Evaluation of PhTX-74 as Subtype-selective Inhibitor of GluA2-containing AMPA Receptors

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Running title: Characterization of philanthotoxins at AMPA receptors

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Abbreviations:
AMPA: α-amino-3-hydroxy-5-methyl-4-isoxazole receptor
GLU: L-glutamate
iGluR: ionotropic glutamate receptor
KA: kainic acid
PhTX: philanthotoxin

S.E.M.: standard error of mean

ANOVA: analysis of variance
ABSTRACT

The α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) receptors are glutamate-gated cation channels that mediate fast excitatory synaptic transmission in the central nervous system (CNS). AMPARs are tetramers formed by homo- or heteromeric assembly of GluA1 to GluA4 subunits to produce multiple subtypes with varying biophysical properties. Polyamine toxins such as joro spider toxins, philanthotoxins and argiotoxins are use-dependent ion channel blockers of AMPARs widely employed as highly potent antagonists of GluA2-lacking receptor subtypes. In addition to this use, recent findings have indicated that a philanthotoxin analogue PhTX-74 can distinguish among GluA2-containing AMPAR subtypes in the presence of the prototypical transmembrane AMPAR regulatory protein γ-2 (or stargazin). Thus, PhTX-74 may be of potential use in studies of the neurobiological role of GluA2-containing subtypes. We have evaluated the pharmacological profile of PhTX-74 and related polyamine toxins at homo- and heteromeric AMPARs in the presence and absence of γ-2. Determination of IC_{50} values for inhibition of glutamate-evoked currents from Xenopus oocytes expressing recombinant homo- or heteromeric combinations of GluA1, GluA2 and GluA3 in the presence of γ-2 show that PhTX-74 inhibits homomeric GluA1 and GluA3 receptors non-selectively with IC_{50} values in the nanomolar range (252 to 356 nM), and heteromeric GluA1/A2 and GluA2/A3 receptors non-selectively with IC_{50} values in the micromolar range (22 µM). Thus, in contrast to earlier findings, we find that PhTX-74 cannot pharmacologically discriminate between GluA2-containing AMPAR subtypes.
INTRODUCTION

AMPA receptors (AMPARs) are glutamate-gated cation-selective channels that mediate fast excitatory neurotransmission in all regions of the CNS (Traynelis et al., 2010). AMPARs are formed by homo- or heterotetrameric assembly of GluA1, GluA2, GluA3 and GluA4 subunits (Traynelis et al., 2010). In each subunit, three of the four membrane-embedded segments (M1 to M3) contribute to forming the central ion channel which is related in structure to potassium channels (Wollmuth and Sobolevsky, 2004). An X-ray crystallographic structure of a homomeric GluA2 AMPAR from rat is available, but providing only limited resolution of the pore region (Sobolevsky et al., 2009). The amino acid sequence of the M1-M3 segments is identical across GluA1-4 and channel properties are highly similar among homomeric AMPAR subtypes. However, in the mature CNS, post-transcriptional editing of GluA2 mRNA changes a codon encoding a neutral glutamine (Q) to a positively charged arginine (R) at the position in M2 that forms the selectivity filter at the core of the ion channel pore (denoted the Q/R site; Fig. 1A) (Burnashev et al., 1992; Verdoorn et al., 1991). Four glutamines thus constitute the selectivity filter in GluA2-lacking AMPARs, whereas subtypes containing edited GluA2 (GluA2R) comprises a mixture of arginine and glutamine residues (Fig. 1A). GluA2R-containing AMPARs exhibit markedly different channel properties compared to GluA2R-lacking AMPARs such as lower single-channel conductance (Swanson et al., 1997), greatly reduced Ca\textsuperscript{2+}-permeability (Geiger et al., 1995; Lomeli et al., 1994), and a linear current-voltage (IV) relationship (Sommer et al., 1991; Verdoorn et al., 1991). In addition to GluA2R content, several classes of AMPAR-interacting transmembrane proteins regulate channel function, acting as auxiliary subunits. These include the transmembrane AMPAR regulatory proteins (TARPs), the cornichons homologs (CNIH) and the cysteine-knot AMPAR modulating protein (CKAMP44) (Diaz, 2010; Jackson and Nicoll, 2011a). Association with TARPs varies in different CNS regions.
and contribute diversification of key properties among native AMPARs (Coombs et al., 2012; Jackson et al., 2011; Jackson and Nicoll, 2011b; Shelley et al., 2012; Tomita et al., 2005).

The AMPAR ion channel contains a binding site for low-molecular weight polyamine-containing natural products that act as channel blockers. These include natural occurring arthropod toxins such as philanthotoxins (PhTXs), joro spider toxins, and argiotoxins. Binding of polyamine toxins is dependent on channel activation and membrane potential and are therefore classified as use- and voltage-dependent blockers. Structure-activity relationship and molecular modeling studies (Andersen et al., 2006; Barygin et al., 2011; Nelson et al., 2009; Tikhonov, 2007) have suggested a binding mode in which the polyamine toxin head group moiety is positioned in the channel vestibule with the polyamine tail permeating the Q/R-site (Fig. 1A). The presence of arginine at the Q/R-site in GluA2R-containing AMPARs is the only molecular determinant found to influence polyamine toxin binding among different AMPAR subtypes. Specifically, the positively charged arginine side chain contributed by GluA2R dramatically lowers toxin affinity for the ion channel binding site (Herlitze et al., 1993). Consequently, polyamine toxins typically display more than 100-fold selectivity for AMPARs lacking GluA2R compared to those containing GluA2R (Brackley et al., 1993; Kromann et al., 2002; Sager et al., 2009b; Washburn and Dingledine, 1996) and have become important pharmacological tools to distinguish among AMPAR subtypes in native receptor populations. In particular, as sensitivity to polyamine channel block often correlate with high Ca\(^{2+}\)-permeability, externally applied polyamine toxin is often used to achieve selective inhibition of Ca\(^{2+}\)-permeable AMPAR subtypes; although certain populations of AMPARs has been reported to exhibit Ca\(^{2+}\) permeability without any appreciable block by polyamines (Meucci et al., 1996; Osswald et al., 2007; Otis et al., 1995).

The PhTX analogue PhTX-74 has recently been reported to distinguish between two subtypes of GluA2R-containing AMPARs, GluA1/A2R and GluA2R/A3 receptors, when these are
expressed in the presence of the prototypical TARP stargazin (\(\gamma-2\)) (Nilsen and England, 2007). GluA1/A2\(_R\) and GluA2\(_R\)/A3 receptors are abundantly expressed subtypes in the mature CNS and are important for synaptic plasticity (Malenka, 2003; Malinow and Malenka, 2002), but cannot be distinguished pharmacologically because of lack of selective ligands. The potential use of polyamine toxins such as PhTX-74 as subtype-selective antagonists of heteromeric AMPARs is therefore of great interest. In the present study, we have evaluated prototypical polyamine toxins as subtype-selective AMPAR antagonists at homo- and heteromeric AMPARs. In particular, we examine the effect of \(\gamma-2\) on the inhibitory potency (IC\(_{50}\)) of PhTX-74 at heteromeric GluA1/A2\(_R\) and GluA2\(_R\)/A3. We find that PhTX-74 is not selective among GluA1/A2\(_R\) and GluA2\(_R\)/A3 receptors regardless of the presence or absence of \(\gamma-2\).
MATERIALS AND METHODS

Materials – PhTX-38, -47, -56, -65, -74, -83, -343 were synthesized as previously described (Kromann et al., 2002). All other chemicals were from Sigma-Aldrich (St Louis, MO) unless otherwise noted. Kainic acid was from Abcam (Cambridge, UK).

Expression of AMPARs in Xenopus oocytes – cRNA for the flip isoforms of rat GluA1, GluA2Q, GluA2R, GluA3 and GluA4 were transcribed from linearized template cDNA (in the pGEM-HE plasmid vector) using the mMESSAGE mMACHINE T7 mRNA-capping kit (Ambion, Austin, TX) according to the protocol supplied by the manufacturer. cRNA for the TARP γ-2 from rat was transcribed from linear cDNA produced by PCR using as template the plasmid vector pBSK-γ-2 (kindly provided by Dr. P. Osten, Cold Spring Harbor Laboratory, NY, USA), the forward primer STZ-T7 (5’-GCATAATACGACTCACTATAGGGAGACTCGAGCCCGGGACCATTGGGCTGTTTGATCGAGG-3’; containing overhang T7 polymerase recognition sequence) and the reverse primer STZ-polyA (5’-TTTTTTTTTTTTTTTTTTTTTTTTCATACCGCGCTGTTTGATCGAGG-3’; containing overhang poly-T sequence). The quality and quantity of the synthesized cRNA was assessed by gel electrophoresis and spectroscopy. Stage V and VI oocytes were surgically removed from the ovaries of Xenopus laevis frogs anaesthetized with 3-aminobenzoic acid ethyl ester (1 g/L). To remove the follicular cell layer, clusters of oocytes were incubated with 300 U/mL type IV collagenase (Worthington, Freehold, NJ, USA) for 1 to 2 h in Ca^{2+}-free solution comprised of (in mM): 82.5 NaCl, 2 KCl, 5 HEPES, 0.82 MgCl_2. Oocytes were then washed extensively and maintained in Barth’s solution comprised of (in mM): 88 NaCl, 1 KCl, 2.4 NaHCO3, 10 HEPES, 0.82 MgSO_4, and 0.41 CaCl_2 and supplemented with 100 μg/mL gentamycin. Oocytes were injected with cRNA within 24 h of isolation. For expression of homomeric and heteromeric AMPARs in the presence or absence of γ-2, oocytes were injected with 25 to 75 nL RNA solutions containing cRNA in the following quantities: Homomeric GluA1, 10 ng; homomeric GluA2Q, 10 ng; homomeric
GluA2, 10 ng; homomeric GluA3, 10 ng; homomeric GluA4, 10 ng; heteromeric GluA1/A2, 5 ng of GluA1 and 10 ng of GluA2, heteromeric GluA2/A3, 10 ng of GluA2 and 5 ng of GluA3. For co-expression with γ-2, an additional 10 ng or 15 ng of γ-2 cRNA was added the cRNA solution for homomeric and heteromeric expressions, respectively. Oocytes were incubated at 17 °C in Barth's solution until time of experiment.

Voltage-clamp recordings from Xenopus oocytes – Two-electrode voltage-clamp (TEVC) recordings were made 1–4 days post-injection. Oocytes were placed in a 500 µL recording chamber with a single perfusion line delivering 5 mL/min perfusion. Voltage and current electrodes were filled with 3 M KCl. Recordings were made using a Warner OC725B two-electrode voltage clamp (Warner Instruments, Hamden, CT) configured as recommended by the manufacturer. Oocytes were perfused with oocyte recording (OR) buffer (in mM: 115 NaCl, 2 KCl, 5 HEPES and 1.8 BaCl2, pH 7.6). All experiments were performed at room temperature (23 °C). Data acquisition and voltage control was accomplished using a CED 1401plus analogue-digital converter (Cambridge Electronic Design, Cambridge, UK) interfaced with a PC running WinWCP software (available from Strathclyde Electrophysiology Software, University of Strathclyde, Glasgow, UK). Concentration-inhibition measurements of polyamine toxin inhibition of the receptors were performed by measuring agonist-evoked currents in step-wise increasing concentrations of polyamine toxin as illustrated in Fig. 1C.

Data analysis – For determination of IC₅₀ values, data were pooled among individual experiments at 5 to 8 individual oocytes and the composite Concentration-inhibition data were fitted using GraphPad Prism software (GraphPad Inc., San Diego, CA, USA) by the equation:

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Response = \frac{1}{1+10^{((\log IC_{50} - X) * n_H)}}
\]
where $Response$ is the agonist-evoked current response measured at a given inhibitor concentration normalized to the agonist-evoked current response in absence of inhibitor, $IC_{50}$ is the concentration of inhibitor that produces a half-maximal inhibition, $X$ is the logarithm of the concentration of the inhibitor concentration, and $n_H$ is the Hill slope. Unless otherwise indicated, statistical differences were analyzed using ANOVA followed by a Dunnett’s Multiple Comparison Test using GraphPad Prism software.
RESULTS

Expression of homomeric and heteromeric AMPARs with and without γ-2 – To enable characterization of polyamine toxins at AMPAR subtypes in presence and absence of the TARP γ-2, we expressed homomeric and heteromeric AMPAR subtypes with and without γ-2 in *Xenopus laevis* oocytes (*Methods*) and measured current responses evoked by L-glutamate (GLU) in the presence of increasing concentrations of toxin to determine compound concentration-inhibition relationships. In general, all compounds were tested on a minimum of six different oocytes for each receptor subtype, and included recordings from oocytes from at least two different frogs. A standard recording protocol and current responses are illustrated in Fig. 1C-D for a representative experiment (PhTX-74 at homomeric GluA1 expressed with γ-2). For AMPAR subtypes co-expressed with γ-2, we determined the ratio of steady-state currents evoked by GLU and the weak partial agonist kainic acid (KA) as a test for functional expression and co-assembly of γ-2 with the AMPAR in oocytes (Kott et al., 2009; Kott et al., 2007; Sager et al., 2009a; Tomita et al., 2005). AMPAR association with γ-2 increases the receptor current response to KA relatively more than GLU; resulting from changed AMPAR kinetics by the co-assembly with γ-2. Therefore, determination of the KA to GLU (KA/GLU) current response ratio provides a convenient and robust test for functional expression of AMPARs in complex with γ-2 (Kott et al., 2009; Kott et al., 2007; Sager et al., 2009a; Shi et al., 2009; Tomita et al., 2005). In corroborations with previous findings (Kott et al., 2009; Kott et al., 2007; Sager et al., 2009a; Shi et al., 2009; Tomita et al., 2005; Turetsky et al., 2005), we found that co-expression of γ-2 increased the KA/GLU ratio more than 20-fold from approximately 1 to 25 for both homomeric GluA1 and GluA3 (Fig. 2A and B). For heteromeric GluA1/A2R and GluA2R/A3 in absence of γ-2 co-expression, we also observed KA/GLU response ratios close to 1 (1.2 ± 0.1 and 0.6 ± 0.1, respectively), which increased to 6 and 7, respectively, upon co-expression of γ-2 (Fig. 2A and B).
These results are in good agreement with previous findings (Korber et al., 2007; Shi et al., 2009; Tomita et al., 2005; Turetsky et al., 2005). For all batches of AMPAR expressing oocytes injected with \( \gamma-2 \), we determined the KA/GLU response ratio to ensure functional co-expression of \( \gamma-2 \). In general, we observed that less than 2% of oocytes expressing homomeric (GluA1 and GluA3) or heteromeric (GluA1/A2\(_{R} \) and GluA2\(_{R}/A3 \)) AMPARs co-injected with \( \gamma-2 \) displayed smaller than 5-fold increases in KA/GLU ratio compared to controls not injected with \( \gamma-2 \) (data not shown); demonstrating a very consistent expression of AMPARs in functional complex with \( \gamma-2 \) across oocyte batches. It should be noted that little biochemical evidence for the stoichiometry of TARP assembly with recombinant or native heteromeric AMPARs currently is available. Therefore, a potential caveat for the use of an increased KA/GLU response ratio as evidence for \( \gamma-2 \) complex formation with heteromeric receptors is that the observed shift in KA/GLU ratio could reflect effects on a contaminating subpopulation of homomeric receptors. We also expressed homomeric GluA2\(_{R} \) in the presence and absence of \( \gamma-2 \). As previously observed (Eugene et al., 1996; Koike et al., 2000), homomeric GluA2\(_{R} \) in the absence of \( \gamma-2 \) did not express to the oocyte cell membrane at levels that allowed detection of agonist-evoked currents by TEVC electrophysiology (data not shown). However, in the presence of \( \gamma-2 \), we measured robust GLU-evoked current responses from all oocyte batches injected with GluA2\(_{R} \) (946 ± 299 nA, \( n = 13 \)). In agreement with previous studies (Kott et al., 2007), we find that the KA/GLU ratio for homomeric GluA2\(_{R} \) co-expressed with \( \gamma-2 \) was close to 1 (1.1 ± 0.1; \( n = 6 \)). For expression of heteromeric AMPARs, we ensured that oocytes expressed a homogeneous population of heteromeric AMPARs devoid of homomeric receptors by determining the current-voltage (IV) relationships. Most GluA2\(_{R} \)-containing AMPARs have low sensitivity to block by intracellular polyamines resulting in a linear IV relationship, whereas AMPARs lacking GluA2\(_{R} \) subunits are highly sensitive to block by intracellular polyamines at positive membrane potentials, resulting in inwardly rectified IV relationships (Pellegrini-
Giampietro, 2003; Verdoorn et al., 1991). Representative IV relationships are shown in Fig. 2C and 2D for homo- and heteromeric AMPARs in the absence and presence of γ-2, respectively. From these, we calculated rectification ratios (RR) as the ratio of current amplitude at +40 mV, which reflects primarily GluA2R-containing heteromeric receptor current, to that at −60 mV. For homomeric GluA1 and GluA3 expressed in the absence of γ-2, RR was 0.04 and 0.08, respectively (Fig. 2E). Co-expression of γ-2 significantly increased RR for GluA1 to 0.15 (Fig. 2E). The decrease in rectification by co-expression of γ-2 that was observed for homomeric GluA1 has previously been reported, and is suggested to result from a slightly decreased sensitivity towards channel block by intracellular polyamines induced by association with γ-2 (Soto et al., 2007). Interestingly, this effect was not present for homomeric GluA3 as RR did not significantly change upon co-expression with γ-2. Expression of heteromeric receptors in the presence or absence of γ-2 showed RR levels close to the theoretically expected value of 0.67 that reflect a linear IV relationship in the −60 to +40 mV range, which is expected for channels insensitive to intracellular polyamine block (Fig 2C-E). This shows that the cRNA co-injection regimen used to co-express GluA1 or GluA3 with GluA2R generates an AMPAR population that is predominantly composed of heteromeric receptors; both in the absence and the presence of γ-2.

**Determination of PhTX-74 inhibitory potency at GluA1/A2R and GluA2R/A3 in the presence and absence of γ-2** — PhTX-74 in the micromolar concentration range has been reported to display selective inhibition between the two major subtypes of GluA2R-containing AMPARs, GluA1/A2R and GluA2R/A3, when these are co-expressed with γ-2 in oocytes (Nilsen and England, 2007). Specifically, PhTX-74 was reported to fully inhibit GluA1/A2R receptors when applied at a concentration of 500 µM while producing ~10% inhibition at GluA2R/A3 receptors (Nilsen and England, 2007), thus suggesting that PhTX-74 could distinguish between these subtypes in native AMPAR populations. To further investigate these aspects, we examined PhTX-74 and other
polyamine PhTX analogues at homo- and heteromeric AMPARs expressed in the presence and absence of γ-2. First, to determine the influence of AMPAR subunit type on PhTX analogue affinity with glutamine at the Q/R-site, we expressed GluA1, GluA2Q, GluA3 and GluA4 as homomeric receptors in oocytes in the absence of γ-2. We compared inhibition of GLU-evoked currents by a series of close PhTX analogues with subtle modifications in the polyamine moiety, previously determined to possess variable AMPAR affinity (Kromann et al., 2002) (Supplemental Fig. S1).

When tested at a single concentration of 1 µM at holding potentials of -60 and -80 mV, all compounds displayed percent inhibition that were identical or differed by less than 20% inhibition at the four homomeric AMPARs (Supplemental Fig. S1). The largest differences were observed for PhTX-47, PhTX-83 and PhTX-74 (Supplemental Fig. S1). Among these we selected PhTX-47 for determination of concentration-inhibition relationships to determine the precise differences in inhibitory potency between homomeric GluA1 to GluA4 (Supplemental Fig. S1). The resulting IC₅₀ values were within 2-fold range of each other (Supplemental Fig. S1). These results suggest that the structural and functional differences that exists among the four AMPAR subunits has marginal influence on the affinities of PhTX analogues for the AMPAR ion channel when glutamines is present in the Q/R site.

We next focused on the influence of γ-2 on affinities of PhTX analogues at homo- and heteromeric AMPARs composed of the GluA1, GluA3 and GluA2ᵦ subunits. Specifically, we expressed GluA1, GluA2ᵦ and GluA3 as homomeric receptors and as heteromeric GluA1/A2ᵦ and GluA2ᵦ/A3 receptors in the presence and absence of γ-2 and determined the concentration-inhibition relationship for PhTX-74 over a concentration range from 10 nM to 300 µM (Fig. 3). At homomeric GluA1 and GluA3, the resulting concentration-inhibition curves produced IC₅₀ values that were not significantly different from each other (296 nM and 263 nM, respectively; P = 0.20; Student's t test) (Table 1). As mentioned previously, we could not express homomeric GluA2ᵦ in
oocytes at levels that allowed measurement of agonist evoked currents (and could therefore not
determine the IC$_{50}$ value of PhTX-74 at this subtype). Co-expression of $\gamma$-2 with homomeric GluA1
and GluA3 did not change the IC$_{50}$ values for PhTX-74 inhibition significantly ($P = 0.62$ for GluA1
with and without $\gamma$-2; $P=0.12$ for GluA3 with and without $\gamma$-2; Student’s $t$ test) (Fig. 3 and Table 1).
The results for homomeric GluA1 and GluA3 receptors are in agreement with previous findings for
homomeric GluA4 receptors that showed co-expression of TARPs, including $\gamma$-2, to have minimal
effect on PhTX-74 inhibition when GLU is used as agonist (Jackson et al., 2011). For heteromeric
GluA1/A2$_{R}$ and GluA2$_{R}$/A3 in the absence of $\gamma$-2, the concentration-inhibition data showed that
PhTX-74 displayed little or no measureable inhibition at toxin concentrations below 1 µM (Fig. 3).
In the micromolar concentrations range, significant inhibition where observed for both heteromeric
subtypes; reaching 80 % and 81% inhibition at the maximum tested concentration (300 µM). The
concentration-inhibition relationships progressed near identical and were fitted well to a
monophasic concentration-inhibition curve defined by a one-binding site model with IC$_{50}$ values of
52 (GluA1/A2$_{R}$) and 49 (GluA2$_{R}$/A3) µM that were not significantly different ($p = 0.59$; Student’s $t$
test) (Fig. 3A). Co-expression with $\gamma$-2 decreased IC$_{50}$ approximately 2-fold at both GluA1/A2$_{R}$ and
GluA2$_{R}$/A3 (both 22 µM; $P < 0.0001$ compared to IC$_{50}$ without $\gamma$-2) (Fig. 3B and Table 1). These
results show that PhTX-74 is non-selective among the tested heteromeric AMPAR subtypes both in
the presence and in the absence of $\gamma$-2. In addition, the presence of $\gamma$-2 has little effect on
GluA1/A2$_{R}$ and GluA2$_{R}$/A3 for PhTX-74 inhibitory potency during activation by GLU.

The robust GLU-evoked currents (946 ± 299 nA, $n = 13$) that were obtained from oocytes
injected with GluA2$_{R}$ and $\gamma$-2 cRNA allowed characterization of PhTX-74 at homomeric GluA2$_{R}$
receptors. In agreement with previous findings (Nilsen and England, 2007), we found that
homomeric GluA2$_{R}$ receptors were virtually insensitive to PhTX-74 when applied in concentrations
up to 300 µM (Fig. 3B). Comparison of this result with the data obtained at homomeric GluA1 and


heteromeric receptors GluA1/A2_R expressed with γ-2 reveal the substantial negative impact on polyamine block that is produced by incremental increase in the number of arginines in the Q/R-site from zero (homomeric GluA1 and GluA3) to two (GluA1/A2_R and GluA2_R/A3) and four (homomeric GluA2_R).
DISCUSSION

Functional diversity among AMPAR subtypes plays important roles for shaping AMPAR signaling in the CNS. In particular, spatiotemporal changes in subtype-composition of synaptic AMPAR populations are critically involved in short- and long term synaptic plasticity. Polyamine toxins have become an indispensable tool in studies of Ca^{2+}-permeable AMPAR subtypes in the CNS by their unique ability to block GluA2R-lacking AMPARs. However, compounds that allow further pharmacological dissection of AMPAR subtypes beyond the major division of Ca^{2+}-permeable AMPARs versus Ca^{2+}-impermeable AMPARs are currently not available. Our results show that polyamine toxins, at least of the PhTX class, are not obvious candidates for achieving such further subtype selectivity. This finding is supported by the 100% sequence identity among GluA1 to GluA4 of the channel-forming M1 to M3 segments. These segments are generally thought to form the polyamine toxin binding site, which therefore is likely to contain little structural difference that can be exploited to develop polyamine-type channel blockers capable of distinguishing further among AMPAR subtypes.

The identical inhibitory potencies of PhTX-74 at GluA1/A2R and GluA2R/A3 observed here are in conflict with the observations reported by Nilsen & England (2007), who found that PhTX-74 when tested at concentrations of 100 and 500 µM displayed pronounced channel block (>80%) of GluA1/A2R, but minimal (<10%) block of GluA2R/A3. This study does not report IC_{50} values for PhTX-74 at GluA1/A2R or GluA2R/A3, but state that PhTX-74 displayed a multiphasic concentration-inhibition relationship at oocytes injected with GluA2R and GluA1 or GluA3, which may indicate a heterogeneous receptor population of homomeric and heteromeric receptors. Thus, the difference in the findings in Nilsen and England (2007) compared to those obtained here can potentially be ascribed to differences between the composition of the expressed AMPAR populations. In addition to heteromeric GluA2R-containing receptors, oocytes co-injected with...
GluA2R and either GluA1 or GluA3 can also express homomeric GluA1, homomeric GluA3 and homomeric GluA2R. In the present study, we used measurements of the rectification ratios to ensure that individual oocytes co-injected with GluA2R and GluA1 or GluA3 express a receptor population devoid of homomeric GluA1 or GluA3. Because of their characteristic inward rectified IV-relationship, the extent of homomeric GluA1 and GluA3 content can be assessed electrophysiologically by measuring this ratio (Kristensen et al., 2011) and used to exclude oocytes expressing detectable amounts of homomeric GluA1 or GluA3 receptors. Furthermore, even when present, homomeric GluA1 and GluA3 are >95% inhibited by PhTX-74 concentration of 10 µM (Fig. 3), and therefore for all practical means do not contribute to the progress of concentration-inhibition relationship in the 10 to 300 µM concentration range. In terms of the potential presence of homomeric GluA2R, we find that these receptors do not express in oocytes in the absence of γ-2 and can therefore be ruled out as a concern for the concentration-inhibition relationships determined in the absence of γ-2. However, in the presence of γ-2, we do find that homomeric GluA2R are readily expressed to the membrane in oocytes to generate ~1000 nA currents. Furthermore, these currents have an IV-relationship very similar to GluA1/A2R or GluA2R/A3 (Fig. 2C and D). Therefore, if a substantial proportion of homomeric GluA2R are present in experiments with co-expression of γ-2, these will contribute current that cannot be distinguished from heteromeric receptors. In particular, in conditions seeking GluA2R/A3 receptors, carefully balanced expression ratios of GluA2R and GluA3 is important for avoiding formation of homomeric GluA2 given the suggestion that GluA3 is trafficked poorly compared to the other AMPAR subunits (Coleman et al., 2010). In the present study, we observe from experiments with oocytes expressing GluA2R alone that homomeric GluA2R is virtually inert to PhTX-74 in the 0.1 to 300 µM concentration range; displaying < 5% inhibition at the maximum tested concentration of 300 µM (Fig. 3B). Together, these findings ensure that the obtained PhTX-74 concentration-inhibition relationships at the 10 to
300 µM PhTX-74 concentration range at oocytes co-injected with GluA2R and GluA1 or GluA3 accurately reflect the inhibitory activity of PhTX-74 at heteromeric GluA1/A2R and GluA2R/A3 receptors. Thus, we conclude that PhTX-74 inhibit these receptors non-selectively with IC50 values around 30 µM; both in the presence and absence of γ-2. By establishing that PhTX-74 is non-selective among these important heteromeric AMPAR subtypes, our findings may have implications for conclusions drawn in previous work utilizing PhTX-74 to study native AMPARs (Argilli et al., 2008; Penn et al., 2012). It should be noted that our work characterizes the potency of PhTX-74 at homomeric and heteromeric AMPAR subtypes under equilibrium conditions (i.e. during continued activation by saturating concentrations of GLU). In relation to the potential use of PhTX-74 in studies of synaptic transmission, it is important to note that synaptic AMPARs are activated by brief millisecond pulses of GLU. The kinetics of external polyamine toxin block under these conditions are essentially unknown and consequently, the amount of block achieved by a given polyamine toxin concentration at synapses may therefore be different than those reported in the present study.

Previous work has shown that TARPs modulate polyamine channel block differentially among AMPAR subtypes (Jackson et al., 2011; Kott et al., 2009; Soto et al., 2007). In general, our present observations that γ-2 increases the potency of externally applied PhTX-74 at heteromeric AMPARs, but not at homomeric AMPARs is in good agreement with previous findings (Jackson et al., 2011; Kott et al., 2009). AMPARs activate the ion channel into multiple subconductance states (Swanson et al., 1997). It has been proposed that the high-affinity polyamine toxin binding site may be less accessible in the channel configurations underlying low-conductance states compared to high-conductance states (Bahring and Mayer, 1998; Jackson et al., 2011). Apparent polyamine toxin affinity may thus increase under conditions that increase the ability of the ion channel to open to high-conductance states (Shelley et al., 2012). Specifically, this has been demonstrated for PhTX-74 at homomeric GluA4 and heteromeric GluA2R/A4 receptors using differential TARP co-expression
and activation by low-efficacy versus high-efficacy agonists to modulate mean channel conductance; hereby establishing a strong correlation between mean channel conductance and apparent PhTX-74 affinity (Jackson et al., 2011). Heteromeric GluA2-containing receptors display very low mean conductance during activation with GLU (Jackson et al., 2011; Kristensen et al., 2011; Swanson et al., 1997). Co-expression with γ-2 and other TARPs increases the mean conductance of heteromeric AMPARs during GLU activation approximately two- to threefold (Jackson et al., 2011; Kristensen et al., 2011). Thus, as previously observed for GluA2δ/A4 (Jackson et al., 2011), our observed increases in PhTX-74 potency upon co-expression by γ-2 for GluA1δ/A2δ and GluA2δ/A3 might therefore reflect an increase in availability of high conductance states. In contrast to the effect on external channel block, γ-2 decreases the extent of internal channel block by endogenous polyamines such as spermine and spermidine (Soto et al., 2007). The molecular basis for these contrasting effects of γ-2 is currently poorly understood, but likely reflects the differences in the chemical structure of intracellular polyamines and polyamine toxins such as PhTX. Specifically, endogenous intracellular polyamines do not contain a bulky head group and can permeate the AMPAR ion channel at high positive membrane potentials (Bowie and Mayer, 1995). TARP-mediated increase in the occurrence of high-conductance states has therefore been suggested to facilitate permeation intracellular polyamines, which will be manifest in a decrease in channel block at high positive potentials (Jackson et al., 2011; Soto et al., 2007).

Our present data suggest that polyamine toxins, at least of the PhTX class, are poor candidates as tools to distinguish among GluA2-containing AMPAR subtypes. At present, two other AMPAR binding sites for inhibitors are known in the form of the orthosteric binding site in the ligand-binding domain that is targeted by competitive antagonists and a binding site for negative allosteric modulators located within the linker region that connect the LBDs to the ion channel domain (Traynelis et al., 2010). Similar to the polyamine toxin blockers, subtype-selective
competitive antagonists have proven difficult to obtain. This is also likely due to a high degree of structural conservation among GluA1 to GluA4 in the orthosteric binding pocket. The binding pocket for negative allosteric modulators has been mapped to the S1-M1 and S2-M4 linker subunit regions that connect the LBD to the ion channel domain (Balannik et al., 2005). These segments contain a higher degree of sequence variation among GluA1-4 compared to the near identical M1-M4 regions and orthosteric binding pockets (Sobolevsky et al., 2009). Thus, non-competitive modulators exemplified by the 2,3-benzodiazepine class of GYKI compounds (Szenasi et al., 2008) and the more potent piraquilone derivatives (Menniti et al., 2000) may constitute a more fruitful framework for development of AMPAR subtype-selective inhibitors. However, further studies of AMPAR molecular pharmacology are necessary to clarify the potential for development of novel subtype-selective inhibitors targeting this site.

In summary, our results show that polyamine toxins belonging to the PhTX class are non-selective among subtypes of Ca\(^{2+}\)-impermeable heteromeric AMPARs; independently of the presence or absence of the prototypical TARP\(\gamma\)-2.
AUTHORSHIP CONTRIBUTIONS

Participated in research design: Poulsen, Strømgaard, Kristensen

Conducted experiments: Poulsen, Kristensen

Contributed new reagents or analytic tools: Lucas

Performed data analysis: Poulsen, Kristensen

Wrote or contributed to writing of the manuscript: Poulsen, Lucas, Strømgaard, Kristensen
REFERENCES


FOOTNOTES

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FIGURE LEGENDS

FIGURE 1. Overview of AMPAR structure and polyamine toxin channel blockers. A. Schematic representation of the AMPAR ion channel showing the location of the Q/R site and the proposed polyamine toxin binding mode. The channel is formed by fourfold symmetrical assembly of the M1-M4 segments from each subunit. Shown are the M1-M3 segments from two subunits with M4 omitted for clarity. Homomeric AMPARs formed from the GluA1, GluA3 and GluA4 subunits contain four Gln (Q) at the Q/R site whereas heteromeric GluA2-containing receptors contain a mixture of Gln and Arg (R) at the Q/R site. B. Structure of the PhTX analog PhTX-74. C. Representative recording traces show concentration-dependent block by PhTX-74 of inward currents evoked by 300 µM GLU at an oocyte co-expressing homomeric GluA1 receptors and γ-2 held at −80 mV. D. Concentration-inhibition curve for PhTX-74 at homomeric GluA1 receptors co-expressed with γ-2. Each point represent the mean ± S.E.M. of the fractional current at a given blocker concentration relative to the current in the absence of blocker obtained in experiments with six to ten oocytes.

FIGURE 2. Characterization of current-voltage relationship and agonist efficacy at hom- and heteromeric AMPARs in the presence and absence of γ-2. A-B. AMPAR current responses evoked by KA and GLU from oocytes expressing homo- or heteromeric AMPARs in the absence of γ-2 are of similar amplitude, whereas co-injection of γ-2 enhances KA current response relative to GLU current response (illustrated for homomeric GluA1 in the representative recording traces in panel A). Panel B summarizes GLU versus KA response ratio calculated as I_{KA}/I_{GLU} where I_{KA} and I_{GLU} are the steady-state current responses evoked by 300 µM GLU and 100 µM KA, respectively. All data points represent the mean ± S.E.M. of experiments with 12 to 20 oocytes. C-D. Current-voltage relationships for GLU (300 µM) evoked currents from oocytes injected with GluA1 (C) or

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GluA3 (D) and/or GluA2R; in the absence and presence of γ-2. Current responses in the -80 mV to +45 mV range are shown normalized to the current response at -80 mV. E. Summary of the +40/-60 current response ratio calculated as $I_{+40}/I_{-60}$ where $I_{+40}$ and $I_{-60}$ are the steady-state currents recorded at membrane potentials of +40 mV and -60 mV, respectively.

**FIGURE 3. Determination of IC$_{50}$ values for PhTX-74 at homomeric and heteromeric AMPARs containing GluA1, GluA2R, and GluA3.** Concentration-inhibition curves for inhibition of GLU-evoked currents from oocytes injected with combinations of cRNAs of GluA1, GluA2R, GluA3 and γ-2 (as described in Methods) and held at membrane potential of -80 mV. Values are means ± SEM of experiments with 4 to 12 oocytes per curve. IC$_{50}$ values derived from the curves are given in Table 1.
### TABLES

#### Table 1. Inhibitory potency of PhTX-74 at recombinant AMPA receptor subtypes in the presence and absence of the TARP γ-2.

<table>
<thead>
<tr>
<th>Receptor subtype</th>
<th>Co-expression</th>
<th>IC$_{50}$ (nM)$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>GluA1</td>
<td>-</td>
<td>296 [266-331]</td>
</tr>
<tr>
<td>GluA1</td>
<td>γ-2</td>
<td>356 [319-399]</td>
</tr>
<tr>
<td>GluA2$_R$</td>
<td>-</td>
<td>N.D.</td>
</tr>
<tr>
<td>GluA2$_R$</td>
<td>γ-2</td>
<td>&gt;100000</td>
</tr>
<tr>
<td>GluA3</td>
<td>-</td>
<td>263 [228-305]</td>
</tr>
<tr>
<td>GluA3</td>
<td>γ-2</td>
<td>252 [229-279]</td>
</tr>
<tr>
<td>GluA1/A2$_R$</td>
<td>-</td>
<td>52150 [44650-60900]</td>
</tr>
<tr>
<td>GluA1/A2$_R$</td>
<td>γ-2</td>
<td>21960 [16620-29030]*</td>
</tr>
<tr>
<td>GluA2$_R$/A3</td>
<td>-</td>
<td>48730 [40050-59290]</td>
</tr>
<tr>
<td>GluA2$_R$/A3</td>
<td>γ-2</td>
<td>22610 [19140-26710]*</td>
</tr>
</tbody>
</table>

Co-expression with γ-2 is described in the Methods. $^a$ IC$_{50}$ values were determined by the nonlinear fitting of concentration-inhibition data collected at 5 to 10 oocytes held at -80 mV (See Methods). Numbers in bracket denote the 95% confidence interval for IC$_{50}$. * $p < 0.01$ significantly different from receptor subtype expressed in absence of γ-2 (unpaired $t$-test with Welch's correction).
Figure 2

A

B

C

D

E

Response ratio

| GluA1 | + | + | - | - | + | + | + | + |
| GluA2_R | - | - | + | + | + | + | + | + |
| GluA3 | - | - | + | + | + | + | + | + |
| γ-2 | - | - | - | - | - | - | - | - |

Response ratio

| GluA1 | + | + | - | - | + | + | + | + |
| GluA2_R | - | - | + | + | + | + | + | + |
| GluA3 | - | - | + | + | + | + | + | + |
| γ-2 | - | - | - | - | - | - | - | - |

Normalized current

Voltage (mV)

Normalized current

Voltage (mV)

Rectification ratio

| GluA1 | + | + | - | - | + | + | + | + |
| GluA2_R | - | - | + | + | + | + | + | + |
| GluA3 | - | - | + | + | + | + | + | + |
| γ-2 | - | - | - | - | - | - | - | - |