; 8, Terlau et al., 1996; 9, Sato et al., 2005.
their efficacy and specificity for specific nAChRs prompted us to test whether a second structurally distinct class of toxins known to act on nAChRs could also retain their specificity when tethered to the cell surface (Figure 2). Thus, tethered conotoxins PnIB (t-PnIB) and MII (t-MII) were tested for their ability to inhibit nAChR currents in oocytes. As shown in Figure 2, t-PnIB retains its specificity for α7 nAChRs, resulting in a block of these currents without affecting either the muscle or neuronal α4β2 nAChR activities. As expected (Luo et al., 1999), t-MII does not inhibit the activity of these receptors.

Inactivation of Voltage-Gated Sodium and Calcium Channels with Tethered Conotoxins

The ability to use tethered conotoxins to manipulate their cognate target molecules presented the possibility of performing an astounding array of genetic experiments to manipulate the properties of cells in vivo. Thus, there are ~500 species of cone snails, each expressing a unique set of 50 to 200 peptide toxins, yielding a collection of nearly 50,000 conotoxins, of which less than 0.2% have been characterized (McIntosh et al., 1999). However, a significant proportion of these molecules (e.g., those that block voltage-gated ion channels) have been shown to block activity by binding in the vestibule of the ion channel to directly block ion flux (Olivera et al., 1994; Terlau and Olivera, 2004). Given the small size of the conotoxin functional domains and the increased distance and rotational flexibility required for a tethered toxin to bind properly within the vestibule of the channel (Figure 1B), it seemed probable that extension of this approach to this class of toxins might be problematic. To investigate this possibility, we next tested tethered toxins directed toward voltage-gated Na⁺ and Ca²⁺ channels. In both cases, we have been able to construct tethered toxins that retained both their activity and specificity against their target channels. Thus, the tethered μ-O-conotoxin MrVIA (t-MrVIA) blocked >90% of the current flux through Na, 1.2 channels in oocytes, without affecting N-type Ca²⁺ channel or shaker K⁺ channel function (Figure 3). In contrast, tethered ω-conotoxin MVIIA (t-MVIIA) coexpression completely blocked Ca,2,2 (N-type) Ca²⁺ channels, without affecting the Na,1.2 Na⁺ or shaker K⁺ channels. Taken together, our data indicate that toxins from several different classes can retain their specificity and activity when expressed as GPI-anchored molecules, effectively inhibiting currents that are fundamental to the physiologic activities of neurons and other cell types. It is important to note, however, that attempts to create tethered toxin constructs using toxin molecules that normally contain noncanonical amino acid residues have not generally been successful. For example, unsatisfactory results were obtained with several toxins in which trans-4-hydroxyproline was present in the venom, which was substituted by proline in our tethered toxin constructs. Thus, tethered toxin constructs for GID, PIIIA, and RIIIK all exhibited reduced or no activity when tested in oocytes (data not shown). We believe that this is due to the requirement for hydroxyproline in these toxins, as has been shown in the case of conotoxin GIIIA (Wakamatsu et al., 1992), although further experimentation will be required to test this idea.

Tethered Toxins Function Cell Autonomously

Maximal utility of the tethered toxin approach for in vivo use requires that the toxins act in a cell-autonomous manner. To demonstrate that the tethered toxins are not released from the cell surface to affect nearby cells, oocytes coexpressing the tethered α7-bungarotoxin construct and neuronal α7 nAChRs were incubated overnight in the presence of oocytes expressing α7 nAChRs only (Figure 4). Each oocyte was then separated from its neighbors, and recordings of nAChR activity in response to acetylcholine were measured. As shown in Figure 4, the receptors on oocytes coexpressing the
tethered toxins had no activity in response to ligand, whereas those coincubated with oocytes expressing only the neuronal \(\alpha_7\) nAChR exhibited normal responses to acetylcholine. These data demonstrate that \(\alpha\) neurotoxins can retain their properties when expressed on the surface of cells via a GPI anchor and that they do not affect neighboring cells through release from the cell surface.

**In Vivo Efficacy of Tethered Toxins**
Zebrafish embryos were chosen to test the efficacy of the tethered toxin approach in vivo because of the ability to visualize single muscle fibers in whole fish and analyze their electrical activity using whole-cell recordings. In vivo block of muscle nAChRs by t-\(\alpha\)Bgtx represents a formidable challenge because of the large number and high density of receptors at the neuromuscular junction.

**Figure 3. Tethered Toxins Specifically Inhibit Voltage-Gated Sodium and Calcium Channels in Xenopus Oocytes**
(Top) Coexpression of tethered MrVIA potently blocks the sodium Na, 1.2 voltage-gated channel, whereas t-MVIIA does not. (Middle) Coexpression of t-MVIIA completely blocks the calcium Ca, 2.2 voltage-gated channel, whereas t-MrVIA has no effect. (Bottom) Potassium Shaker channels are not affected by coexpression of either t-MrVIA or t-MVIIA conotoxins. Representative traces of whole-cell currents recorded from individual oocytes are shown. Voltage steps ranged from \(-70\) to \(40\) mV in steps of \(10\) mV from a holding potential of \(-90\) mV. A prepulse to \(-110\) mV was done for Shaker channel recordings. Bar graphs represent the average \pm SEM of the peak current obtained in ten oocytes for each case.

**Figure 4. Tethered Toxins Are Not Cleaved from the Membrane**
Oocytes injected with only \(\alpha_7\) nAChRs or with \(\alpha_7\) nAChRs plus tethered \(\alpha\)Bgtx were recorded to test the response to ACh. After overnight coincubation of the two types of oocytes, electrophysiology recordings were repeated. Again, three oocytes responded to ACh, and three oocytes did not respond, indicating that the tethered toxin acts only on coexpressed receptors and it does not affect neighboring cells through release from the cell surface.
Zebrafish embryos were injected with dual promoter constructs encoding either t-α-Bgtx or t-κ-Bgtx driven by the muscle-specific α-actin promoter and cytoplasmic EGFP driven by the CMV promoter. As shown in Figure 5A, muscle fibers expressing EGFP were readily identified in fish injected with both tethered toxin constructs. Synaptic sites on EGFP fluorescent muscle cells and their immediate neighbors were identified by labeling with conjugated-fasciculin (FasII) that labels acetylcholinesterase at postsynaptic sites. The positions and morphology of synapses revealed by FasII binding were unaffected in the t-α-Bgtx and t-κ-Bgtx expressing cells, indicating that the expression of tethered bungarotoxins did not interfere overtly with synapse development. However, muscle cells expressing t-α-Bgtx had greatly reduced to nonexistent levels of soluble rhod-Bgtx labeling, demonstrating that the muscle nicotinic receptors in the t-α-Bgtx expressing cells were occupied. t-κ-Bgtx expressing cells retained normal levels of rhod-Bgtx labeling (Figures 5A and 5C). To provide further evidence that the block in rhod-Bgtx labeling was due to the occupation of the muscle nicotinic receptors by t-α-Bgtx, twitch once zebrafish mutants that expressed high levels of the receptor over the entire muscle surface were employed. As shown in Figure 5B, rhod-Bgtx binding is observed over the surface of control EGFP-negative muscle cells and of EGFP-positive cells expressing t-κ-Bgtx, whereas no labeling is detected in EGFP-positive cells expressing t-α-Bgtx. Finally, nAChR function was directly tested in cells expressing t-α-Bgtx by electrophysiological responses to fast application of 10 μM ACh in acutely dissociated muscle cells from these fish. Peak current in nonfluorescent muscle cells revealed robust responses to ACh (mean current 1.4 nA, n = 10), whereas no response was recorded in fluorescent muscle cells coexpressing EGFP and t-α-Bgtx (mean current 0 nA, n = 28 cells) (Figure 5D). Taken together, these data prove that the tethered bungarotoxins retain their specificity in vivo, that they act cell autonomously, and that t-α-Bgtx provides an effective block of its cognate receptor in vivo, even under conditions of extremely high receptor expression. These data also indicate that silencing of muscle nicotinic receptor activity in individual muscle cells during zebrafish development has no gross effect on the development or distribution of neuromuscular synapses.

**Discussion**

We have demonstrated here that peptide neurotoxins from several classes retain their activity and specificity for ligand-gated and voltage-gated ion channels when tethered to the cell membrane via a GPI anchor and that they act cell autonomously in *Xenopus* oocytes and zebrafish muscle fibers. The ability to tether these naturally occurring peptide neurotoxins to the cell surface in a manner that preserves their activity and specificity, combined with the use of BAC transgenic con-
structures to target expression to specific CNS cell types (Gong et al., 2003), allows tremendous flexibility in the genetic dissection of specific factors and pathways that influence the development and function of the mammalian CNS. For example, the expression of tethered bungarotoxins and α-conotoxins can allow simple genetic manipulations of specific nAChR classes to begin to unravel the complex contributions of this diverse group of receptors to the activities of specific cell types and circuits in vivo. Also, selective suppression of neuronal activity can be achieved using t-MVIIA to inhibit neuronal Na⁺ channels that are required for the generation of the action potential or by using t-MVIIA to block Ca²⁺ channels that are required for neurotransmitter release (Terlau and Olivera, 2004). Extension of this approach to other toxins, such as those that block other ion channels, serotonin, and NMDA receptors, offers additional important experimental opportunities. There are many advantages of the tethered toxin approach over other methods for the manipulation of cellular physiology. First, this approach harnesses the impressive functional diversity of the peptide neurotoxins, enabling simple manipulations of ion channels and receptors that mediate important physiologic processes within cells. For example, peptide toxins can differentially block heteromeric channels sharing one or more subunits; conversely, they can block several members of a given ion channel or receptor family. This enables manipulations of currents that would be extremely difficult (or impossible) to achieve using traditional genetic approaches. Second, peptide toxins can act either to block channel activity (as in the cases we have chosen for these studies) or to inhibit channel inactivation resulting in hyperexcitation of the targeted cells (McIntosh et al., 1999; Terlau and Olivera, 2004), offering the possibility of both loss- and gain-of-function studies in vivo. Third, novel toxin activities can be produced using chimeric toxins derived from the fusion of known peptide toxins (Sato et al., 2000). Given the small size of most peptide toxins, we believe that the creation of novel specificities by mutagenesis will extend the use of this approach to receptors and ion channels for which natural toxins have not been identified. Fourth, the small size of most tethered toxins and their simple incorporation into transgenic and viral constructs will allow their use in a wide variety of species for which gene targeting is not yet possible (Gong and Rong, 2003). For example, tethered toxins could be employed to examine the influence of receptor function or neuronal activity on behavior in transgenic rats, which are advantageous for certain studies of CNS function. Two extensions of the present studies are of immediate interest. First, although reversible expression of the tethered toxins can be achieved using established methods (Mansuy and Bujard, 2000), development of strategies for the rapid regulation of these activities for use in short-term experiments remains an important goal. Second, the application of this strategy to other peptides, including hormones and neuropeptides, offers interesting opportunities for the analysis of the roles of these ligands in specific cell types. Thus, we anticipate that tethered toxins and other peptides will become critical instruments for the genetic dissection of CNS cells and circuits.

Experimental Procedures

Generation of Tethered Toxin Constructs

Construction of molecular chimeras between lynx1 and the snake bungarotoxins was done by replacing the sequence encoding lynx1 by the cDNAs of α- or κ-bungarotoxin in-frame between the secretion signal and lynx1 hydrophobic sequence for GPI attachment. A flag epitope sequence was introduced downstream of the secretion signal, and a short nine aa linker was inserted between the toxin and the sequence for GPI attachment. The constructs for tethered conotoxins were prepared as above, except that the flag epitope was inserted downstream of the toxin and that, for toxins against voltage-gated channels, a flexible linker of (asn-gly), joining the mature toxin molecule to the GPI anchoring sequence was inserted between the generate the corresponding conotoxins: PnLb, GGATGTGGAGGT TACCCCTTTGTGACCTAATGTAACCGCGACTATTGT; MIl, GGATGTGGC CAGTAATCGATGTGTCACCTGAAGCTACGTGAACCTTTGC; MrVIA, GCA TGCCCGAAGAAGAGTGACTGCTAGTGCGGCTAAGTGG ATTCATATCATGCTGTGCACCGAGGACCTATATGCGGGTCATTTG; CGTC; MVIA, TGGAGCGAAGGGGGGATCTCCCGCTCTCA TGTATACGTCTGACCGACGTAGTGCGTGGTAAAGTC G.

Injections and Whole-Cell Electrophysiology Recordings

The preparation of Xenopus oocytes, cRNA transcripts, and two-electrode voltage-clamp ACh recordings were done essentially as described (Ibañez-Tallon et al., 2002). cRNA injection mixes containing either receptor/channel subunit cRNAs or the same amount of receptor/channel and t-toxin (t-t) were prepared at the following ratios: for nAChRs [1:1; 0.5:1; 0.5; 0.5:2; 3:3 t-t]; for sodium Nav1.2 [1:1; 4:1; 4:1; 3:3 t-t]; calcium Cav2.2 N-type [1:3; 3:2:1; 1:2:1; 1:2:1] for shaker [1:8; 4:1]. Recordings of voltage-gated channels were performed as described (Lin et al., 1997; Nadal et al., 2001).

Zebrafish Experiments

Tethered bungarotoxins were transiently expressed in zebrafish embryos as described (Ono et al., 2002). The effectiveness of tethered α-Bgtx to bind and block AChRs in muscle was assessed by in vivo labeling with fluorescence-conjugated α-Bgtx and whole-cell recordings (Ono et al., 2001, 2002). Synaptic sites in intact fish were marked by treatment with 0.1°μM Alexa 647-conjugated Fasciculin 2 (Alomone Labs, Israel) for 45 min, which binds to acetylcholinesterase (Peng et al., 1999). Alternatively, muscle was dissociated by incubating skinned fish in 10 mg/ml collagenase (GIBCO) for 2–3 hr prior to gentle Trituration. In some experiments, mutant twitch once fish that expressed large amounts of diffusely distributed acetylcholine receptors were used (Ono et al., 2002). In vivo imaging utilized an inverted Zeiss LSM 510 Meta microscope and a C-Apochromat 63× objective. Simultaneous measurements of EGFP (excitation 488/emission LP505), Alexa-647 (excitation 633/emission LP650), and rhodamine (excitation 543/emission BP560-590) fluorescence were provided by the Meta multitrack mode of acquisition.

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