Functional coupling reactions of human amylin receptor and nicotinic acetylcholine receptors in rat brain neurons

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Abstract: Human amylin (hAmylin) is co-released with insulin from pancreatic B-cells and the actions of this peptide on its target tissues maintain the cell excitability and glucose homeostasis. Inappropriate control of hAmylin secretion may result in human disease, particularly Alzheimer’s disease (AD). It’s unknown that which kind of receptor is activated by human amylin, leading to the neurotoxicity in neurons of brain. Nicotinic acetylcholine receptors (nAChRs) are known to play a critical role in a variety of nervous diseases. In the present study, we sought to determine the inter-relationships between these two receptors by examining the actions of hAmylin and nicotine on whole-cell currents and membrane potential in basal forebrain neurons. Whole cell patch-clamp recordings were performed on enzymatically dissociated neurons of the diagonal band of Broca (DBB), a cholinergic basal forebrain nucleus. The results showed that either hAmylin or nicotine individually caused a dose-dependent (1 nmol/L–20 µmol/L) membrane depolarization and an increase in firing frequency of DBB neurons. Application of AC253, an amylin receptor antagonist, blocked the excitatory effects of not only hAmylin but also nicotine; dihydro-β-erythroidine (DHβE), a nAChR antagonist, also blocked the effects of nicotine and hAmylin. These electrophysiological results suggest that hAmylin receptor and nAChRs on DBB neurons are coupled and may function in a co-operative manner to influence the excitability of DBB neurons. This finding is important for us to understand the cause and mechanisms of AD.

Key words: human amylin receptor; nicotinic acetylcholine receptors; patch clamp; Alzheimer’s disease

人胰淀素受体和烟碱乙酰胆碱受体在大鼠大脑神经元上的功能偶联作用

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摘 要：人胰淀素(hAmylin)是由分泌胰岛素的胰岛B细胞释放，作用于靶组织，维持细胞的兴奋性和葡萄糖在体内的稳态。hAmylin分泌异常会引起人类的疾病，特别是阿尔茨海默氏病(Alzheimer’s disease, AD)。目前对于hAmylin通过激活什么样的受体从而产生脑神经元的神经毒性仍然不清楚。已知烟碱乙酰胆碱受体(nicotinic acetylcholine receptors, nAChRs)是引发多种神经性疾病的关键因素。本研究通过记录hAmylin和烟碱对基底前脑神经元的全细胞电流和膜电位的影响，来确定hAmylin受体和烟碱受体两者之间的相互作用。在酶解分离的基底前脑Broca区(diagonal band of Broca, DBB)胆碱能神经元上进行全细胞膜片钳记录，结果显示，hAmylin和烟碱单独应用，均引起电流(1 nmol/L–20 µmol/L)依赖性膜电位的去极化和DBB神经元放电频率增加，hAmylin受体拮抗剂AC253，不仅阻断hAmylin的兴奋作用，而且也阻断烟碱对神经元的兴奋作用；同样，使用nAChR竞争性拮抗剂二氢-β-刺桐啶碱(dihydro-β-erythroidine, DHβE)，可以阻断烟碱和hAmylin对DBB神经元的兴奋作用。以上结果提示，hAmylin受体和nAChRs受体在DBB神经元可能是功能偶联的，协同影响DBB神经元的兴奋性。

关键词：人胰淀素受体；烟碱乙酰胆碱受体；膜片钳；阿尔茨海默氏病

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Human amylin (hAmylin) is co-released with insulin from pancreatic B-cells and the actions of this peptide on its target tissues maintain the cell excitability and glucose homeostasis. The receptor activity-modifying proteins (RAMPs) are simple transmembrane proteins that transport the calcitonin receptor (CTR) to the cell surface[1]. RAMPs include a family of three proteins (RAMP1, 2 and 3). At the molecular level, it shows that the human calcitonin receptor isotype 2 (hCTR2), co-transfected with either RAMP1 or RAMP3, generated a receptor that demonstrates a high binding affinity for amylin[2].

The nucleus of the diagonal band of Broca (DBB) within the basal forebrain plays an important role in memory and learning processes. The neuropathological hallmarks of Alzheimer’s disease (AD) include a loss of basal forebrain cholinergic neurons and the deposition of β-amyloid peptide (Aβ) in neuritic plaques. The evidence from studies both in vivo and in vitro of animal models supports that the damage of basal forebrain cholinergic neurons is vital in AD process[3].

The central nervous system (CNS) effects of the neurotransmitter acetylcholine are mediated, in part, via nicotinic acetylcholine receptors (nAChRs). nAChRs are significant in the learning and memory functions, neuronal development. The roles of nAChRs are implicated in some neurodegenerative diseases such as AD. Some reports indicate that the effects of Aβ may be expressed through the amylin receptor and non-α7 nAChRs[4,5]. Based on the previous results, we hypothesized that amylin and nicotinic responses might be coupled and therefore sought to examine the inter-relationship between amylin receptor and nAChRs in acutely isolated rat basal forebrain cholinergic neuron using whole cell patch-clamp recordings.

1 Methods

1.1 Reagents and animal
Male Sprague Dawley rats (20–25 day postnatal, from Experimental Animal Center of Henan Province) were used in the present study. They were housed at (22.8 ± 0.8) °C in a 12:12 h light-dark cycle. All chemicals used in electrophysiological recording were purchased from Sigma-Aldrich. The stock solutions of nicotine, hAmylin, AC253 (hAmylin receptor antagonist) and dihydro-β-erythroidine (DHβE, α4-nAChR antagonist) were prepared by dissolving the drugs at 1 mmol/L in deionized water and stored in aliquots at −70 °C. On the day of the experiment, drugs were diluted in perfusate just before the time of application.

1.2 Dissociation procedures
Brains were quickly removed from decapitated male Sprague Dawley rats and placed in cold artificial cerebrospinal fluid (ACSF) that contained (in mmol/L) 140 NaCl, 2.5 KCl, 1.5 CaCl2, 1.2 MgCl2, 10 HEPES, and 33 D-glucose (pH 7.4). Brain slices (350-µm thick) were cut on a vibratome (VT 1000S, Germany), and the area containing the horizontal limb of the DBB was dissected out. Although most of the tissue contained the horizontal limb of the DBB, some slices may have also included the vertical limb of the DBB. Acutely dissociated neurons were prepared by enzymatic treatment of the slice with 0.02% trypsin at 30 °C, followed by mechanical trituration for dispersion of individual cells. The neurons were then plated on poly-L-lysine (0.005% weight/volume)-coated cover slips and viewed under an inverted microscope. All solutions were kept oxygenated by continuous bubbling with pure oxygen.

1.3 Electrophysiological recordings
Whole cell patch-clamp recordings were performed at room temperature (20–22 °C) using an Axopatch-700B amplifier combination with a Digidata 1440A data acquisition system (Axon Instruments). Patch electrodes (World Precision Instruments, thin wall with filament 1.5-mm diameter) were pulled (PC-10, Narishige scientific instrument Lab, Japan) to yield resistances of 3–6 MΩ. Internal patch pipette solution contained (in mmol/L) 140 K-methylsulfate, 10 EGTA, 5 MgCl2, 1 CaCl2, 10 HEPES, 2.2 Na2-ATP, and 0.3 Na-GTP (pH 7.2). All whole-cell recordings were made in current-clamp and voltage-clamp mode, and bridge balance and capacitance compensation were used. After whole cell configuration was established with voltage clamp mode (holding potential, −80 mV), we waited at least 5 min for the cell to stabilize, then started either voltage-clamp studies or switched to current-clamp recording mode. The current and membrane voltages were recorded using a low-pass-filter at 5 kHz and digitized at 10 kHz. Data were collected and analyzed by pCLAMP 10.0 software (Axon Instruments).

1.4 Immunoblotting
Western blotting was performed. The sample protein of acutely dissociated DBB neurons was separated using 5% or 15% SDS polyacrylamide gel electrophoresis (SDS-PAGE) and immobilized on the nitrocellulose
membranes, then probed with RAMP3 and α4-nAChR antibodies (Santa Cruz Biotechnology, dilution 1 : 200).

1.5 Statistical analysis
The results were expressed as means ± SEM. The statistical comparisons were made using one-way analysis of variance and Student’s t-test. P values less than 0.05 were considered significant.

2 Results
The stable whole-cell recordings were obtained from a total of 72 DBB neurons during current clamp configuration. The membrane capacitance of the DDB cells used in these studies ranged from 2.0 to 12.5 pF (6.9 pF ± 0.3 pF). The average series resistance was (8.9 ± 0.3) MΩ.

2.1 Nicotine and hAmylin evoked membrane depolarization in DBB neurons
We examined the effects of nicotine and hAmylin on whole-cell membrane potential in the rat DBB neurons. In a total of 36 neurons, nicotine (10 nmol/L) and hAmylin (10 nmol/L) caused membrane depolarization of (4.7 ± 1.1) mV and (6.25 ± 0.8) mV, respectively (Fig. 1). The effects of nicotine and hAmylin on membrane depolarization were dose-dependent in the range from 1 nmol/L to 20 µmol/L. The EC₅₀ values for nicotine and hAmylin were 0.42 and 0.24 µmol/L separately (Fig. 1). Four DBB neurons did not show the hAmylin- and nicotine-induced membrane depolarization.

2.2 Effects of hAmylin were blocked by DHβE and AC253
Thirty-two cells were recorded to examine the excitatory effect of hAmylin and nicotine on DBB neurons. The repetitive spike discharges were elicited by injection of a 1-s depolarization current pulse (0.2–0.3 nA). In the presence of hAmylin (10 nmol/L), the firing frequency of DBB neurons was significantly increased from (5 ± 2.3) Hz (control group) to (62 ± 8.3) Hz in hAmylin-treated group (n = 9, P < 0.001) (Fig. 2A). Two cells did not show hAmylin-induced excitatory effect. DHβE (10 nmol/L) blocked the increase in excitability induced by hAmylin. The firing rate was decreased from (62 ± 8.3) Hz in hAmylin-treated cells to (6.0 ± 4.1) Hz when using DHβE (P < 0.001). While AC253 was injected, the increase in firing rate induced by hAmylin was reduced from (51 ± 8.8) Hz to (13 ± 0.3) Hz (Fig. 2B, n = 6, P < 0.001).

2.3 Effects of nicotine were blocked by DHβE and AC253
The effect of nicotine on DBB neurons was also tested in a total of 15 DBB neurons. Of these, 6 cells were tested with DHβE, and 6 cells with AC253. Application of nicotine (10 nmol/L) increased the frequency of firing in response to depolarizing current pulses (Fig. 3). On average, the firing rate increased from (1.2 ± 0.4) Hz under control conditions to (66.0 ± 4.5) Hz in the presence of nicotine (P < 0.001). DHβE inhibited the nicotine-induced firing rate from (66.0 ± 4.5) Hz to (2.7 ± 1.0) Hz (P < 0.001). Also, AC253 inhibited the nicotine-induced firing rate from (58 ± 10) Hz to (3.8 ± 2.7) Hz (P < 0.001). In 3 DBB neurons, nicotine did not evoke a significant change in firing rate.

Fig. 1. Nicotine (A) and hAmylin (B) evoked a dose-dependent membrane depolarization of DBB neurons. Upper parts were dose-effect curves. Lower parts were original membrane potential recordings under chemical treatments. EC₅₀ for nicotine and hAmylin were 0.42 and 0.24 µmol/L, respectively.
2.4 The α4 nAChR subunits and RAMP3 proteins were present in neurons
Western blots were performed on tissue containing DBB in order to identify the presence of α4-nAChR subunits and RAMP3, a component of the amylin receptor. Figure 4 showed the results of the blots probed with antibodies of α4-nAChR subunits and RAMP3. It revealed the presence of bands at 50 kDa (α4-nAChR), and 35 kDa (RAMP3 protein).

3 Discussion
Aβ, as a toxic substance, might promote neurodegeneration through interacting with the nAChRs, which is widely expressed throughout the CNS\(^8\). The nAChR may mediate Aβ\(_{1-42}\)-induced cytotoxicity via a high affinity interaction between the two proteins. Loss of nicotinic receptors enhances Aβ oligomer accumulation, exacerbating early-stage cognitive decline\(^7\). Several lines of evidence suggest that amyloid deposition in the brain contributes to neuronal degeneration in AD\(^8\). Despite the presence of large amounts of amyloid in AD, it is not clear how amyloid deposition contributes to the degeneration of neurons. Tucker et al.\(^9\) reported that amylin forms large beta-pleated neurotoxic oligomers which show only 38% sequence similarity to Aβ. Morphologic and quantitative measures of cell death show widespread apoptotic death after amylin treatment. The amylin-induced neuronal death is genetically similar to that of Aβ suggests that these peptides may be neurotoxic through a common mechanism. Some experiments demonstrated that amylin induced the depolarization of membrane potential and increased the action potential frequency in the subfornical organ (SFO) neurons\(^10\). The voltage-clamp recordings have shown that amylin influences a voltage-dependent current activated at depolarized potentials, which may represent actions on the persistent Na\(^+\) current \(^11\). The amylin-induced excitation was blocked by specific amylin receptors antagonist AC-187.

Recent data suggest that many receptors can interact with RAMPs. RAMP interaction with receptors can
lead to a variety of actions that include chaperoning of the receptor protein to the cell surface and the generation of novel receptor phenotypes [12–14]. RAMP3, the single transmembrane spanning molecules, when associated with CTR, altered its pharmacology from CTR to amylin receptor. Further studies have shown that RAMP has a long extracellular N-terminal domain and a short intracellular C-terminal domain (approximately 10 amino acids). The N-terminus of RAMPs probably contains two disulphide bonds; a potential third disulphide is found in RAMP1 and RAMP3. The N-terminus was the principal domain involved in generation of amylin receptors from the CTR. The N-terminus domain is sufficient to maintain a functional interaction between two proteins [12], which indicated RAMP N-terminus could play an important role in reconstituting the coupling of receptor complexes. The new data indicate that the short C-terminal domains are important determinants of function. RAMP3 interacts with N-ethylmaleimide-sensitive factor lead to altered receptor targeting after agonist stimulation [13]. The C-terminus has a unique role in the stimulation of cAMP production by associated with CTR.
the peptide ligands. These results suggest that the C-terminus of the RAMPs can determine what signaling pathway is activated by the coupling receptor complex [14]. More recent work has revealed that the specific RAMP presenting in a heterodimer may modulate other functions such as receptor internalization and recycling and also the strength of activation of downstream signaling pathways [14]. Our results showed the nAChR subunits and RAMP3 proteins are co-expressed on DBB neurons, which suggested that nAChR and hAmylin receptor might reconstitute a novel coupling of receptor complexes via RAMP.

The mechanism of amylin neurotoxicity is largely unknown. Our data showed nicotine and hAmylin depolarized DBB neurons in a dose-dependent manner. The excitability increase evoked by hAmylin can be blocked by nAChR antagonist as well as amylin receptor antagonist. The excitability increase evoked by nicotine can be blocked by amylin receptor antagonist as well as nAChR antagonist. So we can infer that nicotine and hAmylin have cooperation effects on DBB neurons.

Our data suggests that hAmylin receptor and nAChRs are coupling receptor complexes with unusual structure (heterodimer). The underlying interaction mechanisms between these two receptors are unclear. However, it may occur at the membrane level to influence downstream signaling pathways. The stimulation of hAmylin receptor maybe induce a conformation change that is transferred to the nAChRs in the membrane through a direct protein-protein interaction. The N-terminal domains of nAChRs and hAmylin receptor may form a binding site for agonists and antagonists. Agonist or antagonist binds to these active sites and leads to receptor-complexes’ structure changes which mediate cell activity (a downstream or upstream activity in DBB neurons). These observations also indicate that two disparate receptors seem to act synergistically to mediate the effects of Aβ on basal forebrain neurons. The synergistic effect of this receptor-complex can make the difference between survival and death of the neurons.

REFERENCES