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Subtype-selective antagonism of N-Methyl-D-Aspartate receptor ion channels by synthetic conantokin peptides

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Abstract

Conantokin-G (con-G), conantokin-T (con-T), a truncated conantokin-R (con-R[1-17]), that functions the same as wild-type con-R, and variant sequences of con-T, were chemically synthesized and employed to investigate their selectivities as antagonists of glutamate/glycine-evoked ion currents in human embryonic kidney-293 cells expressing various combinations of NMDA receptor (NMDAR) subunits (NR), *viz.*, NR1a/2A, NR1a/2B, NR1b/2A and NR1b/2B. Con-G did not substantially affect ion flow into NR1a,b/NR2A-transfected cells, but potently inhibited cells expressing NR1a,b/NR2B, showing high NR2B selectivity. Con-T and con-R served as nonselective antagonists of all of four NMDAR subunit combinations. C-terminal truncation variants of the 21-residue con-T were synthesized and examined in this regard. While NMDAR ion channel antagonist activity, and the ability to adopt the Ca²⁺-induced α -helical conformation, diminished as a function of shortening the COOH-terminus of con-T, NMDAR subtype selectivity was enhanced in the con-T[1-11], con-T[1-9], and con-T[1-8] variants toward NR2A, NR1b, and NR1b/2A, respectively. Receptor subtype selectivity was also obtained with Met-8 sequence variants of con-T. Con-T[M8A] and con-T[M8Q] displayed selectivity with NR2B-containing subunits, while con-T[M8E] showed enhanced activity toward NR1b-containing NMDAR subtypes. Of those studied, the most highly selective variant was con-T[M8I], which showed maximal NMDAR ion channel antagonism activity toward the NR1a/2A subtype. These studies demonstrate that it is possible to engineer NMDAR subtype antagonist specificity into con-T. Since the subunit composition of the NMDAR varies temporally and spatially in developing brain and in various disease states, conantokins with high subtype selectivities are potentially valuable drugs that may be used at specific stages of disease and in selected regions of the brain.

Keywords

conantokin variants; NMDA receptor subtypes; electrophysiology; peptide conformations; ion channels; conantokin antagonism

1. Introduction

Venoms of predatory marine cone snails (genus, *Conus*) contain many small neuropharmacologically-active peptides (conotoxins) which target specific ligand- or

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voltage-gated ion channel receptors, neurotransmitters, and neurotransmitters (Lewis, 2004). These peptides are effective weapons for survival of the snails, and are also emerging as neurotherapeutic drugs (Alonso et al., 2003; Malmberg et al., 2003). The majority of the conotoxins are built from small superfamilies of disulfide bond-containing structural scaffolds. An exception to this generality is found in the conantokins, four of which have been identified, viz., conantokin (con)-G (McIntosh et al., 1984), con-L (Jimenez et al., 2002), con-R (White et al., 2000), and con-T (Haack et al., 1990), from *Conus geographus*, *Conus lynceus*, *Conus radiatus*, and *Conus tulipa*, respectively, as individual gene products containing 17-27 amino acids. These peptides are disulfide-deficient, but rich in γ -carboxyglutamate (Gla) and other post/co-translationally-derived amino acids.

The conantokins are highly specific antagonists of the ion channels of the NMDA class of ionotropic glutamate receptors (NMDAR). The NMDAR is a ligand-gated neuronal ion channel, which, at resting membrane potentials, is blocked by Mg^{2+} (Mayer et al., 1984). Channel opening occurs through binding of the coagonists, glutamate (or NMDA) and glycine (Jahr and Stevens, 1987; Johnson and Ascher, 1987). Functional NMDARs require the coassembly of two subunit families, viz., NR1, plus one of the NR2 subunits (McIlhinney et al., 2003). The NR1 family consists of a single gene with eight different splice variants, specifically, NR1a-NR1h (Sugihara et al., 1992). Four different gene products (NR2A-NR2D) comprise the NR2 subunit family (Hollmann and Heinemann, 1994). NR1 subunits are expressed throughout the central nervous system (CNS), and the NR2 components display more restricted patterns of expression (Lynch and Guttman, 2001). Another type of NMDAR subunit, NR3A/3B, encoded by two separate genes, that can co-assemble with NR1, is found in other regions of the CNS, leading to Gly-triggered receptors with unique properties, particularly insensitivity to Glu and relative impermeability to Ca^{2+} (Chatterton et al., 2002).

The pharmacology of the NMDAR is influenced by the specific subunit composition of the heterooligomeric receptor assemblage, especially with regard to NR2 subunits that, in turn, vary spatially and temporally in developing brain (Castellino and Prorok, 2000). Depending on the exact subunit composition of the NMDAR, differences have been found in the strength of agonist and antagonist binding (Cheffings and Colquhoun, 2000; Lynch et al., 1994), and sensitivity to polyamines and ifenprodil (Williams, 1993), among other properties (Ehlers et al., 1996; Hrabetova and Sacktor, 1997), including the response to conantokins (Haack et al., 1993).

Many neuropathologies are associated with excitotoxic concentrations of Glu and the resultant disruption of Ca^{2+} homeostasis (Doble, 1995), such as Alzheimer's (Tsai et al., 2002b) and Parkinson's (Tsai et al., 2002a) Diseases. Similar associations have been made with regard to chronic pain (Fisher et al., 2000) and the behavioral effects of alcohol and narcotics (Bisaga and Popik, 2000; Wei et al., 2006). Because of the potential value of NMDAR antagonists in attenuating such types of neuropathologies and to further inform our understanding of the molecular basis of NMDAR subunit selectivity, we have investigated structure-function relationships of conantokin peptides. The present communication summarizes our studies on the electrophysiological responses to variant conantokins of cells transfected with various combinations of NR1 and NR2 subunits in an attempt to uncover subunit-selective peptides, from which drugs that target specific NMDAR subunit combinations can be fashioned.

2. Materials and methods

2.1 Peptide synthesis

The amino acid sequences of the parent conantokins that were chemically synthesized for these studies, are provided below. One of these, con-R[1-17], is a truncated form of con-R with the last 10 COOH-residues residues not included in the synthetic peptide. This amino-terminal-derived variant of con-R was employed because only residues 1-17 are homologous to other conantokins and previous data demonstrated that no differences existed in cation binding properties and NMDA antagonist activities between WT con-R and con-R[1-17] (Blandl et al., 2000; Klein et al., 2003).

Solid phase peptide synthesis was employed throughout using an Applied Biosystems (Foster City, CA) model 433A peptide synthesizer. All reagents and methods have been described previously (Prorok et al., 1996).

2.2. Expression of recombinant (r) NMDAR subtypes

cDNAs encoding rat NR1a, NR1b, NR2A, and NR2B (subcloned into pRC/CMV or pRK5) were provided by Dr. David Lynch (University of Pennsylvania). Plasmid pEGFP-N1, encoding a red-shifted variant of wild-type (WT) green fluorescent protein (GFP), was purchased from Clontech Laboratories (San Diego, CA).

HEK293 cells (from ATCC) were transiently transfected with recombinant NMDAR subunits using calcium phosphate precipitation (Klein et al., 2001). The cells were grown to ~50% confluency on 60 mm poly-D-lysine-coated dishes and transfected with combinations of NR1a, NR1b, NR2A, and NR2B-containing plasmids (10 µg of DNA/dish), along with pEGFP-N1 for positive selection of transfected cells. Plasmid ratios for diheteromeric combinations were 1:3:6 for GFP:NR1a/b:NR2A/B, respectively. Following transfection, the cells were maintained in fresh medium containing 500 µM ketamine to prevent the glutamate that was present in the culture medium, from stimulating cell death. After 48 hr post-transfection, the transfected cells in 60 mm dishes were plated in 35 mm poly-D-lysine-coated dishes for electrophysiological recordings.

2.3. Electrophysiology

Electrophysiological patch clamp whole cell recordings were performed at room temperature (Klein et al., 2001). The cells were bathed in extracellular solution consisting of 140 mM NaCl, 3 mM KCl, 2 mM CaCl₂, 10 mM Na-Hepes, and 20 mM dextrose, pH 7.35. Glycine (10 µM) was also present to saturate its site on the NMDAR. Recording pipettes, constructed from borosilicate glass (Drummond Scientific, Broomall, PA), with a resistance of 2–4 MΩ, were back-filled with a solution containing 140 mM CsF, 1 mM CaCl₂, 2 mM MgCl₂, 10 mM EGTA, 10 mM Cs-Hepes, 2 mM tetraethylammonium chloride, and 4 mM Na₂ATP, pH 7.35. Stock solutions of 10 mM NMDA and 10 mM glycine were prepared and kept frozen at -20 °C. The fresh working solutions were prepared daily.

The GFP fluorescence in cells was visualized using a Nikon eclipse TE200 microscope (Fryer) with a fluorescent detection system, illuminated by a mercury light source with a Mercury-100W short arc DC power supply (Chiu Technical Corporation, Kings Park, NY). The application of agonist solutions was accomplished *via* a rapid solution changer equipped

with a 9 barrel straight-head (RSC-200, Biologic, Calix, France; Molecular Kinetics, Pullman, WA) positioned 200-300 μm from the cell under study. The whole-cell current was recorded using an Axopatch-200B amplifier (Axon Instruments, Foster City, CA), low-pass filtered at 5 kHz by a built-in, eight-pole Bessel filter, digitized at 0.5-2 kHz sampling frequency using a Digidata 1322A (Axon Instruments). The data were acquired on a personal computer using pCLAMP-8 software (Axon Instruments). All measurements were performed with cells voltage-clamped at -70 mV, pH 7.35, at room temperature. The responses in a particular cell were normalized to maximum current evoked in the absence of peptide.

2.4. Data analysis

In order to examine the kinetics of the binding of conantokins to the NMDAR, experiments were conducted to determine time constants for the onset and offset of conantokin-induced responses using the simplest plausible model of the interaction of conantokins with the receptor. In all cases, onsets and recoveries fit well to a single exponential function and we employ a model with a single functional binding site for conantokins to the receptor. The model also assumes that the rates of other events involved in the reactions, e.g., NMDA and glycine binding rates, occur much faster than the perturbations caused by conantokin binding (Fig. 1A). This is a credible assumption, since the on and off rates of conantokins to the NMDAR are on the order of seconds and those that we determined for NMDA, as in Figure 1A, are approximately 12 msec and 15 msec, respectively.

With these assumptions, the time constant (τ_{obs}) of the steady state current relaxation following the perfusion of conantokin is:

$$1/\tau_{\text{obs}} = k_{\text{on}}[\text{conantokin}] + k_{\text{off}}$$

where k_{on} and k_{off} are the conantokin kinetic association and dissociation rate constants, respectively, as measured from their inhibitory response rates toward NMDA/glycine-induced ion channel flow in various recombinant NMDARs. A plot of $1/\tau_{\text{obs}}$ versus the concentration of conantokin allows k_{on} and k_{off} to be calculated from the slopes and intercepts, respectively.

Current measurements of the acquired data were made using pClamp (Axon Instruments, Foster City, CA) and Origin (Microcal Software, Northampton, MA) software. The data were then averaged and presented as the mean $\pm$ S.D. Statistical analyses were performed using the two-tailed Student's t-test and repetitive measures analysis of variance (Sigma Stat, Jandel Scientific, San Rafael, CA and Origin Software). Statistical significance was assigned at $P < 0.05$.

Data analysis for the onset and offset rates of conantokins are most reliable once the steady state with NMDA (+ saturating glycine) is reached before adding the NMDA/glycine + conantokin. In a few cases in our experiments, the current after initial NMDA/glycine application still decayed very slowly even after long application times with these coagonists. When this decay rate slowed considerably, we added conantokins to the perfusion solution since the patch would not continue to hold at longer times. In order to correct for these NMDA/glycine background decay rates in the small number of cases where this occurred, we extrapolated the slow decay rate with NMDA/glycine to the end of the experiment (after NMDA/glycine + conantokin) and subtracted this background decay rate curve from the overall curve obtained after application of NMDA/glycine + conantokin, thus obtaining the corrected conantokin response for analysis.

Since conantokins associate and dissociate slowly from the NMDAR in comparison to other NMDA modulators, there were difficulties in maintaining a stable patch for a sufficiently long period to allow the response to fully recover from inhibition. As a result, more limited data were obtained in offset rate experiments. In most cases, we could readily obtain the τ_{off} values by directly fitting the washing process. However, for those peptides with τ_{off} of many tens of sec e.g., con-G, T, R > 30 sec, we determined these values from plots of k_{obs} against the different peptide concentrations. For those peptides with τ_{off} values between 15 - 30 sec, we measured currents evoked at 5 sec intervals during a wash period after removal of peptide, then plotted the data against time and fit the curves using a single order exponential.

2.5. Circular dichroism (CD)

Solution CD spectra were recorded between 200 and 260 nm on an AVIV Model 202SF spectrometer at 25 °C. The instrument was calibrated with an aqueous solution of *D*-10-(+)-camphorsulfonic acid. Quartz cells (0.2 cm pathlength) were used, and the buffer was the electrophysiology extracellular buffer. The data are reported as mean residue molar ellipticities ($[\theta]$, deg·cm²·dmol⁻¹) and calculated from:

$$[\theta] = [\theta]_{\text{obs}} (\text{mrw}) / 10cl$$

where $[\theta]_{\text{obs}}$ is the ellipticity measured in degrees, mrw is the mean residue molecular mass, *c* is the protein concentration (in g/mL), and *l* is the optical pathlength of the cell in cm.

The α -helical content of peptides in solution was determined from the mean residue ellipticities at 222 nm using the empirical relationship (Chen et al., 1972):

$$\text{fraction}_{\text{helix}} = ([\theta]_{222} - 2340) / 30300$$

Each solution spectrum represents the average of three scans collected at 1.0 nm intervals at a 1.0 nm bandwidth. Any corrections for nonpeptidic contributions to the ellipticity were made by subtracting the averaged spectrum of the peptide-free solution.

3. Results

It has previously been shown in several laboratories that conantokins function as subunit-selective inhibitors of current flow through the NMDAR channel, in major part through interactions with the glutamate site on the NR2 subunit with NR1/NR2B (Donevan and McCabe, 2000; Klein et al., 2001) and NR1/2A,2B (Barton et al., 2004; Klein et al., 2001) subunit combinations providing receptors that are inhibited by conantokins. Conantokins, at least as exemplified by con-G, do not appear to inhibit receptor function through the NR1-specific glycine site, although the potency of inhibition by conantokins of ion flow through NMDAR channels does vary somewhat depending upon the exact NR1 splice variant present (Klein et al., 2001; Ragnarsson et al., 2006). In order to more completely understand the effects of conantokins on the electrophysiology of NMDARs of varying subunit combinations, we undertook a rigorous assessment of the rate constants of the inhibitory responses by these conantokins of ion flow through receptor channels in a model system of HEK293 cells expressing specific recombinant NMDAR subunit combinations.

Since the kinetics of the stimulatory response of NMDA to all of the subunit combinations of NMDARs employed herein have not been reported, and since we wished to establish these values for the current study in our transfection systems, operational EC₅₀ values for NMDA in the presence of a constant and saturating level of 10 μ M glycine were determined.

As exemplified in Fig. 1A for NR1x/NR2x-containing receptors, the onset and offset rates for channel opening evoked by application of 100 μ M NMDA are fast and correspond to less than 20 ms for all receptor combinations tested. NMDA concentration-response curves were generated for the four NMDAR subunit combinations investigated in this study, an example of which is shown for NR1b/2B in Fig. 1B ($EC_{50} = 19.0 \pm 1.5 \mu$ M). The EC_{50} values of NMDA to all receptor subunit combinations employed herein, accomplished by similar experiments as shown in Fig. 1B, are shown in Fig. 1C. The data show that EC_{50} values for NMDA with all receptor combinations are approximately the same ($23.6 \pm 3.2 \mu$ M for NR1a/NR2A, $20.4 \pm 2.6 \mu$ M for NR1a/NR2B, $24.1 \pm 3.7 \mu$ M for NR1b/NR2A, and $19.0 \pm 1.5 \mu$ M for NR1b/NR2B). Because the kinetics and affinity of NMDA are similar across the four NMDAR subunit combinations examined, it can be assumed that the rates of onset and recovery from block that accompany conantokin application are ascribable to the conantokin alone and are not complicated by differential subunit responses to NMDA. We chose a concentration of 100 μ M NMDA, wherein approximately 85-90% saturation of the NMDA response was achieved, for all subsequent experiments.

In a previous study, we assessed the inhibitory properties of con-G, con-T, con-R[1-17], and a variant of con-G containing amino acid substitutions at sequence position 5, on the antagonism of ion flow in HEK 293 cells transfected with different combinations of recombinant NMDAR subunits, specifically, NR1a/NR2A, NR1a/NR2B, NR1b/NR2A, and NR1a/NR2A/NR2B (Klein et al., 2001). These experiments were performed with 50 μ M of each conantokin, thereby allowing rate comparisons at single concentrations of conantokin. To determine the value of that approach toward examination of many other conantokin variants, we first rigorously determined onset and offset rate constants of con-G, con-R[1-17], and con-T induced inhibition of current flow toward NMDARs of varying subunit compositions, by performing electrophysiological experiments at different concentrations of conantokin. An example of the data obtained is shown for con-R[1-17] with cells transfected NR1a/NR2A. The data of Fig. 1D presents a typical trace of current flow at 100 μ M NMDA, 10 μ M glycine, and 50 μ M con-R[1-17], from which the inhibitory onset and offset time constants for conantokin to a variety of NMDARs composed of different subunit combinations can be determined at a single conantokin concentration (Fig. 1E).

From plots of k_{obs} ($1/\tau_{obs}$) against [conantokin], typified by that of Fig. 1E, k_{on} and k_{off} can be determined. Table 1 lists all rate constants obtained by this approach for con-G, con-T, and con-R[1-17] to NMDARs composed of NR1a/NR2A, NR1a/NR2B, NR1b/NR2A, and NR1b/NR2B. These data reveal that con-G does not functionally interact with receptor combinations containing NR2A and that there are few important differences between all three conantokin for subunit combinations that contain NR1a/b and NR2B. Further, kinetic onset constants (k_{on}) of the three conantokin for NR1b/NR2A,B were ~2-fold higher than for NR1a/NR2A,B. This finding suggests that the presence of exon 5 in NR1b plays a role in the binding of the conantokin to the NMDAR, enhancing the binding rate relative to subunit combinations containing NR1a, which lacks this exon. On the other hand, subunit NR2B is related to the dissociation process, since the recovery time of NR1a,b/NR2B subunit combinations from conantokin antagonism was generally longer than for NR1a,b/NR2A receptors. The high potency of inhibition of conantokin for NMDAR subtype NR1b/NR2B demonstrated that this subunit combination offered the best configuration for overall binding within these four dominant NMDAR combinations.

In comparison with previous data obtained at a single conantokin concentration of 50 μ M, there is good agreement for both k_{on} and k_{off} . In an earlier study (Klein et al., 2001), con-G was shown to bind very weakly to NR1b/2A, and this binding was also not detectable in the present experiments. Because of these results, it is appropriate to use a single concentration of 50 μ M conantokin for these types of studies, which should nearly saturate the effects of

conantokins on these receptor subunit combinations and also achieve a more rapid inhibition.

After establishing its validity, this approach was used for electrophysiological study of several conantokin variants, with the aim of discovering features of these peptides that govern inhibition of NMDARs in subunit-selective manners. With regard to the parent conantokins (Table 1), con-G does not interact with subunit combinations containing NR2A and concomitantly does not affect current flow through these recombinant NMDAR channels. Con-R[1-17] and con-T do not show important differences in binding kinetics or inhibition of channels containing binary combinations of NR1a,b/NR2A,B, and con-G is also equivalent with NR1a,b/NR2B subunit combinations.

Since it has been shown that sequence position 5 in con-G is a determinant of selectivity of receptors with NR2B subunits, we focused on con-T, which has not been studied in this regard. Initial experiments centered on COOH-terminal truncation variants of con-T. In these cases, current flow was assessed in receptors containing all combinations of NR1a,b/NR2A,B, with con-T containing the N-terminal 13, 11, 9, and 8 residues. The current flow traces for these experiments are shown in Fig. 2, and the kinetic parameters characterizing the antagonism of various receptor subunit combinations by these con-T variants are listed in Table 2. Operational K_d values were not determined directly, but were estimated from the average values of k_{off}/k_{on} . In all cases of NMDA binary subunit combinations, K_d values increased and current antagonist propensities decreased as the carboxyl-terminal was truncated, from con-T through con-T[1-9], and only minor inhibitory activity was observed in the con-T[1-8] variant. The K_d values for con-T[1-11] and con-T[1-9] are graphically compared with all receptor combinations in Figure 3. Reduced binding strengths in the COOH-truncated variants were primarily due to faster off-rates, which progressed from con-T to con-T[1-9], with the most dramatic change in off-rate between con-T and con-T[1-13]. Con-T[1-13], the product of removing 8 C-terminal residues from the parent peptide, still retains almost the same bioactivity as con-T. Removal of 2 additional C-terminal residues, providing con-T[1-11], showed different specificities, binding more tightly with NR1a,b/2A than NR1a,b/2B, thus displaying a significant trend toward NR2A (Fig. 3A). The even shorter peptides, con-T[1-9] and con-T[1-8], displayed NR1b and NR2A-selective antagonist propensities, respectively (Figure 3B), although both are very weakly active as ion channel antagonists.

Next, the binding and activity of site-specific variants of con-T to binary combinations of NR1a,b/NR2A,B were examined. This current study was focused on substitutions at Met-8, a residue found in both con-R and con-T, but not in the more subunit-selective conantokin, con-G. In order to assess whether the residue at this sequence position was important for the less restricted specificity of con-T to NR2A subunit-containing NMDARs, we examined the electrophysiological responses of a variety of con-T Met-8 variants to NMDARs of various subunit compositions (Fig. 4A,B, Table 3). With regard to the NMDAR that contains the binary subunit compositions of NR1a/NR2A and NR1b/NR2A, con-T[M8Q], like con-G, had greatly diminished inhibitory activity toward with these receptors. However, this variant was active with NR2B-containing subunits (Fig. 4A, Table 3). Similar results were found for the con-T[M8A] variant (Fig. 4B, Table 3), which was even more active with NR2B subunits than con-T[M8Q], primarily as a result of slower off-rates. Con-T[M8E] was found to display NR1b selective character with regard to steady state current antagonism (Fig. 4A, Table 3), but also, in this case, kinetic properties of the binding could not be evaluated due to rates that were too rapid to be measured by this technique. Con-T[M8I] (Fig. 4A, Table 3) showed strong inhibitory activity toward NR1a/NR2A subunits, and weaker activity with NR1/NR2B-containing receptors, thus showing the most restricted activity in this entire set of variants. In the case of con-T[M8F] (Fig. 4B, Table 3), binding and activity were slightly

weaker than for con-T, and no subunit selectivity was observed. Lastly, changing the Met-8 of the more non-specific inhibitor, con-T, to the amino acid residue (Asn) found in the NR2B-selective con-G did, in fact, result in a somewhat diminished inhibitory response to NR2A-containing NMDAR subunits. It appears as though Met-8 partially governs the broader specificity found in con-T. Thus, receptors containing NR2A subunits were the most susceptible to potentiation by the position-8 variants, as observed with con-T[M8E], con-T[M8A], and con-T[M8Q], while for NR2B-containing NMDAR subunits, potentiation did not occur in our experiments using many different conantokin variants.

A hallmark characteristic of the active forms of conantokins is their ability to adopt helical conformations in the presence of divalent cations, with Ca^{2+} as an excellent representative of these cations with regard to this property (Prorok et al., 1996). Thus, CD spectral analysis of the peptides employed in the electrophysiological investigations were accomplished (Fig. 5 and Table 4). Of the truncation variants only con-T[1-13], the most active of this group, contained a significant population α -helical conformers. All Met-8 variants of full-length con-T were capable of adopting α -helical structures. As would be expected the con-T[M8A] variant was most amenable to helical formation and the differences among the other variants was small.

4. Discussion

The conantokins are among the few peptidic allosteric antagonists of the NMDAR and this property allows their use as conformational structure-function probes to investigate the complementary binding site on NMDARs to which these peptides interact. Such knowledge will ultimately lead to the design and development of new drugs that possess NMDAR inhibitory properties in the brain and also are capable of crossing the blood-brain barrier so that they can be systemically administered.

It is now widely accepted that the functional signature of NMDAR in brain is dependent on the subunit composition of the receptor, which varies in a spatial and temporal manner under normal and pathological conditions (Lynch and Guttman, 2001; Stephenson, 2001). From the latter standpoint, it has been shown that the presence of the NR2A and NR2B subunits, primarily expressed in the striatum, may influence the age of onset of Huntington's chorea (Arning et al., 2005). Another study has suggested a link between the NR2A level and the symptomatic progression of this disease (Ali and Levine, 2006). An important role has been ascribed to NR2A/B subunits in human cortical dysplasia-induced epilepsy (Najm et al., 2000). The NR2B subtype has been implicated in Parkinson's disease (Nash et al., 1999) and in the mediation of nociception (Tan et al., 2005). These studies reinforce the importance of developing drugs that are selective for NMDAR subtypes, such that maximal effectiveness and minimal side effects can be obtained.

The conantokins present attractive types of NMDAR antagonists, since they are not use-dependent, thus reducing the many side effects associated with NMDA channel blockers. The question as to whether they function in subunit-selective manners, or can be modified to do so, has not been extensively studied, and is addressed in the current manuscript. One member of conantokin family, con-R, was obviously different from con-G and con-T in primary structure, except for the first four critical amino acids (GE $\gamma\gamma$). In con-R[1-17], a truncation variant with full con-R activity toward the NMDAR (Blandl et al., 2001), there is a high number of hydrophobic amino acids resident in the first 15 amino acids, compared to con-G and con-T. Wild-type con-R and different truncated forms, even the maximum truncation, con-R[1-8], did not show any subunit selectivity (Klein et al., 2001). We believed that this was due to the existence of large number of hydrophobic amino acids, e.g., Ala. This feature allowed the con-R variants to readily interact with NMDARs via

hydrophobic interactions, thus producing the broad subunit selectivity. Therefore, in the current report, we only focused on con-T and its relationship to the structurally similar con-G, investigating the importance of Met at position-8. This residue presents an important difference in the structures of the broadly subunit-selective NMDAR ion channel inhibitor, con-T, and the more specific inhibitor, con-G. The effects of progressive C-terminal truncations on con-T subunit selectivity were also evaluated as an attempt to enable future work with agents as small as possible.

For the truncation variants, the increase in K_d that attends truncation of the parent peptide is primarily attributable to the attending increase in k_{off} (shorter τ_{off}). Previous studies with the conantokins have established that the replacement of Glu residues at sequence positions 10 and 14 (11 and 15 in con-R) increases the rate of recovery from block in murine hippocampal neurons (Klein et al., 2001). Hence, the decrease in τ_{off} observed the progression from full-length con-T to con-T[1-9] can most likely be ascribed to the absence of one, or both, of the C-terminal Glu residues. Less obvious is the trend towards increasing NR2A selectivity upon progressive truncation. Clearly, the con-T[1-11] and con-T[1-9] can assume conformers that are better accommodated in the binding site of NR2A- and NR1b-containing receptors. The reduced helicity and increased conformational flexibility of these variants may be contributing factors in this regard.

Replacement of the Met at position-8 in con-T with different classes of amino acids presented a variety of subunit selectivities to NMDAR channels. However, in most cases, there is one common character, viz., the disappearance of NR2A specific inhibitory function either totally (con-T[M8A] and con-T[M8Q]), or partially (con-T[M8E], con-T[M8I], and con-T[M8N]) towards NMDARs. There is only one amino acid replacement at position 8 (Phe) that still allowed con-T to display broad selectivity to all of four combinations (NR1a,b/NR2A,B) of NMDAR subunits. Another variant, con-T[M8I], presented a finding not seen previously. This peptide showed no activity to NR1b/2A, and only weak activity to NR1a,b/2B. However, con-T[M8I] strongly inhibited NR1a/2A receptor activity, with very rapid onset and offset rate constants. It would logically seem that the specific arrangement of NR1a/NR2A was optimal for NMDAR antagonism by this particular peptide, thus showing that highly specific functions can be engineered into the conantokins. An unexpected outcome of this series of experiments is the potentiation of NR1a/2A steady-state currents effected by M8E, M8A, and M8Q. The latter peptide also exerts slight potentiation of NR1b/2A currents. Con-G and con-G[γ 7A] potentiation of glutamate-evoked responses at NR2A-containing receptors has been noted before in combination with either NR1a or NR1b subunits expressed in *Xenopus* oocytes (Ragnarsson et al., 2006). In that study, as peptide concentration was increased ($> 50 \mu\text{M}$), stimulation was reversed and the inhibition of currents was eventually attained. This biphasic behavior was interpreted in terms of two distinct sites in NR2A-containing receptors that are differentially responsive to con-G binding. Consequently, the stimulatory effect elicited by certain position 8 variants at NR2A-containing receptors may reflect their preferential, or sole, occupancy of the putative stimulatory site. Binding to this site may stabilize the interdimer interface of the NMDA-activated receptor, obviating the desensitization that arises from reorientation of the agonist-bound interface (Sun et al., 2002).

In the absence of structural data, the sites of stimulatory and inhibitory conantokin binding remain speculative. Although an apparent competitive relationship between NMDA and the conantokins has been established (Donevan and McCabe, 2000; Klein, et al., 2003), and mutations in the glutamate locus have been shown to compromise con-G inhibitory potency (Wittekindt, et al., 2001), the possibility of a conantokin binding site incongruent with, but tightly allosterically coupled to, the glutamate site cannot be discounted. An alternative explanation for the potentiating effects of some con-T variants (that invokes overlapping

glutamate and conantokin binding sites) can be rationalized based on the crystal structure of the NR1 ligand-binding core (Furukawa and Gouaux, 2003). Con-T occupation of the glutamate pocket formed by the S1 and S2 polypeptide domains may function as a molecular wedge, stabilizing the open-cleft form of the S1S2 interface in a manner that does not permit channel activation. For potentiating con-T variants, occupation of the agonist site may stabilize the closed conformation of the interlobal clam-shell motif such that channel opening results and rearrangement to the desensitized receptor form is obviated.

In the variant, con-T[M8N], which has the amino acid of con-G at this same position, partial NR2B selectivity is seen. This further suggests that sequence position-8 is partially responsible for NR2A selectivity. To assess whether the Met residue present in con-T and con-R is a determinant for this selectivity or whether specifically Asn from con-G governs this property, we synthesized 2 further variants, viz., con-G[N8M] and con-G[N8A] and examined their NMDAR activities. These peptides still potently inhibited NR2B-containing receptors (data not shown). From this, we conclude that the decreased activity of con-T variants at NR2A-containing receptors originated from removal of Met-8 of con-T, and is not due to the specific placement of Asn at sequence position-8 of con-G. Thus, while position-8 is an important residue for subunit specificity, other amino acids synergistically contribute to this property, and most likely exist in close proximity to sequence position 8.

4.1. Concluding remarks

In conclusion, the conantokins provide a group of small peptides that can be effectively employed to antagonize current flow through the NMDAR and can be manipulated to provide the opportunity to design subunit selective inhibitors. Such drugs will be invaluable to inhibition of specific receptor subunit combinations without affecting the functions of others, a property that would render these agents effective therapeutic and diagnostic drugs.

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Fig. 1.

Time course of current flow through a recombinant NMDARs in transfected HEK293 cells. (A) Representative trace illustrating the electrophysiological response to NR1b/2B to 100 μM NMDA/10 μM glycine, showing the rapid onset and offset responses to NMDA at the beginning and at the conclusion of the agonist applications. (B) Whole-cell currents from cells with different subunit combinations at various concentrations of NMDA, were normalized to the response produced by 100 μM NMDA in the presence of 10 μM glycine. The response at 500 μM NMDA was set to 100%. In this example, the EC_{50} value for NMDA obtained with NR1b/2B was $19.0 \pm 1.5 \text{ μM}$. (C) The EC_{50} values for NMDA obtained with all four different NMDAR subunit combinations. (D) Representative trace illustrating the electrophysiology data obtained to evaluate the τ_{obs} and τ_{off} at 50 μM con-R[1-17] with 100 μM NMDA/10 μM glycine present in all test solutions. The times and duration of application of test solutions are indicated by the solid bars. The onset and offset curves of conantokin inhibition were fit using a single exponential. (E) Plots of k_{obs} values against the concentration of con-R[1-17] used as an inhibitor of current flow through NMDARs composed of NR1b/NR2A transfected into HEK293 cells. The holding potential was maintained at -70 mV.

Fig. 2.

Time course for inhibition of current flow through recombinant NMDAR subunit combinations by con-T[1-13], con-T[1-11], con-T[1-9], and con-T[1-8]. Receptor subunits NR1a/NR2A, NR1b/NR2A, NR1a/NR2B, and NR1b/NR2B were cotransfected into HEK293 cells. Data are shown with 50 μ M of each conantokin applied at a time and for the duration indicated by horizontal line above the traces, with 100 μ M NMDA/10 μ M glycine present in all test solutions. The onset and offset curves of conantokin inhibition were fit using a single exponential. The holding potential was maintained at -70 mV. The solid line above each panel represents the duration of application of the control solution, 100 μ M NMDA + 10 μ M glycine, and the dashed line indicates the duration of application of the relevant conantokin.

Figure 3.

Comparison between K_d values for NR1a/2A, NR1a/2B, NR1b/2A, and NR1b/2B with: (A) con-T[1-11] ($p = 0.006$ for comparison between NR1a/2A and NR1a/2B, $p = 0.004$ for comparison between NR1b/2A and NR1b/2B); (B) con-T[1-9] ($p = 0.006$ for comparison between NR1a/2A and NR1b/2B; $p = 0.003$ for comparison between NR1a/2A and NR1b/2B; $p = 0.006$ between NR1a/2B and NR1b/2B); and (C) con-T[M8N] ($p = 0.02$ for comparison between NR1a/2A and NR1a/2B; $p = 0.02$ for comparison between NR1b/2A and NR1b/2B).

Fig. 4.

Time course for inhibition of current flow through recombinant NMDAR subunit combinations by Met-8 based variants of con-T. Receptor subunits NR1a/NR2A, NR1b/NR2A, NR1a/NR2B, and NR1b/NR2B were cotransfected into HEK293 cells. Data are shown with 40 μ M conantokin under conditions described in Figure 2. A. Current flow data for con-T[M8E], con-T[M8I], con-T[M8Q]. B. Data for con-T[M8F], con-T[M8A], con-T[M8N]. The solid line above each trace represents the duration of application of the control solution, 100 μ M NMDA + 10 μ M glycine, and the dashed line indicates the duration of application of the relevant conantokin.

Fig. 5.

CD analysis of the secondary structure of con-T variants in the presence of Ca^{2+} . (A) CD spectra of truncation variants (100 μM) of con-T. (○) con-T[1-13]; (●) con-T[1-11]; (△) con-T[1-9]; and (◆) con-T[1-8]. (B) CD spectra of sequence position-8 variants of con-T. (☆) extracellular buffer; (●) con-T; (○) con-T[M8A]; (◆) con-T[M8N]; (□) con-T[M8F]; (■) con-T[M8E]; (△) con-T[M8Q]; (▲) con-T[M8I]. The buffer for all spectra was 140 mM NaCl, 3 mM KCl, 2 mM CaCl_2 , 10 mM Na-Hepes, and 20 mM dextrose, pH 7.35, at room temperature (extracellular buffer).

Table 1

Rate constants for conantokin inhibition of current flow in NMDARs of different subunit compositions

Receptors/peptides	τ_{off} (sec)	k_{on} ($10^3 M^{-1} sec^{-1}$)	k_{off}/k_{on} (K_d) (μM)	Inhibition ^d (%)	N ^b
<u>NR1a/NR2A</u>					
con-G	NA (NA) ^c	NA (NA)	NA (NA)	0	10
con-R[1-17] ^d	84 ± 34 (~60) ^e	5.2 ± 0.4 (3.3)	2.3 ± 1.1 (5.0)	93 ± 2 (92)	7
con-T	65 ± 23 (~30)	4.8 ± 0.4 (4.1)	3.2 ± 1.4 (8.2)	90 ± 4 (93)	8
<u>NR1a/NR2B</u>					
con-G	105 ± 47 (>120)	4.9 ± 0.5 (3.8)	1.9 ± 1.0 (2.3)	94 ± 3 (91)	8
con-R[1-17]	110 ± 50 (>120)	5.6 ± 0.4 (3.8)	1.6 ± 0.8 (2.2)	94 ± 2 (88)	8
con-T	83 ± 28 (~60)	4.1 ± 0.3 (3.2)	2.9 ± 1.2 (5.3)	92 ± 4 (95)	7
<u>NR1b/NR2A</u>					
con-G	NA (0.93)	NA (13.8)	NA (77.5)	0 (37.4)	8
con-R[1-17]	63 ± 27 (~60)	9.1 ± 0.8 (7.6)	1.7 ± 0.9 (2.2)	94 ± 3 (98)	6
con-T	45 ± 20 (~30)	10.5 ± 1.2 (10.3)	2.1 ± 1.1 (3.2)	93 ± 2 (98)	6
<u>NR1b/NR2B</u>					
con-G	102 ± 37 (>120)	12.5 ± 2.6 (17.7)	0.8 ± 0.4 (0.5)	96 ± 3 (100)	5
con-R[1-17]	105 ± 40 (>120)	9.6 ± 1.2 (8.8)	1.0 ± 0.5 (1.0)	97 ± 2 (100)	6
con-T	98 ± 36 (>120)	11.4 ± 1.2 (14.3)	0.9 ± 0.4 (0.6)	97 ± 2 (100)	5

^aThe percentage of the steady state current inhibited by conantokins.

^bN, number of cells.

^cNA, not active.

^dThe amino-terminal 17 residues of con-R.

^eThe numbers in parenthesis are data taken from reference (Klein et al., 2001), obtained at a single concentration (50 μM) of conantokin.

Table 2

Rate constants for truncation variants of con-T toward inhibition of current flow in NMDARs of different subunit compositions

Receptors/peptides ^a	τ_{off} (sec)	k_{on} $10^3 M^{-1} sec^{-1}$	k_{off}/k_{on} (K_D) (μM)	Inhibition ^c (%)	N ^d
<u>NR1a/NR2A</u>					
con-T	65 ± 23	4.8 ± 0.4	3.2 ± 1.4	90 ± 4	8
con-T[1-13]	9.2 ± 1.6	6.9 ± 1.0	15.7 ± 3.6	71 ± 8	6
con-T[1-11]	3.2 ± 0.3	8.9 ± 1.9	35 ± 7	67 ± 6	6
con-T[1-9]	1.8 ± 0.2	3.9 ± 0.5	142 ± 36	44 ± 5	5
con-T[1-8]	ND ^e	ND	ND	8 ± 3	4
<u>NR1a/NR2B</u>					
con-T	83 ± 28	4.1 ± 0.3	2.9 ± 1.2	92 ± 4	7
con-T[1-13]	15 ± 3	7.8 ± 1.5	9.2 ± 3.6	82 ± 5	5
con-T[1-11]	<1.0	6.3 ± 3.4	>150	44 ± 5	5
con-T[1-9]	1.8 ± 0.2	3.6 ± 0.6	154 ± 40	61 ± 8	4
con-T[1-8]	NA ^e	NA	NA	0	5
<u>NR1b/NR2A</u>					
con-T	45 ± 20	10.5 ± 1.2	2.1 ± 1.1	93 ± 2	6
con-T[1-13]	9.1 ± 0.8	9.2 ± 1.1	11.9 ± 2.5	86 ± 4	5
con-T[1-11]	2.5 ± 0.4	14.5 ± 2.3	27.6 ± 8.6	76 ± 5	6
con-T[1-9]	3.2 ± 0.2	10.1 ± 0.6	30.9 ± 3.8	75 ± 7	5
con-T[1-8]	ND ^d	ND	ND	10 ± 3	6
<u>NR1b/NR2B</u>					
con-T	98 ± 36	11.4 ± 1.2	0.9 ± 0.4	97 ± 2	5
con-T[1-13]	22 ± 3	13.5 ± 1.8	3.5 ± 1.0	93 ± 3	6
con-T[1-11]	1.1 ± 0.1	8.5 ± 0.9	107 ± 21	42 ± 7	5
con-T[1-9]	1.4 ± 0.3	13.4 ± 2.8	53 ± 22	75 ± 6	6
con-T[1-8]	NA ^f	NA	NA	0	4

^aThe numbers in brackets are the residues from the wild-type peptides that are present in these variants, beginning from the amino-terminus of native con-T.

^b k_{on} was calculated from τ_{obs} ($1/k_{obs}$) and τ_{off} ($1/k_{off}$) at a single concentration (50 μM) of peptide using the relationship $k_{on} = (k_{obs} \cdot k_{off}) / [\text{peptide}]$.

^cThe percentage of the steady state current inhibited by conantokins.

N_p , number of cells

ϵ ND, not evaluated because of rates that were too rapid, and/or the changes in steady state currents were too low.

f_{NA} , not active.

Table 3

Rate constants for Met-8 variants of con-T toward inhibition of current flow in NMDARs of different subunit compositions

Receptors/peptides	τ_{off} (sec)	k_{on} ($10^3 M^{-1} sec^{-1}$)	k_{off}/k_{on} (K_d) (μM)	inhibition ^b (%)	N ^c
NR1a/NR2A					
con-T[M8E]	$1.2 \pm 0.2^{+d}$	$1.3 \pm 0.2^{+d}$	-	$-46 \pm 6^{+d}$	5
con-T[M8I]	ND ^e	ND	ND	96 ± 3	5
con-T[M8Q]	$1.3 \pm 0.1^{+d}$	$1.9 \pm 0.1^{+d}$	-	$-80 \pm 6^{+d}$	6
con-T[M8F]	27 ± 7	2.9 ± 0.7	12.8 ± 6.4	76 ± 6	6
con-T[M8A]	$1.4 \pm 0.2^{+d}$	$1.9 \pm 0.4^{+d}$	-	$90 \pm 10^{+d}$	5
con-T[M8N]	24 ± 6	2.5 ± 0.3	16.7 ± 3.5	48 ± 6	5
NR1a/NR2B					
con-T[M8E]	NA ^f	NA	NA	0	5
con-T[M8I]	5.8 ± 1.3	3.4 ± 0.6	50.7 ± 17.4	58 ± 8	4
con-T[M8Q]	1.5 ± 0.4	4.2 ± 1.1	158 ± 75	28 ± 5	5
con-T[M8F]	13 ± 3	2.2 ± 0.4	35.0 ± 14.2	69 ± 4	5
con-T[M8A]	14 ± 4	5.7 ± 1.3	12.5 ± 2.7	73 ± 8	6
con-T[M8N]	25 ± 7	4.6 ± 0.5	8.7 ± 1.1	82 ± 5	4
NR1b/NR2A					
con-T[M8E]	ND	ND	ND	92 ± 2	6
con-T[M8I]	NA	NA	NA	0	4
con-T[M8Q]	ND ^{+d}	ND ^{+d}	-	$-37 \pm 5^{+d}$	4
con-T[M8F]	19 ± 4	3.0 ± 0.6	17.5 ± 5.3	75 ± 6	6
con-T[M8A]	NA	NA	NA	0	5
con-T[M8N]	20 ± 6	3.4 ± 0.6	14.7 ± 3.4	56 ± 7	4
NR1b/NR2B					
con-T[M8E]	ND	ND	ND	93 ± 2	5
con-T[M8I]	4.1 ± 0.8	9.5 ± 1.8	25.7 ± 9.8	50 ± 7	6
con-T[M8Q]	<1.0	6.2 ± 4.3	>150	32 ± 6	6
con-T[M8F]	28 ± 7	7.6 ± 1.7	4.8 ± 2.1	90 ± 3	6
con-T[M8A]	19 ± 4	6.9 ± 1.4	7.6 ± 3.1	81 ± 5	4

Receptors/peptides	τ_{off} (sec)	k_{on} ($10^3 M^{-1} sec^{-1}$)	k_{off}/k_{on} (K_d) (μM)	inhibition ^b (%)	N ^c
con-T[M8N]	18 ± 4	9.9 ± 1.7	5.6 ± 1.1	90 ± 4	5

^a_{kon} was calculated from τ_{obs} ($1/k_{obs}$) and τ_{off} ($1/k_{off}$) at a single concentration (50 μM) of peptide using the relationship $k_{on} = (k_{obs} - k_{off})/[peptide]$.

^b The percentage of the steady state current inhibited by conantokins.

^c N, number of cells.

^d +represents potentiation of the steady state current. Onset and offset rates, and overall % potentiation (as a negative inhibition) were calculated in most cases.

^e ND, could not be evaluated because of rates that were too rapid, and/or the changes in steady state currents were too low.

^f NA, not active.

Table 4 α -Helicity values for con-T variants in the presence of Ca^{2+}

Peptide	% α -helix
con-T	92 (82) ^a
con-T[1-13]	33 (37) ^a
con-T[1-11]	10 (8) ^a
con-T[1-9]	8 (4) ^a
con-T[1-8]	1 (<2) ^a
con-T[M8A]	58 (51) ^a
con-T[M8N]	45
con-T[M8F]	42
con-T[M8E]	41
con-T[M8Q]	34
con-T[M8I]	31

^afrom (Warder et al., 2001)