The spider toxin, argiotoxin₆₃₆, binds to a Mg²⁺ site on the N-methyl-D-aspartate receptor complex

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1 The mechanism of action of the arylalkylamine spider toxin, argiotoxin₆₃₆, on the N-methyl-D-aspartate (NMDA) receptor was investigated by use of [³H]-dizocilpine binding to well-washed membranes obtained from rat brain.

2 Argiotoxin₆₃₆ decreased [³H]-dizocilpine binding with an apparent potency of about 3 μM. The inhibition of [³H]-dizocilpine by argiotoxin₆₃₆ was insensitive to the concentration of glutamate, glycine and spermidine in the assay.

3 Argiotoxin₆₃₆ alone had no effect on the dissociation of [³H]-dizocilpine. However, argiotoxin₆₃₆ reversed the actions of Mg²⁺ on the dissociation of [³H]-dizocilpine by decreasing the apparent potency of Mg²⁺. Argiotoxin₆₃₆ also reversed the action of Ca²⁺ on the dissociation of [³H]-dizocilpine.

4 These results suggest that argiotoxin₆₃₆ exerts a novel inhibitory effect on the NMDA receptor complex by binding to one of the Mg²⁺ sites located within the NMDA-operated ion channel.

Keywords: NMDA receptor; argiotoxin₆₃₆; [³H]-dizocilpine; Mg²⁺

Introduction

A variety of arylalkylamine toxins have been isolated from the venom of orb-weaving and funnel web spiders (Jackson & Usherwood, 1988; Jackson & Parks, 1989). These include joro spider toxin (JSTX), a family of α-agatoxins from the funnel web spider *Agelenopsis aperta* (Quistad *et al.*, 1990) and a series of argiotoxins from the *Argiope* genus of orb weaving spiders (Adams *et al.*, 1987). Their biological activity is believed to reside in their potent antagonism of arthropod glutamate receptors. These toxins are not competitive with respect to the actions of glutamate and act primarily by blocking glutamate-operated cation channels (Jackson & Usherwood, 1988). Thus, these toxins produce neuromuscular blockade and paralysis.

Glutamate, although not employed at neuromuscular junctions, is an important neurotransmitter in the mammalian central nervous system. Mammalian glutamate receptors are pharmacologically distinct from their arthropod counterparts (Shinozaki, 1988). However, the principal glutamate receptors are also intimately associated with cation-conducting ion channels (Collingridge & Lester, 1989). Thus, it is possible that arylalkylamine spider toxins might also be effective channel blocking agents at mammalian glutamate receptors (Jackson & Usherwood, 1988). Several studies have demonstrated that JSTX selectively and non-competitively blocks AMPA and kainate receptors (Saito *et al.*, 1985; Akaike *et al.*, 1987). In addition, argiotoxin₆₃₆ selectively blocks N-methyl-D-aspartate (NMDA) receptors by a use- and voltage-dependent mechanism, which suggests a site of action within the NMDA-operated ion channel (Priestley *et al.*, 1989).

The NMDA receptor complex has been extensively characterized both electrophysiologically and in ligand binding studies. These studies have demonstrated the presence of several distinct binding sites on the receptor, including recognition sites for glutamate and NMDA, glycine, Zn²⁺, Mg²⁺, phencyclidine-like drugs and most recently polyamines (Collingridge & Lester, 1989). We have previously demonstrated that the action of drugs at each of these binding sites can be differentiated by utilizing [³H]-dizocilpine ([³H]-MK801) binding to the phencyclidine site (Reynolds *et al.*, 1987; Reynolds & Miller, 1988; Reynolds, 1990a,b). The finding that argiotoxin₆₃₆, which incorporates a polyamine-like moiety as a part of its structure (Figure 1), was a specific NMDA receptor antagonist raised the possibility

that argiotoxin₆₃₆ might act by inhibiting the positive modulatory actions of polyamines on the NMDA receptor complex (Ransom & Stec, 1988). To examine this hypothesis, the interaction of argiotoxin₆₃₆ with the NMDA receptor was monitored in ligand binding studies in which [³H]-dizocilpine was used to label the receptor.

Methods

In [³H]-dizocilpine binding experiments well-washed membranes from rat brain were used prepared as previously described (Reynolds & Miller, 1989). Assay tubes contained 100–150 μg membrane protein, 0.2–0.3 nM [³H]-dizocilpine and drugs as appropriate in a final volume of 1 ml 10 mM HEPES-NaOH, pH 7.4. Assays were incubated for 2 h at room temperature (21–25°C), after which tubes were vacuum filtered over glass fibre filters (number 32, Schleicher and Schuell, Keene, NH, U.S.A.). After three 5 ml washes with HEPES buffer, filters were placed in scintillant (Ecolite, ICN Biomedicals, Cleveland, OH, U.S.A.) for at least 90 min before liquid scintillation counting. Assays performed in this way do not reach equilibrium within 2 h unless saturating concentrations of glutamate, glycine and spermidine have been added. This non-equilibrium condition permits the assay of the NMDA receptor activators and positive allosteric modulators (Reynolds *et al.*, 1987; Foster & Wong, 1987; Bonhaus & McNamara, 1988). Solutions of synthetic argiotoxin₆₃₆ (Natural Product Sciences, Salt Lake City, UT, U.S.A.) were prepared in HEPES buffer on the day of use, as stock solutions become discoloured within 24 h of storage at –20°C.

The estimation of the actions of argiotoxin₆₃₆ on the dissociation of [³H]-dizocilpine was performed essentially as previously described (Reynolds & Miller, 1989) with a minor modification. Briefly, [³H]-dizocilpine (15–20 nM) was

Figure 1 Structure of argiotoxin₆₃₆.

incubated with membranes (10 mg ml^{-1} protein) for 2 h. Aliquots ($10 \mu\text{l}$) of this mixture were then added to assay tubes containing $100 \mu\text{M}$ glutamate, $30 \mu\text{M}$ glycine and drugs as appropriate in a final volume of 1 ml HEPES- NaOH , pH 7.4. A smaller volume was used than in the previous study in order to minimize the quantity of argiotoxin₆₃₆ necessary for this series of experiments. However, the dilution factor (100 fold) was similar to that previously employed. Moreover, the addition of dizocilpine after 2 h did not further decrease the levels of binding below that produced by dilution alone, suggesting that the dilution was sufficient to eliminate the association reaction. After a further 60 min incubation, the assay mixture was filtered and radioactivity determined as described above.

Concentration-response curves with argiotoxin₆₃₆ were analysed with the EBDA and LIGAND programmes as previously described. In the presence of $100 \mu\text{M}$ glutamate and $30 \mu\text{M}$ glycine, the dissociation rate of [^3H]-dizocilpine is 0.00384 min^{-1} (Reynolds & Miller, 1989). After 60 min, $56 \pm 3.3\%$ of the total binding remained. Results from these experiments were expressed as a proportion of the binding remaining after 60 min.

Results

Argiotoxin₆₃₆ inhibited basal [^3H]-dizocilpine binding with a potency of about $3 \mu\text{M}$ (Figure 2). The addition of saturating concentrations of glutamate ($100 \mu\text{M}$) and glycine ($30 \mu\text{M}$) did not significantly modify the potency, while spermidine significantly increased the potency of argiotoxin₆₃₆. Interestingly, in the presence of high levels of glutamate and glycine spermidine had no effect on the potency of argiotoxin₆₃₆. A summary of the data for the inhibition of [^3H]-dizocilpine binding by argiotoxin₆₃₆ is given in Table 1. Hill slopes did not significantly differ from unity. The failure of glutamate, glycine and spermidine to decrease the apparent potency of argiotoxin₆₃₆ precludes an action of the toxin at the NMDA, glycine and polyamine sites, in spite of the similarity in structure between argiotoxin₆₃₆ and spermine.

To probe further the mechanism of action of argiotoxin₆₃₆, the effects of the toxin on the dissociation of [^3H]-dizocilpine were monitored. Argiotoxin₆₃₆ had little effect on the dissociation of [^3H]-dizocilpine at concentrations ranging from 0.5 – $200 \mu\text{M}$ (Figure 3a). The addition of 0.5 mM Mg^{2+}

Figure 2 Effect of glutamate, glycine and spermidine (Spd) on the inhibition of [^3H]-dizocilpine binding by argiotoxin₆₃₆. Concentration-response curves were generated in the presence of $0.1 \mu\text{M}$ glutamate and $0.03 \mu\text{M}$ glycine (■), $100 \mu\text{M}$ glutamate and $30 \mu\text{M}$ glycine (●), $0.1 \mu\text{M}$ glutamate, $0.03 \mu\text{M}$ glycine and $50 \mu\text{M}$ spermidine (▲) or $100 \mu\text{M}$ glutamate, $30 \mu\text{M}$ glycine and $50 \mu\text{M}$ spermidine (▼). Non-equilibrium binding assays were performed as described in methods. Results represent the mean of 3–6 experiments performed in duplicate. Vertical bars represent s.e.mean although some error bars are smaller than the appropriate symbols.

Table 1 Effects of argiotoxin₆₃₆ on [^3H]-dizocilpine binding to well-washed rat brain membranes

Condition	IC ₅₀ , μM	nH
$0.1 \mu\text{M}$ Glu + $0.03 \mu\text{M}$ Gly	3.03 ± 0.74	1.22 ± 0.16
$100 \mu\text{M}$ Glu + $30 \mu\text{M}$ Gly	3.36 ± 0.52	1.34 ± 0.13
$0.1 \mu\text{M}$ Glu + $0.03 \mu\text{M}$ Gly + $50 \mu\text{M}$ Spd	$0.84 \pm 0.11^{**}$	1.06 ± 0.08
$100 \mu\text{M}$ Glu + $30 \mu\text{M}$ Gly + $50 \mu\text{M}$ Spd	2.67 ± 0.47	0.96 ± 0.03

Results are the mean ($\pm$ S.E.M.) of 3–5 experiments performed in duplicate as described in methods.

** Value significantly different ($P < 0.05$) from IC₅₀ in the presence of $100 \mu\text{M}$ Glu + $30 \mu\text{M}$ Gly (ANOVA with Bonferroni correction for multiple comparisons).

greatly reduced [^3H]-dizocilpine binding remaining after 60 min. Interestingly, although argiotoxin₆₃₆ had little effect alone, it effectively reversed the actions of Mg^{2+} . Additionally, argiotoxin₆₃₆ also reversed the effects of Ca^{2+} on the dissociation of [^3H]-dizocilpine. Half maximal reversal of the effects of Mg^{2+} were observed at about $30 \mu\text{M}$, and approximately half maximal reversal of the action of Ca^{2+} at $100 \mu\text{M}$. Dizocilpine at concentrations up to $1 \mu\text{M}$ was unable to reverse the actions of Mg^{2+} (data not shown). The concentration-dependence of the actions of Mg^{2+} and Ca^{2+} are illustrated in Figure 3b. These cations increase the

Figure 3 Effects of (a) argiotoxin₆₃₆ and (b) divalent cations on the dissociation of [^3H]-dizocilpine from the NMDA receptor. Data are expressed as a proportion of [^3H]-dizocilpine binding remaining after 60 min. At this time $56 \pm 3.3\%$ of the total binding remained. Curves in (a) were performed in the absence of cations (■) or in the presence of 0.5 mM Mg^{2+} (●) or 0.5 mM Ca^{2+} (▲). Curves in (b) were performed using Mg^{2+} (squares) and Ca^{2+} (circles) in the absence (closed symbols) and presence (open symbols) of $50 \mu\text{M}$ argiotoxin₆₃₆ as indicated. The results represent the mean of 3–4 experiments performed in duplicate as described in methods. Vertical bars represent s.e.mean.

dissociation of [³H]-dizocilpine at similar concentrations to those at which they inhibit non-equilibrium [³H]-dizocilpine binding (Reynolds & Miller, 1988). The addition of 50 μ M argioToxin₆₃₆ decreased the apparent potency of both Mg²⁺ and Ca²⁺ in this assay although Mg²⁺ was more sensitive to the effects of the toxin.

Discussion

A primary goal of this study was to determine the site and mechanism of action of the arylalkylamine spider toxin, argioToxin₆₃₆. These experiments permit several conclusions to be drawn regarding the likely site of action of the toxin. It is unlikely that argioToxin₆₃₆ binds to the NMDA or glycine binding sites on the NMDA receptor as glutamate and glycine did not alter the apparent potency of the toxin. Polyamines exert two prominent effects on [³H]-dizocilpine binding. Low concentrations (5–100 μ M) increase [³H]-dizocilpine binding, while higher concentrations have an inhibitory effect (Reynolds & Miller, 1989; Williams *et al.*, 1989). The concentration of spermidine used is sufficient to occupy fully the polyamine site responsible for increasing [³H]-dizocilpine binding and will significantly decrease the apparent potency of competitive polyamine antagonists such as arcaine (Reynolds, 1990a). This concentration of spermidine did not decrease the potency of argioToxin₆₃₆, thus precluding an action at this polyamine site. Indeed, in the presence of low levels of glutamate and glycine, spermidine actually increased the potency of argioToxin₆₃₆. The mechanism underlying this effect is not clear.

The investigation of the actions of argioToxin₆₃₆ on the dissociation of [³H]-dizocilpine was most revealing. ArgioToxin₆₃₆ alone had little effect on the dissociation of [³H]-dizocilpine. This distinguishes it from Zn²⁺-like drugs. The latter agents, by closing the NMDA-operated ion channel, delay the egress of [³H]-dizocilpine and thereby result in more binding remaining after 60 min, compared to control. In addition, argioToxin₆₃₆ does not simply mimic the actions of Mg²⁺. As demonstrated in Figure 3, Mg²⁺ profoundly decreases the levels of [³H]-dizocilpine binding remaining after 60 min, presumably as a consequence of an allosteric interaction between the Mg²⁺ and the phenylcyclidine binding sites. It is also unlikely that argioToxin₆₃₆ mimics dizocilpine as, unlike argioToxin₆₃₆, dizocilpine was unable to reverse the actions of Mg²⁺.

On the basis of these inferences it seems probable that argioToxin₆₃₆ binds to the Mg²⁺ site on the receptor that is responsible for accelerating the dissociation of [³H]-dizocilpine, but without exerting an allosteric effect on the phenylcyclidine site. ArgioToxin₆₃₆ might therefore be considered as a Mg²⁺-like compound that lacks efficacy, that is, a Mg²⁺ antagonist. However, this term should be used

with caution, as argioToxin₆₃₆ itself is an antagonist of the function of the receptor. If argioToxin₆₃₆ binds to a Mg²⁺ site but does not produce an allosteric effect it is reasonable to ask how argioToxin₆₃₆ inhibits non-equilibrium [³H]-dizocilpine binding as shown in Figure 2. The mechanism for this inhibition has not been determined. However, as the Mg²⁺ site, like the phenylcyclidine site, is located within the ion channel and the argioToxin₆₃₆ molecule is relatively large, it is possible that inhibition of binding occurs through steric hindrance.

This conclusion is consistent with several prior observations. Priestley and colleagues (1989) showed that argioToxin₆₃₆ was a use- and voltage-dependent antagonist of the NMDA receptor. The finding that argioToxin₆₃₆ interacts with a Mg²⁺ site is fully consistent with these observations. Moreover, the potency of argioToxin₆₃₆ was similar to that found in this study. Preliminary studies that monitored the actions of argioToxin₆₃₆ on [³H]-glycine binding showed that argioToxin₆₃₆ increased [³H]-glycine binding (Gullak *et al.*, 1989). This is consistent with the lack of a competitive interaction between argioToxin₆₃₆ and glycine observed in this study. Interestingly, Mg²⁺ also stimulates glycine binding (Marvizon & Skolnick, 1988; Gullak *et al.*, 1989). Moreover, Mg²⁺ effectively displaced [¹²⁵I]-argioToxin₆₃₆ binding to rat brain membranes (Mena *et al.*, 1989). In a recent study, it was concluded that the low affinity inhibitory actions of polyamines were mediated by an action at a Mg²⁺-like binding site (Reynolds, 1990a). The structural similarity between argioToxin₆₃₆ and spermine support the possibility that the inhibitory effects of the two compounds are mediated by the same binding site.

The precise identity of the Mg²⁺ site to which argioToxin₆₃₆ binds cannot be clearly ascertained. The most prominent action of Mg²⁺ is to effect a voltage-dependent blockade of the NMDA-operated ion channel (Nowak *et al.*, 1984; Mayer *et al.*, 1984), and it has generally been assumed that the actions of Mg²⁺ observed in [³H]-dizocilpine and [³H]-TCP (N-(1-thienyl)-cyclohexyl-3,4-piperidine binding assays are a consequence of Mg²⁺ binding to this site. However, a second Mg²⁺ binding site within the channel was recently reported. Mg²⁺ approaches this site from inside the cell, and displays a reversed voltage-dependence compared to the external site (Johnson & Ascher, 1990). It is not clear which of these sites is responsible for the actions of Mg²⁺ on [³H]-dizocilpine binding, nor is it clear to which Mg²⁺ site argioToxin₆₃₆ binds. However, the voltage-dependence observed by Priestley *et al.* (1989) is not consistent with an action only at the internal Mg²⁺ site. Further studies will be necessary to resolve this issue.

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References

- ADAMS, M.E., CARNEY, R.L., ENDERLIN, F.E., FU, E.T., JAREMA, M.A., LI, J.P., MILLER, C.A., SCHOOLEY, D.A., SHAPIRO, M.J. & VENEMA, V.J. (1987). Structures and biological activity of three synaptic antagonists from Orb Weaver spider venom. *Biochem. Biophys. Res. Commun.*, **148**, 678–683.
- AKAIKE, N., KAWAI, N., KISKIN, N.I., KLJUCHKO, E.M., KRISHTAL, O.A. & TSYNDRENKO, A.Y. (1987). Spider toxin blocks excitatory amino acid responses in isolated hippocampal pyramidal neurons. *Neurosci. Lett.*, **79**, 326–330.
- BONHAUS, D.W. & McNAMARA, J.O. (1988). N-methyl-D-aspartate receptor regulation of uncompetitive antagonist binding in rat brain membranes: kinetic analysis. *Mol. Pharmacol.*, **34**, 250–255.
- COLLINGRIDGE, G.L. & LESTER, R.A.J. (1989). Excitatory amino acid receptors in the vertebrate central nervous system. *Pharmacol. Rev.*, **41**, 143–210.
- FOSTER, A.C. & WONG, E.H.F. (1987). The novel anticonvulsant MK801 binds to the activated state of the N-methyl-D-aspartate receptor in rat brain. *Br. J. Pharmacol.*, **91**, 403–409.
- GULLAK, M.F., PAGNOZZI, M.J., RICHTER, K.E. & MENA, E.E. (1989). Effects of polyamine spider venom components on [³H]Glu and [³H]Gly binding to rat CNS membranes. *Soc. Neurosci.*, **153**, 1168. (Abstract)
- JACKSON, H. & PARKS, T.N. (1989). Spider toxins: recent applications in neurobiology. *Annu. Rev. Neurosci.*, **12**, 405–414.
- JACKSON, H. & USHERWOOD, P.N.R. (1988). Spider toxins as tools for dissecting elements of excitatory amino acid transmission. *Trends Neurosci.*, **11**, 279–283.
- JOHNSON, J.W. & ASCHER, P. (1990). Voltage dependent block by intracellular Mg²⁺ of N-methyl-D-aspartate activated channels. *Biophys. J.*, **57**, 1085–1090.
- MARVIZON, J.C. & SKOLNICK, P. (1988). [³H]glycine binding is modulated by Mg²⁺ and other ligands of the NMDA receptor-cation channel complex. *Eur. J. Pharmacol.*, **151**, 157–158.
- MAYER, M.L., WESTBROOK, G.L. & GUTHRIE, P.B. (1984). Voltage-dependent block by Mg²⁺ of NMDA responses in spinal cord neurones. *Nature*, **309**, 261–263.
- MENA, E.E., GULLAK, M.F., PAGNOZZI, M.J., PHILLIPS, D. & SACCOMANO, N. (1989). CNS binding sites of the novel NMDA antagonist Arg636. *Soc. Neurosci.*, **15**, 1168. (Abstract)

- NOWAK, L., BREGESTOVSKI, P., ASCHER, P., HERBERT, A. & PROCHIANZ, A. (1984). Magnesium gates glutamate-activated channels in mouse central neurones. *Nature*, **307**, 462–465.
- PRIESTLEY, T., WOODRUFF, G.N. & KEMP, J.A. (1989). Antagonism of responses to excitatory amino acids on rat cortical neurones by the spider toxin argitoxin₆₃₆. *Br. J. Pharmacol.*, **97**, 1315–1323.
- QUISTAD, G.B., SUWANRUMPHA, S., JAREMA, M.A., SHAPIRO, M.J., SKINNER, W.S., JAMIESON, G.C., LUI, A. & FU, E.W. (1990). Structures of paralytic acylpolyamines from the spider *Agelenopsis aperta*. *Biochem. Biophys. Res. Commun.*, **169**, 51–56.
- RANSOM, R.W. & STEC, N.L. (1988). Cooperative modulation of [³H]MK-801 binding to the N-methyl-D-aspartate receptor-ion channel complex by L-glutamate, glycine and polyamines. *J. Neurochem.*, **51**, 830–836.
- REYNOLDS, I.J. (1990a). Arcaine uncovers dual interaction of polyamines with the N-methyl-D-aspartate receptor. *J. Pharmacol. Exp. Ther.*, **255**, 1001–1007.
- REYNOLDS, I.J. (1990b). Arcaine is a competitive antagonist of the polyamine site on the NMDA receptor. *Eur. J. Pharmacol.*, **177**, 215–216.
- REYNOLDS, I.J. & MILLER, R.J. (1988). Multiple sites for the regulation of the N-methyl-D-aspartate receptor. *Mol. Pharmacol.*, **33**, 581–584.
- REYNOLDS, I.J. & MILLER, R.J. (1989). Ifenprodil is a novel type of NMDA receptor antagonist: interaction with polyamines. *Mol. Pharmacol.*, **36**, 758–765.
- REYNOLDS, I.J., MURPHY, S.N. & MILLER, R.J. (1987). ³H-labelled MK-801 binding to the excitatory amino acid receptor complex from rat brain is enhanced by glycine. *Proc. Natl. Acad. Sci., U.S.A.*, **84**, 7744–7748.
- SAITO, M., KAWAI, N., MIWA, A., PAN-HOU, H. & YOSHIOKA, M. (1985). Spider toxin (JSTX) blocks glutamate synapse in hippocampal pyramidal neurons. *Brain Res.*, **346**, 397–399.
- SHINOZAKI, H. (1988). Pharmacology of the glutamate receptor. *Prog. Neurobiol.*, **30**, 399–435.
- WILLIAMS, K., ROMANO, C. & MOLINOFF, P.B. (1989). Effects of polyamines on the binding of [³H]MK801 to the N-methyl-D-aspartate receptor: pharmacological evidence for the existence of a polyamine recognition site. *Mol. Pharmacol.*, **36**, 575–581.

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