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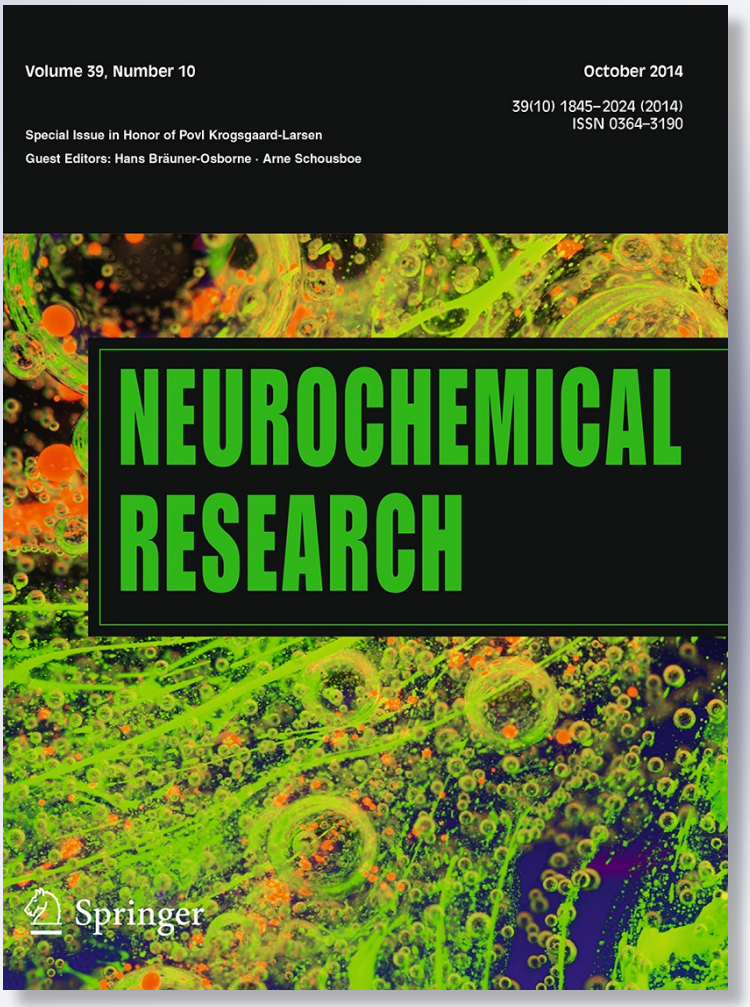

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ORIGINAL PAPER

Inhibition of AMPA Receptors by Polyamine Toxins is Regulated by Agonist Efficacy and Stargazin

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Abstract The α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid receptors (AMPARs) are glutamate-gated cation channels mediating the majority of fast excitatory synaptic transmission in the central nervous system (CNS). Polyamine toxins derived from spiders and wasps are use- and voltage-dependent channel blockers of Ca^{2+}-permeable AMPARs. Recent studies have suggested that AMPAR block by polyamine toxins is modulated by auxiliary subunits from the class of transmembrane AMPAR regulatory proteins (TARPs), which may have implications for their use as tool compounds in native systems. We have explored the effect of the TARP γ-2 (also known as stargazin) on the inhibitory potency of three structurally different polyamine toxins at Ca^{2+}-permeable homomeric GluA1 AMPARs expressed in oocytes. We find that polyamine toxin IC_{50} is differentially affected by presence of stargazin depending on the efficacy of the agonists used to activate GluA1. Co-assembly of GluA1 receptors with stargazin increases the potency of the polyamine toxins when activated by the weak partial agonist kainate, but has no effect in presence of full-agonist L-glutamate (Glu) and partial agonist (*RS*)-willardiine.

Special Issue: In honor of Krogsgaard-Larsen.

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Keywords Ca^{2+}-permeable AMPARs · Polyamine toxins · Auxiliary subunits · *Xenopus* oocytes

Introduction

α-Amino-3-hydroxy-5-methyl-4-isoxazole propionic acid receptors (AMPARs) are ligand-gated cation channels belonging to the ionotropic glutamate receptor superfamily and mediate fast excitatory synaptic transmission in all major regions of the central nervous system (CNS); being implicated in brain functions such as cognition, perception, learning and memory [1, 2]. AMPARs are formed by tetrameric assembly of four subunits (GluA1–4) to produce a range of homo- or heteromeric receptor subtypes of varying subunit composition [2]. GluA1 to GluA4 subunits are highly similar and share the same overall structural topology (Fig. 1a). Each subunit contains an extracellular agonist-binding domain (ABD), which upon binding of Glu undergoes conformational changes that are relayed to the ion channel to drive channel opening [2]. The central channel is formed by equal contribution of M2 and M3 membrane segments from each subunit (Fig. 1a). The M2 segments line the ion permeation pore, whereas the extracellular facing parts of the M3 segments form gates that control ion accessibility to the channel (Fig. 1a) [3, 4]. The gating segment of M3 is connected to the ABD via a short linker; allowing agonist-induced conformational change in the ABD to be translated into rearrangement of the M3 gating segment in each subunit [4]. In the receptor, each of the four subunit-associated gates are thought to operate semi-autonomously such that AMPARs can assume multiple open states depending on the number (1, 2, 3 or 4) of gates that are open simultaneously [5]. These open states have discrete ion permeation properties, which underlie the up to four

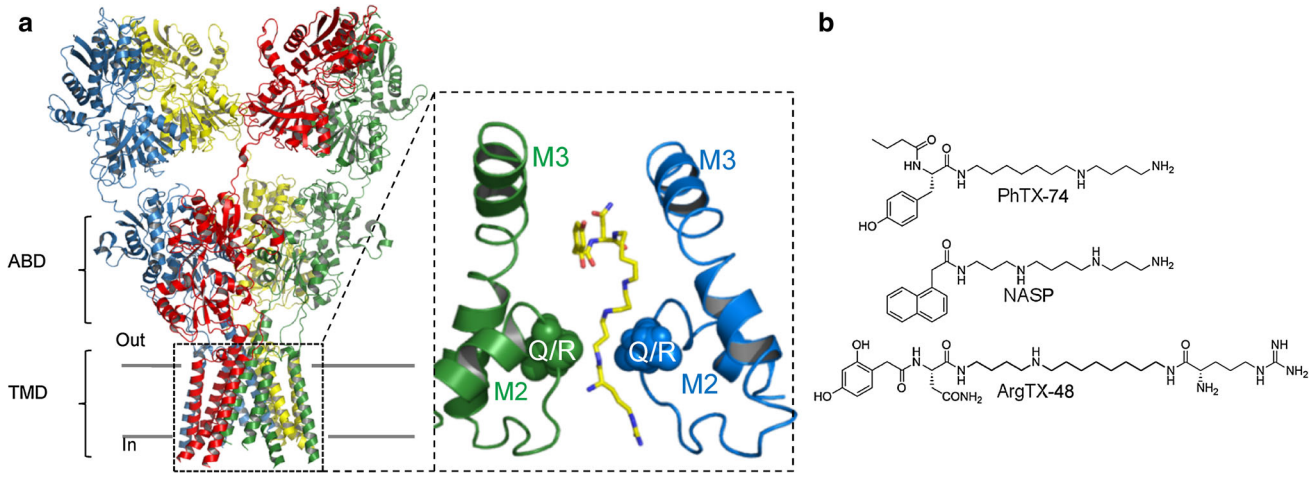

Fig. 1 Overview of AMPAR structure and polyamine toxin channel blockers. **a** Molecular structure of homomeric GluA2 receptor (PDB code 3KG2). The surface contours of subunits are colored in *different colors* to illustrate tetrameric subunit organization (*right*). Molecular modeling of ArgTX-636 docked into a homology model of the GluA1 ion channel [29] illustrating the proposed polyamine toxin binding mode. Shown are the M2–M3 segments from two subunits with M1 and M4 of the transmembrane domain (TMD) omitted for clarity. ArgTX is shown as *yellow sticks* and the selectivity filter *highlighted ball representation* (*left*). **b** Structures of selected polyamine toxins included in the study

conductance levels that are observed in recordings of AMPAR single-channel currents [5–10]. However, insight into the structural basis underlying multiple conductance states is limited as the presently single existing full-length AMPAR X-ray crystal structure represent an antagonist-bound closed conformation [3]. In addition to subunit composition, AMPAR properties depend also on subunit splice isoform [11–13], posttranscriptional modifications [7, 14, 15] and co-assembly with a range of interacting proteins [6, 16–18]. Overall, AMPAR subtypes can be divided into two functional groups depending on the presence of the GluA2 subunit in the receptor complex. In the mature CNS, mRNA editing changes a Gln to Arg at position 602 (the Q/R site) located at the tip of the M2 segment that forms the channel selectivity filter (Fig. 1a). GluA2-containing AMPARs thus contain positively charged Arg side chains at the selectivity filter, which lowers channel conductance levels [9], Ca^{2+} permeability and sensitivity to channel blockers [19–22].

In contrast to GluA2-containing AMPARs, GluA2-lacking AMPARs are Ca^{2+}-permeable and subject to high-affinity channel block by polyamine toxins isolated from the venom of spiders and wasps that act as use- and voltage-dependent ion channel blockers [22–25]. These are low-molecular weight compounds that share an aromatic head-group and a polyamine tail moiety (Fig. 1b) [26]. No X-ray crystal structure is available of an AMPAR in complex with a polyamine toxin, but molecular modeling and structure–activity relationship studies have suggested a binding mode in which the polyamine toxin head-group is positioned in the ion channel vestibule external to the selectivity filter with the polyamine tail permeating the selectivity filter (Fig. 1a) [27–30]. Polyamine toxins are widely used as tool compounds in studies of native AMPARs because of their ability to selectively inhibit GluA2-lacking AMPARs. These polyamine toxins include the philanthotoxins (PhTXs), joro spider toxins (JSTXs), and argiotoxins (ArgTXs) (Fig. 1b), which all are well characterized at recombinant AMPARs [29, 31–34]. However, there is now wide consensus and good evidence that the majority of native AMPARs exist in stable complex with various types of transmembrane proteins that modify AMPAR channel function and pharmacology, including polyamine toxin block [35]. At present, three major classes of such auxiliary subunits have been identified; the TARPs, the cornichon homologs (CNIH), and the cysteine-knot AMPAR modulating proteins (CKAMP) [35–37]. Among these, members of the TARPs and CHINs families have been found to modify AMPAR channel properties; including rectification, subconductance levels and pharmacology [6, 16, 18, 38–40]. So far, auxiliary subunits from the TARP class have been found to modulate AMPAR polyamine channel block [38, 39, 41]. In particular, TARPs enhance the inhibitory efficacy of toxins from the PhTX class under conditions where a low efficacy agonist is used to activate GluA2-lacking AMPARs [39].

In the present study, we have explored the influence of the prototypical TARP stargazin on inhibitory potency of different types of polyamine toxins at homomeric GluA1 AMPARs. We find that the modulatory effect of stargazin vary among types of polyamine toxins and is dependent on agonist efficacy. Specifically, we observe modest or no effect of stargazin on all tested types of polyamine toxins when a high-efficacy agonist is used to activate GluA1. In contrast, stargazin decreases IC_{50} for all toxins when the weak partial agonist kainate (KA) is used; most dramatically producing an 80-fold decrease in the IC_{50} for the ArgTX analogue ArgTX-48.

Materials and Methods

Chemicals

PhTX-74 and ArgTX-48 were synthesized as previously described [32, 33], KA and willardiine (WIL) were from Abcam (Cambridge, UK). All other chemicals were from Sigma-Aldrich (St Louis, MO, USA).

Expression of AMPARs in *Xenopus* oocytes

The pGEM-HE plasmid vector containing the cDNA encoding the flip isoform of rat GluA1 was linearized using NheI restriction enzyme and followed by cRNA transcription using AmpliCap-Max^{TM} T7 High Yield Message Maker kit (VWR, PA, USA) using the protocol supplied by the manufacturer. The pBSK plasmid containing the cDNA encoding the rat TARP stargazin was kindly provided by Dr. P. Osten (Cold Spring Harbor Laboratory, NY, USA) and linearized using PCR (Forward primer containing overhang T7 polymerase recognition sequence; 5′-GCATAATACGA CTCACTATAGGGAGACTCGAGCCCGGGACCAT-GG GGCTGTTTGATCGAGG-3′, and the reverse primer containing overhang poly-T sequence; (5′-TTTTTTTTTTTTTT TTCATACGGGCGTGGTCCGGC-3′). Spectroscopy and gel electrophoresis was performed to quantify the quality and quantity of the synthesized cRNA. Stage V and VI oocytes were surgically removed, prepared and maintained as described by Poulsen et al. [34]. Oocytes were injected with 20–70 nL RNA solutions containing 10 ng GluA1 cRNA or a mixture of 10 ng GluA1 and 10 ng of stargazin RNA. Oocytes were incubated at 17 °C in standard Barth solution (in mM: 88 NaCl, 1 KCl, 0.33 $Ca(NO_3)_2$, 0.41 $CaC1_2$, 0.82 $MgSO_4$, 2.4 $NaHCO_3$, 10 HEPES; pH 7.4) applied with gentamicin (0.10 mg/mL) for approximately 24–48 h until the time of experiment.

Two-Electrode Voltage-Clamp Recordings and Data Analysis

Oocytes were placed in a recording chamber and voltage-clamped by the use of a two-electrode voltage clamp (Dagan Corporation, Minneapolis, USA) with both microelectrodes filled with 3 M KCl and immersed in the oocyte. During recordings the oocyte was continuously superfused with Ringer solution (in mM: 115 NaCl, 2 KCI, 1.8 $BaC1_2$, 5 HEPES; pH 7.6) and the test compounds (dissolved in Ringer solution) were added by bath application. Generation of concentration-inhibition curves were performed by applying increasing concentrations of polyamine toxins in the continuous presence of saturating concentrations of the appropriate agonist. Current response in the presence of a given polyamine toxin concentration was normalized to the current in absence of toxin for individual oocytes. Concentration–response data sets from 3 to 8 individual oocytes were pooled and used to determine IC_{50} by curve fitting using GraphPad Prism 6.0 software (GraphPad, San Diego, USA) to the equation:

$$I = \frac{1}{1 + \left(\frac{[antagonist]}{IC_{50}}\right)^{nH}},$$

where I is the agonist-evoked current at a given concentration of polyamine toxin, [antagonist] is the concentration of polyamine toxin and n_H is the Hill slope using.

Results

Auxiliary subunits belonging to the TARP family have recently been found to modulate polyamine toxin inhibition of Ca^{2+}-permeable AMPARs [18, 38–40]. Specifically, co-expression of the prototypical TARP stargazin (also known as γ-2) and γ-8 with homomeric GluA4 was shown to increase extent of block by PhTX analogues of currents evoked by the partial agonist KA, while not affecting block of currents evoked by the full agonist Glu [39]. To systematically examine the generality of the effect of stargazin and agonist efficacy on the inhibitory potency of polyamine toxins, we expressed homomeric GluA1 receptors in the presence and absence of stargazin in *Xenopus* oocytes. IC_{50} values for polyamine toxins were determined from full concentration-inhibition curves for inhibition of agonist-evoked currents using two-electrode voltage clamp electrophysiology at holding potentials of −80 and −60 mV ("Materials and Methods" section) (Figs. 2, 3). The standard recording protocol used for measurement of polyamine toxin inhibition is illustrated in Fig. 2a. Also, to verify functional complex formation of GluA1 receptors and stargazin in oocytes ("Materials and Methods" section), we determined the ratio of steady-state currents induced by KA and Glu. Potentiation of steady-state current induced by association with stargazin is larger for KA than for Glu. Thus, measurements of the ratio between Glu and KA-evoked currents can be employed as an assessment of stargazin presence in the AMPAR complex at a given oocytes [16, 17, 39, 41–44]. We found that oocytes injected with GluA1 and stargazin consistently displayed KA/Glu response ratios more than 20-fold higher than the ratio observed in oocytes injected with GluA1 alone (Fig. 2b), in agreement with previous studies [16, 17, 34, 41, 43] and ensuring functional association of stargazin with GluA1.

In addition to PhTX-74, two structurally diverse polyamine toxin analogues in the form of 1-naphtyl acetyl spermine (NASP) [45] and ArgTX-48 [32] were included. NASP is structurally similar to PhTX-74, with a related polyamine and aromatic head-group, but has the unique

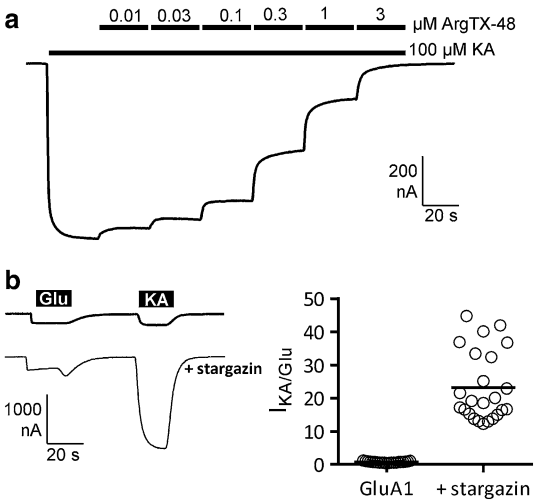

Fig. 2 Electrophysiological characterization of polyamine toxin channel block of GluA1 currents in *Xenopus* oocytes. **a** Current recording illustrating the protocol for obtaining concentration-inhibition relationship of polyamine toxin block of agonist-evoked steady-state currents at GluA1 receptors. **b** Current responses evoked by Glu and KA from oocytes expressing homomeric GluA1 receptors in the absence (*upper trace*) and presence of stargazin (*lower trace*) (*left*). Scatterplot summarizing the KA versus Glu response ratio calculated as I_{KA}/I_{Glu} where I_{KA} and I_{Glu} are the steady-state current responses evoked by 100 μM KA and 300 μM Glu, respectively (*right*). Each data point represents an unique oocyte

property among polyamine toxins of having very low dependence on membrane potential for high-affinity binding in the AMPAR channel. Both are popular tools for achieving selective block of GluA2-lacking AMPAR subtypes in native cells and tissue, for which homomeric GluA1 receptors should be a good representative. ArgTX-48 is a close analog of the prototypical ArgTX-636 and has recently been identified as one of the most potent and selective inhibitors of AMPARs [32]. For activation of GluA1 receptors, we used three different agonists, chosen to cover a range of efficacies at GluA1, in the form of the endogenous full agonist Glu (representing a high-efficacy agonist), the medium efficacy partial agonist WIL and the low efficacy partial agonist KA [5, 7].

First, at holding potential of −80 mV, we observed no or less than twofold difference between the IC_{50} values obtained in the absence and presence of stargazin for any of the three blockers when the high-efficacy agonist Glu was used to activate GluA1 (Fig. 3; Table 1). For PhTX-74, this result is in good agreement with previous findings by Jackson et al. [39], who found that stargazin had little effect on the percent inhibition of Glu-evoked currents at GluA4 produced by 100 nM PhTX-433. In contrast, when the low-efficacy agonist KA was used, we observed differences between IC_{50} values obtained in absence and presence of stargazin for all three compounds (Fig. 3; Table 1). As summarized in Fig. 4, these changes all followed the same trend where co-expression of stargazin increased inhibitory potency of the polyamine toxin analogues. However, the magnitude of this effect of stargazin varied among the compounds. Specifically, the IC_{50} value of PhTX-74 and NASP decreased by six- and twofold, respectively, upon co-expression with stargazin, whereas a much larger 80-fold

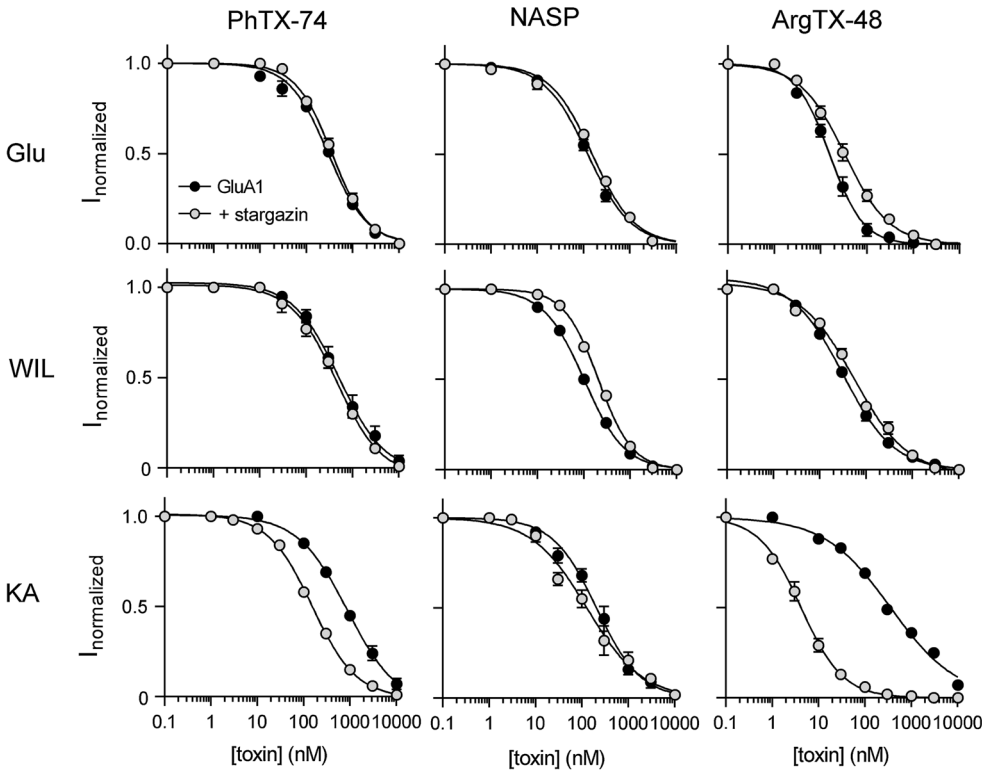

Fig. 3 Determination of IC_{50} for PhTX-74, NASP and ArgTX-48 at homomeric GluA1 receptors in presence and absence of stargazin. Concentration-inhibition curves for inhibition by PhTX-74 (*left column*), NASP (*middle column*), and ArgTX-48 (*right column*) of currents evoked by Glu (300 μM; *upper row*), WIL (30 μM; *middle row*) and KA (100 μM; *lower row*) from oocytes expressing GluA1 in presence (*gray circles*) and absence (*black circles*) of stargazin and held at holding potential (V_H) of −80 mV. Values are mean ± SEM of experiments with 4–12 oocytes. IC_{50} values derived from the curves are given in Table 1. Data for PhTX-74 inhibition of Glu evoked currents are from Ref. [34]

Table 1 Dependence of inhibitory potency of polyamine toxins at homomeric GluA1 on agonist type and presence of stargazin at $V_H = -80$ and -60 mV

Agonist	Stargazin[a]	IC_{50} (nM)[b]					
		PhTX-74		NASP		ArgTX-48	
		−60 mV	−80 mV	−60 mV	−80 mV	−60 mV	−80 mV
Glu	–	934	350[c]	107	130	27	15
		(576–1,514)	(289–424)	(86–132)	(101–156)	(12–59)	(12–18)
	+	1,717	376[c]	133	170	45	28
		(1,442–2,045)*	(319–444)	(109–163)	(147–198)*	(36–56)	(22–38)*
WIL	–	1,324	498	146	103	123	32
		(743–2,358)	(332–793)**	(99–214)	(92–115)	(95–160)**	(26–38)**
	+	1,927	435	159	221	99	55
		(1,506–2,467)	(357–564)**	(128–196)	(199–245)*	(82–120)**	(43–70)*,**
KA	–	4,236	879	570	241	965	323
		(2,836–6,259)**	(686–1,127)**	(371–877)**	(178–323)**	(473–1,968)**	(262–407)**
	+	583	151	188	110	8	4
		(463–733)*,**	(131–174)*,**	(119–297)*	(76–159)*,**	(6–10)*,**	(3–5)*,**

* $p < 0.05$ Significantly different from compound IC_{50} obtained in absence of stargazin (Unpaired *t* test with Welch's correction). ** $p < 0.05$ Significantly different from compound IC_{50} obtained using Glu as agonist (Unpaired *t* test with Welch's correction)

[a] Co-expression with stargazin is described in the ("Materials and Methods" section)

[b] IC_{50} values were determined by the nonlinear fitting of composite concentration-inhibition data collected at 4–12 oocytes held at −60 and −80 mV (See "Materials and Methods" section)

[c] Data derived from Poulsen et al. [34]

decrease was observed for ArgTX-48 (Fig. 4). We also determined the IC_{50} values of all three polyamine blockers when WIL was used as agonist. WIL has efficacy in-between the efficacy of Glu and KA [5, 7, 46] (Fig. 3; Table 1). For this agonist, there was no or less than twofold difference between the IC_{50} values obtained in the absence and presence of stargazin for any of the three compounds (Fig. 4). These effects are similar to that observed when using Glu as agonist; indicating that the modulatory effect of stargazin on polyamine toxin inhibitory potency is constrained to the low-efficacy agonist KA. We also evaluated the effects of stargazin and agonist type on voltage-dependency of polyamine toxin block by determining IC_{50} values at a holding potential of −60 mV (Table 1). In general, for all conditions the IC_{50} values of PhTX-74 and ArgTX-48 at GluA1 were found to increase approximately two- to fourfold when the holding potential was lowered from −80 to −60 mV, whereas NASP displayed similar IC_{50} values at −60 and −80 mV; in agreement with previous findings that this polyamine toxin analogue has limited voltage dependency [45]. The relative increase in IC_{50} values when KA was used as agonist compared to Glu and its rescue by stargazin, observed for PhTX-74 and ArgTX-48, was also maintained at −60 mV. These results show that stargazin does not influence the voltage-dependency of polyamine toxin channel block of AMPARs.

Discussion

In addition to subunit composition, mRNA editing and alternative splicing, functional heterogeneity of native AMPARs is now widely recognized to also be controlled by differential association with auxiliary subunits, which have varied expression among different CNS regions and neuronal cell types [35, 47]. These findings have expanded the spectrum of possible functional phenotypes among native AMPARs and added a new layer of complexity to our understanding of the neurobiological role of AMPARs in the mature and developing CNS and in diverse neurological conditions. Furthermore, the realization that most auxiliary subunits can have profound influence on important aspects of AMPAR pharmacology have prompted renewed characterizations of the pharmacology of classical AMPAR ligands at receptors expressed in the presence of auxiliary subunits, including polyamine toxins [17, 41–43]. Specifically, members of the TARP and CNIH classes of auxiliary subunits are now known to modulate properties such as the mean conductance of the channel [6, 16, 18], which has been found important for polyamine block [38, 39]. Overall, our results show that the prototypical TARP stargazin does not change the inhibitory potency and voltage-dependence of PhTX-74, NASP and ArgTX-48

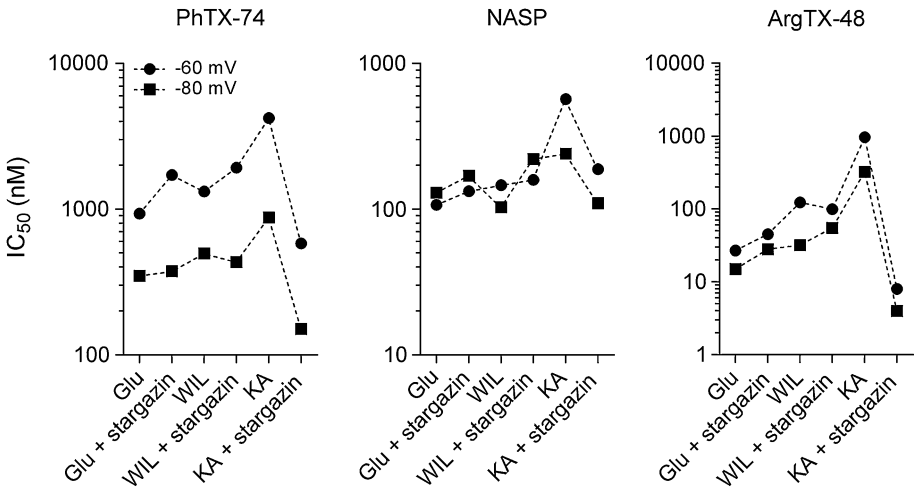

Fig. 4 Graphical summary of the effect of agonist type and stargazin on polyamine toxin inhibitory potency. IC_{50} values for PhTX-74 (*left*), NASP (*middle*) and ArgTX-84 (*right*) obtained at membrane potentials of −60 mv (*filled circle*) and −80 mV (*filled square*) are shown as function of presence or absence of stargazin and agonist type; connected by stipulated lines to visualize toxin-specific patterns of sensitivity of IC_{50} to agonist type and stargazin presence

inhibition of currents evoked by the endogenous AMPAR agonist Glu. Together with previous results showing little or no effect of stargazin and other TARP subtypes (γ-3, -4, -8) on inhibition by PhTX-433 [39], PhTX-74 [32] and NASP [41] of Glu-evoked steady-state currents at homomeric GluA1, GluA3 and GluA4, our results suggest that TARPs do not modulate the pharmacology of externally applied polyamine toxins at GluA2-lacking receptors. Thus, experimental protocols utilizing polyamine toxins that were devised prior to the realization of the important role of TARPs as auxiliary AMPAR subunits may still be considered valid.

For recombinant AMPARs in the absence of auxiliary subunits, it has previously been observed that polyamine toxins can block AMPAR currents activated by high-efficacy agonists to a higher extent than currents activated by low-efficacy agonists [39, 48]. This has been attributed to differential affinity of polyamine toxins for the ion channel binding site among the distinct structural conformations that underlie different conductance levels. Even at saturating agonist conditions where all four AMPAR subunits are agonist-bound, low-efficacy agonists such as KA only activate channel openings of very low conductance [5, 9]. Within the model for AMPAR partial agonism proposed by Jin et al. [5], low-conductance states correspond to one or two subunit gates being open simultaneously. When the ion channel resides in these low-conductance states, polyamine toxins may have lower affinity for their binding site as compared to high-conductance states that are frequently visited during activation by high-efficacy agonists [39]. Our present results provide the first systematic quantification of the dependence of toxin IC_{50} on agonist type. As summarized in Fig. 4, in the absence of stargazin, IC_{50} increases significantly for all tested toxins when a saturating concentration of the weak partial agonist KA is employed to activate GluA1 compared to when the full agonist Glu is used as agonist. We observed the largest decrease in IC_{50} for ArgTX-48, which is approximately 20-fold less potent when KA is used opposed to Glu, whereas IC_{50} of NASP was least dependent (Fig. 4). Thus, agonist-dependent IC_{50} appears to be a general feature for polyamine toxins, but the effect of agonist type vary in extent among different toxin classes. Furthermore, our results confirm the initial observation by Jackson et al. [39] for PhTX-433, which showed co-expression of stargazin to have a dramatic effect on agonist-dependence. Specifically, this study found PhTX-433 to become equipotent at GluA4 currents evoked by Glu and KA. At GluA1, we also observe this effect of stargazin for PhTX-74, NASP and ArgTX-48: Co-expression with stargazin "rescue" the loss-of-potency observed at KA-evoked currents compared to Glu-evoked currents, most dramatically for ArgTX-48, where stargazin produce an 80-fold increase in potency for inhibition of KA-evoked currents (Table 1; Fig. 4). Interestingly, for both PhTX-74 and ArgTX-48, we observe higher toxin potency with KA as agonist compared to Glu and WIL when stargazin is present in the GluA1 complex. The profound enhancement by stargazin of polyamine toxin potency for inhibition of KA-evoked currents has been attributed to TARP enhancement of AMPAR gating efficiency [39]; hereby increasing the probability that multiple gates are open simultaneously and thus increasing the frequency of high-conductance states (3–4 open gates) at the expense of decreased frequency of low-conductance states (1–2 open gates). Specifically, TARPs dramatically increase the apparent efficacy of KA [16, 17, 41–44]. Given the previous finding that extent of PhTX-433 inhibition correlates with AMPAR mean conductance [39], our findings can be explained by a shift towards high-conductance states for which the polyamine toxins may have higher affinity. It is noteworthy that we observe large differences among PhTX-74, NASP and ArgTX-48 with

regards to the fold decrease in IC_{50} for KA-evoked currents upon stargazin co-expression. PhTX-74 and NASP experience approximately 22- to 10-fold decrease in IC_{50}, whereas the decrease for ArgTX-48 is close to 100-fold (Table 1; Fig. 4). Thus, within the above described framework of state-dependent polyamine toxin binding, ArgTX-48 appears much more dependent on high- versus low-conductance state configurations of the AMPAR ion channel compared to PhTX-74 and NASP (Fig. 4). ArgTX-48 is structurally different from PhTX-74 and NASP by having a longer polyamine moiety, an additional tail amino acid (Arg), as well as a linker amino acid (Asn) between the polyamine tail and the aromatic head-group (Fig. 1b), and its increased sensitivity towards low-conductance channel configurations might indicate a unique binding mode of ArgTX-48 compared to PhTX-74 and NASP. These different properties could prove useful in future studies aimed to probe the molecular conformation of the AMPAR ion channel.

In general, the realization that auxiliary subunit modulation of receptor function can influence polyamine toxin pharmacology might warrant that other mechanisms that shape AMPAR kinetics are systematically re-evaluated for their influence on polyamine toxin potency. These could include additional characterization of the interplay between auxiliary subunits and alternative splicing of the flip/flop exons, which shape desensitization and deactivation kinetics, as well as receptor phosphorylation states that can control receptor single-channel kinetics and open-probability.

In conclusion, we find little or no influence of stargazin on potency of voltage-dependent polyamine toxin block of homomeric GluA1 AMPARs activated by Glu. In contrast, we observe significant differences among the dependence of polyamine toxin potency on type of agonist, which is influenced by presence of stargazin in the receptor complex.

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