Cellular/Molecular

β-Amyloid Regulation of Presynaptic Nicotinic Receptors in Rat Hippocampus and Neocortex

John J. Dougherty, Jianlin Wu, and Robert A. Nichols
Department of Pharmacology and Physiology, Drexel University College of Medicine, Philadelphia, Pennsylvania 19102

Alteration by β-amyloid (Aβ) of signaling via nicotinic acetylcholine receptors (nAChRs) has been implicated in the early stages of Alzheimer's disease. nAChRs function both post- and presynaptically in the nervous system; however, little is known about the functional consequence of the interaction of Aβ with these receptors, particularly those on presynaptic nerve terminals. In view of the strong correlation between loss of synaptic terminals and dementia, together with the reduction in nAChRs in Alzheimer's disease, the possibility exists that presynaptic nAChRs may be targets for Aβ. To explore this possibility, we assessed the effect of Aβ peptides on nicotine-evoked changes in presynaptic Ca^{2+} level via confocal imaging of isolated presynaptic nerve endings from rat hippocampus and neocortex. $A\beta_{1-42}$ appeared to inhibit presynaptic nAChR activation by nicotine. Surprisingly, picomolar $A\beta_{1-42}$ was found to directly evoke sustained increases in presynaptic Ca^{2+} via nAChRs, revealing that the apparent inhibitory action of $A\beta_{1-42}$ was the result of an occlusion of nicotine to further stimulate the receptors. The direct effect of Aβ was found to be sensitive to α-bungarotoxin, mecamylamine, and dihydro-β-erythroidine, indicating involvement of α7-containing nAChRs and non-α7-containing nAChRs. Prior depolarization strongly attenuated subsequent Aβ-evoked responses in a manner dependent on the amplitude of the initial presynaptic Ca^{2+} increase, suggesting that nerve activity or Ca^{2+} channel density may control the impact of Aβ on presynaptic nerve terminal function. Together, these results suggest that the sustained increases in presynaptic Ca^{2+} evoked by Aβ may underlie disruptions in neuronal signaling via nAChRs in the early stages of Alzheimer's disease.

Key words: **nicotinic receptor; amyloid; presynaptic; hippocampus; calcium imaging; Alzheimer's disease**

Introduction

One prominent feature of Alzheimer's disease is the presence of neuritic plaques containing β-amyloid (Aβ) peptides. Aβ peptides (39–43 amino acids in length) are generated by proteolytic cleavage of the Aβ precursor protein, a transmembrane glycoprotein present in multiple isoforms (Selkoe, 1998; Walter et al., 2001). The dominant peptide fragment present within the neuritic plaques, as insoluble fibrils, is the 42-residue species $A\beta_{1-42}$ (Iwatsubo et al., 1994; Gravina et al., 1995; Selkoe, 1998). The Aβ fibrils most likely result from self-aggregation of the $A\beta_{1-42}$ (Teplow, 1998; Huang et al., 2000). There is, however, only a weak correlation between fibrillar Aβ content and cognitive dysfunction (Lue et al., 1999; McLean et al., 1999). In contrast, the severity of dementia does correlate with the degree of loss of presynaptic terminals (Terry et al., 1991; Sze et al., 1997) as well as with the total load of soluble Aβ (Lue et al., 1999; McLean et al., 1999). Moreover, transgenic strains exist (e.g., Tg2576) wherein Aβ levels are elevated without plaque formation or nerve cell loss, yet learning and memory deficits are evident (Irizarry et al., 1997; Kotilinek et al., 2002; Westerman et al., 2002). Consequently, it has been hypothesized that Aβ may be largely acting in a soluble form (dimers and/or small oligomers, also referred to as ADDLs) (Garzon-Rodriguez et al., 1997) to disrupt neuronal signaling (Lambert et al., 1998; Klein et al., 2001), particularly at an early stage in Alzheimer's disease, with nicotinic acetylcholine receptors (nAChRs) as a major target (Auld et al., 1998). Other possible targets have been suggested, such as microglial scavenger receptors (El Khoury et al., 1998) and the receptor for advanced glycation end products (Lue et al., 2001).

Evidence for a strong interaction between Aβ and specific nAChRs, particularly α7 subunit-containing nAChRs, has accumulated (Dineley et al., 2001; Liu et al., 2001; Pettit et al., 2001; Wang et al., 2000a,b). Studies examining binding of $A\beta_{1-42}$ to nAChRs expressed on clonal cell lines indicated that Aβ has a picomolar affinity for α7-containing nAChRs (Wang et al., 2000a). In both rat hippocampal slices (Pettit et al., 2001) and cultured neurons (Liu et al., 2001), nanomolar Aβ was shown to inhibit nicotine-evoked currents, including α7-AChRs and non-α7-AChRs, in a reversible, apparently noncompetitive manner. In addition, acute treatment with Aβ was found to activate the MAP kinase cascade in mouse hippocampal slices via α7-nAChRs, whereas chronically elevated Aβ in a mouse model of Alzheimer's disease led to downregulation of MAP kinase with concomitant upregulation of α7-nAChRs in aged animals (Dineley et al., 2001). These latter data suggest that Aβ may first activate and then inhibit nAChRs, although no direct activation by Aβ of nAChRs was noted in any of the aforementioned reports using primary tissue.

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Correspondence should be addressed to Dr. Robert A. Nichols, Department of Pharmacology and Physiology, Drexel University College of Medicine (formerly MCP Hahnemann University), 245 North 15th Street, Philadelphia, PA 19102. E-mail: robert.nichols@drexel.edu.

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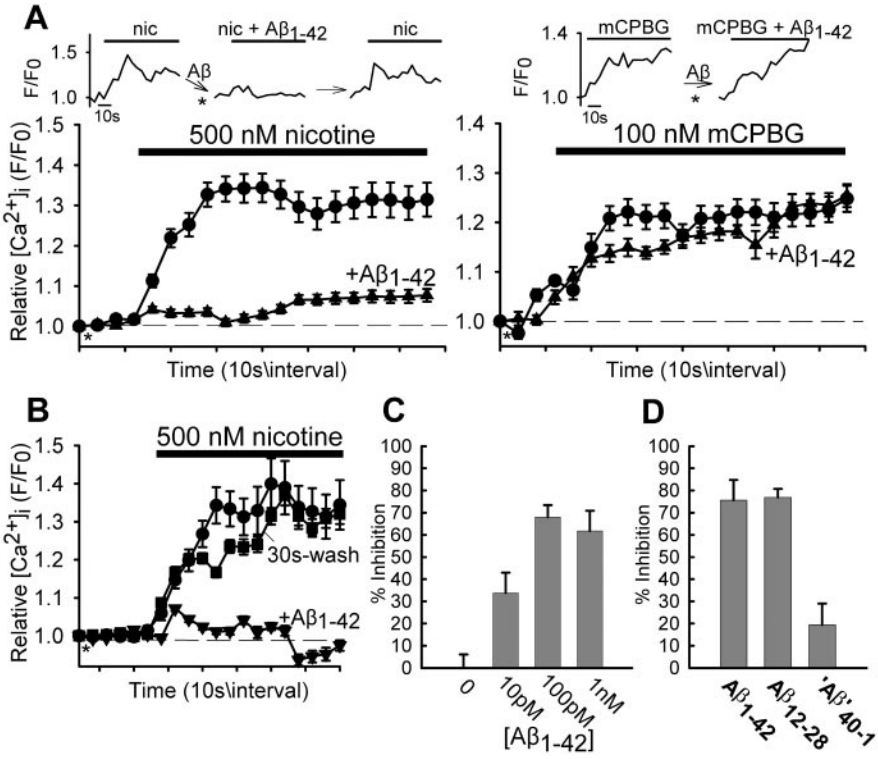

Figure 1. Inhibitory effect of Aβ on nicotine-evoked increases in Ca^{2+} level in individual isolated hippocampal nerve endings. *A*, Successive stimulation with 500 nM nicotine (left) or 100 nM of the 5-HT_3 agonist *m*-chlorophenyl biguanide (mCPBG; right) in the absence or presence of 100 nM $A\beta_{1-42}$ during an intervening 10-min wash period and the second stimulation. Top, Representative successive Ca^{2+} responses in individual synaptosomes. Bottom, Averaged responses expressed as means ± SEM before and after incubation of $A\beta_{1-42}$. Left graph, $n = 45$; Right graph, $n = 15$. Reversibility (*B*), concentration dependence (*C*), and peptide specificity (*D*) of the inhibitory effect of 100 nM $A\beta_{1-42}$ were also examined. In *D*, peptides were present at 100 nM. Note that for *A*, *B*, Ca^{2+} responses to the second stimulation were renormalized (*), although a maintained Ca^{2+} increase occurred with Aβ (Fig. 3). In *C*,*D*, data are expressed as percentage of control plateau values (% inhibition). *B*, $n = 13$; *C*: 0 pM, $n = 26$; 10 pM, $n = 8$; 100 pM, $n = 5$; 1 nM, $n = 8$; *D*: $A\beta_{1-42}$, $n = 73$; $A\beta_{12-28}$, $n = 15$; $'A\beta'_{40-1}$, $n = 6$.

To determine the consequence of Aβ action on presynaptic nicotinic receptors on nerve terminal signaling, we investigated the effects of Aβ peptides on nicotine-induced Ca^{2+} responses in individual isolated nerve terminals from rat hippocampus and neocortex.

Materials and Methods

Materials. Synthetic Aβ peptides were purchased from BACHEM (King of Prussia, PA). For all experiments, Aβ peptides were suspended in physiological saline at 100–1000× stock concentration, thoroughly bath sonicated, and then immediately diluted for use. Under these conditions, the final solutions (picomolar–nanomolar peptide) remain clear, with no visible particles when viewed by phase–contrast microscopy (Lorenzo and Yankner, 1994), for the course of an experiment (15–30 min). Fluo-3 and fluo-4 were obtained from Molecular Probes, Inc. (Eugene, OR) and are kept as 1 mM stock solutions in DMSO at −20°C. Cell-Tak was purchased from Collaborative Biomedical Products (Bedford, MA). Nicotine, *m*-chlorophenyl biguanide, α-bungarotoxin, mecamylamine, and dihydro-β-erythroidine were purchased from Sigma (St. Louis, MO). Conotoxins and agatoxin-TK were purchased from Alomone Labs (Jerusalem, Israel). All drugs and toxins were suspended in physiological saline just before use.

Synaptosome preparation. Hippocampi, striata, or cortices were dissected out of brains from adult male Sprague Dawley rats (Taconic Farms, Germantown, NY) and immediately placed in ice-cold 0.32 M sucrose, following a protocol approved by the Drexel University College of Medicine (formerly MCP Hahnemann University) Institutional Animal Care and Use Committeee. Tissue was then homogenized in 0.32 M sucrose using a glass Teflon tissue grinder. Synaptosomes were isolated according to the method described by Dunkley et al. (1986). The preparations were washed into oxygenated HEPES-buffered saline [HBS; composition (in mM): 142 NaCl, 2.4 KCl, 1.2 K_2PO_4, 1 $MgCl_2$, 5 D-glucose, and 10 HEPES, pH 7.4]. This procedure yields synaptosomes, ~90% of which have shown to be intact and functional, based on the stability and consistency of Ca^{2+} responses of dye-loaded synaptosomes to multiple rounds of stimulation as gauged using confocal imaging (Nayak et al., 2000, 2001).

Calcium imaging. Synaptosomes were loaded with fluorescent Ca^{2+} indicator dye (fluo-3 or fluo-4) at 5 μM by incubating with the acetoxymethyl ester derivative in HBS for 30–45 min at 37°C. Dye-loaded preparations were washed in HBS containing 1 mM $CaCl_2$ and then plated onto Cell-Tak-coated coverslips. Relative changes in internal Ca^{2+} in individual synaptosomes were assessed using confocal imaging (Rondé and Nichols, 1998; Nayak et al., 2001) via a Nikon PCM 2000 laser-scanning confocal imaging system connected to a Nikon Diaphot 300 microscope. In brief, the preparations on coverslips were mounted in a rapid-exchange Warner (36 μl volume) perfusion system attached to the microscope and subjected to perfusion with HBS containing Ca^{2+} at 3–5 ml/min. Imaging was commenced, and after obtaining a baseline series of five images, stimulatory agents were applied by rapid switching between manifolds on the perfusion system. Complete exchange of the perfusion chamber took place in <1 sec. Images were typically collected at 4-sec intervals, although in several experiments 15-sec intervals were used. A given experiment corresponded to a series of images captured from a single preparation. The fluorescent intensities associated with a given structure, determined from digitized images using OPTIMAS image analysis software (Optimas Co., Seattle, WA), were expressed as normalized values (F/F_0; where F_0 = fluorescence intensity at t_0). All time series were corrected for photobleaching.

Statistics. Data sets were compared using matched Student's *t* tests. Significance was indicated when *p* was minimally <0.05.

Results

Nicotine induces robust increases in Ca^{2+} level in a subpopulation (10–25%) of isolated nerve terminals (synaptosomes), which slowly decay (several minutes) depending on concentration and receptor subtype (Nayak et al., 2001). The nicotine-induced Ca^{2+} responses in striatal synaptosomes were found to be effectively independent of voltage-gated Ca^{2+} channels, indicating that the sustained changes in internal Ca^{2+} likely parallel, although certainly with some delay, the influx of Ca^{2+} via the nicotinic receptor channel. Nicotine-induced Ca^{2+} responses in hippocampal or cortical synaptosomes, in contrast, display significant dependence on voltage-gated Ca^{2+} channels, as gauged by sensitivity to Ca^{2+} channel toxins wherein responses were inhibited to 30–40% of controls, indicating a strong depolarizing component to the responses in the nerve terminals in these brain regions. Sustained elevation in internal Ca^{2+} seems to be a common feature underlying responses in nerve terminals to activation of presynaptic nicotinic receptors (McGehee et al., 1995; Gray et al., 1996; Coggan et al., 1997; Léna and Changeux, 1997; Mansvelder and McGehee, 2000; Kiyosawa et al., 2001; Díaz-

Hernández et al., 2002), as well as the closely related 5-HT_3 serotonin receptors when expressed on presynaptic nerve endings (Rondé and Nichols, 1998, 2001).

Treatment with $A\beta_{1-42}$ appeared to strongly inhibit nicotine-induced Ca^{2+} responses in individual hippocampal synaptosomes in a readily reversible manner (Fig. 1*A*, left), with the nicotine-induced Ca^{2+} responses recovering to a significant degree as soon as 30 sec after washing out the $A\beta_{1-42}$ (Fig. 1*B*). The action of $A\beta_{1-42}$ on nicotinic receptors present on the presynaptic terminals appears to be specific, because it had no significant effect on presynaptic 5-HT_3 serotonin receptor-induced Ca^{2+} responses in the same synaptosomes (Fig. 1*A*, right), as assessed using the highly selective 5-HT_3 receptor agonist *m*-chlorophenyl biguanide. The latter conclusion was made based on the finding that 5-HT_3 serotonin receptors colocalize with nicotinic receptors at presynaptic sites (Nayak et al., 2000). The lack of significant effect of $A\beta_{1-42}$ on 5-HT_3 receptors is also consistent with previous reports (Wang et al., 2000a; Liu et al., 2001). In addition, $A\beta_{1-42}$ has been shown to have no effect on glutamate receptors in a hippocampal preparation (Pettit et al., 2001). The inhibitory effect of $A\beta_{1-42}$ was concentration dependent, with low nanomolar levels producing near complete blockade of the nicotine-induced Ca^{2+} responses, whereas significant inhibition was evident down into the picomolar range (IC_{50}, ~10 pM; Fig. 1*C*). Finally, a control peptide having a reversed sequence (40–1; $'A\beta'_{40-1}$ used at 100 nM) from that of $A\beta_{1-42}$ had no significant effect on nicotine-induced Ca^{2+} responses in the hippocampal synaptosomes (Fig. 1*D*). In contrast, 100 nM $A\beta_{12-28}$ was nearly as effective as 100 nM $A\beta_{1-42}$ in inhibiting the nicotine-induced responses (Fig. 1*D*). $A\beta_{12-28}$ was previously shown to interact strongly with α7-nAChRs (Wang et al., 2000b), suggesting that it may contain the nicotinic receptor-binding motif of the Aβ peptides. $A\beta_{12-28}$ was also shown to block nicotine-induced currents in hippocampal neurons, as, if not more, effectively as $A\beta_{1-42}$ (Pettit et al., 2001). These results indicate that presynaptic nicotinic receptors are selectively inhibited by soluble Aβ. However, because each Ca^{2+} response is normalized to baseline, the question arises as to whether Aβ has a direct effect on presynaptic Ca^{2+} levels, resulting in an occlusion of the nicotine-induced Ca^{2+} responses, in contrast to direct inhibition of the presynaptic nicotinic receptor. As described later, the inhibitory effect of Aβ was indeed because of an occlusion, i.e., full activation of the nAChRs, preventing further activation by nicotine.

Nicotine-induced Ca^{2+} responses in a subset of hippocampal synaptosomes (12 ± 6% SD) seem to involve both α7-containing nAChRs and non-α7-containing nAChRs (Fig. 2*A*), as gauged by sensitivity of the responses to various nicotinic receptor antagonists (α7, α-bungarotoxin; non-α7, dihydro-β-erythroidine or mecamylamine), consistent with studies of nicotine-induced neurotransmitter release (Clarke and Reuben, 1996; Gray et al., 1996; Radcliffe et al., 1999; Fabian-Fine et al., 2001; Kulak et al., 2001). The profiles of sensitivity of presynaptic nicotine-induced Ca^{2+} responses were similar between hippocampal and cortical synaptosomes (data not shown). Interestingly, the apparent inhibitory effect of $A\beta_{1-42}$ was more pronounced with hippocampal and cortical synaptosomes as compared with striatal synaptosomes (Fig. 2*B*). Although $A\beta_{1-42}$ has been shown to inhibit non-α7-containing nAChRs (Pettit et al., 2001), it displays the highest affinity for α7-containing nAChRs (Wang et al., 2000b), and α7-containing nAChRs appear to be a minor presence, at best, on striatal nerve terminals (Nayak et al., 2000; cf. Marchi et al., 2002), perhaps explaining the much smaller effect of $A\beta_{1-42}$ on striatal synaptosomes, particularly when used at 100 pM. Evidence for involvement of α7-containing nAChRs on hippocampal synaptosomes is indicated by α-bungarotoxin sensitivity of direct effects of $A\beta_{1-42}$ to increase synaptosomal Ca^{2+} (Fig. 5).

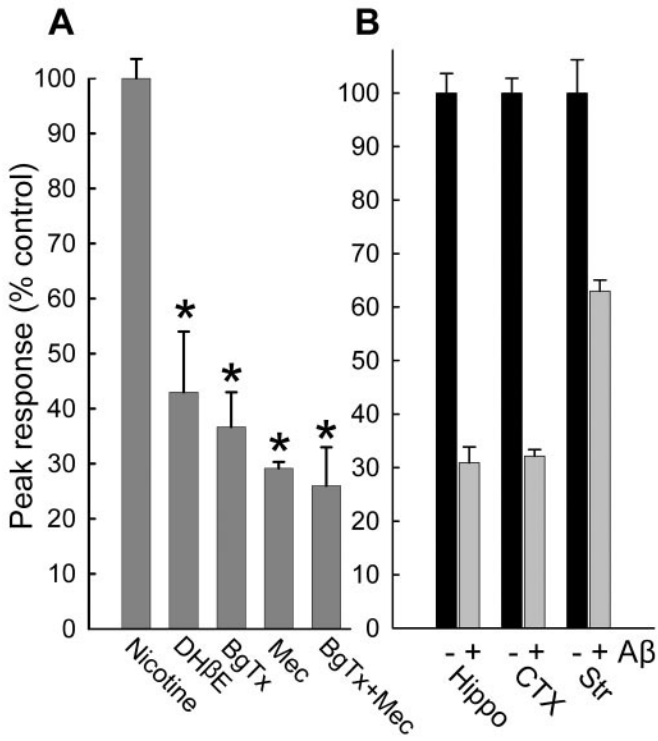

Figure 2. Sensitivity of nicotine-evoked presynaptic Ca^{2+} increases to various nicotinic antagonists and to Aβ for preparations from various regions of the brain. *A*, Average maximal Ca^{2+} responses to 500 nM nicotine in hippocampal synaptosomes in the absence ($n = 34$) or presence of 5 μM dihydro-β-erythroidine (DHβE; $n = 26$), 500 nM α-bungarotoxin (BgTx; $n = 9$), 10 μM mecamylamine (Mec; $n = 18$), or BgTx plus Mec ($n = 11$) during the second stimulation, using the successive stimulation protocol described in the legend to Figure 1. *B*, Inhibitory effects of 100 nM $A\beta_{1-42}$ on nicotine-evoked Ca^{2+} responses in synaptosomes from hippocampus (Hippo; $n = 17$), cortex (CTX; $n = 13$), or striatum (Str; $n = 10$). Qualitatively similar results were obtained using 100 pM $A\beta_{1-42}$. $^*p < 0.05$; *t* test with paired control.

$A\beta_{1-42}$ was found to induce directly substantial increases in Ca^{2+} level (Fig. 3) in a subset of hippocampal synaptosomes (17 ± 10% SD), with sustained elevation in Ca^{2+} level evident for over 10 min of incubation (Fig. 3*A*). $A\beta_{1-42}$-induced synaptosomal Ca^{2+} responses were comparable in number and average magnitude to those evoked by nicotine stimulation. The direct effect of $A\beta_{1-42}$ was concentration dependent, with measurable responses in the low picomolar range (Fig. 3*C*). Similar findings were obtained using cortical synaptosomes; however, the increases in Ca^{2+} level in response to 100 pM $A\beta_{1-42}$ (F/F_0, 1.13 ± 0.03 SEM; $n = 23$) were substantially lower than those seen in response to 100 nM $A\beta_{1-42}$ (F/F_0, 1.45 ± 0.03 SEM; $n = 30$), in contrast to what was observed for $A\beta_{1-42}$-evoked responses in hippocampal synaptosomes at these same concentrations, indicating a significantly lower potency of Aβ for cortical presynaptic terminals. These direct effects of Aβ were unaltered by filtration of the $A\beta_{1-42}$-containing HBS perfusion solution (Fig. 3*E*), indicating that the Aβ was not acting via an aggregated form. $A\beta_{12-28}$ was also able to induce directly increases in synaptosomal Ca^{2+} (Fig. 3*B*). Interestingly, synaptosomal Ca^{2+} responses to picomolar $A\beta_{1-42}$ were dependent on external Ca^{2+}, whereas they were only partially dependent on external Ca^{2+} for concentrations of $A\beta_{1-42}$ in the high nanomolar to micromolar range (Fig. 4*A*). However, Zn^{2+} (50 μM), known to block alleged Aβ chan-

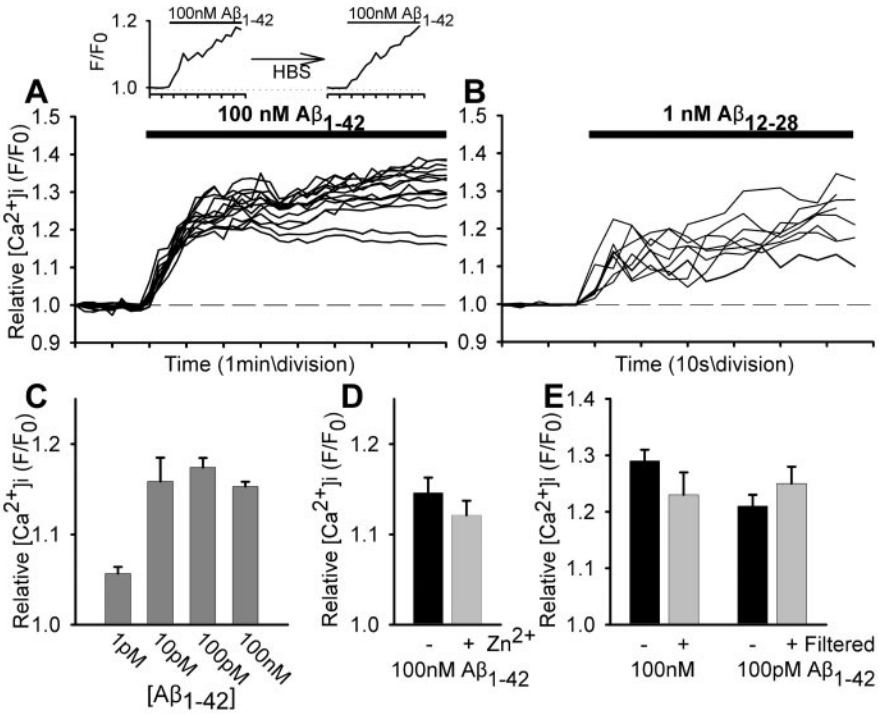

Figure 3. Aβ-evoked Ca^{2+} increases in individual hippocampal nerve endings. Composites of Ca^{2+} responses in individual synaptosomes to $A\beta_{1-42}$ (*A*) and $A\beta_{12-28}$ (*B*) over an extended time course. Top, Representative initial phases of successive Ca^{2+} responses in an individual synaptosome to 100 nM $A\beta_{1-42}$. Data sampling were 4-sec intervals. *C*, Concentration dependence: 1 pM, $n = 26$; 10 pM, $n = 20$; 100 pM, $n = 116$; 100 nM, $n = 175$. *D*, Insensitivity to prior treatment with 50 μM $ZnCl_2$ ($n = 15$). *E*, Average maximal Ca^{2+} responses to 100 nM $A\beta_{1-42}$ ($n = 11$) or 100 pM $A\beta_{1-42}$ ($n = 14$) before or after filtration of the Aβ solutions through 0.2 μm filters.

nels that form at relatively high (micromolar) concentrations of $A\beta_{1-42}$ (Lin et al., 2001), had no effect on synaptosomal Ca^{2+} responses to $A\beta_{1-42}$ at any concentration (Fig. 3*D*). Moreover, the stimulatory effect of $A\beta_{1-42}$ was partially dependent on voltage-gated Ca^{2+} channels at 100 pM but not at 100 nM (Fig. 4*B*), as gauged by the insensitivity of the $A\beta_{1-42}$-induced Ca^{2+} responses to inhibition by a mixture of Ca^{2+} channel blockers (agatoxin-TK, o-conotoxin MVIIC, o-conotoxin GVIA) shown previously to block K^+-induced synaptosomal Ca^{2+} responses (Rondé and Nichols 1998). Similar results were obtained when Ca^{2+} channels were blocked with micromolar Cd^{2+} plus Co^{2+} (data not shown). The relative sensitivity of nicotine-induced Ca^{2+} responses to Ca^{2+} blockers was similar to that seen for 100 pM $A\beta_{1-42}$-induced responses (data not shown). Finally, the direct effect of $A\beta_{1-42}$ was sensitive to α-bungarotoxin (Fig. 5*A*), the open-channel blocker mecamylamine (Fig. 5*B*), and dihydro-β-erythroidine (Fig. 5*D*), to extents comparable with the inhibitory effects of these nicotinic antagonists on nicotine-induced Ca^{2+} responses (compare Fig. 2), indicating a significant involvement of α7-containing nAChRs and non-α7-containing nAChRs. Interestingly, the effect of α-bungarotoxin was more pronounced when $A\beta_{1-42}$ was used at 100 pM, whereas the effect of dihydro-β-erythroidine was more pronounced when $A\beta_{1-42}$ was used at 100 nM. Combining α-bungarotoxin with mecamylamine led to an inhibition profile similar to that of α-bungarotoxin alone (Fig. 5*C*). These results indicate that soluble Aβ at picomolar concentration directly activates presynaptic nAChRs predominantly of the α7-containing subtype to increase nerve terminal Ca^{2+} in a manner dependent on entry of Ca^{2+} through both the nAChR receptor channel and voltage-gated Ca^{2+} channels, whereas soluble Aβ at nanomolar concentration directly activates presynaptic nAChRs predominantly of the non-α7-containing subtype to increase nerve terminal Ca^{2+} largely via the nAChR receptor channel.

Because the apparent inhibitory effect of $A\beta_{1-42}$ on nicotine-induced responses (Fig. 1) was observed under conditions in which each response was necessarily normalized to the initial baseline, owing to uncertainty over baseline values after extended treatment periods without imaging, sequential addition of Aβ and nicotine was performed while imaging. The addition of 500 nM nicotine just after the initial application of $A\beta_{1-42}$ to hippocampal synaptosomes resulted in no further increase in Ca^{2+} level over that obtained in response to $A\beta_{1-42}$ alone (Fig. 6*A*), indicating that $A\beta_{1-42}$ strongly attenuates Ca^{2+} responses to nanomolar concentrations of nicotine in these nerve endings. In contrast, increasing nicotine to the micromolar range revealed nicotine-induced Ca^{2+} responses on top of the initial $A\beta_{1-42}$-induced responses. The control reverse peptide, $'A\beta'_{40-1}$, caused no significant change in the synaptosomal Ca^{2+} level and did not significantly inhibit responses to nicotine added on top of the $'A\beta'_{40-1}$ (Fig. 6*A*). Likewise, the addition of $A\beta_{1-42}$ just after the initial application of 500 nM nicotine had no significant effect on nicotine-induced Ca^{2+} responses when applied at 10 pM to 1 nM on top of nicotine, but it did increase synaptosomal Ca^{2+} when applied at 10–100 nM (Fig. 6*C*,*D*). In either case, in which relatively high concentrations of the second stimulatory agent were used (Fig. 3*C*), all synaptosomes responding to Aβ also responded to nicotine. Taken together, these results indicate that activation of presynaptic nicotinic receptors by picomolar $A\beta_{1-42}$ occludes the action of nicotine on the same receptors when applied at typical maximal concentration (500 nM) and vice versa. That the occlusion was not the result of a saturating Ca^{2+} response or dye saturation is evident in the ability of elevated concentrations to elicit responses (Fig. 6*B*,*D*). Thus, the apparent inhibitory effect of Aβ on nicotine-induced Ca^{2+} responses (Fig. 1) was a consequence of prior activation of the nAChRs by Aβ.

Prior depolarization with elevated external K^+ resulted in significant attenuation of subsequent $A\beta_{1-42}$-evoked Ca^{2+} responses (Fig. 7*A*) in a manner that was dependent on the amplitude of the initial K^+-evoked response (Fig. 7*B*). Under any condition, application of 30 mM (or higher) KCl will substantially depolarize all of the synaptosomes in the preparation; however, a wide range of response amplitudes are observed (cf. Nichols and Mollard, 1996). When Ca^{2+} responses were first evoked with $A\beta_{1-42}$ and followed by subsequent depolarization, the K^+-evoked Ca^{2+} responses were correspondingly attenuated (Fig. 7*C*) in a manner somewhat dependent on the amplitude of the initial $A\beta_{1-42}$-evoked response (Fig. 7*D*). In this case, even the largest K^+-evoked Ca^{2+} responses were substantially smaller after initial $A\beta_{1-42}$ stimulation in comparison with typical Ca^{2+} responses evoked by K^+ alone (compare Fig. 7*A*). These results

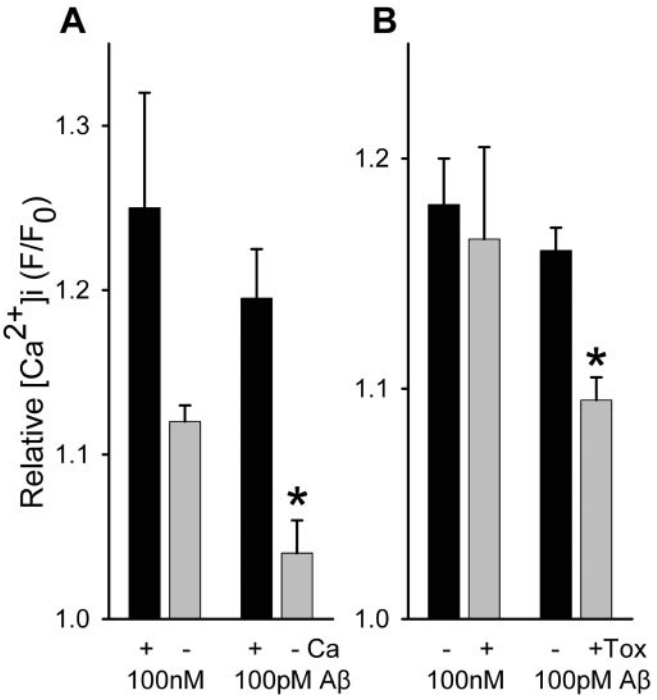

Figure 4. Dependence of Aβ-evoked Ca^{2+} increases in isolated hippocampal nerve endings on Ca^{2+} entry and voltage-gated Ca^{2+} channels. *A*, Average maximal responses Ca^{2+} responses to 100 nM ($n = 16$) or 100 pM ($n = 7$) $A\beta_{1-42}$ in the absence or presence of 1 mM external Ca^{2+}. *B*, Average maximal Ca^{2+} responses to 100 nM $A\beta_{1-42}$ ($n = 22$) or 100 pM $A\beta_{1-42}$ ($n = 17$) in the absence or presence of a mixture of voltage-gated Ca^{2+} channel blockers (toxins: 200 nM agatoxin TK; 500 nM conotoxin GVIA; 500 nM conotoxin MVIIC), which have previously been shown to block completely K^+ depolarization-induced synaptosomal Ca^{2+} responses (Rondé and Nichols, 1998). $^*p < 0.05$; *t* test with paired control.

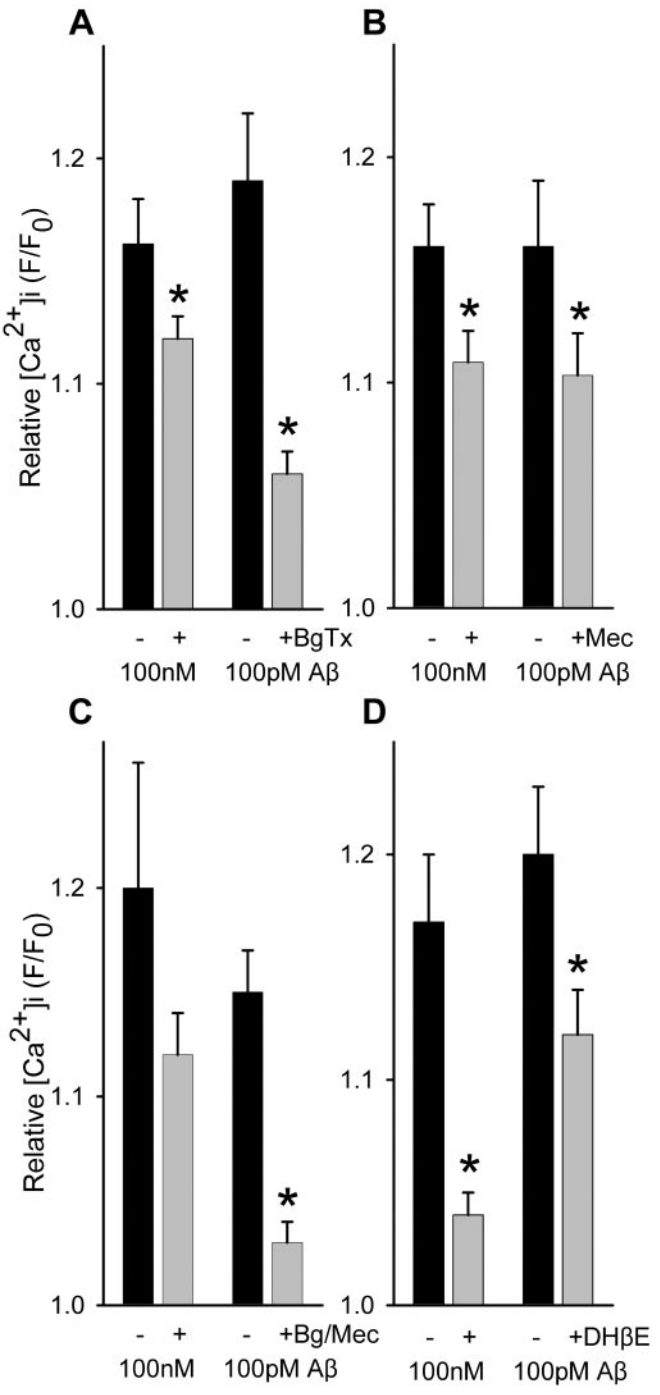

Figure 5. Sensitivity of Aβ-evoked Ca^{2+} increases in isolated hippocampal nerve endings to nicotinic receptor antagonists. Average maximal responses Ca^{2+} responses to $A\beta_{1-42}$ in the absence or presence of 500 nM α-bungarotoxin (BgTx; *A*) (100 pM, $n = 14$; 100 nM, $n = 22$), 10 μM mecamylamine (Mec; *B*) (100 pM, $n = 14$; 100 nM, $n = 14$), 500 nM α-bungarotoxin plus 10 μM mecamylamine (Bg/Mec; *C*) (100 pM, $n = 12$; 100 nM, $n = 6$), or 5 μM dihydro-β-erythroidine (DHβE; *D*) (100 pM, $n = 22$; 100 nM, $n = 16$). $^*p < 0.05$; *t* test with paired control.

suggest that the effect of nerve activity on presynaptic responses to Aβ is strictly dependent on the extent of the increase in Ca^{2+} level in response to presynaptic depolarization. In contrast, the presence of Aβ can attenuate presynaptic Ca^{2+} increases in response to nerve activity. To some degree, these effects may have resulted from prior activation of voltage-gated Ca^{2+} channels by either K^+ depolarization or Aβ-induced depolarization via nAChRs, with the contribution of voltage-gated Ca^{2+} channels to the evoked Ca^{2+} signals varying widely across synaptosomes (Nichols and Mollard, 1996).

Discussion

Aβ accumulates in plaques near dystrophic neurites and nerve endings (Brendza et al., 2003). Moreover, synaptic loss is strongly correlated with severity of dementia (Terry et al., 1991; Sze et al., 1997). As yet, effects of Aβ on the presynaptic nerve terminal have not been defined clearly. The present study undertook to explore the possibility that soluble Aβ peptide may modulate presynaptic Ca^{2+} levels, evaluating first effects on nicotine-induced responses, as an extension of previous work demonstrating an inhibitory interaction between Aβ and nAChRs, and later direct effects of Aβ itself. The observed sustained increases in presynaptic Ca^{2+} in response to soluble $A\beta_{1-42}$ raise the question as to whether such an effect would ultimately be toxic. Interestingly, prior depolarization attenuated the effect of Aβ, indicating that the degree of nerve activity and/or relative density of voltage-gated Ca^{2+} channels on the nerve endings may also play a role in modulating the action of soluble Aβ at presynaptic sites, in particular its toxicity.

Several previous reports have demonstrated an action of soluble Aβ peptides on nicotinic receptors (Dineley et al., 2001; Liu et al., 2001; Pettit et al., 2001). In particular, Aβ was found to block nicotine-stimulated increases in spontaneous neurotransmitter release from cultured hippocampal neurons (Liu et al., 2001), as the first clear indication of an action of Aβ on presynaptic nAChRs. In contrast, a recent report demonstrates a direct activation of nAChR-mediated currents in an oocyte expression

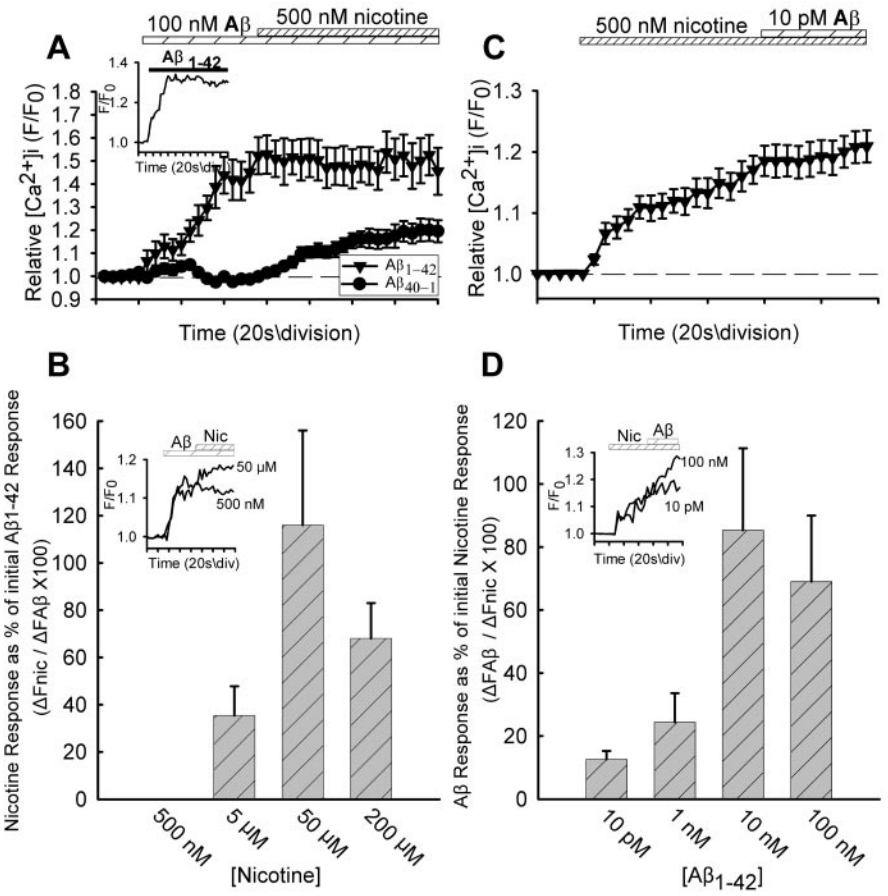

Figure 6. Ca^{2+} responses in isolated nerve endings to Aβ, followed by various concentrations of nicotine (*A*, *B*) compared with Ca^{2+} responses to nicotine, followed by various concentrations of Aβ (*C*,*D*). Averaged responses in *A* ($n = 6$) and *C* ($n = 13$) are means ± SEM. Averaged maximal responses in *B* (500 nM, $n = 2$; 5 μM, $n = 4$; 50 μM, $n = 3$; 200 μM, $n = 5$) and *D* (10 pM, $n = 14$; 1 nM, $n = 9$; 10 nM, $n = 9$; 100 nM, $n = 8$) denote the second agent as a percentage of the maximal response to the first agent. Insets, Representative responses.

system by Aβ (Dineley et al., 2002). Our work confirms and extends these previous findings, showing specifically that Aβ can directly induce increases in presynaptic Ca^{2+}. At picomolar Aβ concentration, the resultant Ca^{2+} responses appeared to largely involve α7-containing nAChRs, owing to the sensitivity of the Ca^{2+} responses to nicotinic antagonist α-bungarotoxin. Interestingly, however, prior treatment with Aβ inhibited subsequent action of high nanomolar nicotine, a typical maximal concentration for presynaptic nAChR-evoked responses (Nayak et al., 2001), by occlusion. That relatively high concentrations of nicotine could overcome, to some degree, the occlusion effect of Aβ, in the same synaptosomes, also confirms the involvement of nAChRs. Under the conditions used, these experiments cannot distinguish whether the action of Aβ is competitive or noncompetitive at presynaptic nAChRs, however, but do indicate significant overlap with the α-bungarotoxin site in the case of α7-nAChRs. Previous results indicated that Aβ may be noncompetitive for acetylcholine (Liu et al., 2001), which is consistent with findings of at least two binding sites for Aβ on α7-nAChRs in ligand-binding studies (Wang et al., 2000b), and the results presented here do not exclude this possibility. Further study will be needed to determine how Aβ specifically interacts with presynaptic nAChRs.

Although α7-containing nAChRs seem to be largely involved in the presynaptic actions of Aβ at picomolar concentration, the results indicate that the action of Aβ when present at high nanomolar concentration on presynaptic Ca^{2+} may be largely at non-α7-containing nAChRs, owing to a more pronounced sensitivity of the responses to the nicotinic antagonist dihydro-β-erythroidine. The results demonstrating that relatively high concentration of Aβ can overcome the occlusion by nicotine of the action of picomolar levels of Aβ are, in contrast, not inconsistent with the possibility that Aβ may be activating a pathway in the nerve endings that is independent of nAChRs. Other indications of direct stimulatory effects of Aβ that are most likely independent of nicotinic receptors have also been noted. For example, Aβ peptide was found to evoke a nonselective inward ion current in rat cortical neurons (Furukawa et al., 1994). The Aβ peptide-induced current was proposed to have resulted from the formation of cation channels, reported previously, wherein Aβ was used at particularly elevated concentrations (micromolar) (Lin et al., 2001). Here, however, Aβ induced increases in presynaptic Ca^{2+} in a reversible manner that was insensitive to Zn^{2+}, ruling out formation of channels.

An important question is whether Aβ-induced alterations in presynaptic Ca^{2+} affect the release of neurotransmitter. In one study, Aβ was actually found to have an inhibitory effect on K^+-evoked acetylcholine release from hippocampal slices (Kar et al., 1996, 1998), although the time frame of these measurements was rather extended. In view of the sustained increases in presynaptic Ca^{2+} in response to Aβ that were observed here, it may be that neurotransmitter release evoked subsequent to Aβ treatment would be inhibited perhaps as a consequence of a longer-term depressive and/or toxic effect on the nerve terminal. In contrast, it would be predicted that neurotransmitter release would be initially evoked by Aβ as a result of the increased presynaptic Ca^{2+}. Previous observations of an inhibitory effect of Aβ peptide on nicotine-stimulated increases in spontaneous neurotransmitter release in cultured hippocampal neurons did not, however, note a direct effect of Aβ alone (Liu et al., 2001). Effects, or lack thereof, of Aβ on exocytosis in individual nerve terminals from brain would require imaging using, for example, amphipathic fluorescent dyes such as FM1–43 (Rondé and Nichols, 1998).

Another issue is whether sustained presynaptic Ca^{2+} in response to Aβ will activate certain intracellular signaling pathways, such as protein kinases. Previous observations using hippocampal slices demonstrated extracellular signal-regulated kinase (ERK) activation in response to Aβ (Dineley et al., 2001). Whether Aβ activates protein kinase pathways, such as ERK, in the presynaptic nerve terminal is currently under investigation.

Despite several lines of evidence, including our own, indicating the possibility of a direct interaction between Aβ and nAChRs, such an interaction remains to be proven. It remains possible that Aβ interacts with a protein that associates with

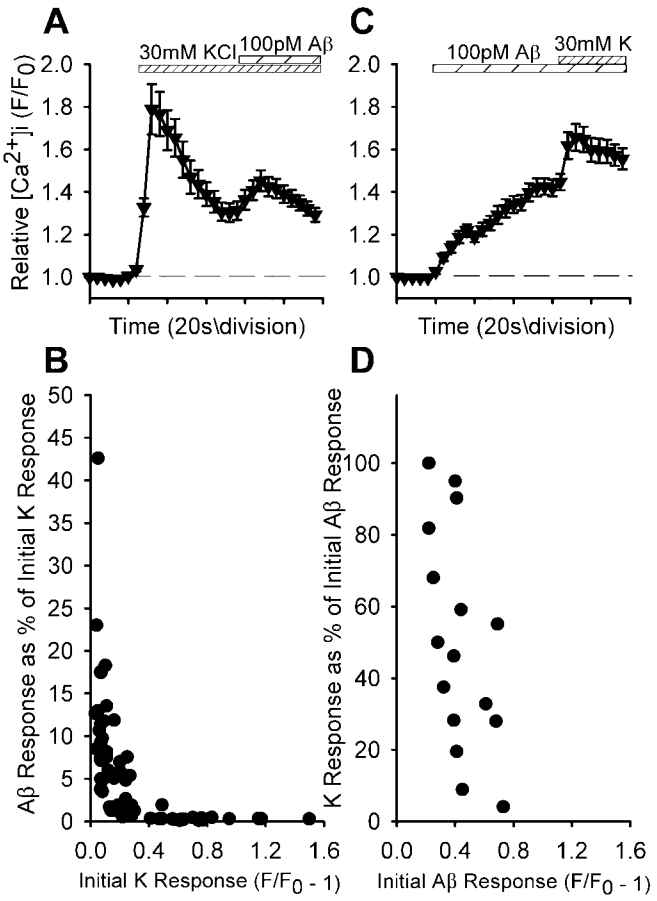

Figure 7. Ca^{2+} responses in isolated hippocampal nerve endings to Aβ after prior depolarization by KCl compared with Ca^{2+} responses to depolarization by KCl after Aβ. The averaged responses in *A* ($n = 26$) and *C* ($n = 18$) are means ± SEM. Individual peak responses to the second agent in *B* ($n = 71$) and *D* ($n = 16$) are correlated to the initial peak responses of the first agent, with each response to the second agent normalized to the response to the first agent as a percentage.

nAChRs and perhaps other signaling molecules. In contrast, nicotinic antagonists were able to inhibit Aβ-evoked presynaptic Ca^{2+} increases, indicating, as noted previously, some overlap of Aβ interaction with the ligand binding site(s). Sorting out the actual presynaptic targets for Aβ will entail a series of detailed molecular studies using, for example, preparations containing nAChRs modified by site-directed mutagenesis, especially in view of differences noted in the structural components of nAChRs that are essential for agonist binding as compared with antagonist binding (Arias, 1997).

Although speculative at present, our results might suggest that as Aβ begins to accumulate near synaptic sites, nicotine-mediated presynaptic regulation will be initially disrupted, although in a manner dependent on nerve activity-coupled presynaptic Ca^{2+} changes. Nicotinic receptors, particularly presynaptic nAChRs, have been implicated in long-term potentiation in the hippocampus (Fujii et al., 1999; Matsuyama et al., 2000) as well as the ventral tegmental area (Mansvelder and McGehee, 2000), and, thus, Aβ disruption of signaling via these receptors could have consequences for cognitive function. As Aβ further accumulates, toxic effects on nerve terminals may arise. For example, prolonged Aβ has been shown to alter nerve terminal mitochondrial function (Mattson et al., 1998). A key question is to what extent such toxicity might be a consequence of sustained presynaptic Ca^{2+} as compared with Aβ gaining entry into the nerve terminal (Kienlen-Campard et al., 2002; Nagele et al., 2002). It is likely that disruption of neuronal function at multiple levels via several pathways ultimately underlies the pathology arising over the course of Alzheimer's disease.

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