Human Amylin Actions on Rat Cholinergic Basal Forebrain Neurons: Antagonism of Beta-Amyloid Effects

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Jhamandas, Jack H., Kim H. Harris, Caroline Cho, Wen Fu, and David MacTavish. Human amylin actions on rat cholinergic basal forebrain neurons: antagonism of beta-amyloid effects. J Neurophysiol 89: 2923–2930, 2003. First published February 5, 2003; 10.1152/jn.01138.2002. Human amylin (hAmylin), a 37-amino acid pancreatic peptide, and amyloid β protein (Aβ), a 39–43 amino acid peptide, abundantly deposited in the brains of Alzheimer’s patients, induce neurotoxicity in hippocampal and cortical cultures. Although the mechanism of this neurotoxicity is unknown, both peptides are capable of modulating ion channel function that may result in a disruption of cellular homeostasis. In this study, we examined the effects of hAmylin on whole cell currents in chemically identified neurons from the rat basal forebrain and the interactions of hAmylin-induced responses with those of Aβ. Whole cell patch-clamp recordings were performed on enzymatically dissociated neurons of the diagonal band of Broca (DBB), a cholinergic basal forebrain nucleus. Bath application of hAmylin (1 nM to 5 μM) resulted in a dose-dependent reduction in whole cell currents in a voltage range between −30 and +30 mV. Single-cell RT-PCR analysis reveal that all DBB neurons responding to hAmylin or Aβ were cholinergic. Using specific ion channel blockers, we identified hAmylin and Aβ effects on whole cell currents to be mediated, in part, by calcium-dependent conductances. Human amylin also depressed the transient outward (I\text{O}) potassium currents. The hAmylin effects on whole cell currents could be occluded by Aβ and vice versa. Human amylin and Aβ responses could be blocked with AC187 (50 nM to 1 μM), a specific antagonist for the amylin receptor. The present study indicates that hAmylin, like Aβ, is capable of modulating ion channel function in cholinergic basal forebrain neurons. Furthermore, the two peptides may share a common mechanism of action. The ability of an amylin antagonist to block the responses evoked by hAmylin and Aβ may provide a novel therapeutic approach for Alzheimer’s disease.

INTRODUCTION

Alzheimer’s disease (AD), the most prevalent form of dementia, is a progressive neurodegenerative disease characterized by the loss of memory and higher cognitive functions (Selkoe 2001). The neuropathological features associated with AD include the presence of extracellular neuritic plaques composed of amyloid β protein (Aβ), intracellular neurofibrillary tangles, and the loss of basal forebrain cholinergic neurons that innervate the hippocampus and the cortex (Price 1986; Selkoe 1999). The principal constituents of the amyloid deposits is Aβ, which is a 39–43 amino acid peptide generated by proteolytic cleavage of a larger amyloid precursor protein. The in vitro neurotoxic potential of Aβ peptides when applied to either primary rat or human neuronal cultures has been advanced in the “amyloid hypothesis” to explain a central role for Aβ in neuronal death in AD (Kar et al. 1996; Mattson et al. 1992).

Human amylin (hAmylin) is a 37-amino acid peptide that is a major constituent of protein deposits identified in the islets of Langerhans of patients with noninsulin-dependent diabetes mellitus. Despite limited primary sequence homology, hAmylin shares several biophysical properties with Aβ including an ability to aggregate into β-pleated sheets in aqueous solutions. Importantly, hAmylin, but not rat amylin, has a profile of neurotoxicity in hippocampal and cortical neurons that is strikingly similar to that of Aβ in several aspects including that related to the time- and concentration-dependent induction of apoptotic genes (May et al. 1993; Tucker et al. 1998). A possible mechanism of such toxicity is the ability of both peptides to form cation-selective channels in membranes that would allow for unregulated calcium entry across such channels (Kawahara et al. 2000).

We have recently shown that Aβ selectively inhibits a suite of potassium conductances and increases the excitability of cholinergic neurons of the diagonal band of Broca (DBB), a basal forebrain nucleus (Jhamandas et al. 2001). On the basis of similarities in secondary structure in solution and neurotoxic profiles in vitro, we hypothesize that Aβ and hAmylin may share a similar ionic mechanism of action on cholinergic basal forebrain neurons. In this study, we first examined and characterized the effects of hAmylin on whole cell currents in chemically identified neurons of the DBB. We next investigated the possibility that hAmylin may occlude the Aβ effects on DBB neurons and vice versa. Finally, we tested the ability of AC 187, a specific antagonist of the amylin receptor, to block the effects of both hAmylin and Aβ on DBB neurons.

METHODS

Dissociation procedures

Details of the procedure for acute dissociation of neurons from the DBB are described in Jassar et al. (1999). Briefly, brains were quickly

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removed from decapitated male Sprague Dawley rats (15–25 day postnatal) and placed in cold artificial cerebrospinal fluid (ACSF) that contained (in mM) 140 NaCl, 2.5 KCl, 1.5 CaCl₂, 1.2 MgCl₂, 10 HEPES, and 33 d-glucose (pH 7.4). Brain slices (350–500 μm thick) were cut on a vibratome, and the area containing the DBB was dissected out. Although most of the tissue contained the horizontal limb of the DBB, some slices may have included a component of the vertical limb of the DBB. Acutely dissociated neurons were prepared by enzymatic treatment of the slice with trypsin (0.65 mg/ml) at 30°C, followed by mechanical trituration for dispersion of individual cells. Cell were C, followed by

Electrophysiological recordings

Whole cell patch-clamp recordings were performed at room temperature (20–22°C) using an Axopatch-1D amplifier. Patch electrodes (World Precision Instruments, thin wall with filament,1.5-mm diam) were flame polished to yield resistances of 3–6 MΩ. Internal patch pipette solution contained (in mM) 140 K-methylsulfate, 10 EGTA, 5 MgCl₂, 1 CaCl₂, 10 HEPES, 2.2 Na₂-ATP, and 0.3 Na-GTP (pH 7.2). Junction potential was nullled with the pipette tip immersed in the bath. Putative acutely dissociated DBB neurons were initially identified for recording by visual inspection. Current-voltage relationships and excitability characteristics were used to distinguish neurons from glial or other cell types (Jassar et al. 1999). The membrane currents under voltage-clamp conditions were recorded and analyzed on computer using pCLAMP software (version 6.0.3).

After whole cell configuration was established, we waited ≥5 min for steady-state currents to stabilize. The filter was set at 20 kHz during data acquisition. Cells were held in voltage-clamp at −80 mV, which was close to the resting membrane potential (RMP) observed in earlier studies on neurons from basal forebrain slices (Alonso et al. 1994; Easaw et al. 1997). Based on the previous observations (Jassar et al. 1999), we utilized a voltage-ramp protocol where the cells were held at −80 mV and subjected to voltage ramps from −110 to +30 mV at the rate of 20 mV/s. A 1-s long hyperpolarizing command to −110 mV was applied to remove inactivation of K⁺ channels so that the maximum current could be activated during the subsequent slow voltage ramp to +30 mV. No obvious tail currents were observed at the end of the ramp when the command potential was returned to −80 mV, suggesting that the ramp elicited mainly steady-state currents.

Cell size was estimated electronically using the whole cell capacitance compensation circuit on the Axopatch-1D amplifier. Series resistance compensation was continuously adjusted to >80% and monitored and readjusted as necessary during the course of each experiment. The average series resistance (electrode plus access resistance) was 7.3 ± 0.5 (SE) MΩ (n = 51). Maximum voltage-clamp error in recording a current of 10 nA using a patch electrode with an electrode resistance of 8 MΩ was 16 mV. This reflects the average maximum error because the currents recorded were usually <10 nA.

To record currents through calcium channels, we used Ba²⁺ as a charge carrier as previously described (Easaw et al. 1999). The external solution contained (in mM) 150 tetraethylammonium chloride, 2 BaCl₂, 10 HEPES, and 30 glucose (pH to 7.4 with TEA-OH). The internal patch pipette solution consisted of (in mM) 130 Cs-methanesulfonate, 2 MgCl₂, 10 HEPES, 10 BAFTA, 4 Mg-ATP, 0.3 Na-GTP, and 0.1 leupeptin (pH to 7.2 with CsOH). Depolarizing voltage steps from −80 to +70 mV (increment: 10 mV/step; 20-ms duration) were applied to voltage-clamped DBB neurons under control conditions and in the presence of hAmylin. Leak currents were minimal under our recording conditions. They did not change during the recordings and were not affected by application of hAmylin. Therefore we did not subtract these in subsequent measurements of steady-state barium currents.

Drugs and solutions

Human amylin and AC 187, an amylin receptor antagonist (Ac-Val-Leu-Gly-Lys-Leu-Ser-Gln-Glu-Leu-His-Leu-Gln-Thr-Tyr-Pro-Arg-Thr-Asn-Thry-Gly-Ser-Asn-Thr-Tyr-NH₂) (Young et al. 1994), were obtained from SynPep (Dublin, CA), Aβ25–35 from QCB Biosource International (Camarillo, CA), and ibotenic acid from Sigma Chemical (St. Louis, MO). All the agents were dissolved in distilled water to make 1,000× stock solution (stored at −70°C) and diluted in external perfusing medium just before the time of application. All drugs and chemicals were applied via bath perfusion at the rate of 3–5 ml/min, which allowed complete exchange in less than half a minute. Data are presented as mean ± SE. Student’s two-tailed t-test was utilized for determining significance of effect.

Single-cell RT-PCR for chemical phenotyping

Neurons were harvested after electrophysiological recordings were completed and readied for RT-PCR according to a previously described protocol (Surmeier et al. 1996). In brief, contents of the electrode containing the cell and 5 μl of internal solution were expelled into a 0.2 ml PCR tube containing 5 μl sterile water (Sigma water W-4502) 0.5 μl dithiothreitol, 0.1 M (DTT), 0.5 μl RNasin (10 U/μl), and 1 μl oligo-dT (0.5 μg/μl). The tube was then placed on ice. Single-stranded cDNA was then synthesized by adding a solution containing 1 μl SuperScript II (RT) (200U/μl), 2 μl 10× PCR buffer, 2 μl 25 mM MgCl₂, 1.5 μl 0.1 M DTT, 1 μl 10 mM dNTPs, and 0.5 μl RNasin (10U/μl). The PCR tube was gently mixed and incubated in a Techne Progene thermal cycler at 42°C for 50 min. The process was then terminated by heating to 72°C for 15 min, and the tube cooled to 4°C. Subsequently 2 μl of the RT product was taken and combined with 5 μl 10× PCR buffer, 5 μl 25 mM MgCl₂, 0.5 μl Taq polymerase (5 U/μl), 31.5 μl sterile water (Sigma water W-4502), 1 μl 25 mM dNTP mixture, and a 1.5 μl of a specific set of primers (15 μM). All reagents were purchased from GibcoBRL. Primer sequences for choline acetyltransferase (ChAT) and for glutamate decarboxylase (GAD) have been previously described (Surmeier et al. 1996; Tkatch et al. 1998), and that for β-actin was obtained from GenBank (the lower primer: 5'-GAT AGA GCC ACC AAT CCA C, the upper primer: 5'-CCA TGT ACG TAG CCA TCC A). All primers were synthesized at the University of Alberta Department of Biochemistry. The contents were mixed together and placed in the thermal cycler. The PCR amplification protocol was as follows: step 1: 94°C 4 min, step 2: 94°C 1 min, 53°C 1 min, 72°C 45 s, (step 2 was repeated 35 times) step 3: 72°C 15 min, step 4: held at 4°C. A portion of the product was then run on a 2% TEA agarose gel, and the gel was then placed in a bath containing 2 μg/ml of ethidium bromide after 10 min DNA bands were visualized with UV light box and photographed with a Polaroid camera.
rons, application of hAmylin resulted in a decrease in the outward currents in the voltage range from −30 to +30 mV (Fig. 1A). In comparison to control conditions, the amplitude of the currents at +30 mV was significantly decreased in the presence of hAmylin (control = 6.44 ± 0.43 nA, hAmylin = 5.54 ± 0.39 nA, recovery = 6.04 ± 0.45, n = 51, P < 0.001). The maximal effects of hAmylin on whole cell currents were observed within 90–180 s of the drug application, and the response did not significantly desensitize with repeated applications of the peptide (Fig. 1, inset). hAmylin inhibited peak whole cell currents of DBB neurons in a dose-dependent manner with an EC_{50} of 71.1 nM (Fig. 1B). Thus to ensure maximal responses, in all subsequent experiments, we used a concentration of 1 μM, which is also consistent with other electrophysiological studies examining the actions of this peptide (Riediger et al. 1999, 2001). In 14 DBB neurons, hAmylin did not evoke a significant change in peak current at +30 mV (control = 6.96 ± 0.13 nA, hAmylin = 6.86 ± 0.13 nA, P = 0.09).

**Chemical phenotype of human amylin responsive neurons**

There are two main chemical neurotransmitter phenotypes represented in the DBB neurons—GABAergic and cholinergic. We have previously reported that a definitive determination of these chemical phenotypes in isolated DBB neurons can be performed using single-cell RT-PCR analysis (Jhamandas et al. 2001, 2002). To chemically identify DBB neurons that responded to hAmylin, ChAT and GAD primers were used as a specific markers for cholinergic neurons and GABAergic neurons, respectively. Figure 1C shows the photograph of a gel indicating RT-PCR products from an hAmylin-responsive cell and also a hAmylin-nonresponsive neuron. The hAmylin responsive cell on the right reveals a band corresponding to the molecular weight of the ChAT primer and the hAmylin non-responsive cell on the left side of the gel shows a band corresponding to the molecular weight of GAD primer. In all gels, β-actin served as a positive control. Of the 35 cells that responded to hAmylin with a reduction in whole cell currents and that were tested for RT-PCR, 16 were ChAT positive and none were GAD positive. For the 11 hAmylin-nonresponsive neurons that were tested for RT-PCR, 6 were GAD positive and none were ChAT positive.

**Effects of human amylin on calcium-activated potassium currents**

The outward potassium currents are a mixture of calcium- and non-calcium-activated components. Calcium-activated currents include the voltage-sensitive conductances called maxi g_{K(Ca)} (I_{C}) and the voltage-insensitive ones that underlie action potential afterhyperpolarization (I_{AHP}). Of the two main Ca^{2+}-activated potassium currents, under whole cell recording conditions from DBB neurons, I_{AHP} makes little contribution.

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FIG. 1. Effects of Human amylin (hAmylin) on whole cell currents in cholinergic DBB neurons. A: current-voltage relationship from 51 diagonal band of Broca (DBB) neurons showing whole cell currents evoked under control conditions, in the presence of 1 μM hAmylin, after recovery. B: dose-response curve for hAmylin inhibition of peak whole cell currents. C: examples of single-cell RT-PCR analysis from 2 DBB neurons tested with hAmylin. The hAmylin responsive neuron is cholinergic [choline acetyltransferase (ChAT) positive], whereas the neuron that does not respond to hAmylin is GABAergic [glutamate decarboxylase (GAD) positive]. For each cell, the presence of β-actin acts as a positive control. Molecular weight for β-actin ~606, ChAT ~290, and GAD ~400.

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and majority of the currents flow through \( I_C \) channels (Jassar et al. 1999).

To elucidate the degree to which hAmylin effects are mediated via \( I_C \), we examined actions of hAmylin under conditions where cells were perfused with iberiotoxin (IBTX), a specific blocker of \( I_C \) channels. Figure 2A shows the average of current-voltage relationships obtained from six neurons under control conditions, in the presence of IBTX (25 nM) and hAmylin application in the presence of IBTX. IBTX applied alone reduced the outward currents. Application of hAmylin in the presence of IBTX resulted in an additional but smaller reduction of the currents than evoked by hAmylin alone (at \( +30 \) mV, control = 7.33 \( \pm \) 1.04 nA, IBTX = 6.26 \( \pm \) 1.04 nA, IBTX and hAmylin = 5.85 \( \pm \) 1.04 nA, recovery = 6.92 \( \pm \) 1.09 nA). In comparison to reduction in whole cell currents evoked by hAmylin alone (normalized to 100%), inclusion of IBTX with hAmylin in the same group of neurons resulted in a significant block of the hAmylin response (Fig. 2A inset, \( P < 0.01 \)).

**FIG. 2.** hAmylin effects on calcium and potassium dependent currents. **A:** current-voltage relationship from 6 DBB neurons under control conditions, in the presence of iberiotoxin (IBTX, 50 nM), hAmylin (1 \( \mu \)M) with IBTX, and recovery. Human amylin causes a partial but persistent reduction in whole cell currents in the presence of IBTX, a blocker of calcium-activated potassium conductance (BK channels). Histogram on the right depicts the response to hAmylin at \( +30 \) mV under control conditions (done in the same group of cells prior to the data depicted in the current-voltage plot above and the response is normalized to 100%) vs. hAmylin response in the presence of IBTX (33.0 \( \pm \) 8.9%); *, significant difference between 2 responses at \( P < 0.01 \), **B:** average current-voltage relationship of barium currents (\( I_{Ba} \)) from 8 DBB neurons under control conditions, with hAmylin (1 \( \mu \)M) and recovery. Human amylin significantly inhibits barium currents. Sample \( I_{Ba} \) current from a single neuron at \( +10 \) mV command potential is shown at the right. Voltage protocol employed is shown in the top inset. **C** and **D:** the effects of hAmylin (1 \( \mu \)M) on \( I_K \) and \( I_A \) in DBB neurons, respectively. In **C**, hAmylin causes a small reduction in \( I_K \) (delayed rectifier) currents. The voltage protocol for recording \( I_K \) is shown between the 1st 2 traces with holding potential of the cell at \( -80 \) mV and a 150-ms conditioning pulse to \( -40 \) mV. In this protocol, outward currents are mediated predominantly through \( I_K \). **D:** hAmylin causes a decrease in transient outward \( K^+ \) currents (\( I_A \)). \( I_A \) was obtained by subtracting the currents obtained by the 2 voltage protocols shown between the 1st and 2nd traces. Note that the in the 1st voltage protocol, cells were initially held at \( -80 \) mV and a 150-ms conditioning pulse to \( -120 \) mV was applied. The 2nd voltage protocol is identical to that shown in **C**.
Effects of human amylin on calcium currents

Because hAmylin reduction of whole cell currents is significantly attenuated by in the presence of IBTX, Ca$^{2+}$-activated currents appear to play an important role in the response of DBB neurons to hAmylin application. This can result from either a direct effect on Ca$^{2+}$-dependent conductances i.e., $I_C$ or, a more upstream effect on Ca$^{2+}$ channels, which in turn may activate potassium conductances. To examine this issue, we recorded barium currents ($I_{Ba}$) flowing through Ca$^{2+}$ channels. Figure 2B shows the average current-voltage relationships of $I_{Ba}$ recorded from eight neurons. hAmylin significantly reduced the $I_{Ba}$ compared with control conditions (control $= 2.32 \pm 0.27 \text{nA}$, hAmylin $= 1.80 \pm 0.23 \text{nA}$, recovery $= 2.18 \pm 0.20 \text{nA}$, $P < 0.01$, at $-10 \text{mV}$).

Effects of human amylin on transient outward ($I_A$) and the delayed rectifier ($I_K$) potassium currents

$I_A$ and $I_K$ are voltage-sensitive currents, and their activation and inactivation are strongly voltage dependent. $I_A$ requires the holding potential to be relatively hyperpolarized (approximately $-110 \text{mV}$) for removal of its inactivation, whereas it is inactivated at $-40 \text{mV}$. On the other hand, $I_K$ is not inactivated at $-40 \text{mV}$. These biophysical properties of $I_A$ and $I_K$ can thus be utilized to isolate these currents. Therefore a conditioning pulse to $-40 \text{mV}$ will activate $I_K$ without any significant contamination by $I_A$ (Connor and Stevens 1971; Easaw et al. 1999). A conditioning pulse to $-120 \text{mV}$ will activate both $I_A$ and $I_K$. The difference currents obtained by subtracting the currents evoked by depolarizing pulses following a conditioning pulse to $-40 \text{mV}$ from those evoked following a conditioning pulse to $-120 \text{mV}$ provide an accurate estimate of $I_A$ (Connor and Stevens 1971; Easaw et al. 1999). Figure 2C shows the currents recorded from a neuron with a conditioning pulse to $-40 \text{mV}$ for 150 ms, representing mainly $I_K$, under control conditions, in the presence of hAmylin and recovery on washout of hAmylin. hAmylin reduced $I_K$ by $8.4 \pm 2.9\%$ (control $= 4.46 \pm 0.79 \text{nA}$, hAmylin $= 4.10 \pm 0.79 \text{nA}$, $n = 5$, $P < 0.05$ at $+30 \text{mV}$). Figure 2D shows the difference currents recorded from the same neuron representing mainly $I_A$ under control conditions, in the presence of hAmylin and on washout. $I_A$ was reduced significantly by hAmylin by $16.5 \pm 3.7\%$ (control $= 1.56 \pm 0.16 \text{nA}$, hAmylin $= 1.31 \pm 0.17 \text{nA}$, $n = 7$, $P < 0.01$). We have previously shown that the residual sustained current remaining at the end of the 100-ms test pulse (shown in Fig. 2D) consists mainly of $I_K$ and $I_C$ (Easaw et al. 1999), both of which are also reduced by hAmylin.

Occulsion of human amylin effects by Aβ and vice versa

Because the profile of hAmylin effects on whole cell currents in DBB neurons is similar to that we have previously observed for Aβ, we investigated the possibility that the actions of the two peptides may occur through a shared mechanism. In cells where individual applications of hAmylin and Aβ evoked a reduction in whole cell currents, we attempted to occlude the Aβ response in the presence of hAmylin. Data obtained from five cells that responded to hAmylin and Aβ applications are illustrated in Fig. 3, A and B. Subsequent to the application of hAmylin alone, application of Aβ in conjunction with hAmylin did not cause an additional reduction of whole cell currents (at $+30 \text{mV}$, hAmylin $= 5.85 \pm 0.73 \text{nA}$ vs. hAmylin and Aβ $= 5.74 \pm 0.73 \text{nA}$; $P = 0.3$, Fig. 3A). This is illustrated another way in the histogram (Fig. 3B), where the depression of whole cell currents by Aβ alone (normalized for each cell to 100%) is compared with the reduction of current by Aβ in the presence of hAmylin in the same group of neurons (at $+30 \text{mV}$, reduction of the currents with hAmylin in Aβ $= 21.2 \pm 2.5\%$; $n = 5$, $P < 0.05$). We have previously shown that the repeated applications of Aβ in DBB neurons cause a reduction in whole cell currents that is time independent and does not sensitize appreciably (Jhamandas et al. 2001).

We repeated the same experiment but in reverse, this time examining the hAmylin response alone and in the presence of Aβ. Figure 3C illustrates the data from six DBB cells where the response to hAmylin in the presence of Aβ is almost completely occluded (at $+30 \text{mV}$, Aβ $= 5.75 \pm 0.72 \text{nA}$ vs. Aβ and hAmylin $= 5.71 \pm 0.76 \text{nA}$, $P = 0.6$). Figure 3D shows a comparison of the reduction in normalized whole cell currents by hAmylin alone and hAmylin with Aβ in the same group of neurons (at $+30 \text{mV}$, hAmylin $= 100\%$ vs. hAmylin in Aβ $= 18.4 \pm 5.5\%$, $P < 0.01$).
AC 187 blockade of human amylin and Aβ responses

AC 187 is a selective peptidergic amylin receptor antagonist, which in the dose range of $10^{-3}$–$10^{-6}$ range has been shown to block the actions of hAmylin (Riediger et al. 1999). We investigated whether the effects of hAmylin or Aβ on DBB neurons could also be blocked using this amylin receptor antagonist. Application of 50 nM AC 187 blocked the hAmylin response (at +30 mV, control = 5.22 ± 0.66; AC 187 = 4.85 ± 0.51 nA vs. hAmylin and AC 187 = 4.74 ± 0.46 nA; n = 8; P = 0.3, Fig. 4, A and B). In Fig. 4B, the hAmylin response for each cell is normalized to 100% and compared with the response of hAmylin in the presence of AC 187 (21.8 ± 4.3%, n = 8; P < 0.01) in the same group of cells. AC 187, while still blocking the effects of hAmylin across a range of doses (50 nM to 1 μM) caused a small but significant reduction in whole cell currents when applied alone [control = 4.78 ± 0.39, AC 187 (50 nM) = 4.47 ± 0.31 nA, n = 15, P < 0.05].

FIG. 4. Blockade of hAmylin and Aβ responses in the presence of AC 187, an amylin receptor antagonist. A: current-voltage plots from 8 DBB neurons under control conditions, in the presence of AC187 (50 nM), and hAmylin (1 μM) in the presence of AC187. hAmylin applied alone also caused a significant reduction in whole cell current (not shown), hAmylin applied in the presence of AC 187 does not cause a significant reduction in peak whole cell current. B: histograms showing the hAmylin response on peak whole cell currents (+30 mV) when applied alone and normalized to 100% vs. hAmylin response in the presence of AC 187 (21.8 ± 4.3%); *, significant difference between the 2 responses in the same group of cells at P < 0.01. C: current-voltage plots from 8 DBB neurons under control conditions, in the presence of AC187 (50 nM), and Aβ (1 μM) in the presence of AC187. Aβ applied alone also caused a significant reduction in whole cell current (not shown). Aβ applied in the presence of AC 187 does not cause a significant reduction in peak whole cell currents. B: histograms showing the Aβ response on peak whole cell currents (+30 mV) when applied alone and normalized to 100% vs. Aβ response in the presence of AC 187 (19.2 ± 5.6%); *, significant difference between the 2 responses in the same group of cells at P < 0.01.

AC 187 (50 nM) also blocked the reduction in whole cell currents evoked by Aβ (at +30 mV, control = 4.28 ± 0.22, AC 187 = 4.05 ± 0.22, Aβ and AC 187 = 3.95 ± 0.23; n = 7; P = 0.4; Fig. 4, C and D). In Fig. 4D, the Aβ response for each cell is normalized to 100% and compared with the response of Aβ in the presence of AC 187 (19.2 ± 5.6%, n = 7; P < 0.01) in the same group of cells.

DISCUSSION

We have shown here for the first time the ability of hAmylin to modulate specific ionic conductances in central mammalian neurons of the basal forebrain. Specifically, we demonstrate that hAmylin causes a reduction of calcium-activated potassium (BK) conductance. The hAmylin-induced depression of calcium currents may be an upstream event that in turns results in a decreased activation of calcium-activated potassium channels. Single-cell RT-PCR data indicate that, like Aβ, the actions of hAmylin are specific to cholinergic neurons of the DBB and not their GABAergic counterparts, which are the other major chemical phenotype in the rat basal forebrain. We also provide evidence that hAmylin and Aβ may share common mechanism of action because each peptide can occlude the effects of the other. Moreover, AC 187, a specific amylin receptor antagonist, blocks not only the effects of hAmylin but also those of Aβ on cholinergic basal forebrain neurons.

Cellular actions of human amylin actions on central neurons

Amylin immunoreactivity and binding sites have been reported in widespread brain regions of the rat including the basal forebrain (Sexton et al. 1994; Skofitsch et al. 1995; van Rossum et al. 1994, 1997). Data from in vivo experiments suggest a role for amylin as a neuromodulator in the CNS to partially explain its anorectic, episodic, and inhibitory effects on gastric emptying (Baldo and Kelley 2001; Young 1997). However, currently there is relatively little information on the actions of this peptide in the brain at a cellular level. Virtually all studies examining amylin’s effects on neuronal activity have been performed on neurons of the area postrema and subfornical organ, which are circumventricular structures that lack a blood brain barrier (Riediger et al. 1999, 2001). In these regions, amylin elicits excitatory effects via formation of cGMP. In our study, hAmylin caused a dose-dependent reduction of whole cell currents in the voltage range normally associated with activation of calcium-dependent conductances. Pharmacological blockade of the calcium-activated potassium BK type of channels with iberiotoxin markedly reduces the hAmylin response, indicating that most of the effects of this peptide on whole cell currents are mediated via BK channels. Because the activation of BK channels requires the entry of Ca$^{2+}$ into the cell, we also examined the effects of hAmylin on voltage-sensitive calcium channels (VSCCs). We have previously shown that the majority of the Ca$^{2+}$ current in DBB neurons flows through N- and L-type VSCCs (Chin et al. 2002; Jassar et al. 1999), and therefore the hAmylin-induced depression of barium currents likely occurs through these two moieties of VSCCs. In hippocampal neurons, the activity of N-type Ca$^{2+}$ channels is coupled to a specific activation of BK types of channels (Marriott and Tavalin 1998). It is possible that the hAmylin effects on Ca$^{2+}$ entry through N-type channels could
account for the downstream effects of this peptide on the BK channels.

BK channels play an important role in governing the excitability of neurons through their effects on spike repolarization and accommodation (Vergara et al. 1998). In previous studies from our laboratory, we have shown that applications of specific blockers of calcium conductances such as the N-type channel blocker omega-conotoxin or iberiotoxin, which selectively blocks the BK channels, result in an increase in excitability of DBB neurons (Jhamandas et al. 1999; Jhamandas et al. 2002). Thus the hAmylin-induced reduction of such calcium-dependent conductances reported here would be predicted to result in a general increase in excitability of DBB neurons and is consistent with the previously reported excitatory actions of this peptide on area postrema and subfornical organ neurons (Riediger et al. 1999, 2001).

The inhibitory effects of hAmylin on fast-inactivating (I_A) and the slower inactivating (I_K) potassium channels are virtually identical to those which we have observed for Aβ on the same channels (Jhamandas et al. 2001). These potassium conductances are important in regulating neuronal excitability, in part, through their effects on the early phase of spike repolarization. Therefore hAmylin-induced reduction of I_A and I_K would be expected to contribute to the overall increase in the excitability of DBB neurons and consistent with its effects on BK channels discussed earlier.

Central administrations of amylin have been shown to affect memory through undetermined mechanisms (Morley et al. 1995). Cholinergic neurons of the DBB are intimately involved in memory and spatial learning through their anatomical connections with the hippocampus, and lesions of the DBB result in a disruption of these cognitive processes (Roman et al. 1993). Our single-cell RT-PCR data indicate that the effects of amylin appear to be selective to cholinergic DBB cells and may provide a cellular basis for its behavioral and memory-related effects when injected intracerebroventricularly (Morley et al. 1995).

**hAmylin interactions with Aβ**

hAmylin and Aβ demonstrate a low sequence homology, but both peptides share the ability to acquire β-pleated structures and form α-helical domains in water solutions. These physiochemical properties of the peptides in aqueous solutions have been deemed to be important in their ability to form de novo cation channels in plasma membranes of immortalized hypothalamic GnRH neurons (GT1-7 cells) (Kawahara et al. 2000). hAmylin, Aβ, or prion protein applied individually induce increased Ca^{2+} influx through such channels that may eventually result in Ca^{2+} overload and cell death. Alternately, it has been proposed that the presence of the β-pleated aggregates formed by hAmylin or Aβ near cell surface could cause membrane disruption possibly by cross-linking receptors and transmembrane proteins (Tucker et al. 1998). Formation of de novo cation channels or a disruption of membrane structure seem less likely possibilities in our system given that the responses to hAmylin and Aβ were reversible. With respect to the conformation of Aβ (and hAmylin) used in our experiments, recent studies have characterized conditions and procedures that produce structurally distinct assemblies of Aβ, i.e., the soluble oligomeric form versus the fibrillar aggregates (Stine et al. 2002). These authors have also correlated the formation of each specific form of Aβ species to their ability to produce toxicity in vitro (Dahlgren et al. 2002). Based on data from these studies and our own protocol, whereby we prepared fresh solutions of Aβ immediately prior to each experiment, it is likely that the Aβ we used was in the soluble oligomeric rather than the aggregated form.

Our results provide evidence that hAmylin and Aβ may share the same mechanism of action on cholinergic basal forebrain neurons because each peptide can occlude the response of the other. This interplay between the two peptides may occur either at the level of the ion channels they activate or shared intracellular signal transduction mechanisms. The block of hAmylin and Aβ responses by the amylin receptor antagonist AC 187 suggests that the interaction between the two peptides occurs at the receptor level. The ability of an amylin receptor antagonist to block the Aβ response at a cellular level is particularly interesting and suggests that the amylin receptor may be a new therapeutic target in AD. In this regard, we are currently investigating the effects of AC 187 in blocking the Aβ-induced neurotoxicity in vitro in primary septal cultures.

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