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COMMUNICATIONS

Published on Web 03/29/2007

A Subtype-Selective, Use-Dependent Inhibitor of Native AMPA Receptors

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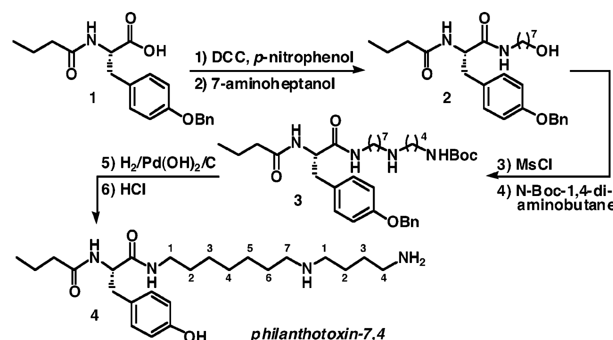
AMPA (α-amino-3-hydroxy-5-methyl-4-isooxazole) receptors (AMPARs) are glutamate-gated ion channels that play central roles in the mammalian brain, mediating fast excitatory synaptic transmission and underlying several forms of synaptic plasticity.[1] In particular, activity-dependent changes in the number of synaptic AMPARs modulate the strength of synaptic transmission. For example, the insertion of additional AMPARs into synapses following brief periods of high-frequency activity underlies hippocampal long-term potentiation (LTP), a strengthening of synaptic transmission thought to share processes related to learning and memory.[2] Discreet, subtype-specific phases of AMPAR trafficking may underlie this potentiation.

Two types of AMPARs are primarily expressed on excitatory neurons in adult animals—heteromeric channels composed of GluR1 and GluR2 subunits (GluR1/2 receptors) and channels composed of GluR2 and GluR3 subunits (GluR2/3 receptors).[3] Efforts to distinguish between the roles these receptors play at synapses (the subcellular fraction of AMPARs mediating communication between neurons) have relied on genetic approaches and have produced conflicting results. Experiments utilizing virus-driven overexpression of mutant receptor subunits in brain slices, providing a unique biophysical and/or optical signature for AMPARs containing specific combinations of subunits, suggested that GluR1/2 and GluR2/3 receptors play distinct roles at synapses.[4] However, in genetically engineered mice lacking any single AMPAR subunit or combinations of subunits, synaptic transmission and plasticity are intact, implying redundant roles for GluR1/2 and GluR2/3 receptors.[5] These observations underscore the importance of developing a subtype-selective AMPAR antagonist to probe native receptors and, along with an intriguing report describing the solid-phase synthesis of polyamine toxins shown to be active against AMPARs,[6] prompted us to synthesize and characterize the selectivity of philanthotoxin-7,4 against a panel of AMPAR subtypes.

Philanthotoxin-7,4 (PhTx-74) is a synthetic analogue of the naturally occurring wasp venom toxin philanthotoxin-4,3,3 (PhTx-433), differing in the number of amines and intervening methylene units contained in the polyamine tail.[7] Here we show that PhTx-74 is a subtype-selective AMPAR antagonist, inhibiting GluR1/2, but not GluR2/3 receptors. Inhibition is use-dependent (only open channels are blocked), allowing for the selective block of synaptic receptors activated by neurotransmitter released at nerve terminals. The molecular basis for the observed selectivity and a highly efficient synthesis of this toxin are also presented.

PhTx-74 was synthesized in six steps, as shown in Scheme 1. Acid **1** was activated as the *p*-nitrophenol ester and coupled to 7-aminoheptanol to form alcohol **2**, which was subsequently converted to the mesylate and treated with *N*-Boc-1,4-diaminobutane to form the protected toxin **3**. The benzyloxy ether protecting group was removed using hydrogenolysis and the *tert*-butyl carbamate hydrolyzed with hydrochloric acid, to provide PhTx-74 **4**. Notably, this solution-phase route provides gram quantities of PhTx-74 in 78% overall yield, a marked improvement over the previously reported synthesis[6] in terms of overall yield, cost per gram, and scalability.

Scheme 1



To evaluate the selectivity of PhTx-74, we carried out standard two-electrode voltage clamp current recordings from *Xenopus* oocytes heterologously expressing individual AMPAR subtypes. First, we examined the effect of PhTx-74 on homomeric AMPARs by injecting oocytes with GluR1, GluR2, or GluR3 subunit mRNA. Generally, polyamine toxins are potent inhibitors of AMPARs lacking the GluR2 subunit.[1b,7] Indeed, we found that PhTx-74 blocks glutamate-evoked currents from homomeric GluR1 and GluR3 receptors, having no effect on homomeric GluR2 receptors (Figure 1). The observed inhibition is use-dependent, consistent with the generally accepted binding mode of polyamine toxins—within the pore of open channels.[8] Next, we examined the effect of PhTx-74 on heteromeric GluR1/2 and GluR2/3 receptors by co-injecting oocytes with GluR2 and either GluR1 or GluR3 subunit mRNAs in a 1:1 ratio. In these experiments, we observed that PhTx-74 (100 μM) inhibits GluR1/2 receptors, but not GluR2/3 receptors even at concentrations as high as 500 μM (Figure 1). Unfortunately, efforts to measure an IC_{50} for PhTx-74 at GluR1/2 receptors met with little success (see Supporting Information for a detailed discussion).

GluR1 and GluR3 AMPAR subunits appear to preferentially assemble with GluR2 subunits to form heteromeric GluR2-containing receptors.[9] The subunit stoichiometry (fixed versus variable) is a matter of debate, though significant evidence points to assembly of tetrameric receptors as dimers of heteromeric subunit dimers.[10] Nonetheless, to rule out the possibility that the observed block is due to the formation of a substantial population of homomeric GluR1 receptors in oocytes co-injected with GluR1 and GluR2 subunits, we monitored current−voltage (*I*/*V*) curves in these cells. Linear *I*/*V* curves are indicative of the formation of GluR2-containing receptors, whereas inwardly rectifying *I*/*V* curves are diagnostic of receptors lacking the GluR2 subunit (e.g., GluR1 homomers).[1b] We consistently observed linear *I*/*V* curves in these experiments, confirming the predominant formation of GluR2-containing AMPA receptors (Figure 1). Moreover, 1-naphthyl acetyl spermine (NASPM, 100 μM), a potent inhibitor of GluR1 ho-

10.1021/ja0705801 CCC: $37.00 © 2007 American Chemical Society

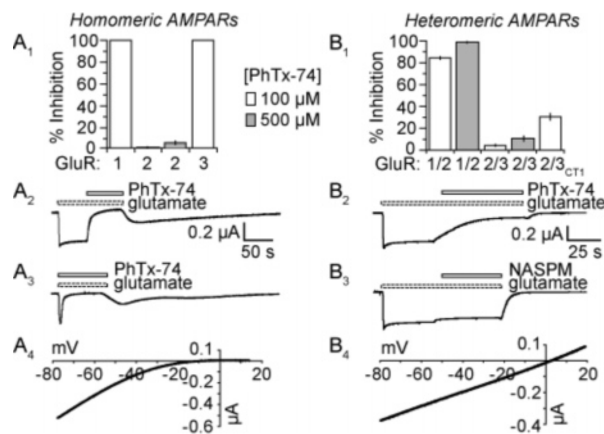


Figure 1. PhTx-74 is a subtype-selective inhibitor of AMPARs. (A) Effect of PhTx-74 on homomeric AMPARs (GluR1, GluR2, and GluR3). (A_1) Bar graph showing PhTx-74 inhibits GluR1 and GluR3 receptors, but not GluR2 receptors. Percent inhibition (mean ± SEM) was calculated for each cell ($n \geq 5$) by measuring the steady-state glutamate-evoked (100 μM) current before and after co-applying PhTx-74 (100, 500 μM). (A_2) Representative trace showing the inhibitory effect of PhTx-74 (100 μM) on steady-state glutamate-evoked (100 μM) currents from GluR1 receptors. (A_3) Representative trace from the same cell in (A_2) showing the effect of co-applying glutamate (100 μM) and PhTx-74 (100 μM) on GluR1 receptors. The peak current is comparable to the steady-state current observed in (A_1), consistent with use-dependent, open-channel block. (A_4) Representative inwardly rectifying current−voltage curve measured from the same cell as in (A_2) and (A_3). (B) Effect of PhTx-74 on heteromeric AMPARs (GluR1/2, GluR2/3, GluR2/3_{CT1}). (B_1) Bar graph showing that PhTx-74 inhibits GluR1/2, but not GluR2/3 receptors. Partial inhibition is observed at GluR2/3_{CT1} receptors comprised of GluR2 and chimeric GluR3 subunits in which the GluR3 C-terminal domain has been replaced by the GluR1 C-terminus. Percent inhibition was calculated for each cell ($n \geq 10$, across two different batches of oocytes) as in (A_1). (B_2) Representative trace showing the inhibitory effect of PhTx-74 (100 μM) on steady-state glutamate-evoked (100 μM) currents from GluR1/2 receptors. See Figure S2 for a representative trace showing the block is use-dependent. (B_3) Representative trace from the same cell in (B_2), showing the absence of an effect of NASPM (100 μM) on glutamate-evoked currents from GluR1/2 receptors. (B_4) Representative linear current−voltage curve measured from the same cell as in (B_2) and (B_3).

momers ($IC_{50} = 0.3$ μM), had an insignificant effect on AMPA receptor-mediated currents from these oocytes (Figure 1).

The apparent subtype-selective block of GluR1/2 but not GluR2/3 receptors by PhTx-74 is quite remarkable. Sequences lining the pore region of AMPARs are generally thought to be the key determinant of polyamine toxin binding (e.g., the mRNA-edited Q/R site residue).[1b] However, the membrane spanning sequences of GluR1 and GluR3 (i.e., GluR1/2 and GluR2/3 receptors), including those lining the channel pore, are identical (Figure S1). In contrast, the GluR1 and GluR3 C-terminal domains, which have been shown to influence the channel conductance (i.e., rate of ion permeation),[1c] are quite dissimilar and could give rise to quaternary structural differences between the GluR1/2 and GluR2/3 open-channel pores, thus resulting in distinct affinities for PhTx-74. To investigate this possibility, we replaced the GluR3 C-terminal domain with the terminus from GluR1 and coexpressed the resulting GluR3 subunit chimera (GluR3_{CT1}) with GluR2 in oocytes. In these experiments, we found that, unlike wild-type GluR2/3 receptors, GluR2/3_{CT1} receptors are partially inhibited by PhTx-74, consistent with the idea that the C-terminal domains impact AMPAR biophysics, including the binding to polyamine toxins within the channel pore (Figure 1).

In summary, we have identified and developed a highly efficient synthesis of the first subtype-selective inhibitor of *native* AMPARs. Notably, since PhTx-74 is a use-dependent inhibitor, it provides a means of selectively blocking synaptic GluR1/2 receptors and thus directly monitoring subtype-specific changes in the composition of synaptic AMPA receptors proposed to underlie synaptic plasticity, including hippocampal LTP.

Acknowledgment. We thank Ning Bao and Ming An for technical assistance. This work was supported by grants from the Sandler Foundation and McKnight Foundation.

Supporting Information Available: Synthetic and electrophysiological methods. This material is available free of charge via the Internet at http://pubs.acs.org.

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JA0705801