JPET Fast Forward. Published on October 25, 2005 as DOI: 10.1124/jpet.105.095505

JPET #95505

Apolipoprotein E-derived peptides block α7 neuronal nicotinic ACh receptors expressed in *Xenopus* oocytes[†]

Elaine A. Gay, Rebecca C. Klein and Jerrel L. Yakel

Laboratory of Neurobiology, National Institute of Environmental Health Sciences, National Institutes of Health, Department of Health and Human Services, P.O. Box 12233, Research Triangle Park, N.C. 27709 (E.A.G., R.C.K., J.L.Y.)

JPET #95505

Running Title: ApoE peptide inhibition of α7 nAChRs

Corresponding author: Jerrel L. Yakel
NIEHS, F2-08, P.O. Box 12233
111 T.W. Alexander Drive
Research Triangle Park, N.C. 27709
Tel. (919) 541-1407
Fax. (919) 541-1898
E-Mail: yakel@niehs.nih.gov

Number of text pages: 16
Number of tables: 1
Number of figures: 7
Number of references: 40
Number of words in Abstract: 236
Number of words in Introduction: 691
Number of words in Discussion: 1475

Abbreviations: AChE, acetylcholinesterase; CGRP, calcitonin gene-related peptide; ChAT, choline acetyltransferase; DMSO, Dimethyl sulfoxide; MLA, methyllycaconitine; NMDA, N-methyl-D-aspartate;

Recommended section assignment: Neuropharmacology

JPET Fast Forward. Published on October 25, 2005 as DOI: 10.1124/jpet.105.095505
This article has not been copyedited and formatted. The final version may differ from this version.

Abstract

For decades the pathology of Alzheimer's disease has been associated with dysfunction of cholinergic signaling; however, the cellular mechanisms by which nicotinic acetylcholine receptor (nAChR) function is impaired in Alzheimer's disease are as yet unknown. The most significant genetic risk factor for the development of Alzheimer's disease is inheritance of the ε4 allele of apolipoprotein E (apoE). Recent data has demonstrated the ability of apoE-derived peptides to inhibit nAChRs in rat hippocampus. In the current study, the functional interaction between nAChRs and apoE-derived peptides was investigated in *Xenopus* oocytes expressing selected nAChRs. Both a 17 amino acid peptide fragment, $apoE_{133-149}$, and an 8 amino acid peptide, $apoE_{141-148}$, were able to maximally block ACh-mediated peak current responses for homomeric α7 nAChRs. ApoE peptide inhibition was dose-dependent, and voltage- and activity-independent. The current findings suggest that apoE peptides are non-competitive for acetylcholine and do not block functional α-bungarotoxin binding. ApoE peptides had a significantly decreased ability to inhibit ACh-mediated peak current responses for α4β2 and α2β2 nAChRs. Amino acid substitutions in the apoE peptide sequence suggest that the arginines are critical for peptide blockade of the α7 nAChR. The current data suggests that apoE fragments can disrupt nAChR signaling through a direct blockade of α7 nAChRs. These results may be useful in elucidating the mechanisms underlying memory loss and cognitive decline seen in Alzheimer's disease, as well as aid in the development of novel therapeutics using apoE-derived peptides.

Introduction

Apolipoprotein E (apoE) is the principal apolipoprotein synthesized in the brain, and is implicated as a risk factor in a variety of CNS disorders including Alzheimer's disease (AD), and response to traumatic brain injury. ApoE is a 299 amino acid protein that, in the brain, is synthesized and secreted primarily by astrocytes (Pitas et al., 1987). ApoE binds to low density lipoprotein (LDL) receptors and historically is known to be involved with lipid metabolism and cholesterol transport. There are three isoforms of apoE (apoE2, apoE3 and apoE4), of which the apoE4 gene is associated with an increased risk of developing both familial and sporadic late-onset AD (Corder et al., 1993; Rebeck et al., 1993). ApoE4 has been shown to co-localize with both Aβ plaques and neurofibrillary tangles, and evidence suggests that apoE4 may be associated with the progressive loss of cognitive function in AD (for review see Marques and Crutcher, 2003). Several hypotheses have emerged to account for apoE in the development of AD (Bales et al., 2002; Harris et al., 2003); however, none of these has yet provided a clear understanding of the role of apoE in the pathology of AD. ApoE is also a risk factor in several other conditions including cognitive impairment: after traumatic brain injury, due to the progression of Parkinson's disease, and during normal aging (Friedman et al., 1999; Tang et al., 2002; Howieson et al., 2003).

Proteolytic fragments of apoE, including the n-terminal thrombin cleavage fragment of 22 kDa, have been shown to be increased in the brain and CSF of AD patients (Marques et al., 1996). Both the full length apoE, after proteolysis, and this n-terminal truncated apoE have been shown to cause neurotoxicity under a variety of experimental conditions (Marques et al., 1996; Michikawa and Yanagisawa, 1998). In addition,

synthetic peptides derived from the LDL receptor binding domain of apoE have been shown to demonstrate similar neurotoxic effects (Clay et al., 1995; Tolar et al., 1997). Previous work has shown that apoE peptides can also mimic the actions of the holoprotein in terms of binding to LDL receptor related protein and protecting against NMDA-mediated excitotoxicity (Aono et al., 2003; Croy et al., 2004). In addition, apoE mimetic peptides have demonstrated potential therapeutic usefulness in both head trauma and following ischemic injury (Lynch et al., 2005; McAdoo et al., 2005); however, the cellular mechanisms underlying this benefit have not been identified in detail.

Neuronal nicotinic acetylcholine receptors (nAChRs) are involved in a variety of normal brain functions including cognitive tasks, reward systems, and neuronal development (Jones et al., 1999). Dysregulation of nAChR signaling has long been associated with multiple pathologies including AD, schizophrenia, epilepsy and Parkinson disease (for review see Levin, 2002; Picciotto and Zoli, 2002; Raggenbass and Bertrand, 2002). For example, selective neurodegeneration of cholinergic neurons occurs in AD and is evident by decreases in both ChAT and AChE activity as well as a decrease in nAChR number in the brains of AD patients (Davies and Maloney, 1976; Araujo et al., 1989). In AD patients, administration of either nicotine or nAChR agonists can enhance performance on cognitive tasks (Jones et al., 1992; White and Levin, 1999). Moreover, of the drugs approved to date to treat AD, all are AChE inhibitors with the exception of memantine, a NMDA receptor antagonist (for review see Lleo et al., 2005). Despite the past few decades of investigation, the direct cellular mechanisms by which nAChR function is impaired in AD are as yet unknown.

Recent work has demonstrated that apoE-derived peptides from the LDL receptor binding region inhibit native α7-containing nAChRs expressed on interneurons in rat hippocampal slices, and that this inhibition was specific for excitatory receptors in the superfamily of ligand-gated ion channels (Klein and Yakel, 2004). The current study probes the functional interaction between apoE-derived peptides and nAChRs expressed in *Xenopus* oocytes. The selectivity of apoE peptides for α7- and non α7-containing nAChRs was investigated, as well as the sequence specificity for apoE peptide interaction with nAChRs. The nature of the apoE peptide/nAChR interaction was also explored. The current data supports the hypothesis that apoE-derived peptides disrupt cholinergic signaling through a direct blockade of α7 nAChRs.

Methods

Peptide Synthesis–ApoE-derived peptides were synthesized by Sigma-Genosys (The Woodlands, TX) at a purity of 95% and reconstituted in either sterile, deionized water or DMSO, yielding stock concentrations of 15-20 mM. Stock solutions were stored at –20°C and diluted to desired concentrations on the day of the experiment. The peptides used in this study were acetylated at the amino terminus and amide-capped at the carboxyl terminus, except for $ApoE_{133-140}$ which contained a free amino terminus. Pentalysine was purchased from Sigma (St. Louis, MO) and stored at –20ºC (50 mM).

Oocyte Preparation–Female *Xenopus laevis* frogs were anesthetized in cold water containing 0.2% metaaminobenzoate and the spinal cord severed. Oocytes were dissected and defolliculated by treatment with collagenase B (2 mg/mL, Roche Diagnostics) and trypsin inhibitor (1 mg/ mL, Gibco) for 2 h. Oocytes were maintained in solution containing: 82.5 mM NaCl, 2.5 mM KCl, 1 mM Na_2HPO_4, 3 mM NaOH, 5 mM HEPES, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 2.5 mM pyruvic acid, and 0.05 mg/mL Gentamycin sulfate with constant rotation at 18°C. mRNA for each of the nAChR subunits was transcribed from plasmids using mMeassage mMachine 17 kit from Ambion (Austin, TX) according to the manufacturer's instructions. The total amount of RNA injected for α7 nAChR subunits was 50 ng, and for α4, α2 and β2 subunits was 12.5 ng each. Recordings were made 3-7 days post RNA injection.

Oocyte Electrophysiology–Current responses were obtained by two-electrode voltage clamp recording at a holding potential of –60 mV (unless otherwise stated) using a Geneclamp 500 and pClamp 8 software. Electrodes contained 3 M KCl and had a resistance of <1 MΩ. ACh and peptides were prepared daily in bath solution (96 mM

NaCl, 2 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM HEPES) from frozen stocks. ACh was applied for various time periods using a synthetic quartz perfusion tube (0.7 mm i.d.) operated by a computer-controlled valve. Peptides were bath applied. Data were analyzed using pClamp 8, Excel (Microsoft) and GraphPad Prism4. Peak current responses to each dose of apoE peptide or ACh were averaged, and the mean ± S.E.M were analyzed by nonlinear regression using a logistic equation (Y = bottom + (top – bottom))/(1 + 10^($LogEC_{50}$ – X))). For dose-response curves the bottom limit was set to zero and IC_{50} values are presented with 95% confidence intervals. Data for ACh dose-response curves were normalized to the peak current response at 1 mM ACh control. Multiple group comparisons were preformed by one-way ANOVA followed by a Tukey post hoc analysis to make specific comparisons between individual values (Origin 6, Microcal Software). Significance was defined at $p< 0.05$. Data are reported as mean ± S.E.M of multiple experiments (see results for n values). For α-bungarotoxin (α-BgTx) competition experiments, apoE peptides (10 μM) or MLA (10 nM) were bath applied for ten minutes, followed by co-application of α-BgTx (10 nM) with either apoE peptides or MLA for an additional ten minutes, and subsequently followed by washout with bath solution. The concentrations of antagonists were chosen using a two ligand receptor occupancy equation (Kenakin, 2004), with KDs for α-BgTx and MLA of approximately 5 and 2 nM respectively (http://pdsp.cwru.edu/pdsp.asp), so that approximately 89 % of nAChRs would be occupied by $apoE_{133-149}$, 77 % by $apoE_{141-148}$, and 71 % by MLA when in combination with α-BgTx.

Circular Dichroism Spectroscopy–CD spectra were recorded between 195 nm and 260 nm on a *JASCO* 810 spectrometer using 0.1 cm pathlength cells. Peptides were

JPET Fast Forward. Published on October 25, 2005 as DOI: 10.1124/jpet.105.095505
This article has not been copyedited and formatted. The final version may differ from this version.

diluted from stock to 150 μM in buffer containing 20 mM sodium phosphate (pH 6.0), 100 mM sodium chloride, and 40% trifluoroethanol (TFE). The α-helical content of the peptides was determined from the ellipticities at 222 nm using the empirical relationship, $fraction_{helix} = (-[\Theta]222- 2340)/30,300$.

JPET Fast Forward. Published on October 25, 2005 as DOI: 10.1124/jpet.105.095505
This article has not been copyedited and formatted. The final version may differ from this version.

Results

ApoE peptides inhibit α7 nAChRs expressed in Xenopus oocytes–The ability of synthetic apoE peptides, containing the LDL receptor binding region, to modulate nAChRs expressed in *Xenopus* oocytes was examined. Homomeric α7 nAChRs were expressed and the effects of apoE peptides on ACh-induced responses were determined. Receptors were activated by the rapid application of ACh (2 mM) at a holding potential of –60 mV (Figure 1). The 17 amino acid peptide $apoE_{133-149}$ (3 μM) inhibited ACh-induced α7 nAChR peak current responses by 91 ± 3 % (n = 12), which was reversible upon wash out (Figure 1a). This inhibition was dose-dependent with an IC_{50} value of 445 nM (95%CI: 349 nM – 566 nM, Figure 2).

In order to determine the active sequence of this 17mer apoE peptide, two peptides of 8 amino acids, $apoE_{133-140}$ and $apoE_{141-148}$, were tested. Interestingly, the n-terminal portion of the peptide, $apoE_{133-140}$, caused significantly less inhibition of ACh-mediated responses (16 ± 3 % at 3 μM; n = 8) as compared to $apoE_{133-149}$, while the c-terminal portion of the peptide, $apoE_{141-148}$, was able to inhibit α7 nAChR-mediated responses similar to $apoE_{133-149}$ (81 ± 2 % at 3 μM; n = 15) (Figure 1b, c). Correspondingly, the ability of $apoE_{141-148}$ to block α7 nAChR function was dose-dependent (IC_{50} = 1.30 μM, 95%CI: 1.07 μM – 1.56 μM, Figure 2) and the peak current response returned upon wash out of the peptide (Figure 1b). Maximum peptide inhibition generally occurred within the time period between agonist applications (i.e. 2 min), a time required for full recovery from α7 receptor desensitization. Application of apoE peptides did not affect the baseline current responses (data not shown).

JPET Fast Forward. Published on October 25, 2005 as DOI: 10.1124/jpet.105.095505
This article has not been copyedited and formatted. The final version may differ from this version.

ApoE peptides inhibit α7 nAChR function in a non-competitive and voltage-independent manner–ACh dose-response curves were generated in the presence or absence of apoE peptides (0.3 μM) in order to determine the mechanism of interaction between the apoE peptide and the α7 nAChR. For each ACh concentration tested, both $apoE_{133-149}$ and $apoE_{141-148}$ displayed comparable inhibition of peak current responses, as well as similar EC_{50} values (control = 124 μM, $apoE_{133-149}$ = 102 μM, and $apoE_{141-148}$ = 118 μM) for activation of the α7 nAChR by ACh (Figure 3b, n = 2-11 oocytes for each ACh concentration). These data suggest that both $apoE_{133-149}$ and $apoE_{141-148}$ are interacting with the channel in a non-competitive manner.

In order to determine if apoE peptides were competing with the α-BgTx binding site, we used a method similar to previous studies demonstrating that pre-incubation with ligands competitive for the α-BgTx binding site can preclude the very slow recovery from α-BgTx functional block (Ellison et al., 2003). A ten minute bath application of 10 nM α-BgTx was enough to block peak ACh current responses at α7 nAChRs by 91 ± 3 % (n = 3, Figure 4a). This inhibition was slow to wash out with only 15 ± 3 % of the peak response returning after 20 min. However, when oocytes were pretreated for ten min. with 10 nM MLA, a known reversible competitive antagonist of α7 nAChRs, followed by 10 min. co-application of MLA and α-BgTx, the peak ACh current response recovered more quickly (i.e. by 78 ± 6 % (n = 4) in 20 min; Figure 4b). These data suggest that MLA and α-BgTx are competing for the same site on α7 nAChRs, as expected. However, when oocytes were pretreated with either 10 μM $apoE_{133-149}$ or $apoE_{141-148}$ followed by co-application with α-BgTx, the peak current response did not

JPET Fast Forward. Published on October 25, 2005 as DOI: 10.1124/jpet.105.095505
This article has not been copyedited and formatted. The final version may differ from this version.

recover upon wash out (6 ± 2 % and 18 ± 6 % respectively, n = 3, Figure 4c and d), suggesting that these apoE peptides are blocking at a site distinct from α-BgTx.

Another potential mechanism of block was an open-channel block, which is generally considered to be highly voltage- and use-dependent (Colquhoun and Ogden, 1988; Maconochie and Knight, 1992). The ability of apoE peptides to block α7 nAChR function was not significantly different at +30 versus –60 mV. $ApoE_{133-149}$ (3 μM) blocked the peak ACh current response at +30 mV by 95 ± 1 % (n = 6) and $apoE_{141-148}$ (3 μM) blocked 77 ± 3 % (n = 6) (data not shown), similar to the block at -60 mV, suggesting that these peptides inhibit through a voltage-independent mechanism. Furthermore, when $apoE_{133-149}$ was applied for 10 min. before ACh application, the initial current response showed maximal inhibition, indicating that there was no use-dependent component of the peptide block (data not shown). Together these data suggest that apoE peptides are not blocking α7 nAChRs through an open channel block mechanism.

ApoE peptides preferentially inhibit α7-versus non α7- containing nAChRs–In order to determine the specificity of the apoE peptide interaction with α7- versus non α7-containing receptors, α4β2 and α2β2 nAChRs were expressed in *Xenopus* oocytes. Compared to homomeric α7 nAChRs, both α4β2 and α2β2 nAChRs activate more slowly and do not desensitize in the continued presence of agonist. Both $apoE_{133-149}$ and $apoE_{141-148}$ showed significantly less inhibition of ACh-mediated peak current responses for α4β2 and α2β2 nAChRs (Figure 5). $ApoE_{133-149}$ (3 μM) blocked α4β2 and α2β2 nAChRs by 43 ± 6 % (n = 9) and 71 ± 4 % (n = 6) of control values, respectively. Interestingly, the shorter 8 amino acid peptide, $apoE_{141-148}$, demonstrated more selectivity for α7 nAChRs, inhibiting ACh-induced peak currents by only 23 ± 6 % (n = 9) for α4β2

JPET Fast Forward. Published on October 25, 2005 as DOI: 10.1124/jpet.105.095505
This article has not been copyedited and formatted. The final version may differ from this version.

receptors and 8 ± 4 % (n = 6) for α2β2 nAChRs. These data suggest that the LDLR-derived apoE peptides are less effective at inhibiting non-α7 receptor mediated responses, with $apoE_{141-148}$ demonstrating pronounced selectivity for α7 nAChRs.

ApoE peptide sequence requirements for nAChR inhibition–In order to determine the minimally active peptide, as well as peptide sequence specificity, synthetic peptides of varying length and sequence were tested. A five amino acid peptide, $apoE_{144-148}$, had nominal ability to block ACh peak current responses for α7 nAChRs (25 ± 4 % at 3 μM; n = 6; Figure 6). Both random and non-random scrambled peptides also demonstrated limited inhibition (35 ± 7 %, and 23 ± 4 %, n = 10 and n = 8, respectively; Figure 6). Similarly, pentalysine displayed minimal block of α7 nAChRs function (10 ± 2 % at 3 μM, n = 6). These data suggest that both peptide sequence and length are important for block of α7 nAChRs.

In addition, substitutions at certain residues of both $apoE_{133-149}$ and $apoE_{141-148}$ were tested to probe the key amino acid residues responsible for apoE inhibition of α7 nAChR function. First, the two positively charged lysines (positions 143 and 146) were substituted with leucines in both $apoE_{133-149}$ and $apoE_{141-148}$. These altered peptides were able to block ACh peak current responses for α7 nAChRs similar to their native counterparts (93 ± 1 %, and 74 ± 8 %, n = 7 and n = 8, respectively; Figure 6). While replacing these basic lysines with the nonpolar leucines had no effect on maximal inhibition, this change dramatically decreased the rate of block for α7 nAChRs from less than 2 min to over 10 min. For both of these peptides, the inhibition was reversible upon wash out (Figure 7). Next, the introduction of a negative charge at these two lysine positions by substitution with glutamate completely abrogated the ability of the peptide to

inhibit α7 nAChRs responses (2 ± 1 % at 3 μM; n = 5; Figure 6). Finally, there are three positively charged arginines in the active 8mer peptide. Altering two of the three arginines to leucines (positions 142 and 147) significantly reduced the ability of $apoE_{141-148}$ to inhibit peak α7 nAChR responses (7 ± 2 % at 3 μM; n = 4).

Circular Dichroism Measurements–The apoE peptides used in this study are derived from an α-helical portion of the apoE protein that includes the LDL receptor binding region. In order to determine the α-helical propensity of the peptides, we assessed the helical content in the presence of 40 % TFE, a solvent which can stabilize α-helical regions of a peptide (supplemental Figure 1). $ApoE_{133-149}$ exhibited α-helicity of 47 %, while $apoE_{141-148}$ only demonstrated 13 % helicity in the presence of TFE. $ApoE_{133-140}$, along with both of the scrambled peptides, had minimal α-helical tendencies (< 15 %). Interestingly, while unable to block ACh responses, the altered apoE peptide containing glutamate residues had a propensity to helicity of 37 %, similar to the active $apoE_{133-149}$ (Table 1).

Discussion

The present study demonstrates that peptides derived from the LDL receptor binding domain of apoE inhibit α7-containing nAChRs expressed in *Xenopus* oocytes. Both a 17 amino acid peptide fragment of apoE ($apoE_{133-149}$) and a shorter 8 amino acid peptide ($apoE_{141-148}$) were able to block ACh-mediated peak current responses for α7 nAChRs. ApoE peptide inhibition was dose-dependent, with IC_{50} values in the high nM to μM range, and similar to those found in hippocampal interneurons (Klein and Yakel, 2004). Neither a peptide containing amino acids 133-140, nor a 5 amino acid peptide, $apoE_{144-148}$, were able to block ACh peak current responses for α7 receptors, indicating activity is preserved within the 8 residue fragment, $apoE_{141-148}$.

Similar apoE peptides have been used in several studies investigating various physiological effects. Studies have demonstrated that apoE-derived peptides can inhibit lymphocyte proliferation without loss of cell viability (Clay et al., 1995), induce neurite degeneration (Tolar et al., 1997), and block both neuronal death and NMDA-mediated calcium influx (Aono et al., 2003). Interestingly, it was shown that $apoE_{133-149}$ could block NMDA-induced excitotoxicity, while the shorter peptide, $apoE_{139-149}$, did not retain this protective function (Aono et al., 2003). This is in contrast to the data presented here with the α7 nAChR where $apoE_{141-148}$ preserved activity. Intriguingly, there is recent evidence that as an alternative to the cholinergic hypothesis of Alzheimer's disease glutamatergic dysfunction may play a role in the etiology of the disease (for review see Doraiswamy, 2003). Perhaps most important has been the demonstration that $apoE_{133-149}$ can improve both motor and cerebellar function following closed head injury in mice (Lynch et al., 2005), as well as reduce brain injury following perinatal hypoxia-ischemia

in rats (McAdoo et al., 2005). These data suggest the immense potential therapeutic usefulness of apoE-derived peptides.

Additional studies by other groups have been performed using synthetic peptides that are tandem repeats of the LDL receptor binding region of the protein. These experiments have somewhat contradictory results to the monomeric peptide data since tandem repeat peptides cause neurite degeneration and neuronal cell death in multiple cell preparations (Crutcher et al., 1994; Tolar et al., 1997; Tolar et al., 1999; Qiu et al., 2003), and increase $[Ca^{2+}]_i$ through NMDA receptors (Tolar et al., 1999; Qiu et al., 2003). These discrepancies could be due both to the precise peptide used, as well as differences in peptide exposure time. In addition, the majority of apoE peptides used to date do not include the polymorphic sites of the apoE protein and therefore do not address directly apoE isoform specific affects. However, differences at the polymorphic sites (positions 112 and 158) of apoE have been demonstrated to affect the LDL receptor binding region (Weisgraber et al., 1982), suggesting that apoE genotype may still play a role in peptide action.

The current data support the hypothesis that the apoE peptides derived from the LDL receptor binding domain interact directly with the nAChR to modulate its function. ApoE peptide inhibition of α7 nAChR responses was unaltered by changes in voltage; in addition, the ability of $apoE_{133\text{-}149}$ to inhibit ACh-mediated peak current responses was unaffected by previous activity of the channel. These data indicate that apoE peptide inhibition of nAChR function is not activity dependent and that the peptides are not functioning as open channel blockers. The relative inhibition of apoE peptides for ACh-mediated peak current responses at α7 nAChR was similar across a range of ACh

concentrations, suggesting the peptides do not compete for the ACh binding site. These experiments were conducted under conditions in which the ligand and receptor were not at equilibrium, and therefore the possibility that apoE peptides interact with nAChRs in a competitive manner could not be ruled out. However, further data were also consistent with a non-competitive interaction. ApoE peptides were unable to block functional α-BgTx binding, indicating that the peptides do not interact with α7 nAChRs in a manner that is competitive for the α-BgTx binding site. These data would suggest that apoE peptides interact with α7 nAChRs either at a site other than the traditional ligand binding site, or at the interface between subunits at a distinct microsite that does not preclude α-BgTx binding. In this manner, apoE peptides may interact with α7 nAChRs in a mode similar to the α-conotoxin ImII (Ellison et al., 2003). Together, the current data suggest that $apoE_{133-149}$ and $apoE_{141-148}$ act as non-competitive antagonists of α7 nAChRs.

To investigate the specificity of the apoE peptides' actions at various subtypes of nAChRs, the ability of the peptides to block α4β2 and α2β2 nAChRs in *Xenopus* oocytes was also tested. Both $apoE_{133-140}$ and $apoE_{141-148}$ (3 μM) had a significantly decreased ability to inhibit α4β2 and α2β2 peak ACh current responses, suggesting apoE peptides are somewhat selective for α7-containing nAChRs over non α7 receptors. Interestingly, compared to $apoE_{133-149}$, the shorter $apoE_{141-148}$ had a more pronounced selectivity for α7-containing over non α7-containing nAChRs. This suggests that there may be more than one mode of interaction for apoE peptides and nAChRs.

To further probe the amino acid requirements and sequence specificity of the apoE peptide necessary for inhibition of α7 nAChR responses, several peptides with

substitutions in particular residues were assessed. Scrambled (both random and non-random) and shorter ($apoE_{144-148}$) peptides had a significantly reduced ability to inhibit α7 nAChRs. However, the scrambled peptides retained minimal activity, denoting that while the presence of basic amino acids is not the defining characteristic for peptide activity, positive charges may contribute to the ability of apoE peptides to block α7 nAChR function. Substitutions in the apoE peptide sequence suggest that the arginines are critical for peptide blockade of the ACh peak response, while the lysines are not. In addition, the decreased rate of inhibition for the lysine to leucine substituted peptides again suggests the possibility of multiple modes of interaction between apoE peptides and α7 nAChRs. This may be a sign of multiple binding sites or peptide specific interactions with particular receptor residues at a single binding site. Over all, these data indicate that both the sequence and charge of amino acids in the peptide play a role in receptor blockade; however, the specific amino acid sequence is critical for complete inhibition of α7 mediated responses.

Similar to earlier work (Clay et al., 1995; Aono et al., 2003), CD measurements revealed that apoE peptides are capable of adopting an α-helical structure, with the longer $apoE_{133-149}$ peptide demonstrating a higher propensity for α-helical formation than the shorter $apoE_{141-148}$. However, the current data suggest that α-helicity is not required for blockade of nAChR function since the inactive arginine to leucine substitution displayed a higher % α-helicity than the maximally active $apoE_{141-148}$. It should be noted that all CD measurements were made in the presence of α-helical promoting TFE, and that the 2-dimensional structure of these peptides has yet to be determined under physiological conditions or upon binding to nAChRs.

There are an increasing number of reports of small brain-derived peptides interacting with neuronal nAChRs to modulate function. Similar to the data presented here, β-amyloid$_{1-42}$ has been shown to block α4β2 receptors in a non-competitive fashion and at higher concentrations to block α7 receptors (Wu et al., 2004). CGRP fragments have been shown to either inhibit or facilitate non α7-containing nAChR responses depending on the peptide (Di Angelantonio et al., 2002). Recently, a peptide derived from the c-terminal region of acetylcholinesterase (AChE) was demonstrated to modulate α7 but not α4β2 nAChRs expressed in oocytes. In contrast to the apoE peptides, this AChE peptide potentiated ACh responses at low concentrations (1 nM), while blocking ACh-mediated currents at higher concentrations (1 μM) (Greenfield et al., 2004). Together these findings suggest that the interaction between small peptides and nAChRs may be a unique way to modulate nAChR signaling in the brain. Specific peptide entities may be useful both as scientific tools as well as potential therapeutic agents. For instance, after insult, apoE4 has been demonstrated to decrease microglial activation less than apoE2 or E3. However, apoE peptides containing the LDL receptor binding region can suppresses microglial activation, potentially compensating for the apoE4 genotypic deficits (Laskowitz et al., 2001). Also, as mentioned above, the use of apoE-derived peptides may represent a novel therapeutic strategy (Lynch et al., 2005; McAdoo et al., 2005), and the current results may provide insight into the mechanisms underlying the potential therapeutic benefit of these peptides. In addition, protein fragments created *in vivo* may in part underlie the progressive pathology of multiple neurodegenerative processes, and use of peptides that mimic these fragments may help to elucidate the etiology of the disease. The data presented here demonstrates that apoE-derived peptides

disrupt nAChR signaling by directly inhibiting ion channel activation. The current findings may have considerable implications both in elucidating the mechanisms underlying the memory loss and cognitive decline seen in AD, as well as in the development of novel therapeutics through the use of apoE-derived peptides to regulate nAChR signaling.

Acknowledgements

We would like to thank Patricia Lamb for her assistance preparing the mRNA and oocytes, Robert Petrovich for his assistance with the circular dichroism spectroscopy, and Christian Erxleben and Joseph Lundquist for advice in preparing the manuscript.

References

Aono M, Bennett ER, Kim KS, Lynch JR, Myers J, Pearlstein RD, Warner DS and Laskowitz DT (2003) Protective effect of apolipoprotein E-mimetic peptides on N-methyl-D-aspartate excitotoxicity in primary rat neuronal-glial cell cultures. *Neuroscience* **116**:437-445.

Araujo DM, Lapchak PA, Collier B and Quirion R (1989) N-[3H]methylcarbamylcholine binding sites in the rat and human brain: relationship to functional nicotinic autoreceptors and alterations in Alzheimer's disease. *Prog Brain Res* **79**:345-352.

Bales KR, Dodart JC, DeMattos RB, Holtzman DM and Paul SM (2002) Apolipoprotein E, amyloid, and Alzheimer disease. *Mol Interv* **2**:363-375, 339.

Clay MA, Anantharamaiah GM, Mistry MJ, Balasubramaniam A and Harmony JA (1995) Localization of a domain in apolipoprotein E with both cytostatic and cytotoxic activity. *Biochemistry* **34**:11142-11151.

Colquhoun D and Ogden DC (1988) Activation of ion channels in the frog end-plate by high concentrations of acetylcholine. *J Physiol* **395**:131-159.

Corder EH, Saunders AM, Strittmatter WJ, Schmechel DE, Gaskell PC, Small GW, Roses AD, Haines JL and Pericak-Vance MA (1993) Gene dose of apolipoprotein E type 4 allele and the risk of Alzheimer's disease in late onset families. *Science* **261**:921-923.

Croy JE, Brandon T and Komives EA (2004) Two apolipoprotein E mimetic peptides, ApoE(130-149) and ApoE(141-155)2, bind to LRP1. *Biochemistry* **43**:7328-7335.

Crutcher KA, Clay MA, Scott SA, Tian X, Tolar M and Harmony JA (1994) Neurite degeneration elicited by apolipoprotein E peptides. *Exp Neurol* **130**:120-126.

Davies P and Maloney AJ (1976) Selective loss of central cholinergic neurons in Alzheimer's disease. *Lancet* **2**:1403.

Di Angelantonio S, Costa V, Carloni P, Messori L and Nistri A (2002) A novel class of peptides with facilitating action on neuronal nicotinic receptors of rat chromaffin cells in vitro: functional and molecular dynamics studies. *Mol Pharmacol* **61**:43-54.

Doraiswamy PM (2003) The role of the N-methyl-D-aspartate receptor in Alzheimer's disease: therapeutic potential. *Curr Neurol Neurosci Rep* **3**:373-378.

Ellison M, McIntosh JM and Olivera BM (2003) Alpha-conotoxins ImI and ImII. Similar alpha 7 nicotinic receptor antagonists act at different sites. *J Biol Chem* **278**:757-764.

Friedman G, Froom P, Sazbon L, Grinblatt I, Shochina M, Tsenter J, Babaey S, Yehuda B and Groswasser Z (1999) Apolipoprotein E-epsilon4 genotype predicts a poor outcome in survivors of traumatic brain injury. *Neurology* **52**:244-248.

Greenfield SA, Day T, Mann EO and Bermudez I (2004) A novel peptide modulates alpha7 nicotinic receptor responses: implications for a possible trophic-toxic mechanism within the brain. *J Neurochem* **90**:325-331.

Harris FM, Brecht WJ, Xu Q, Tesseur I, Kekonius L, Wyss-Coray T, Fish JD, Masliah E, Hopkins PC, Scearce-Levie K, Weisgraber KH, Mucke L, Mahley RW and Huang Y (2003) Carboxyl-terminal-truncated apolipoprotein E4 causes Alzheimer's disease-like neurodegeneration and behavioral deficits in transgenic mice. *Proc Natl Acad Sci U S A* **100**:10966-10971.

Howieson DB, Camicioli R, Quinn J, Silbert LC, Care B, Moore MM, Dame A, Sexton G and Kaye JA (2003) Natural history of cognitive decline in the old. *Neurology* **60**:1489-1494.

Jones GM, Sahakian BJ, Levy R, Warburton DM and Gray JA (1992) Effects of acute subcutaneous nicotine on attention, information processing and short-term memory in Alzheimer's disease. *Psychopharmacology (Berl)* **108**:485-494.

Jones S, Sudweeks S and Yakel JL (1999) Nicotinic receptors in the brain: correlating physiology with function. *Trends Neurosci* **22**:555-561.

Kenakin T (2004) *A pharmacology primer: theory, application, and methods*. Elsevier Academic Press, San Diego.

Klein RC and Yakel JL (2004) Inhibition of nicotinic acetylcholine receptors by apolipoprotein E-derived peptides in rat hippocampal slices. *Neuroscience* **127**:563-567.

Laskowitz DT, Thekdi AD, Thekdi SD, Han SK, Myers JK, Pizzo SV and Bennett ER (2001) Downregulation of microglial activation by apolipoprotein E and apoE-mimetic peptides. *Exp Neurol* **167**:74-85.

Levin ED (2002) Nicotinic receptor subtypes and cognitive function. *Journal of Neurobiology* **53**:633-640.

Lleo A, Greenberg SM and Growdon JH (2005) Current Pharmacotherapy for Alzheimer's Disease. *Annu Rev Med*.

Lynch JR, Wang H, Mace B, Leinenweber S, Warner DS, Bennett ER, Vitek MP, McKenna S and Laskowitz DT (2005) A novel therapeutic derived from

apolipoprotein E reduces brain inflammation and improves outcome after closed head injury. *Exp Neurol* **192**:109-116.

Maconochie DJ and Knight DE (1992) Markov modelling of ensemble current relaxations: bovine adrenal nicotinic receptor currents analysed. *J Physiol* **454**:155-182.

Marques MA and Crutcher KA (2003) Apolipoprotein E-related neurotoxicity as a therapeutic target for Alzheimer's disease. *J Mol Neurosci* **20**:327-337.

Marques MA, Tolar M, Harmony JA and Crutcher KA (1996) A thrombin cleavage fragment of apolipoprotein E exhibits isoform-specific neurotoxicity. *Neuroreport* **7**:2529-2532.

McAdoo JD, Warner DS, Goldberg RN, Vitek MP, Pearlstein R and Laskowitz DT (2005) Intrathecal administration of a novel apoE-derived therapeutic peptide improves outcome following perinatal hypoxic-ischemic injury. *Neurosci Lett* **381**:305-308.

Michikawa M and Yanagisawa K (1998) Apolipoprotein E4 induces neuronal cell death under conditions of suppressed de novo cholesterol synthesis. *J Neurosci Res* **54**:58-67.

Picciotto MR and Zoli M (2002) Nicotinic receptors in aging and dementia. *Journal of Neurobiology* **53**:641-655.

Pitas RE, Boyles JK, Lee SH, Foss D and Mahley RW (1987) Astrocytes synthesize apolipoprotein E and metabolize apolipoprotein E-containing lipoproteins. *Biochim Biophys Acta* **917**:148-161.

Qiu Z, Crutcher KA, Hyman BT and Rebeck GW (2003) ApoE isoforms affect neuronal N-methyl-D-aspartate calcium responses and toxicity via receptor-mediated processes. *Neuroscience* **122**:291-303.

Raggenbass M and Bertrand D (2002) Nicotinic receptors in circuit excitability and epilepsy. *J Neurobiol* **53**:580-589.

Rebeck GW, Reiter JS, Strickland DK and Hyman BT (1993) Apolipoprotein E in sporadic Alzheimer's disease: allelic variation and receptor interactions. *Neuron* **11**:575-580.

Tang G, Xie H, Xu L, Hao Y, Lin D and Ren D (2002) Genetic study of apolipoprotein E gene, alpha-1 antichymotrypsin gene in sporadic Parkinson disease. *Am J Med Genet* **114**:446-449.

Tolar M, Keller JN, Chan S, Mattson MP, Marques MA and Crutcher KA (1999) Truncated apolipoprotein E (ApoE) causes increased intracellular calcium and may mediate ApoE neurotoxicity. *J Neurosci* **19**:7100-7110.

Tolar M, Marques MA, Harmony JA and Crutcher KA (1997) Neurotoxicity of the 22 kDa thrombin-cleavage fragment of apolipoprotein E and related synthetic peptides is receptor-mediated. *J Neurosci* **17**:5678-5686.

Weisgraber KH, Innerarity TL and Mahley RW (1982) Abnormal lipoprotein receptor-binding activity of the human E apoprotein due to cysteine-arginine interchange at a single site. *J Biol Chem* **257**:2518-2521.

White HK and Levin ED (1999) Four-week nicotine skin patch treatment effects on cognitive performance in Alzheimer's disease. *Psychopharmacology (Berl)* **143**:158-165.

Wu J, Kuo YP, George AA, Xu L, Hu J and Lukas RJ (2004) beta-Amyloid directly inhibits human alpha4beta2-nicotinic acetylcholine receptors heterologously expressed in human SH-EP1 cells. *J Biol Chem* **279**:37842-37851.

Footnotes

[†]This research was supported by the Intramural Research Program of the NIH, National Institute of Environmental Health Sciences.

Reprints**:** Jerrel L. Yakel, NIEHS, F2-08, P.O. Box 12233, 111 T.W. Alexander Drive, Research Triangle Park, N.C. 27709; E-Mail: yakel@niehs.nih.gov

Figure Legends

Figure 1. Homomeric α7 nAChRs expressed in *Xenopus* oocytes are inhibited by apoE-derived peptides. The α7 nAChR-mediated responses were elicited by application of 2 mM ACh for 200-250 ms (bar) at 2 min intervals. Representative traces (left) for ACh-evoked current responses before and during bath application of peptide are illustrated, with timecourse of effects on the right. (a) $ApoE_{133-149}$ and (b) $apoE_{141-148}$ produced marked inhibition, whereas $apoE_{133-140}$ had minimal effect (c). Peak ACh current responses returned with washout of the peptide.

Figure 2. ApoE peptides inhibit nAChR responses in a dose-dependent manner. Plot of % inhibition of ACh-evoked peak current responses versus increasing concentrations of $apoE_{133-149}$ (■) and $apoE_{141-148}$ (●) yielded IC_{50} values of 445 nM (95%CI: 349 nM – 566 nM) and 1.30 μM, (95%CI: 1.07 μM – 1.56 μM), respectively. Responses were elicited as described in Figure1. Data are mean ± S.E.M. of 5-12 oocytes per data point for $apoE_{133-149}$ and 3-15 oocytes for $apoE_{141-148}$.

Figure 3. ApoE peptides inhibit α7 nAChR responses in a non-competitive manner. (a) Representative traces of $apoE_{133-149}$ inhibition of nAChR responses for varying concentrations of ACh (bar; 200 ms application pulses). (b) Dose-response curves for ACh peak current responses for α7 nAChRs were generated in the presence and absence of apoE peptides (0.3 μM). In the presence of $apoE_{133-149}$ (●) and $apoE_{141-148}$ (▲), ACh displayed similar EC_{50} values as control (■), suggesting a non-competitive interaction. Data were normalized to the peak current response at 1 mM ACh control.

Figure 4. ApoE peptides do not block α-bungarotoxin inhibition of α7 nAChR responses. Timecourse of percent peak ACh current before, during and following application of either (a) α-BgTx (10 nM) alone, or (b) MLA (10 nM), (c) $apoE_{133-149}$ (10 μM), and (d) $apoE_{141-148}$ (10 μM) each followed by co-application with α-BgTx. MLA prevents functional block of nAChRs by subsequent α-BgTx exposure. Neither $apoE_{133-149}$ nor $apoE_{141-148}$ competes for α-BgTx binding sites as demonstrated by the inability for peak responses to recover during washout. Data are the mean ± S.E.M. of 3-4 oocytes per point.

Figure 5. ApoE-derived peptides are less potent blockers of heteromeric α4β2 and α2β2 nAChRs expressed in *Xenopus* oocytes. The α4β2 and α2β2 nAChR-mediated responses were elicited by application of 1 mM ACh for 0.5s (bar) at 2 - 3 min. intervals. Representative traces for ACh-evoked responses for α4β2 (a) and α2β2 (b) nAChRs before and during bath application of 3 μM $apoE_{133-149}$ (left) and 3 μM $apoE_{141-148}$ (right) are illustrated. (c) 3 μM $apoE_{133-149}$ inhibited α7 ACh responses by 91 ± 3%, α4β2 ACh responses by 43 ± 6%, and α2β2 ACh responses by 71 ± 4% of control values. 3 μM $apoE_{141-148}$ inhibited α7 ACh responses by 81 ± 2%, α4β2 ACh responses by 23 ± 6%, and α2β2 ACh responses by 8 ± 4% of control values. Data are mean ± S.E.M. of at least 6 oocytes for each receptor subtype (* = $p<0.05$, compared to α7 nAChRs).

Figure 6. Effect of apoE peptide amino acid substitutions and sequence on inhibition of α7 nAChRs. $ApoE_{133-149}$ and the c-term portion of the peptide ($apoE_{141-148}$)

dramatically inhibited ACh-evoked responses, while the n-terminal portion of the peptide ($apoE_{133-140}$) was almost completely inactive. A shorter 5 amino acid peptide ($apoE_{144-148}$) was unable to significantly inhibit α7 nAChR mediated responses. Both random and non-random scrambled peptides had a significantly reduced ability to block α7 nAChR function. Pentalysine was unable to inhibit α7 nAChR-mediated responses. The introduction of glutamate residues significantly decreased peptide activity, while replacing arginine with leucine residues also dramatically reduced the ability of the apoE peptide to inhibit α7 nAChRs. (* = $p<0.05$, compared to both $apoE_{133-140}$ and $apoE_{141-148}$). All peptide effects are shown at 3 μM.

Figure 7. Effect of apoE peptide lysine substitutions on inhibition of α7 nAChRs. Substitution of the lysines to leucines (at positions 143 and 146, designated 2K/2L) did not reduce the ability of either $apoE_{133-140}$2K/2L or $apoE_{144-148}$2K/2L to reduce peak ACh responses, however the rate of block and recovery was dramatically slowed compared to control. (a) Representative traces of ACh-evoked responses before and during bath application of $apoE_{133-149}$2K/2L (3 μM ; bar = 200ms). (b) The rate of block by both peptides was noticeably decreased. Peak ACh current responses returned with washout of the peptides.

JPET Fast Forward. Published on October 25, 2005 as DOI: 10.1124/jpet.105.095505
This article has not been copyedited and formatted. The final version may differ from this version.

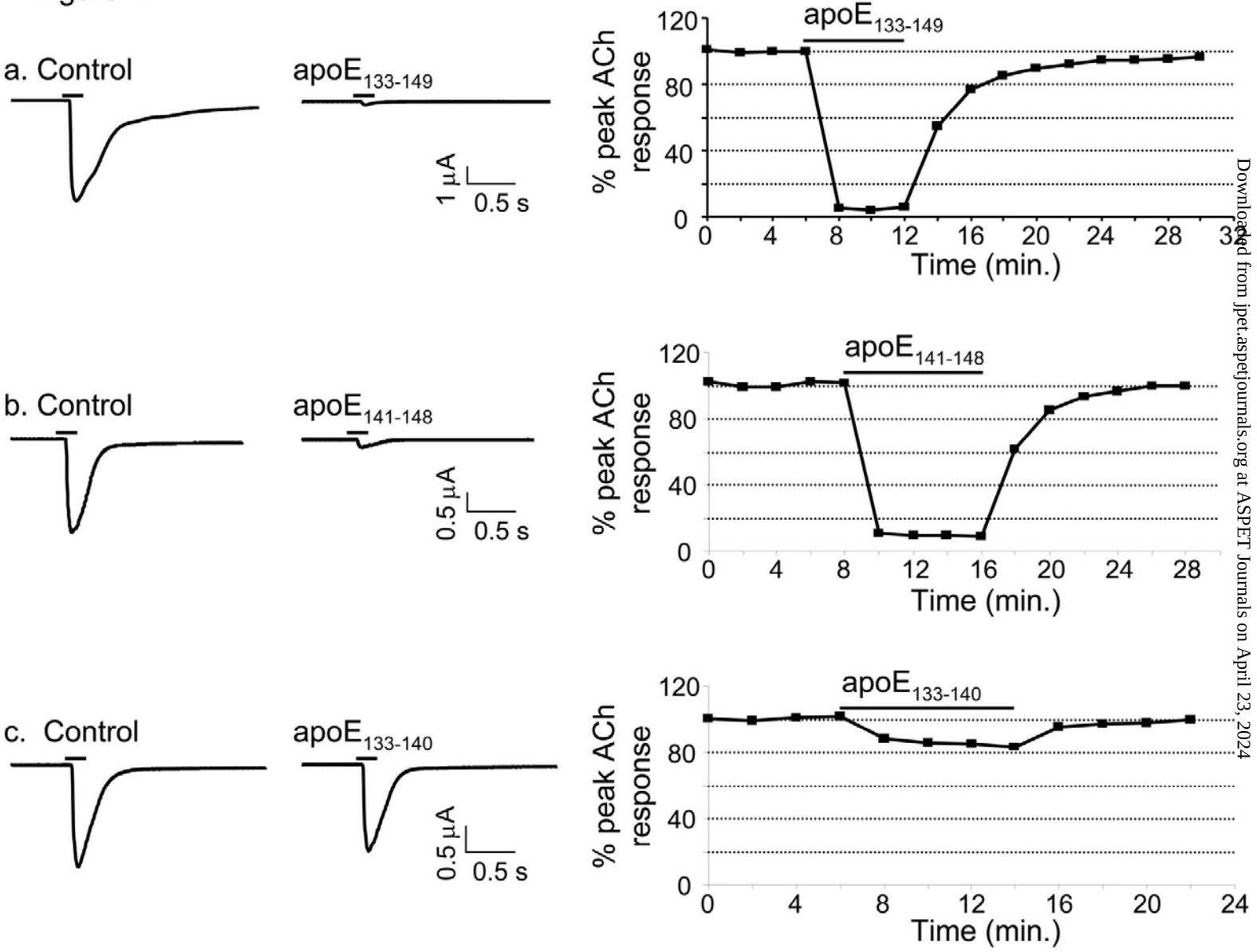
Table I. Efficacy, potency and helicity of distinct apoE peptides for a7 nAChR inhibition

Peptide	Maximal Inhibition (3 μM) [a]	IC_{50} (μM)	% Helicity [b]
$ApoE_{133-149}$	91 ± 3	.45 (.35-.57)	46.5
$ApoE_{133-140}$	16 ± 3		2.6
$ApoE_{141-148}$	85 ± 1	1.3 (1.1-1.6)	12.8
$ApoE_{144-148}$	25 ± 4		
RLKKLRLR	35 ± 4		4.5
KKLLLRRR	23 ± 4		6.5
KKKKK	10 ± 2		
LRVRLASH-LRLLRLRLL	93 ± 7	.62 (.38-1.0)	N.D. [c]
LRLLRLRL	74 ± 8	2.2 (.44-10)	N.D.
LRELRERL	2 ± 1		37.4
LLKLRKLL	7 ± 2		N.D.

[a]Data represent the mean ± S.E.M. of 5-15 oocytes per peptide. IC_{50} values are presented with 95% confidence intervals. [b]Percent helicity was measured in the presence of 40 % TFE (see methods). [c]N.D.: not determined due to suspension in DMSO.

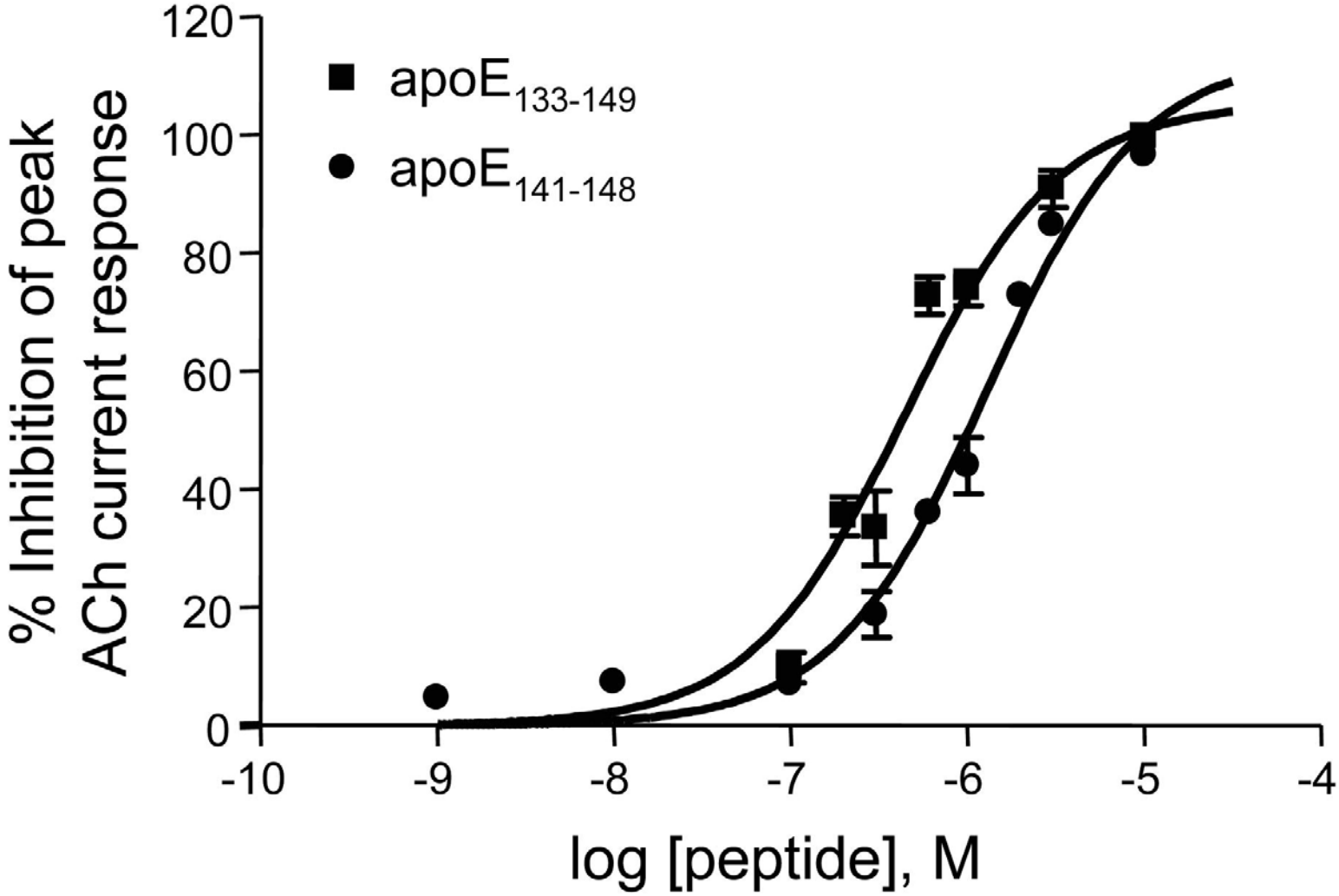
JPET Fast Forward. Published on October 25, 2005 as DOI: 10.1124/jpet.105.095505
This article has not been copyedited and formatted. The final version may differ from this version.

Figure 1



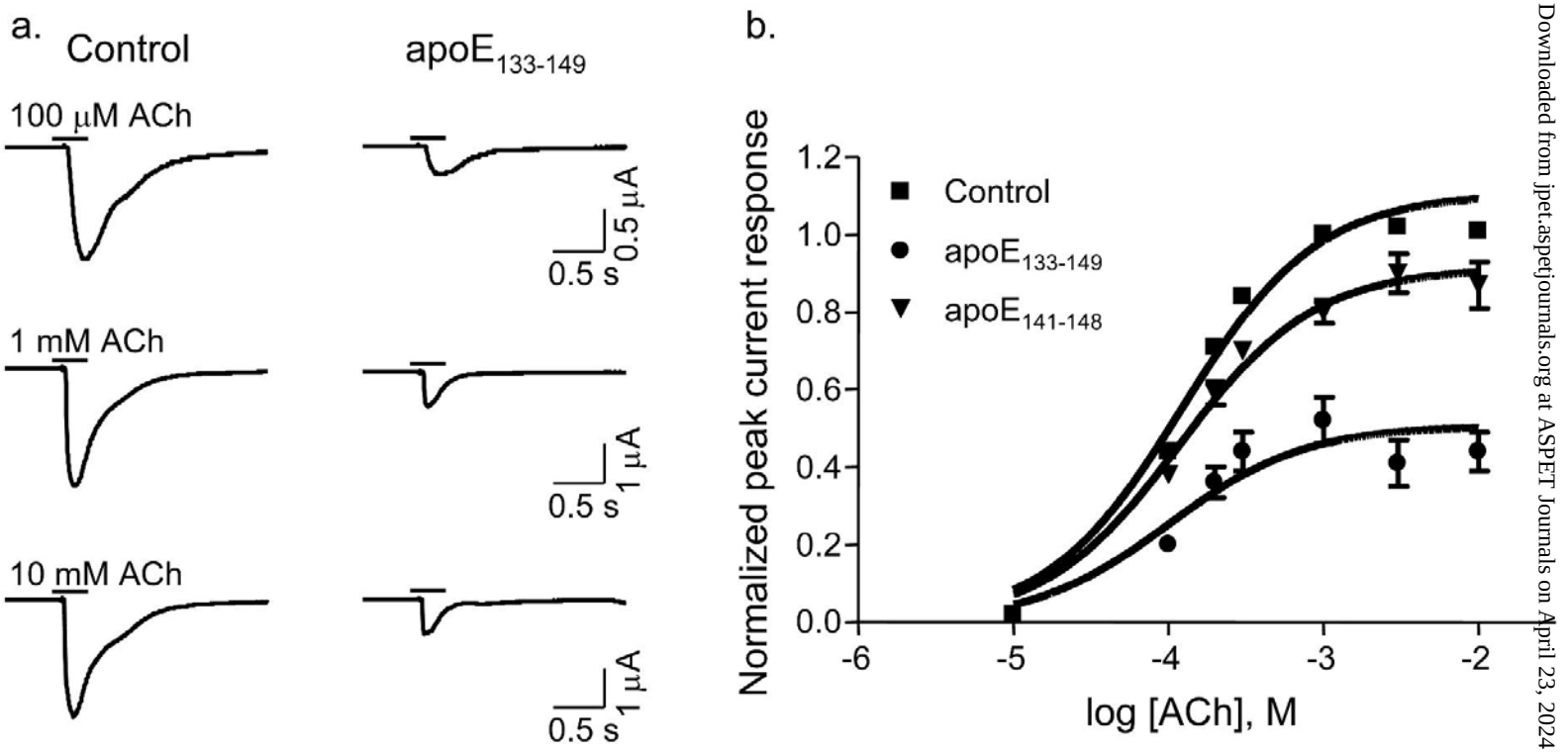
JPET Fast Forward. Published on October 25, 2005 as DOI: 10.1124/jpet.105.095505
This article has not been copyedited and formatted. The final version may differ from this version.

Figure 2



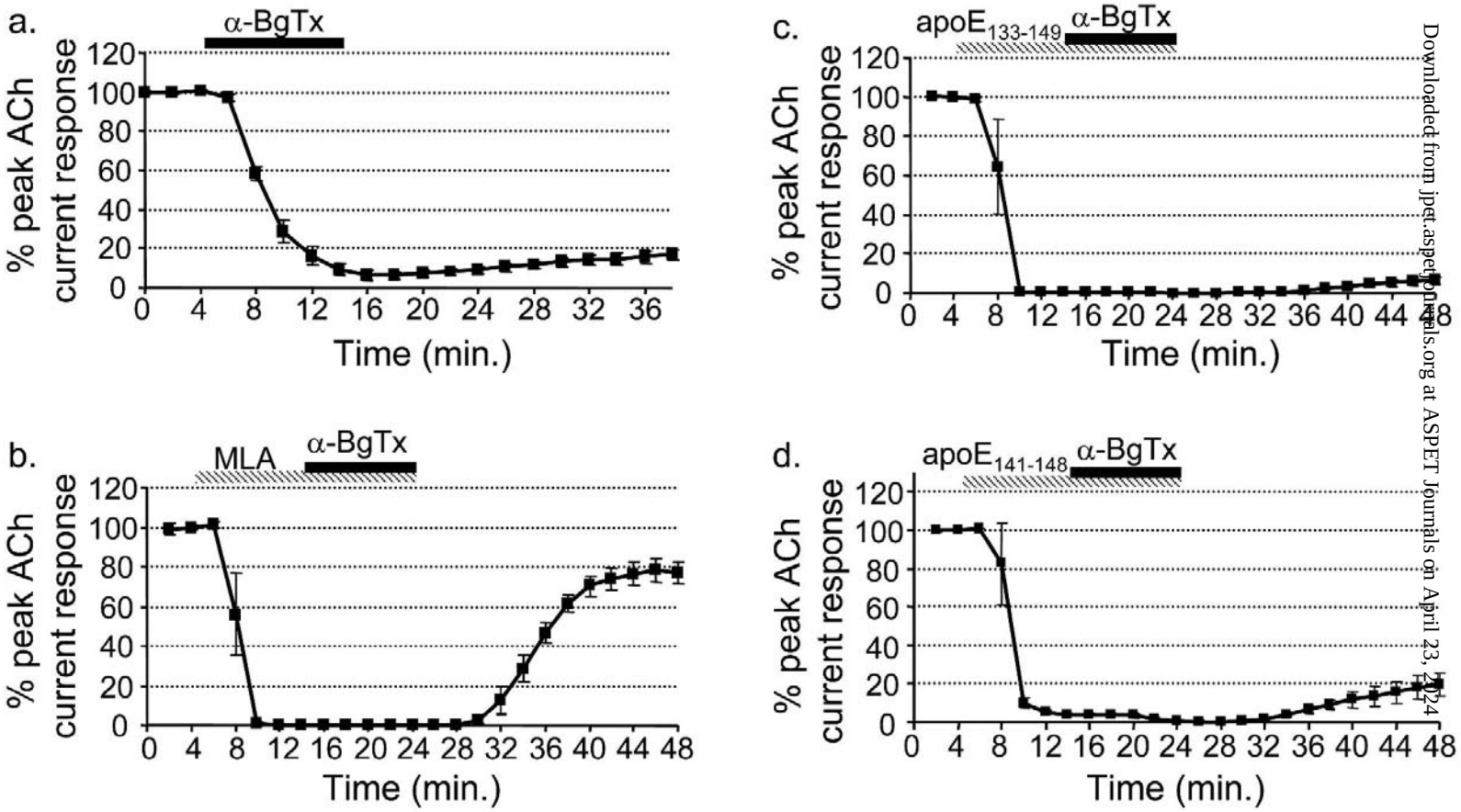
JPET Fast Forward. Published on October 25, 2005 as DOI: 10.1124/jpet.105.095505
This article has not been copyedited and formatted. The final version may differ from this version.

Figure 3



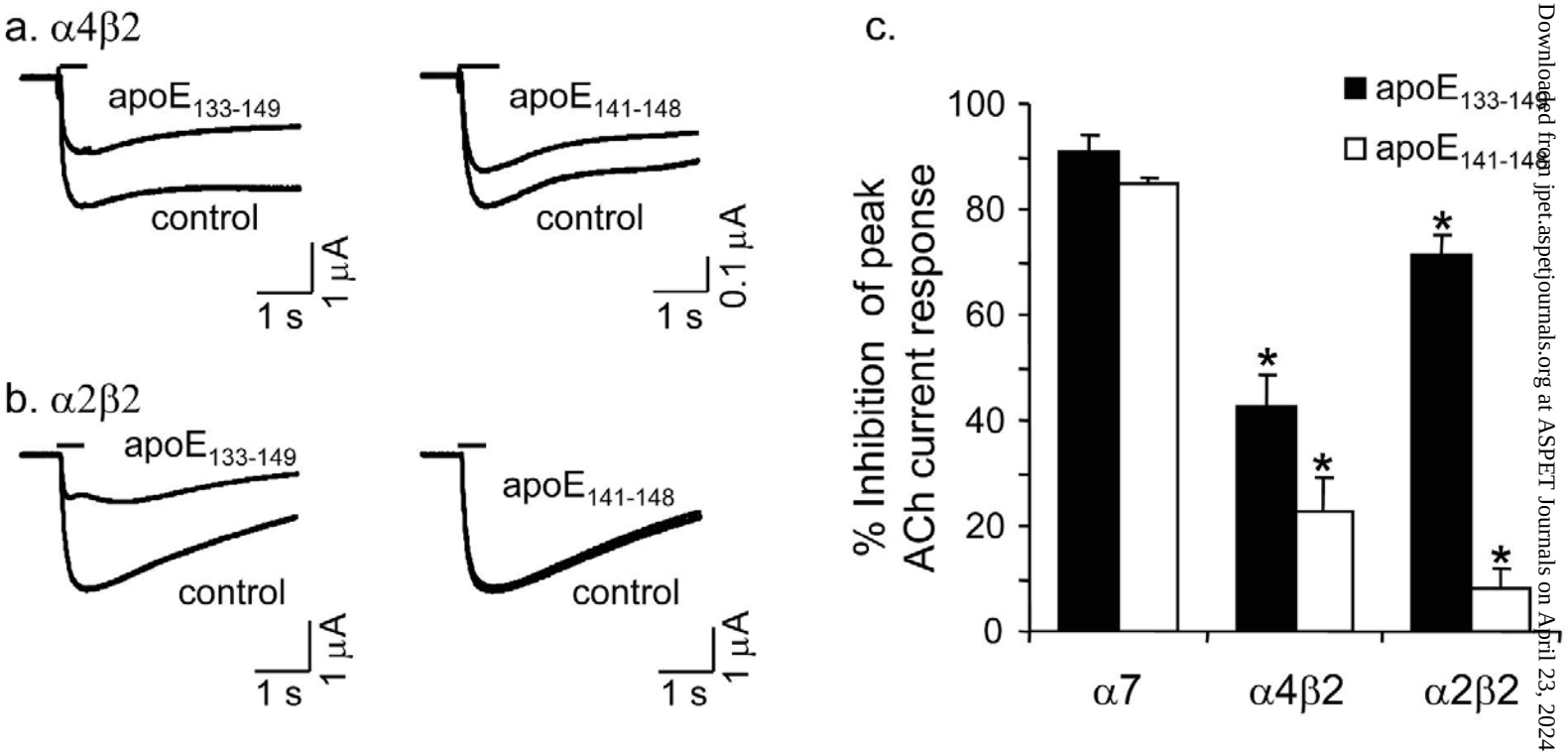
JPET Fast Forward. Published on October 25, 2005 as DOI: 10.1124/jpet.105.095505
This article has not been copyedited and formatted. The final version may differ from this version.

Figure 4



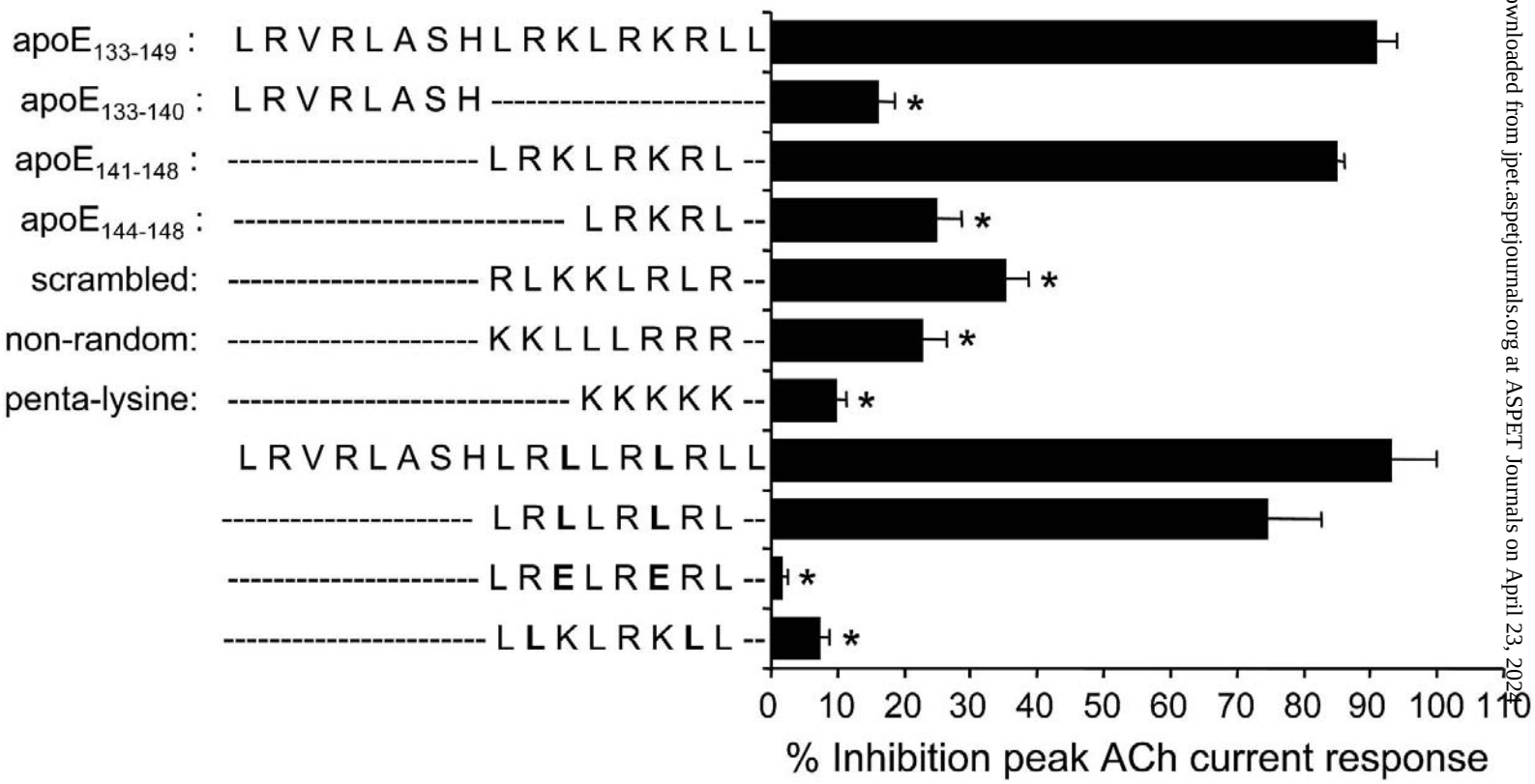
JPET Fast Forward. Published on October 25, 2005 as DOI: 10.1124/jpet.105.095505
This article has not been copyedited and formatted. The final version may differ from this version.

Figure 5



JPET Fast Forward. Published on October 25, 2005 as DOI: 10.1124/jpet.105.095505
This article has not been copyedited and formatted. The final version may differ from this version.

Figure 6



JPET Fast Forward. Published on October 25, 2005 as DOI: 10.1124/jpet.105.095505
This article has not been copyedited and formatted. The final version may differ from this version.

Figure 7