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Inhibition of Native and Recombinant Nicotinic Acetylcholine Receptors by the Myristoylated Alanine-Rich C Kinase Substrate Peptide

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Abstract

A variety of peptide ligands are known to inhibit the function of neuronal nicotinic acetylcholine receptors (nAChRs), including small toxins and brain-derived peptides such as β -amyloid_{1–42} and synthetic apolipoproteinE peptides. The myristoylated alanine-rich C kinase substrate (MARCKS) protein is a major substrate of protein kinase C and is highly expressed in the developing and adult brain. The ability of a 25-amino acid synthetic MARCKS peptide, derived from the effector domain (ED), to modulate nAChR activity was tested. To determine the effects of the MARCKS ED peptide on nAChR function, receptors were expressed in *Xenopus laevis* oocytes, and two-electrode voltage-clamp experiments were performed. The MARCKS ED peptide completely inhibited acetylcholine (ACh)-evoked responses from $\alpha 7$ nAChRs in a dose-dependent manner, yielding an IC₅₀ value of 16 nM. Inhibition of ACh-induced responses was both activity- and voltage-independent. The MARCKS ED peptide was unable to block α -bungarotoxin binding. A MARCKS ED peptide in which four serine residues were replaced with aspartate residues was unable to inhibit $\alpha 7$ nAChR-mediated currents. The MARCKS ED peptide inhibited ACh-induced $\alpha 4\beta 2$ and $\alpha 2\beta 2$ responses, although with decreased potency. The effects of the MARCKS ED peptide on native nAChRs were tested using acutely isolated rat hippocampal slices. In hippocampal interneurons, the MARCKS ED peptide was able to block native $\alpha 7$ nAChRs in a dose-dependent manner. The MARCKS ED peptide represents a novel antagonist of neuronal nAChRs that has considerable utility as a research tool.

An assortment of small peptide ligands has been identified that inhibit the function of neuronal nicotinic acetylcholine receptors (nAChRs), including naturally occurring snail and snake toxins. Some of these peptides demonstrate robust selectivity, making them ideal scientific tools for investigating the functional role of particular receptor subtypes. In addition to the toxin peptides, there are an increasing number of reports of small brain-derived peptides interacting with neuronal nAChRs to modulate function. β -Amyloid_{1–42} blocks $\alpha 4\beta 2$ receptors in a noncompetitive and voltage- and use-independent fashion (Wu et al., 2004). There are discrepancies as to whether A β _{1–42} can inhibit or activate $\alpha 7$ nAChRs, and studies at native and expressed receptors have found varied results, from almost complete antagonism (Liu et al., 2001; Grassi et al., 2003), minimal to no antagonism (Pettit et al., 2001; Lamb et al., 2005), and agonism (Dineley et al., 2002; Dougherty et al., 2003). Calcitonin gene-related peptide fragments either inhibit or facilitate non- $\alpha 7$ -containing nAChR responses, depending on the particular peptide sequence (Di Angelantonio et al., 2002). A peptide derived from the

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C-terminal region of acetylcholinesterase modulates $\alpha 7$ but not $\alpha 4\beta 2$ nAChRs (Greenfield et al., 2004). We demonstrated recently that peptides derived from the low-density lipoprotein receptor binding domain of apolipoproteinE can inhibit $\alpha 7$ nAChRs, both in recombinant systems and native tissue (Klein and Yakel, 2004; Gay et al., 2006). These brain-derived peptides not only represent novel tools for investigating nAChR function, but they also provide unique therapeutic utility. For example, apolipoproteinE mimetic peptides have been suggested as potential therapeutic agents after head injury and ischemic events and in multiple sclerosis (Lynch et al., 2005; McAdoo et al., 2005; Li et al., 2006).

The myristoylated alanine-rich C kinase substrate (MARCKS) protein is a major substrate of protein kinase C (PKC) and is enriched in the brain and in activated immune cells (Aderem, 1992; Blackshear, 1993). MARCKS has been implicated in regulating actin polymerization and spine morphology, sequestering phosphatidylinositol 4,5-bisphosphate in membranes, and modulating cell migration (Sundaram et al., 2004). Heterozygous MARCKS knockout mice show a decrease in long-term potentiation but not short-term potentiation in the mossy fiber-CA3 pathway (Hussain et al., 2006). In addition, MARCKS knockout animals demonstrate gross abnormalities in central nervous system development (Stumpo et al., 1995), and both MARCKS heterozygous mutant mice and transgenic mice overexpressing MARCKS have impairment in spatial learning acquisition but not contextual fear conditioning (McNamara et al., 1998, 2005).

In neurons, MARCKS is localized to dendrites in association with microtubules and to axonal terminals in association with synaptic vesicles (Ouimet et al., 1990). MARCKS plays a role in the maintenance of dendritic spines and PKC-dependent changes in spine morphology (Calabrese and Halpain, 2005). Inside neurons, MARCKS can be cleaved into amino- and carboxyl-terminal fragments by cathepsin B, potentially regulating the amount of active MARCKS in the cell (Spizz and Blackshear, 1997). The effector domain of MARCKS has been identified as the region of the protein involved in membrane interactions, phosphorylation, Ca^{2+} /calmodulin binding, and F-actin binding. This portion of the protein has no secondary structure and remains unfolded (for review, see Arbuzova et al., 2002).

Interestingly, the effector domain of MARCKS has a primary sequence comparable with some of the other brain-derived peptides that modulate nAChR function in that the peptide contains a high percentage of positively charged amino acids, such as lysine. A synthetic 25-amino acid peptide derived from the effector domain (ED) of MARCKS was tested for its ability to modulate nAChR activity at recombinant receptors in *Xenopus laevis* oocytes using two-electrode voltage clamp experiments and at native receptors expressed on interneurons in hippocampal slice recordings using patch-clamp experiments.

Materials and Methods

Oocyte Preparation

All procedures with *X. laevis* and rats were approved by the Animal Care and Use Committee of the National Institute of Environmental Health Sciences. Female frogs housed under a 12-h day/night cycle were anesthetized in cold water containing 0.2% meta-aminobenzoate. Oocytes were dissected and defolliculated by treatment with collagenase B (2 mg/ml; Roche Diagnostics, Indianapolis, IN) and trypsin inhibitor (1 mg/ml; Invitrogen, Carlsbad, CA) for 2 h. The total amount of RNA injected was approximately 50 ng for $\alpha 7$ nAChR subunits or 12 to 25 ng each for $\alpha 4$, $\alpha 2$, and $\beta 2$. For injection of MARCKS peptides internally, 50 nl of MARCKS ED or MARCKS S/D peptide (100 μM) was injected for an approximate final concentration of 10 μM peptide in the oocyte intracellular space.

¹²⁵I- α -Bungarotoxin Competition Binding

For binding assays, oocytes injected with wild-type $\alpha 7$ nAChRs were incubated for 30 min, with various concentrations of either the MARCKS ED peptide or methyllycaconitine (MLA) in bath solution (96 mM NaCl, 2 mM KCl, 1.8 mM CaCl₂, 1 mM MgCl₂, 5 mM HEPES) plus 1 mg/ml bovine serum albumin. After the addition of 5 nM ¹²⁵I- α -bungarotoxin (α -BgTx), oocytes were incubated at room temperature with shaking for 1 to 1.5 h. The reaction was stopped by washing oocytes three times with bath solution. Each condition was run in triplicate, and oocytes were counted individually. Bound ¹²⁵I- α -BgTx was measured by gamma counting on a Packard Cobra Quantum (Global Medical Instrumentation, Inc., Ramsey, MN). Nonspecific binding was determined in the presence of 1 μ M MLA and was similar to binding in oocytes not injected with the $\alpha 7$ nAChR. Data were initially expressed as femtomoles of binding per oocyte and then converted to percentage specific binding for analysis by nonlinear regression (see below).

Oocyte Electrophysiology

Current responses were obtained by two-electrode voltage clamp recording at a holding potential of -60 mV using a Geneclamp 500 and pClamp 8 or 10 software (Molecular Devices, Sunnyvale CA). All electrophysiological experiments were done at room temp, ~24°C. Electrodes contained 3 M KCl and had a resistance of <1 M Ω . ACh and MARCKS peptides were prepared daily in bath solution (96 mM NaCl, 2 mM KCl, 1.8 mM CaCl₂, 1 mM MgCl₂, 5 mM HEPES) from frozen stocks. ACh was applied using a synthetic quartz perfusion tube (0.7-mm i.d.) operated by a computer-controlled valve. MARCKS peptides were bath applied. Activity dependence of the MARCKS ED peptide block was tested by rapidly applying ACh every 2 min to obtain control data, then bath application of the MARCKS ED peptide for 10 min in the absence of ACh stimulation. Finally, in the continued presence of the MARCKS ED peptide, ACh was again rapidly applied every 2 min for up to 10 min.

Slice Electrophysiology

Standard techniques were used to prepare 350- μ m-thick acute hippocampal slices from 14 to 21-day-old rats (Khiroug et al., 2003). Whole-cell patch-clamp recordings were performed from CA1 stratum radiatum interneurons as described by Klein et al. (2004). In brief, cells were clamped using an Axopatch 200B amplifier at a holding potential of -70 mV. The nAChR-mediated responses were elicited at 30-s intervals by pressure application of ACh via a glass pipette. Currents were recorded using pClamp software.

Data Analysis and Statistical Procedures

Data were analyzed using pClamp 8 or 10 and Excel 5.1 (Microsoft, Redmond, WA). Dose-response data for MARCKS peptide inhibition and MLA competition binding were analyzed by nonlinear regression using a logistic equation with variable slope ($Y = \text{bottom} + (\text{top} - \text{bottom}) / (1 + 10^{(\log EC_{50} - X)})$). For dose-response curves, the bottom limit was set to zero, and comparisons of IC₅₀ values were done using an *F* test. IC₅₀ values are presented with 95% confidence intervals (GraphPad Prism4; GraphPad Software Inc., San Diego, CA).

Peptide Synthesis and Other Materials

The MARCKS ED and MARCKS S/D peptides were synthesized by Sigma-Genosys (The Woodlands, TX) at a purity of 95%. The MARCKS ED peptide consisted of amino acids 151 to 175 from the wild-type protein, KKKKKRFSFKKSFKLSGFSFKKNK, and the mutated MARCKS S/D had a sequence KKKKKRFDKFKDFKLDGFDKFKKNK. The MARCKS₁₅₄₋₁₆₅ and MARCKS₁₅₉₋₁₆₅ peptides were purchased from AnaSpec, Inc. (San Jose, CA). The peptides used in this study were acetylated at the amino terminus and amide-capped at the carboxyl terminus. Peptides were reconstituted in phosphate-buffered saline,

yielding stock concentrations of 1 mM. Stock solutions were stored at -20°C and diluted to desired concentrations on the day of the experiment. ^{125}I - α -Bungarotoxin was purchased from PerkinElmer Life and Analytical Sciences (Waltham, MA). MLA and bovine serum albumin were purchased from Sigma-Aldrich (St. Louis, MO).

Results

The MARCKS ED Peptide Inhibits $\alpha 7$ nAChRs Expressed in *X. laevis* Oocytes

The ability of a synthetic MARCKS peptide (MARCKS_{151–175}), derived from the ED of the protein to modulate nAChRs expressed in *X. laevis* oocytes, was examined. Homomeric $\alpha 7$ nAChRs were expressed, and the effect of the MARCKS ED peptide on ACh-induced responses was investigated. Receptors were activated by the rapid application of ACh (1–2 mM, an ACh concentration that elicits a maximal current response under these experimental conditions) at a holding potential of -60 mV, and the MARCKS ED peptide was bath applied (Fig. 1A). The MARCKS ED peptide (1 μM) inhibited ACh-induced $\alpha 7$ nAChR peak current responses by $99 \pm 0.2\%$ ($n = 7$). The onset of inhibition was rapid, but washout was slow, with $82 \pm 3\%$ inhibition still present after 10 min (Fig. 1B). Inhibition of ACh-mediated $\alpha 7$ responses by the MARCKS ED peptide was dose-dependent, with an IC_{50} value of 16 nM (95% CI, 14–19 nM; Fig. 1C). The injection of the MARCKS ED peptide (~ 10 μM) intracellularly either acutely or 24 h before recording did not significantly effect ACh-mediated $\alpha 7$ nAChR currents (data not shown).

The MARCKS ED Peptide Inhibition of $\alpha 7$ nAChR Function Is Activity- and Voltage-Independent

To test for the activity dependence of MARCKS ED peptide inhibition, peptide was bath applied in the absence of ACh stimulation for 10 min (see Materials and Methods). When ACh was applied, the initial current response demonstrated maximal inhibition, suggesting that there was not a use-dependent component of the peptide block (Fig. 2A).

To test for voltage dependence of MARCKS ED peptide inhibition of $\alpha 7$ nAChRs, the effect of the MARCKS ED peptide was tested at two different holding potentials. The ability of the MARCKS ED peptide to inhibit ACh-mediated responses at $\alpha 7$ nAChRs was similar at $+30$ and -60 mV (Fig. 2B). The MARCKS ED peptide (1 μM) blocked the peak ACh current response at $+30$ mV by $85 \pm 2\%$ ($n = 6$). Although this was statistically different from the inhibition at -60 mV, the data indicate that the majority of MARCKS ED peptide inhibition is voltage-independent (only 14% of the inhibition was voltage-dependent). These results suggest that most of the inhibition by the MARCKS ED peptide (86%) is not because of open channel block, which would be expected to be both voltage- and use-dependent (Colquhoun and Ogden, 1988; Maconochie and Knight, 1992). However, as noted above, a small portion of the MARCKS ED effect is voltage-dependent (although not use-dependent); therefore, the peptide also may be acting as an open channel blocker.

The MARCKS ED Peptide Does Not Block α -BgTx Binding

Direct binding experiments using α -BgTx were carried out to determine whether the MARCKS ED peptide binds competitively with the toxin for $\alpha 7$ nAChRs. Increasing concentrations of the MARCKS ED peptide did not effect the initial rate of binding for ^{125}I - α -BgTx, whereas MLA was able to dose-dependently block ^{125}I - α -BgTx binding to $\alpha 7$ nAChRs expressed in *X. laevis* oocytes ($K_{0.5} = 1.54 \pm 0.14$ nM, Hill slope = 0.87 ± 0.12 , $n = 3$; Fig. 3).

A Mutated and Two Truncated MARCKS Peptides Do Not Inhibit $\alpha 7$ nAChR Responses

All four serine residues present in the MARCKS ED peptide were mutated to aspartate (MARCKS S/D). This mutant peptide was unable to inhibit ACh-mediated $\alpha 7$ nAChR responses (Fig. 4, A and D). When bath applied at 100 nM, the MARCKS S/D peptide inhibition was $2 \pm 2\%$, whereas wild-type MARCKS ED peptide inhibited $\alpha 7$ responses by $90 \pm 2\%$ ($n = 5$ and 11 , respectively; see Fig. 1 for MARCKS ED dose-response curve). In addition, truncated forms of the MARCKS ED peptide were unable to significantly inhibit $\alpha 7$ nAChRs; the percentage block by MARCKS_{154–165} (1 μ M) was $12 \pm 3\%$ and by MARCKS_{159–165} (1 μ M) was $5 \pm 2\%$ (Fig. 4, B–D). In addition, the intracellular injection of the mutant MARCKS S/D peptide (~ 10 μ M) acutely or 24 h before recording did not significantly effect ACh-mediated $\alpha 7$ nAChR currents (data not shown).

The MARCKS ED Peptide Preferentially Inhibits $\alpha 7$ -versus Non- $\alpha 7$ -Containing nAChRs

The most common heteromeric nAChRs expressed in hippocampal interneurons are the $\alpha 4\beta 2$ and $\alpha 2\beta 2$ subtypes (Sudweeks and Yakel, 2000). To determine the specificity of the MARCKS ED peptide interaction with $\alpha 7$ - versus non- $\alpha 7$ -containing receptors, $\alpha 4\beta 2$ and $\alpha 2\beta 2$ nAChRs were expressed in *X. laevis* oocytes. Compared with homomeric $\alpha 7$ nAChRs, both $\alpha 4\beta 2$ and $\alpha 2\beta 2$ nAChRs activate and desensitize more slowly in the continued presence of agonist. The MARCKS ED peptide inhibited $\alpha 4\beta 2$ and $\alpha 2\beta 2$ nAChRs in a dose-dependent fashion (Fig. 5C). The potency for the MARCKS ED peptide block for $\alpha 4\beta 2$ and $\alpha 2\beta 2$ nAChRs was significantly decreased compared with $\alpha 7$ nAChRs ($\alpha 4\beta 2 = 223$ nM, 95% CI, 112–447 nM; $\alpha 2\beta 2 = 72$ nM, 95% CI, 57–91 nM; $n = 2$ –8 for each data point).

Nicotinic $\alpha 7$ AChRs in Acute Hippocampal Slices Are Inhibited by the MARCKS ED Peptide

To test whether the MARCKS ED peptide could inhibit native nicotinic receptors, nAChR-mediated responses in rat CA1 stratum radiatum interneurons were measured in hippocampal slices. Pressure application of ACh (2 mM) principally activates $\alpha 7$ -containing nAChRs (Khiroug et al., 2003). Bath application of the MARCKS ED peptide was able to inhibit these responses in a dose-dependent manner (1 μ M, $59 \pm 13\%$; 3 μ M, $92 \pm 2\%$; 10 μ M, $100 \pm 0\%$; $n = 3$; Fig. 6). As is typical of slice data, the onset of inhibition was slow. In addition, similar to the lack of rapid washout in oocytes, recovery of responses from peptide inhibition was negligible, with no significant rescue within 20 min of washout. There was no effect of the MARCKS ED peptide on baseline current in hippocampal interneurons.

Discussion

The present study demonstrates that a peptide derived from the effector domain of human MARCKS protein inhibits $\alpha 7$, $\alpha 4\beta 2$, and $\alpha 2\beta 2$ nAChRs expressed in *X. laevis* oocytes, and $\alpha 7$ -containing nAChRs in hippocampal slices. This is the first study showing a direct effect of a MARCKS-derived peptide on nAChR function. The MARCKS ED peptide inhibition of $\alpha 7$ nAChRs was dose-dependent, with an IC_{50} value of 16 nM and slow recovery during washout. The MARCKS ED peptide had no effect on the initial rate of α -bungarotoxin binding and was both voltage- and activity-independent. In addition, MARCKS ED peptides injected intracellularly into *X. laevis* oocytes did not effect nAChR function, demonstrating that the MARCKS ED peptide is a potent antagonist that binds directly to the extracellular domain of $\alpha 7$ nAChRs and acts primarily as an allosteric antagonist rather than as an open channel blocker.

A mutated form of the MARCKS ED peptide that mimics the phosphorylated protein was unable to inhibit ACh responses at $\alpha 7$ nAChRs, suggesting that the serines within the MARCKS ED peptide are important either for blockade of nAChRs or for an unknown secondary structure necessary for peptide interaction with the receptor. Alternatively, the introduction of a negative charge may make the peptide repulsive to the receptor. MARCKS is a natively unfolded protein,

and the MARCKS ED peptide does not appear to have any distinct secondary structure. However, a crystal structure of a similar MARCKS peptide in the presence of calmodulin reveals that a portion of the ED peptide takes on an α helical conformation when in contact with Ca^{2+} /calmodulin, suggesting that the peptide may take on a secondary structure when interacting with other proteins (Yamauchi et al., 2003). It is interesting that PKC phosphorylation of the MARCKS ED peptide inhibits the direct interaction of the peptide with Ca^{2+} /calmodulin (Graff et al., 1989, 1991), similar to the lack of inhibition of nAChR responses by the MARCKS S/D peptide. The lack of effect by the pseudophosphorylated version of the MARCKS ED peptide raises the possibility of a reversible inhibitor under physiological conditions. Although not tested directly here, if the MARCKS S/D peptide acts in a competitive manner with the MARCKS ED peptide for binding to the nAChR, then under physiological conditions, the phosphorylation state of MARCKS could potentially regulate nAChR signaling. In addition, shorter MARCKS ED peptides were also unable to significantly inhibit $\alpha 7$ nAChRs, suggesting that the longer 25-amino acid peptide is required for interaction with nACh receptors. The sequence of the MARCKS ED peptide contains a significant number of positively charged amino acids, including five lysines at the N-terminal and four at the C-terminal; however, pentyllysine has been tested previously and does not block $\alpha 7$ nAChR currents (Gay et al., 2006), demonstrating that a series of positive charges alone is not enough to block nAChR function.

The MARCKS ED peptide produced less potent inhibition of ACh-stimulated $\alpha 4\beta 2$ and $\alpha 2\beta 2$ nAChR responses, demonstrating some selectivity for the homomeric $\alpha 7$ receptors over the heteromeric non- $\alpha 7$ nAChR subtypes. In addition, the MARCKS ED peptide appears to dissociate more rapidly from heteromeric nAChRs. These data suggest that the binding of the MARCKS ED peptide to nAChRs is different to some extent between distinct neuronal nAChR subtypes. Understanding the structural differences between $\alpha 7$ versus non- $\alpha 7$ nAChR interactions with the MARCKS ED peptide will help to elucidate differences between these receptor subtypes and may also reveal novel approaches for the development of ligands with specificity for the various nAChRs. In addition, it may be of interest to test whether the MARCKS ED peptide inhibits muscle-type nAChRs with the same potency as neuronal nAChRs.

It remains to be determined whether MARCKS or fragments of the MARCKS protein interact with nAChRs in vivo. MARCKS is distributed throughout the rat brain and enriched in particular areas including the piriform and entorhinal cortices, the amygdala, and some catecholaminergic and serotonergic nuclei. In addition, MARCKS is expressed both in neurons, pre- and postsynaptically, and in glial cells (Ouimet et al., 1990). Although the full MARCKS protein is expressed intracellularly, the current data reveal that the MARCKS ED peptide interacts directly with the extracellular domain of nAChRs. MARCKS is cleaved by cathepsin B as a way of regulating MARCKS intracellular concentrations (Spizz and Blackshear, 1996; Spizz and Blackshear, 1997). Nevertheless, it seems unlikely that fragments of the MARCKS protein would be available to interact with the extracellular domain of nAChR receptors unless under pathological conditions, where cell death could release MARCKS into the extracellular space. It is interesting that if fragments of the MARCKS protein do, in fact, interact with nAChRs under physiological conditions, then the inability of the MARCKS S/D peptide to block nAChR signaling suggests that this interaction could be regulated in a phosphorylation-dependent manner.

An assortment of small peptides is known to interact with nAChRs, such as naturally occurring toxins and brain-derived protein fragments; these include peptides from β -amyloid, acetylcholinesterase, apolipoprotein E, and now MARCKS (for a list of additional neuropeptides, see Di Angelantonio et al., 2003). The majority of these peptides are noncompetitive, and some are both voltage- and activity-independent, similar to the MARCKS

ED peptide. The low nanomolar potency of inhibition demonstrated by the MARCKS ED peptide at the $\alpha 7$ nAChR makes it a practical antagonist of nAChR function for both scientific and perhaps clinical utility. As a research tool, the MARCKS ED may be of particular interest because it appears to bind to the $\alpha 7$ nAChR at a location distinct from the α -BgTx binding site, which is known to be competitive for ACh. Therefore, the MARCKS ED peptide may help to define a unique allosteric binding site for nAChRs. In addition, the distinct specificity of the MARCKS ED peptide for the various neuronal nAChRs may be particularly useful compared with other known peptides such as α -BgTx and the α -conotoxins that inhibit a limited number of nAChRs, whereas the MARCKS ED peptide can block specifically $\alpha 7$ - or also non- $\alpha 7$ -containing nAChRs, depending on the concentration used. In terms of clinical utility, nAChR agonists are regarded generally to provide the largest therapeutic potential. However, antagonists, such as this MARCKS ED peptide, provide distinctive therapeutic possibilities as well. For example, nAChR antagonists may provide therapeutic utility in nicotine addiction, schizophrenia, or chronic pain (Dwoskin and Crooks, 2001) or when nAChR hyperactivity is toxic (Lukas et al., 2001). In addition, because of the rapid agonist-induced desensitization of nAChRs, nAChR antagonists may also turn out to be therapeutically useful in other neuropathologies, including Alzheimer's disease, Parkinson's disease, and memory and learning disabilities (Arneric et al., 2007). However, it is unlikely that this highly charged peptide will cross the blood-brain barrier; therefore, the MARCKS ED peptide may be more useful as a foundation for structure/activity endeavors that lead to rational drug design.

The current data demonstrate that the MARCKS ED peptide disrupts nAChR signaling by directly inhibiting ion channel activation and may be used as a modulator of nAChR function. In addition, characterizing the mechanism of nAChR block by this unique peptide may help to elucidate structural elements of the $\alpha 7$ nAChR involved in receptor antagonism and differences between the $\alpha 7$ and the $\alpha 4\beta 2$ and $\alpha 2\beta 2$ receptors. Finally, the MARCKS ED peptide represents a high-potency antagonist of neuronal nAChR signaling that may prove beneficial as a starting point in the development of drugs for pathological conditions in which nicotinic hyperfunction underlies neurotoxicity. The MARCKS ED peptide represents a novel, noncompetitive antagonist of neuronal nAChRs that has considerable utility as a research tool. In addition the interaction between the MARCKS ED peptide and neuronal nAChRs may help to elucidate a unique ligand binding site by which synaptic transmission can be modulated during both normal and pathological conditions.

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ABBREVIATIONS

nAChR, nicotinic ACh receptor; MARCKS, myristoylated alanine-rich C kinase substrate; PKC, protein kinase C; ED, effector domain; MLA, methyllycaconitine; α -BgTx, α -bungarotoxin; ACh, acetylcholine; CI, confidence interval..

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Fig. 1. MARCKS ED peptide inhibition of $\alpha 7$ nAChRs. The MARCKS ED peptide completely inhibited ACh-evoked responses from $\alpha 7$ nAChRs expressed in *X. laevis* oocytes. A, representative traces for ACh-evoked current responses before (control) and during bath application of peptide are illustrated. B, time course of peptide inhibition illustrating that the MARCKS ED peptide was slow to recover with washout (mean $\pm$ S.E.M., $n = 5$). The MARCKS ED peptide inhibited in a dose-dependent manner (C), yielding an IC_{50} value of 16 nM (95% CI, 14–19 nM; $n = 3$ –11 oocytes per data point). The $\alpha 7$ nAChR-mediated responses were elicited by application of 2 mM ACh for 200 to 250 ms (bar) at 1 to 2-min intervals.

Fig. 2.

MARCKS ED peptide inhibition of ACh-induced responses was activity- and voltage-independent. With a 10-min washin of the MARCKS ED peptide in the absence of repetitive ACh stimulation, the MARCKS ED peptide inhibited $\alpha 7$ nAChR responses upon subsequent ACh application. A, peak ACh response over time (mean $\pm$ S.E.M., $n = 3$, left); 1 μ M MARCKS ED peptide inhibited $\alpha 7$ nAChRs by $99 \pm 0.2\%$ with continued ACh application (total inhibition, $n = 7$) and by $98 \pm 2\%$ with a 10-min washin in the absence of ACh (activity-independent inhibition, $n = 5$, right). Inhibition of $\alpha 7$ currents by the MARCKS ED peptide was similar at both -60 and $+30$ mV. B, representative traces of the peak ACh response at $+30$ mV both before (control) and during bath application of the MARCKS ED peptide (left) (bar, application of 2 mM ACh, 250 ms). Mean $\pm$ S.E.M. of MARCKS ED peptide inhibition of ACh-mediated responses at holding potentials of -60 and $+30$ mV ($n = 4$ and 6, respectively, right).

Fig. 3. MARCKS ED peptide does not compete for ^{125}I - α -bungarotoxin binding. The MARCKS ED peptide was unable to inhibit the initial rate of ^{125}I - α -BgTx binding to $\alpha 7$ nAChRs expressed in *X. laevis* oocytes. In contrast, MLA blocked ^{125}I - α -BgTx binding in a concentration-dependent manner. Each data point represents the mean $\pm$ S.E.M. of three experiments conducted in triplicate. The MLA curve was generated by nonlinear regression using a logistic equation with variable slope.

Fig. 4.

Mutated and truncated MARCKS ED peptides do not inhibit $\alpha 7$ nAChR responses. A mutant MARCKS ED peptide, in which the four serine residues were replaced with aspartate residues, and two truncated versions of the MARCKS ED peptide were unable to inhibit ACh-induced $\alpha 7$ nAChR-mediated currents. Representative traces for ACh-evoked current responses before (control) and during bath application of MARCKS S/D (A), MARCKS₁₅₄₋₁₆₅ (B), and MARCKS₁₅₉₋₁₆₅ (C) are illustrated (bar, application of 2 mM ACh, 200 ms for A; and 1 mM ACh, 2 s for B and C). D, MARCKS ED peptide (1 μ M) inhibited $\alpha 7$ ACh responses by $99 \pm 1\%$ (see Fig. 1, A and C), whereas MARCKS S/D (100 nM) inhibited $\alpha 7$ ACh responses by $2 \pm 2\%$, MARCKS₁₅₄₋₁₆₅ (1 μ M) by $12 \pm 3\%$, and MARCKS₁₅₉₋₁₆₅ (1 μ M) by $5 \pm 2\%$. Data are mean $\pm$ S.E.M. of at least four oocytes for each peptide.

Fig. 5. MARCKS ED peptide inhibits heteromeric $\alpha 4\beta 2$ and $\alpha 2\beta 2$ nAChRs expressed in *X. laevis* oocytes. nAChR-mediated responses were elicited by application of 1 mM ACh for 1 to 1.5 s (bar) at 1- to 2.5-min intervals. Representative traces for ACh-evoked responses at $\alpha 4\beta 2$ nAChRs (A) and $\alpha 2\beta 2$ (B) before (control) and during bath application of 1 μ M MARCKS ED peptide. C, plot of percentage inhibition of ACh-evoked peak current responses versus increasing concentrations of MARCKS ED peptide yielded IC_{50} values of 223 nM (95% CI, 112–447 nM) for $\alpha 4\beta 2$ and 72 nM (95% CI, 57–91 nM) for $\alpha 2\beta 2$. Dose-response curve for $\alpha 7$ nAChR is included for reference (from Fig. 1). Data are from two to eight oocytes for each data point.

Fig. 6.

Nicotinic AChRs in acute hippocampal slices are inhibited by MARCKS ED peptide. A, representative traces for ACh-evoked responses before (control) and during bath application of peptide are illustrated. The nAChR-mediated responses were elicited at 30-s intervals by pressure application of 2 mM ACh (bar). B, peak ACh current responses over time indicating that ACh-mediated responses do not recover after MARCKS ED peptide inhibition (data are mean $\pm$ S.E.M. of two to three interneurons, sweeps were 30 s apart). The MARCKS ED peptide inhibited nAChR responses in a dose-dependent manner. C, bar graph of percentage inhibition of ACh-evoked responses at increasing concentrations of the MARCKS ED peptide; inhibition was 59% for 1 μ M, 92% for 3 μ M, and 100% for 10 μ M peptide. Data are from three interneurons for each concentration.