Available online at www.sciencedirect.com
SCIENCE DIRECT®
European Journal of Pharmacology 468 (2003) 1–8
www.elsevier.com/locate/ejphar

6β-Acetoxy nortropane regulated processing of amyloid precursor protein in $CHOm_1$ cells and rat brain

Yu Qiu, Hong-zhuan Chen*, Xing-jun Wu, Zheng-jun Jin

Department of Pharmacology, Drug Research Institute, Shanghai Second Medical University, Shanghai 200025, China

Received 12 November 2002; received in revised form 17 March 2003; accepted 25 March 2003

Abstract

The effects of the muscarinic receptor agonist 6β-acetoxy nortropane on amyloid precursor protein (APP) processing were studied in both transfected Chinese hamster ovary cells stably expressing muscarinic M_1 receptors (denoted as $CHOm_1$ cell line) and in cerebral cortical and hippocampal slices. Exposure of $CHOm_1$ cells to 6β-acetoxy nortropane for 1 h significantly increased the secretion of secretory amyloid precursor protein (derived from α-secretase cleavage) in a concentration-dependent manner. In the same system, 6β-acetoxy nortropane reduced the β-amyloid peptide production. Similar results were obtained in hippocampal and cerebral cortical slices, with 6β-acetoxy nortropane administration resulting in an increase in secretory amyloid precursor protein and a decrease in β-amyloid peptide release. The increase of secretory amyloid precursor protein secretion was abolished by preincubation with selective muscarinic M_1 receptor antagonist pirenzepine, but not by preincubation with selective muscarinic M_2 receptor antagonist methoctramine, suggesting that 6β-acetoxy nortropane promotes secretory amyloid precursor protein release in the brain via muscarinic M_1 receptor activation. These results suggest that 6β-acetoxy nortropane could exert a beneficial effect on the progress of Alzheimer's disease by promoting amyloid precursor protein processing through α-secretase.

Keywords: Muscarinic receptor; Amyloid precursor protein; Alzheimer's disease

1. Introduction

Alzheimer's disease is a neurodegenerative condition characterized by a progressive decline in cognitive function. The neuropathology of Alzheimer's disease is characterized by the degeneration of neurons in the nucleus basalis of Meynert, and the formation of neurofibrillary tangles and senile plaques (Terry et al., 1991). Approximately 94% of the neurons in nucleus basalis of Meynert are cholinergic neurons (Mesulam and Mufson, 1984) that project into hippocampus and cerebral cortex (Mesulam and Geyla, 1988). Many studies have shown that degeneration of neurons in nucleus basalis of Meynert may play an important role in pathogenesis of Alzheimer's disease (Mesulam and Mufson, 1984; Mesulam and Geyla, 1988; Bierer et al., 1995).

On the other hand, the deposition of β-amyloid peptide, a 4-kDa peptide composed of 39–43 amino acids, is thought to be a critical factor in the formation of senile plaques. Several lines of evidence suggest that β-amyloid peptide, which is derived from proteolytic processing of amyloid precursor protein (APP), has neurotoxic effects. These effects may be mediated by the generation of free radical species, by activation of microglial cells, or by induction of apoptosis (Behl et al., 1994; El Khoury et al., 1996; Yan et al., 1999). APP is a ubiquitous glycosylated transmembrane protein but is most abundant in the brain. Full-length APP isoforms vary from 651 to 770 amino acid long, and β-amyloid peptide is an internal peptide that begins 99 residues from the carboxy terminus and extends 12–15 residues into the hydrophobic transmembrane domain. Cleavage of APP can occur via several pathways (Mattson, 1997; Mills and Reiner, 1999). The major route is by α-secretase, an enzyme that cleaves within β-amyloid peptide sequence thereby preventing the formation of β-amyloid peptide, while generating soluble secretory APP (APPs), which has neurotrophic effects (Mattson et al., 1993; Qui et al., 1995). This pathway is called

* Corresponding author. Tel.: +86-21-63846590x776451; fax: +86-21-63842916.
E-mail address: chz@shsmu.edu.cn (H. Chen).

0014-2999/03/$ - see front matter © 2003 Elsevier Science B.V. All rights reserved.
doi:10.1016/S0014-2999(03)01665-0

nonamyloidogenic secretory pathway. Alternative to α-secretase cleavage and APPs secretion, full-length APP can be reinternalized from the cell surface via clathrin-coated pits that are targeted for the acidic endosomal/lysosomal compartment. In late endosomes and in lysosomes, APP is processed by β- and γ-secretase at the N- and C-terminal ends of β-amyloid peptide, respectively, and generates β-amyloid peptide and soluble secretory APP with less neurotrophic functions (Furukawa et al., 1996). In Alzheimer's disease, altered processing of the APP and its overexpression are associated with the production of β-amyloid peptide, and thus the formation of senile plaques.

A growing body of evidence indicates that there is mutual facilitation between degeneration of cholinergic neurons and β-amyloid peptide deposit in Alzheimer's disease. It has been shown that hypocholinergic activity in cortex and hippocampus elevated APP expression but reduced APPs secretion (Rossner et al., 1997; Leanza, 1998). In contrast, repeated in vivo administration of β-amyloid peptide fragment 25–35 decreases muscarinic receptors in cerebral cortex (Pavia et al., 2000). Many studies have demonstrated the neurotoxic effects of β-amyloid peptide to cholinergic neurons such as suppression of acetylcholine synthesis (Allen et al., 1997; Hoshi et al., 1997), inhibition of high-affinity choline uptake and acetylcholine release (Kar et al., 1996, 1998), acetylcholinesterase expression increase (Sberna et al., 1997), promotion of potassium outflux (Colom et al., 1998) and disruption of carbachol-induced muscarinic cholinergic signal transduction (Kelly et al., 1996). Therefore, hypocholinergic activity can induce β-amyloid peptide formation, which further aggravates cholinergic deficiency. On the other hand, the cellular APP processing can be regulated by cholinergic activity. In particular, α-secretase processing of APP can be accelerated by the stimulation of muscarinic M_1 receptors (Nitsch et al., 1992; Roberson and Harrell, 1997; Hellstrom-Lindahl, 2000). The formation of β-amyloid peptide is concomitantly decreased. The study of Beach et al (2001) showed that muscarinic M_1 receptor agonists decreased β-amyloid peptide concentrations in cerebrospinal fluid. Postmortem analyses of the brains of Alzheimer patients indicate no change in postsynaptic muscarinic M_1 receptor density in cortex and hippocampus (Pearce and Potter, 1991). Thus, treatment with muscarinic M_1 receptor agonists may have the added benefit of affecting the progress of Alzheimer's disease by inhibiting β-amyloid peptide deposition.

6β-Acetoxy nortropane (6β-AN) (see Fig. 1 for structural formula) is an analogue of Bao Jia Sou (2β-hydroxy-6β-acetoxy nortropane) isolated from a Chinese herb, Erycibe Obtusifolia Benth. 6β-AN, which was first synthesized in Shanghai Second Medical University, structurally belongs to the cholinergic tropane family and is closely related to atropine. Previous studies showed that 6β-AN was a potent muscarinic receptor agonist (Yu and Sun, 1990) and could significantly improve scopolamine-induced cognitive impairments (Tang, 1992). In the present study, both a Chinese hamster ovary (CHO) cell line stably expressing

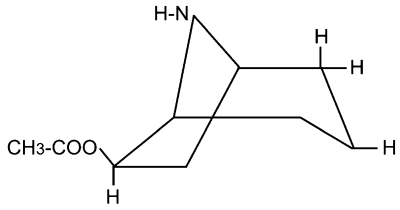

Fig. 1. Structure of 6β-AN, an analogue of Bao Jia Sou (2β-hydroxy-6β-acetoxy nortropane) isolated from the Chinese herb, Erycibe Obtusifolia Benth.

muscarinic M_1 receptors (denoted as $CHOm_1$ cell line) and freshly prepared rat cerebral cortex and hippocampus slices were used to explore the possible effects of 6β-AN on APPs and β-amyloid peptide secretion.

2. Materials and methods

2.1. Materials

6β-AN was synthesized in the Department of Chemistry of Drug Research Institute in Shanghai Second Medical University (Shanghai, China). For the experiments described in this report, 6β-AN was dissolved in distilled water.

Carbachol, pirenzepine, atropine, 1-(5-isoquinolinesulfonyl)-2-methylpiperazine (H-7), aprotinin (Sigma), methoctramine (RBI), phenylmethylsulfonyl fluoride, EGTA (Amresco) and monoclonal antibody 6E10 (Senetek) which recognizes the amino acid sequence 1–17 of β-amyloid peptide were used in this study.

2.2. Cell culture

Chinese hamster ovary cell line stably transfected with human cDNA for muscarinic M_1 receptors (denoted as $CHOm_1$ cell line) was originally from Dr. Buckley. Receptor densities were determined in saturation binding experiments with [^{3}H]quinuclidinyl benzilate and found to be 2290 fmol/mg protein. Cells were cultured at 37 °C in a humidified CO_2 (5%) incubator in Dulbecco's modified Eagle's medium (Gibco BRL) supplemented with fetal bovine serum (10%), penicillin (100 U/ml), streptomycin (100 U/ml) and glutamine (4 μM). Cells were grown to confluency and were then washed twice with Dulbecco's phosphate-buffered saline plus 1 g/l glucose (D-PBS/G). Cells were incubated in D-PBS/G containing various concentrations of 6β-AN or carbachol. Then the media were collected and centrifuged to remove detached cells.

2.3. Brain slice preparation

Male Sprague–Dawley rats weighing from 400 to 500 g rats (Certificate no. 02-23-4) were obtained from the Animal Center of Shanghai Second Medical University and were strictly treated in accordance with the National

Institutes of Health Guide for the Care and Use of Laboratory Animals. Rats were anesthetized with ketamine and decapitated. The brains were rapidly removed from the cranium and transferred into ice-cold Krebs–Ringer buffer (KRB) (1.2 mM $CaCl_2$, 120 mM NaCl, 3.5 mM KCl, 1.2 mM $MgSO_4$, 1.2 mM NaH_2PO_4, 25 mM $NaHCO_3$, 10 mM glucose, pH 7.4, equilibrated with 95% O_2/5% CO_2). After careful removal of meninges and blood vessels, the hippocampus and cerebral cortex were dissected from both hemispheres, and slices (300 μm) were prepared at 4 °C on a Mc^{2}lwain tissue chopper (3K18, China). Slices were washed with cold buffer three times to remove debris and were equilibrated for 50 min in oxygenated KRB at 37 °C. Approximately 8–10 slices were transferred into a chamber with fresh KRB containing 100 μg/ml phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin and various concentrations of 6β-AN or carbachol were added. The slices were incubated for 1 h at 37 °C. At the end of the incubation period, the media were collected and centrifuged at 15,000 × *g* for 10 min at 4 °C to remove debris before APPs and β-amyloid peptide determinations.

2.4. Quantitation of APPs secretion

The protein content of the collected media was measured. The media were desalted by dialysis, lyophilized, and then reconstituted in gel loading buffer (100 mM Tris, pH 6.8, 4% sodium dodecyl sulfate (SDS), 20% glycerol, 200 mM dithiothreitol, 0.2% bromophenol blue). Reconstituted media proteins were boiled for 3 min and loaded on 10% acrylamide/SDS minigels and blotted onto nitrocellulose sheets. Remaining binding sites were blocked for 1 h with 10% (w/v) powdered milk in phosphate-buffered saline containing 0.05% Tween-20 (PBST). Membranes were immunoblotted with monoclonal antibody 6E10 (1:200) for 2 h at room temperature, washed four times in PBST, incubated with sheep anti-mouse horseradish peroxidase-linked secondary antibody, and visualized using Chemiluminescence Reagent (Pierce, USA) followed by exposure to an X-ray film (Kodak). Quantitative determination of the bands was achieved by Tanon image densitometry analysis software (Tanon Technology, China).

2.5. Quantitation of β-amyloid peptide secretion

The collected media were immunoprecipitated with monoclonal antibody 6E10 and protein G-agarose (Roche, Germany) overnight at 4 °C. The protein G-agarose with antigen–antibody complex was centrifuged for 20 s at 12,000 × *g* at 4 °C, washed in 10 mM Tris–HCl (pH 7.4), reconstituted in gel loading buffer (100 mM Tris, pH 6.8, 1% SDS, 4% 2-mercaptoethanol, 0.02% Coomassie brilliant blue G-250, 24% glycerol), and separated on 16.5% Tris/tricine gels and blotted onto nitrocellulose sheets. Following steps were the same as that in quantitation of APPs secretion.

2.6. Statistical analysis

Data are presented as means ± S.E.M. The statistical analysis between two groups was performed using two-tailed Student's *t*-test and differences between groups were assessed as statistically significant when $P < 0.05$.

3. Results

3.1. Effects of 6β-AN on APPs and β-amyloid peptide secretion in $CHOm_1$ cells

Stimulation of human muscarinic M_1 receptors stably expressed in $CHOm_1$ cells for 1 h with 6β-AN significantly increased the secretion of APPs (Fig. 2). The increase was concentration-dependent, and it was statistically significant from 0.01 to 100 μM. The maximum effect was at 10 μM and the median effective concentration (EC_{50}) value was 1.32 μM. In contrast, the full

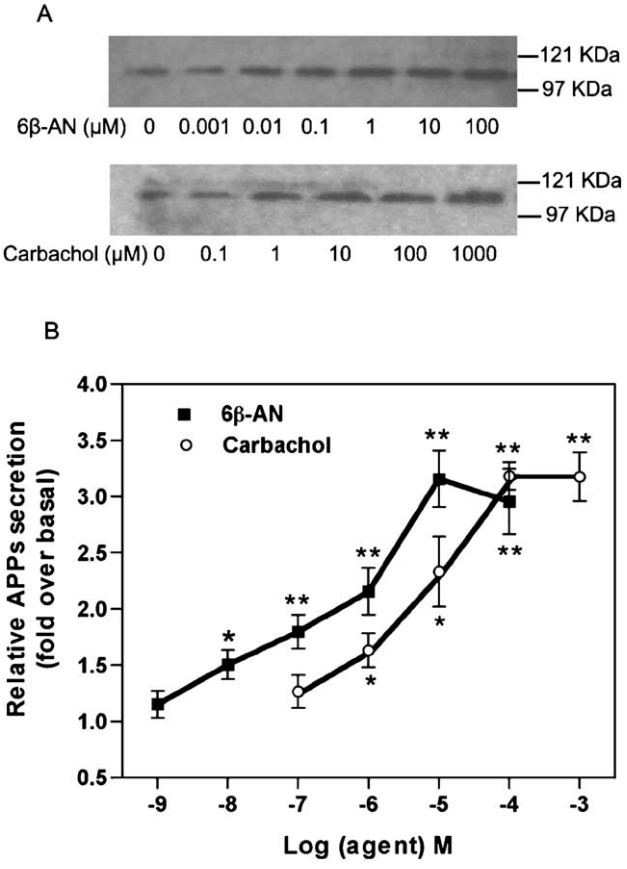

Fig. 2. Concentration-dependent responses of 6β-AN and carbachol on APPs secretion from $CHOm_1$ cells. (A) $CHOm_1$ cells were stimulated for 1 h with various concentrations of 6β-AN (upper panel) or carbachol (lower panel), and medium APPs content was measured by Western blotting with antibody 6E10. (B) The relative APPs release levels were plotted as fold over basal. Data are means ± S.E.M. of three independent experiments. *$P < 0.05$, **$P < 0.01$ versus basal.

muscarinic receptor agonist carbachol failed to change basal APPs secretion at doses less than 1 μM. The EC_{50} value for carbachol was 1.56 μM. The maximal effects for 6β-AN and carbachol were almost the same, which suggests that the intrinsic activity of 6β-AN at recombinantly expressed muscarinic M_1 receptors is the same as that of carbachol.

We measured the APPs secretion after exposure to 6β-AN or carbachol for 10, 20, 30 and 60 min (Fig. 3). The results revealed that the effects of 6β-AN and carbachol on APPs release were time-dependent. For 6β-AN, the half-maximal stimulation was achieved within 20 min, and the maximum stimulation was attained within 30 min. Whereas, longer incubation period was needed to increase the APPs secretion for carbachol.

To detect the effect of 6β-AN on β-amyloid peptide production, the $CHOm_1$ cells were incubated with 10 μM 6β-AN for 3, 6 and 12 h, and the β-amyloid peptide production was determined by immunoprecipitation and Western blotting (Fig. 4). As shown in the figure, significant reduction in β-amyloid peptide secretion during 6β-AN incubation was observed. Similarly, 1 mM carbachol also reduced β-amyloid peptide production after 3, 6 and 12 h of incubation. Based on the results obtained from APPs release, $CHOm_1$ cells were exposed to 0.01 μM, 1 μM and 100 μM of 6β-AN, or 0.1 μM, 10 μM and 1 mM carbachol. As shown in Fig. 5, 6β-AN and carbachol decreased β-amyloid peptide release in a concentration-dependent manner, and it was statistically significant at 1 μM and 100 μM for 6β-AN and at 10 μM and 1 mM for carbachol.

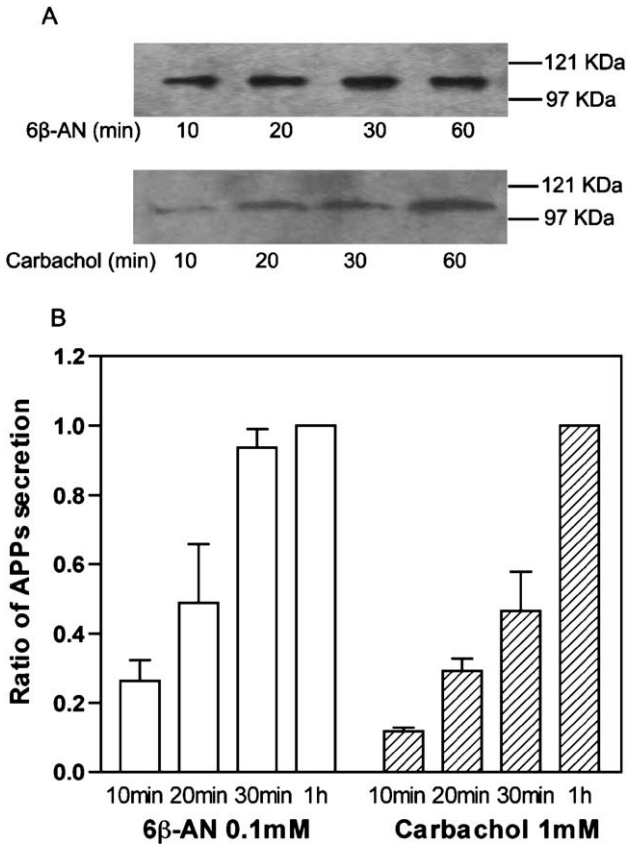

Fig. 3. Time-dependent responses of 6β-AN and carbachol on APPs secretion from $CHOm_1$ cells. (A) $CHOm_1$ cells were stimulated for various time with 0.1 mM 6β-AN (upper panel) or 1 mM carbachol (lower panel), and medium APPs content was measured by Western blotting with antibody 6E10. (B) The relative APPs release levels were plotted as a ratio of the response after 1-h incubation. Data are means ± S.E.M. of three independent experiments.

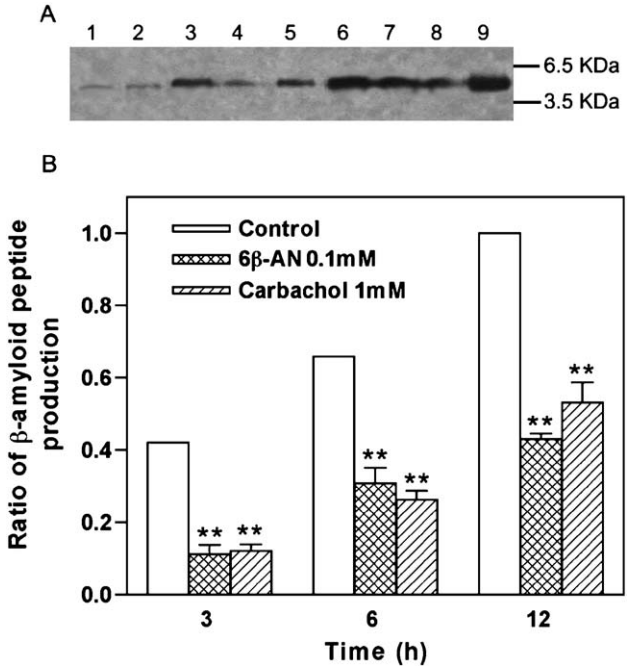

Fig. 4. Time-dependent responses of 6β-AN and carbachol on β-amyloid peptide production from $CHOm_1$ cells. (A) β-Amyloid peptide in medium was immunoprecipitated and measured by Western blotting with antibody 6E10. $CHOm_1$ cells were incubated for 3 h (lanes 1–3), 6 h (lanes 4–6) and 12 h (lanes 7–9). Lanes 1, 4 and 7: $CHOm_1$ cells were treated with 0.1 mM 6β-AN; lanes 2, 5 and 8: $CHOm_1$ cells were treated with 1 mM carbachol; lanes 3, 6 and 9: Control. (B) The relative β-amyloid peptide production levels were plotted as a ratio of 12-h control. Data are means ± S.E.M. of three independent experiments. $^{**}P < 0.01$ versus respective control.

As shown in Table 1, 10 μM atropine completely blocked the APPs release stimulated by 6β-AN and carbachol. To test the possibility that Ca^{2+} influx and protein kinase C (PKC) are involved sequentially in APPs release, EGTA was used to chelate extracellular Ca^{2+} and H-7 was used to inhibit the activity of PKC. Both EGTA and H-7 partially blocked the effects of 6β-AN and carbachol.

3.2. Effects of 6β-AN on APPs and β-amyloid peptide secretion in hippocampal and cerebral cortical slices

In order to test whether 6β-AN can modulate APP processing in a physiologically relevant brain tissue envi-

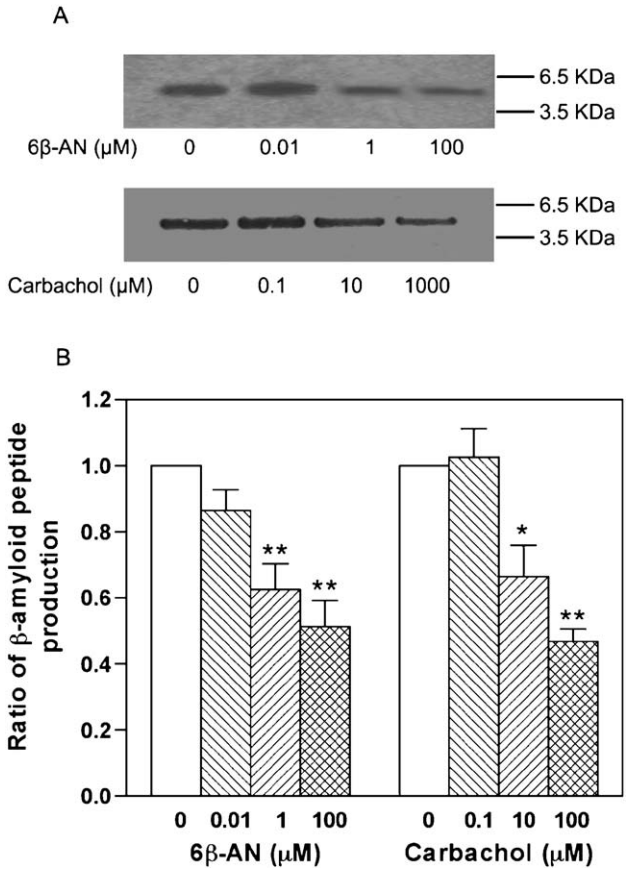

Fig. 5. Concentration-dependent responses of 6β-AN and carbachol on β-amyloid peptide production from $CHOm_1$ cells. (A) $CHOm_1$ cells were stimulated for 6 h with various concentrations of 6β-AN (upper panel) or carbachol (lower panel), and β-amyloid peptide in medium was immunoprecipitated and measured by Western blotting with antibody 6E10. (B) The relative β-amyloid peptide production levels were plotted as a ratio of control. Data are means ± S.E.M. of three independent experiments. $^{*}P<0.05$, $^{**}P<0.01$ versus control.

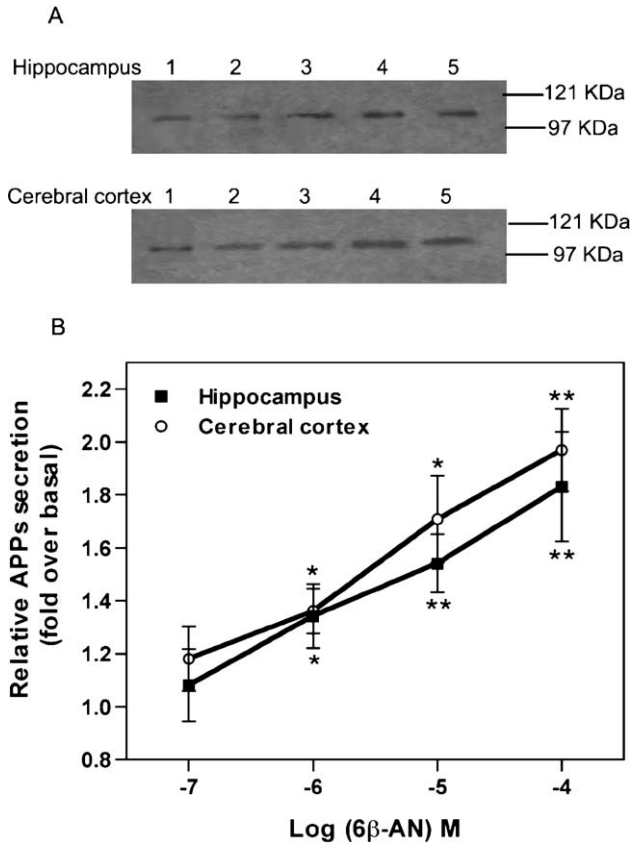

Fig. 6. Concentration-dependent responses of 6β-AN on APPs secretion from rat hippocampal and cerebral cortical slices. (A) Hippocampal (upper panel) and cerebral cortical (lower panel) slices were stimulated with 0, 0.1, 1, 10 and 100 μM (lanes 1–5) 6β-AN, and medium APPs content was measured by Western blotting with antibody 6E10. (B) The relative APPs release levels were plotted as fold over basal. Data are means ± S.E.M. of three independent experiments. $^{*}P<0.05$, $^{**}P<0.01$ versus basal.

ronment, we exposed freshly prepared hippocampal and cerebral cortical slices to various concentrations of 6β-AN by adding it to the medium (Fig. 6). In comparison to unstimulated control slices, 6β-AN clearly increased APPs secretion in both hippocampal and cerebral cortical slices after 1 h incubation. The effect of 6β-AN was concentration-dependent from 1 to 100 μM. The increases caused by 1 μM of 6β-AN were statistically significant ($P<0.05$). The increase in APPs secretion by 6β-AN (100 μM) in hippocampal and cerebral cortical slices was 1.83 and 1.97 times of basal levels, respectively.

The nonselective antagonist atropine (10 μM) prevented the increase in APPs secretion in response to 6β-AN. This inhibitory effect was also observed when hippocampus

Table 1
Effects of atropine, EGTA and H-7 on carbachol- and 6β-AN-stimulated APPs secretion from $CHOm_1$ cells

	APPs release (fold over basal)			
	Control	Atropine	EGTA	H-7
Carbachol	3.18 ± 0.22	1.16 ± 0.20[a]	1.57 ± 0.11[a]	1.75 ± 0.10[a]
6β-AN	2.95 ± 0.29	1.01 ± 0.18[a]	1.59 ± 0.16[b]	1.61 ± 0.17[b]

$CHOm_1$ cells were treated with 10 μM atropine, 0.3 mM EGTA or 50 μM H-7 for 10 min, then stimulated with 1 mM carbachol or 0.1 mM 6β-AN for another 1 h. $CHOm_1$ cells were treated with no Ca^{2+} Dulbecco's PBS when EGTA was administered. Data are means ± S.E.M. of three independent experiments.

[a] $P<0.01$ versus group treated with carbachol or 6β-AN alone.

[b] $P<0.05$ versus group treated with carbachol or 6β-AN alone.

Table 2
Effects of muscarinic antagonists on 6β-AN-stimulated APPs release

Groups	APPs release (fold over basal)
6β-AN	1.83 ± 0.21
6β-AN + Atropine (10 mM)	1.09 ± 0.05[a]
6β-AN + Pirenzepine (1 mM)	1.08 ± 0.13[a]
6β-AN + Methoctramine (1 mM)	1.77 ± 0.15

Rat hippocampal slices were treated with atropine, pirenzepine or methoctramine for 10 min, then treated with 100 μM 6β-AN for another 1 h. Data are means ± S.E.M. of three independent experiments.

[a] $P<0.05$ versus group treated with 6β-AN alone.

slices were preincubated with selective muscarinic M_1 receptor antagonist pirenzepine at 1 μM concentration, while preincubation with the selective muscarinic M_2 receptor antagonist methoctramine did not abolish the effect of 6β-AN (Table 2).

Incubation of hippocampal slices with 6β-AN at concentration of 1 μM for 1 h also significantly reduced the β-amyloid peptide release ($P<0.05$) with inhibition rate of $27.37 \pm 7.95\%$. Higher concentration of 6β-AN at 100 μM resulted in an inhibition rate of $66.98 \pm 4.70\%$ ($P<0.01$). These results were obtained from three independent experiments (data not shown).

4. Discussion

In agreement with previous studies (Nitsch et al., 1992; Eckols et al., 1995), exposure of transfected cells stably expressing human muscarinic M_1 receptors to the muscarinic receptor agonist carbachol resulted in increased APPs release and decreased β-amyloid peptide production. These effects were blocked by the muscarinic antagonist atropine. Here we demonstrated that 6β-AN increased APPs release and decreased β-amyloid peptide secretion in $CHOm_1$ cells. These effects can be blocked by atropine, implying that the effects of 6β-AN on APP processing are muscarinic M_1 receptor-mediated.

Activation of muscarinic M_1 receptors stimulates phospholipase C activity in various cells, leading to the activation of PKC as well as the release of Ca^{2+} from intracellular stores. At the same time, Ca^{2+} influx also occurs after muscarinic M_1 receptor activation. To detect the role of PKC and Ca^{2+} influx played in the muscarinic M_1 receptor-mediated APP processing, the PKC inhibitor H-7 and the extracellular Ca^{2+} chelator EGTA were administered 10 min before the addition of carbachol or 6β-AN (Table 1). H-7 and EGTA decreased the release of APPs, suggesting that activation of PKC and elevation in intracellular Ca^{2+} concentration are involved in the stimulation of APPs release by carbachol or 6β-AN. Since the elevation of intracellular Ca^{2+} after muscarinic M_1 receptor activation is primarily mobilized from intracellular stores, and extracellular Ca^{2+} influx only contributes a small portion in the elevation of intracellular Ca^{2+} (Dolezal et al., 1997), the elevation of intracellular Ca^{2+} secondary to increased Ca^{2+} influx might play a relatively important role in the stimulation of APPs release. Petryniak et al. (1996) showed that Ca^{2+} influx increased secretory processing of APP by initiating PKC-independent and tyrosine phosphorylation-dependent pathways.

In this study, the effects of 6β-AN on APPs release and β-amyloid peptide production were also studied in slices of hippocampus and cerebral cortex. We found that 6β-AN effectively increased APPs release in both hippocampal and cerebral cortical slices. We also demonstrated that 6β-AN reduced β-amyloid peptide production in hippocampal slices. The nonselective muscarinic antagonist, atropine, abolished the effects of 6β-AN. On the other hand, 6β-AN shows weak affinity for the nicotine receptor (3000-fold less than that of nicotine (Pei et al., 1998)). These results suggest that the effects of 6β-AN are mediated through muscarinic receptors. Although previous studies suggested that 6β-AN is a selective muscarinic M_2 receptor agonist (Yu and Sun, 1990; Yao et al, 1996), 6β-AN does activate muscarinic M_1 receptors, its K_i value versus [^{3}H]quinuclidinyl benzoate for rat cortex–hippocampus (mainly muscarinic M_1 receptors) is 0.158 μM (Yu and Sun, 1990). In addition, muscarinic M_1 receptor is mainly located postsynaptically in cerebral cortex and hippocampus, whereas muscarinic M_2 receptor is located in central cholinergic presynaptic terminals, where it functions as an autoreceptor (Kasa et al., 1997). Thus, in order to observe the role of muscarinic M_1 and M_2 receptors in the stimulation of APPs release by 6β-AN, the selective muscarinic M_1 receptor antagonist, pirenzepine, or the selective muscarinic M_2 receptor antagonist, methoctramine, was administered 10 min before the addition of 6β-AN. Pirenzepine blocked the increase of APPs secretion in response to 6β-AN, whereas methoctramine did not. These data suggest that 6β-AN promote APPs release in brain via muscarinic M_1 receptor activation.

Previously published studies showing increased APPs release from transfected cells and brain slices (Eckols et al., 1995; Muler et al., 1997) have utilized the monoclonal antibody 22C11, which recognizes an amino terminal epitope on APP and cannot distinguish nonamyloidogenic forms (derived from α-secretase cleavage) from amyloidogenic forms (derived from β-secretase cleavage). Therefore, it is uncertain from such studies to what extent nonamyloidogenic forms of APPs may contribute to muscarinic receptor-stimulated APPs release. In our study we utilized monoclonal antibody 6E10, which recognizes the amino acid sequence 1–17 of β-amyloid peptide (DeLapp et al, 1998). Thus the APPs detected here was the nonamyloidogenic form of APPs. Furthermore, our results were supported by the observed β-amyloid peptide reduction from $CHOm_1$ cells and hippocampal and cerebral cortical slices due to muscarinic M_1 receptor activation. This study thus provides further evidence to the notion that the increase in APPs release and the reduction in β-amyloid peptide production are mediated through activation of muscarinic M_1 receptors. To our knowledge, this is the first evidence that muscarinic M_1 receptor-stimulated APP processing occurs via α-secretase pathway in brain slices.

Our results have several implications for the use of muscarinic receptor agonists in the treatment of Alzheimer's disease. Apart from being a neurotransmitter replacement for improvement of cognitive function, muscarinic receptor agonists may delay the progress of Alzheimer's disease by increasing the secretion of APPs, which has neurotrophic and neuroprotective functions, as well as by decreasing β-amyloid peptide formation and thus slowing down amyloid

deposition. The experiment of Yan et al (2000) showed that the muscarinic receptor agonist carbachol time- and dose-dependently blocked apoptosis induced by β-amyloid peptide fragment 31–35 in cultured cortical neurons. In addition, recent study suggested that long-term use of mucarinic M_1 receptor agonists decreased tau hyperphosphorylation, which played a major role in the formation of neurofibrillary tangles (Fisher et al, 2002). All these studies demonstrate other beneficial roles of muscarinic receptor agonists in inhibiting the processes of Alzheimer's disease pathogenesis.

In conclusion, we demonstrated that 6β-AN effectively stimulated APPs secretion and decreased β-amyloid peptide formation in both transfected cells stably expressing human muscarinic M_1 receptor and in hippocampal and cerebral cortical slices. 6β-AN could accelerate APP processing by increasing α-secretase proteolytic cleavage of APP via muscarinic M_1 receptor activation. These results suggest that 6β-AN may be useful for replacement of acetylcholine in Alzheimer's disease as well as for modulating the progress of the disease by promoting APP processing through α-secretase.

Acknowledgements

This study was supported by grant from the National Natural Science Foundation of China (no. 30070860). The authors thank Professor Ya-er Hu (Department of Nuclear Medicine, Drug Research Institute, Shanghai Second Medical University) for kindly providing $CHOm_1$ cells. The authors thank Dr. Maureen Riedl (Department of Neuroscience, University of Minnesota) for literal review of manuscript.

References

Allen, D.D., Galdzicki, Z., Brining, S.K., Fukuyama, R., Rapoport, S.I., Smith, Q.R., 1997. Beta-amyloid induced increase in choline flux across PC12 cell membranes. Neurosci. Lett. 4, 71–73.

Beach, T.G., Walker, D.G., Potter, P.E., Sue, L.I., Fisher, A., 2001. Reduction of cerebrospinal fluid amyloid beta after systemic administration of M1 muscarinic agonists. Brain Res. 29, 220–223.

Behl, C., Davis, J.B., Lesley, R., Schubert, D., 1994. Hydrigen peroxide mediates amyloid protein toxocity. Cell 77, 817–827.

Bierer, L.M., Haroutunian, V., Gabriel, S., Knott, P.J., Carlin, L.S., Purohit, D.P., Perl, D.P., Schmeidler, J., Kanof, P., Davis, K.L., 1995. Neurochemical correlates of dimentia severity in the Alzheimer's disease: relative importance of the cholinergic deficits. J. Neurochem. 64, 749–760.

Colom, L.V., Diaz, M.E., Beers, D.R., Neely, A., Xie, W.J., Appel, S.H., 1998. Role of potassium channels in amyloid-induced cell death. J. Neurochem. 70, 1925–1934.

DeLapp, N., Wu, S., Belagaje, R., Johnstone, E., Little, S., Shannon, H., Bymaster, F., Calligaro, D., Mitch, C., Whitesitt, C., Ward, J., Sheardown, M., Fink-Jensen, A., Jeppesen, L., Thomsen, C., Sauerberg, P., 1998. Effects of the M_1 agonist xanomeline on processing of human beta-amyloid precursor protein (FAD, Swedish mutant) transfected into Chinese hamster ovary-m_1 cells. Biochem. Biophys. Res. Commun. 244, 156–160.

Dolezal, V., Lisa, V., Tucek, S., 1997. Differential effects of the M_1–M_5 muscarinic acetylcholine receptor subtypes on intracellular calcium and on the incorporation of choline into membrane lipids in genetically modified Chinese hamster ovary cell lines. Brain Res. Bull. 42, 71–78.

Eckols, K., Bymaster, F.P., Mitch, C.H., Shannon, H.E., Ward, J.S., DeLapp, N.W., 1995. The muscarinic M_1 agonist xanomeline increases soluble amyloid precursor protein release from Chinese hamster ovary-m_1 cells. Life Sci. 57, 1183–1190.

El Khoury, J., Hickman, S.E., Thomas, C.A., Cao, L., Silverstein, S.C., Loike, J.D., 1996. Scavenger receptor-mediated adhesion of microglia to β-amyloid fibrils. Nature 382, 716–722.

Fisher, A., Brandeis, R., Bar-Ner, R.H., Kliger-Spatz, M., Natan, N., Sonego, H., Marcovitch, I., Pittel, Z., 2002. AF150(S) and AF267B: M1 muscarinic agonists as innovative therapies for Alzheimer's disease. J. Mol. Neurosci. 19, 145–153.

Furukawa, K., Sopher, B.L., Rydel, R.E., Begley, J.G., Pham, D.G., Martin, G.M., Fox, M., Mattson, M.P., 1996. Increased activity-regulating and neuroprotective efficacy of alpha-secretase-derived secreted amyloid precursor protein conferred by a C-terminal heparin-binding domain. J. Neurochem. 67, 1882–1896.

Hellstrom-Lindahl, E., 2000. Modulation of β-amyloid precursor protein processing and tau phosphorylation by acetylcholine receptors. Eur. J. Pharmacol. 393, 255–263.

Hoshi, M., Takashima, A., Murayama, M., Yasutake, K., Yoshida, N., Imahori, K., 1997. Nontoxic amyloid beta peptide 1–42 suppresses acetylcholine synthesis. Possible role in cholinergic dysfunction in Alzheimer's disease. J. Biol. Chem. 272, 2038–2041.

Kar, S., Seto, D., Gaudreau, P., Quirion, R., 1996. β-Amyloid-related peptides inhibit potassium-evoked acetylcholine release from rat hippocampal formation. J. Neurosci. 16, 1034–1040.

Kar, S., Issa, A.M., Seto, D., Auld, D.S., Collier, B., Quirion, R., 1998. Amyloid beta-peptide inhibits high-affinity choline uptake and acetylcholine release in rat hippocampal slices. J. Neurochem. 70, 2179–2187.

Kasa, P., Rakonczay, Z., Gulya, K., 1997. The cholinergic system in Alzheimer's disease. Prog. Neurobiol. 52, 511–535.

Kelly, J.F., Furukawa, K., Barger, S.W., Rengen, M.R., Mark, R.J., Blanc, E.M., Mattson, M.P., 1996. Amyloid beta-peptide disrupts carbachol-induced muscarinic cholinergic signal transduction in cortical neurons. Proc. Natl. Acad. Sci. U. S. A. 93, 6753–6758.

Leanza, G., 1998. Chronic elevation of amyloid precursor protein expressing in the neocortex and hippocampus of rats with selective cholinergic lesions. Neurosci. Lett. 257, 53–56.

Mattson, M.P., 1997. Cellular aspects of β-amyloid precursor protein and its soluble and fibrillogenic derivatives. Physiol. Rev. 77, 1081–1132.

Mattson, M.P., Cheng, B., Culwell, A.R., Esch, F.S., Lieberburg, I., Rydel, R.E., 1993. Evidence for the excitoprotective and interneuronal calcium-regulating roles for secreted forms of the β-amyloid precursor protein. Neuron 10, 243–254.

Mesulam, M.M., Geyla, C., 1988. Nucleus basalis (Ch4) and cortical cholinergic innervation in the human brain: observations based on the distribution on acetylcholinesterase and choline acetyltransferase. J. Comp. Neurol. 274, 216–240.

Mesulam, M.M., Mufson, E.J., 1984. Neural inputs into the nucleus basalis of the substantial innominata (Ch4) in the rhesus monkey. Brain 107, 253–274.

Mills, J., Reiner, P.B., 1999. Regulation of amyloid precursor protein cleavage. J. Neurochem. 72, 443–460.

Muler, D.M., Mendla, K., Farber, S.A., Nitsch, R.M., 1997. Muscarinic M_1 receptor agonists increase the secretion of the amyloid precursor protein ectodomain. Life Sci. 60, 985–991.

Nitsch, R.M., Slack, B.E., Wurtman, R.J., Growdon, J.H., 1992. Release of Alzheimer amyloid precursor derivatives stimulated by activation of muscarinic acetylcholine receptors. Science 258, 304–307.

Pavia, J., Alberch, J., Alvarez, I., Toledano, A., de Ceballos, M.L., 2000. Repeated intracerebroventricular administration of beta-amyloid(25–35) to rats decreases muscarinic receptors in cerebral cortex. Neurosci. Lett. 278, 69–72.

Pearce, B.D., Potter, L.T., 1991. Coupling of M_1 muscarinic receptors to G protein in Alzheimer disease. Alzheimer Dis. Assoc. Disord. 5, 163–172.

Pei, X.F., Gupta, T.H., Badio, B., Padgett, W.L., Daly, J.W., 1998. 6beta-Acetoxynortropane: a potent muscarinic agonist with apparent selectivity toward M_2-receptors. J. Med. Chem. 41, 2047–2055.

Petryniak, M.A., Wurtman, R.J., Slack, B.E., 1996. Elevated intracellular calcium concentration increases secretory processing of the amyloid precursor protein by a tyrosine phosphorylation-dependent mechanism. Biochem. J. 320, 957–963.

Qui, W., Ferreira, A., Miller, C., Koo, E.H., Selkoe, D.J., 1995. Cell-surface β-amyloid precursor protein stimulates neurite outgrowth of hippocampal neurons in an isoform-dependent manner. J. Neurosci. 15, 2157–2167.

Roberson, M.R., Harrell, L.E., 1997. Cholinergic activity and amyloid precursor protein metabolism. Brain Res. Rev. 25, 50–69.

Rossner, S., Ueberham, U., Yu, J., Kirazov, L., Schliebs, R., Bigl, V., 1997. In vivo regulation of amyloid precursor protein secretion in rat neocortex by cholinergic activity. Eur. J. Neurosci. 9, 2125–2134.

Sberna, G., Sez Valero, J., Beyreuther, K., Masters, C.L., Small, D.H., 1997. The amyloid beta-protein of Alzheimer's disease increases acetylcholinesterase expression by increasing intracellular calcium in embryonal carcinoma P19 cells. J. Neurochem. 69, 1177–1184.

Tang, Y.P., 1992. Effects of 6β-acetoxy nortropane on mouse learning and memory. Acta Univ. Med. Secondae Shanghai 12, 219–221.

Terry, R.D., Maslian, E., Salmon, D., Butters, N., De Teresa, R., Hansen, L., Katzman, R., 1991. Physiological basis of cognitive alterations in Alzheimer disease: synapse loss is the major correlate of cognitive impairment. Ann. Neurol. 39, 572–580.

Yan, X.Z., Qiao, J.T., Dou, Y., Qiao, Z.D., 1999. β-Amloid peptide fragment 31–35 induces apoptosis in cultured cortical neurons. Neuroscience 92, 177–184.

Yan, X.Z., Xiao, R., Dou, Y., Wang, S.D., Qiao, Z.D., Qiao, J.T., 2000. Carbachol blocks beta-amyloid fragment 31–35-induced apoptosis in cultured cortical neurons. Brain Res. Bull. 51, 465–470.

Yao, B., Ge, X.Q., Zheng, J.L., Qin, W., Bian, C.F., 1996. Effects of M1 and M2 receptor agonists and blockers on dog respiration. Acta Pharmacol. Sin. 17, 267–270.

Yu, A.Y., Sun, C., 1990. 6β-Acetoxy nortropane and its muscarinic receptor kinetics. Acta. Pharmacol. Sin. 11, 394–400.