BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **244,** 156–160 (1998)
ARTICLE NO. RC988235

Effects of the M1 Agonist Xanomeline on Processing of Human β-Amyloid Precursor Protein (FAD, Swedish Mutant) Transfected into Chinese Hamster Ovary-m1 Cells

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Received January 31, 1998

Complimentary DNA (cDNA) encoding human β-amyloid precursor protein familial Alzheimer's disease (FAD) Swedish mutant (βAPP$_{SM}$) form was cloned into a mammalian expression vector (PK255) containing the CMV promoter. The vector was transfected into Chinese hamster ovary cells containing human muscarinic m1 receptors (CHO-m1), and clonal cells stably expressing βAPP$_{SM}$ were isolated. The effects of m1-receptor activation by the selective m1 agonist xanomeline and the non-selective muscarinic agonist carbachol on processing of βAPP$_{SM}$ to release soluble APP (APPs) and β-amyloid peptide (Aβ) were compared. Xanomeline stimulated APP release with a potency 1000-fold greater than that observed for carbachol. Concentrations of carbachol and xanomeline producing maximal effects on APPs release reduced the secretion of Aβ by 28 and 46%, respectively. These results extend previous studies with xanomeline and suggest that cholinergic replacement therapy for Alzheimer's disease may reduce amyloid deposition. © 1998 Academic Press

One of the characteristics of Alzheimer's Disease (AD) is the deposition of amyloid in senile plaques and in the walls of cerebral blood vessels (1,2). AD amyloid is composed of a 39 to 43 residue amyloid β peptide (Aβ) that is derived from a set of precursor proteins 695 to 770 amino acids long collectively referred to as the β-amyloid precursor protein (βAPP). βAPP is processed either by a constitutive secretory or endosomal-lysosomal pathway (3–7). The secretory pathway which is associated with neuroprotection and neuroplasticity produces a large secreted soluble derivative (APPs), and an 8.7 Kd membrane associated fragment neither of which can produce amyloid plaque (3–5). The alternative endosomal-lysosomal pathway produces a complex set of C-terminal derivatives which include potentially amyloidogenic forms with the entire Aβ sequence at or near their N-terminus (6, 7).

The identification of rare familial AD (FAD) kindreds in which the AD phenotype cosegregates with mutations in the βAPP gene strongly suggests that amyloid deposition is critically important in the development of AD. Three of the FAD linked βAPP mutations convert valine 717 located three residues carboxyl to Aβ_{43} to isoleucine, phenylalanine or glycine (6). A fourth double mutation (FAD, Swedish, βAPP$_{SM}$) changes the lysine 670 and methionine 671 located immediately N-terminal to Aβ_1 to asparagine and leucine (7). These mutations in close proximity to Aβ further suggest that they may cause AD by altering βAPP processing in a way that is amyloidogenic.

Stimulation of muscarinic receptor subtypes m1 and m3 that are coupled to PKC activation via phosphatidylinositol hydrolysis has been shown to increase the release of APPs and decrease the release of Aβ into the medium of cultured cells (9–11). Xanomeline is a novel muscarinic receptor agonist with selectivity for m1 receptors and the ability to readily cross the blood-brain barrier (12–14). It is currently undergoing clinical trials as a cholinergic replacement therapy for AD. In a previous study it was shown that xanomeline potently stimulated the release of endogenous APPs from CHO-m1 cells (15). The present study extends previous work by examining the effect of xanomeline, in comparison to carbachol, on the release of human APPs as well as Aβ from CHO-m1 cells transfected with βAPP$_{SM}$.

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0006-291X/98 $25.00

MATERIALS AND METHODS

Reagents. Xanomeline (LY246708/NNC11-0232, [3(3-hexyloxy-1,2,5-thiadiazol-4-yl)1,2,5,6-tetrahydro-1-methylpyridine)] was synthesized at Eli Lilly and Co. (Indianapolis, IN) or at Novo Nordisk (Måløv, Denmark). Carbachol and atropine sulfate were from Research Biochemicals (Natick, MA). Monoclonal antibodies 6E10 and 4G8 were obtained from Senetek (Maryland Heights, MO). Plasmid PK255 was from Nagaraja Rao at Lilly Research Laboratories (Indianapolis, IN).

Production of the βAPP_{SM} expression vector. The gene coding for βAPP_{SM} (695 isoform) was isolated by PCR from a cDNA clone as described by Kang et. al. (16). Oligonucleotides corresponding to bp -97 to -78 (P1, sense), 895 to 925 (P2, antisense), 900 to 930 (P3, sense), 2521 to 2552 (P4, antisense) and 1763 to 1804 (P5, sense) were synthesized and used as primers to amplify DNA fragments I (1018 bp), II (1630 bp) and III (789 bp) corresponding to the BAPP gene. The primer P1 contained additional recognition sequences for S*pe* I, A*sc* I restriction enzymes whereas the primer P5 contained the codons for Swedish mutations. PCR amplifications were performed for 30 cycles using Taq polymerase (94°C for 1 min., 65°C for 1 min., and 72°C for 2 min.). The amplified fragments I and II were digested with S*pe* I/X*ho* I and X*ho* I/B*cl* I restriction enzymes respectively and cloned into a modified pUC19 plasmid (pRB9.27) between the X*ba* I and B*cl* I restriction sites to form the plasmid pRB9.159 containing the entire coding sequence for βAPP_{SM}. The nucleotide sequences of cloned genes were confirmed by DNA sequencing. For expression of βAPP_{SM}, the A*sc* I/C*la* I restriction fragment (2.63 KB) containing the desired coding sequences was isolated from the plasmid pRB9.159 and then inserted into a mammalian expression vector, pK255 which uses the cyto megalovirus (CMV) promoter to drive the expression, a tetracycline operator and repressor to control gene expression, and a hygromycin resistance gene to select clones in mammalian cells. The resulting plasmid was designated pK255.βAPP_{SM}.

Cell culture and transfection. Cells were grown in Dulbecco's Modified Eagle Medium (DMEM) supplemented with D-glucose, L-glutamate, 1% non-essential amino acids, 10% fetal bovine serum, 100 μg/ml of Geneticin. For selection medium hygromycin at 200 μg/ml was added. Cells were maintained at 37°C under 5%/95% CO_2/O_2 . The pK255.βAPP_{SM} expression vector was transfected into cells using calcium phosphate precipitation and clones were selected by resistance to hygromycin. Clones expressing human APP were further identified by measuring APPs secreted into the medium using western blot analysis employing the monoclonal antibody 6E10.

Quantification of APPs secretion. Cells were grown to confluency in T-25 flasks and were then washed two times with Dulbecco's Phosphate Buffered saline plus 1g/L glucose (D-PBS/G). Cells were incubated in D-PBS/G ± agonists for 30 minutes at 37°C after which the medium was collected and centrifuged to remove detached cells. Supernatants were concentrated and desalted using Centricon 30 centrifugal concentrators (Amicon, Beverly, MA). Cells remaining in flasks were solubilized with 1N NaOH and protein was determined by the Lowry method (17). Sample volumes adjusted to equal amounts of total cell protein per flask were electrophoresed on 10% Tris-glycine gels (Novex, San Diego, CA) and were transferred to nitrocellulose membranes. Samples were immunoblotted with 6E10 using goat anti-mouse IgG horseradish peroxidase-coupled second antibody and the ECL detection system of Amersham (Arlington Heights, IL.).

Bands on X-ray film were quantified using a laser densitometer (Molecular Dynamics, Sunnyvale, CA).

Quantification of Aβ release. Cells were grown to confluence in T-75 flasks. The medium was removed and the cells were incubated for 30 minutes at 37°C in serum and methionine-free medium. The medium was then replaced with serum and methionine-free medium containing 273 μCi/ml of ^{35}S-methionine and the cells were labeled for four hours at 37°C. The labeled medium was removed, the cells were washed once with D-PBS/G, and were then incubated for three hours in serum-free medium containing excess cold methionine with or without muscarinic agonist. After the three hour time interval

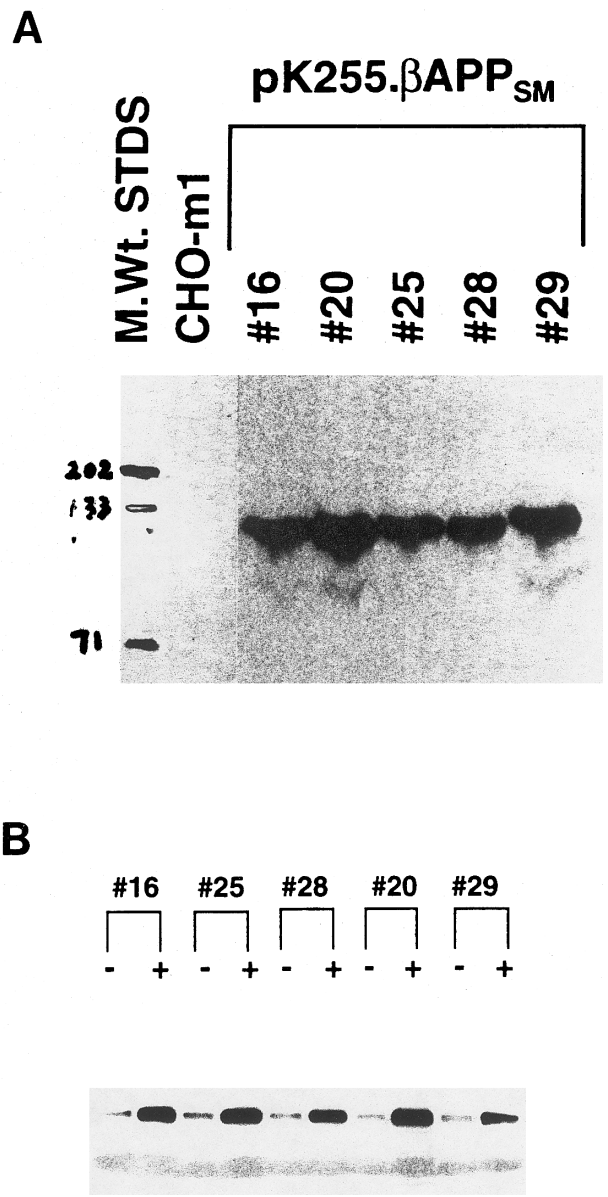

FIG. 1. Basal and stimulated release of APPs from clones stably expressing βAPP_{SM}. The expression vector PK255.βAPP_{SM} was transfected into CHO m1 cells by calcium phosphate precipitation and resulting clones were selected by resistance to hygromycin. Clonal pools were subcloned and individual clones were analyzed for secretion of APPs by western blot analysis with monoclonal antibody 6E10 as described in MATERIALS AND METHODS. In order to examine the effect of m1 receptor stimulation, cells were grown to near confluence and were stimulated with 100 μM carbachol for 30 minutes followed by analysis of APPs released into the medium by western blot analysis with 6E10. (A) Basal overnight release of APPs by clones expressing $BAPP_{SM}$. (B) Basal and carbachol-stimulated APPs release over a 30 minute time interval from clones expressing βAPP_{SM}. −, basal; +, carbachol-stimulated.

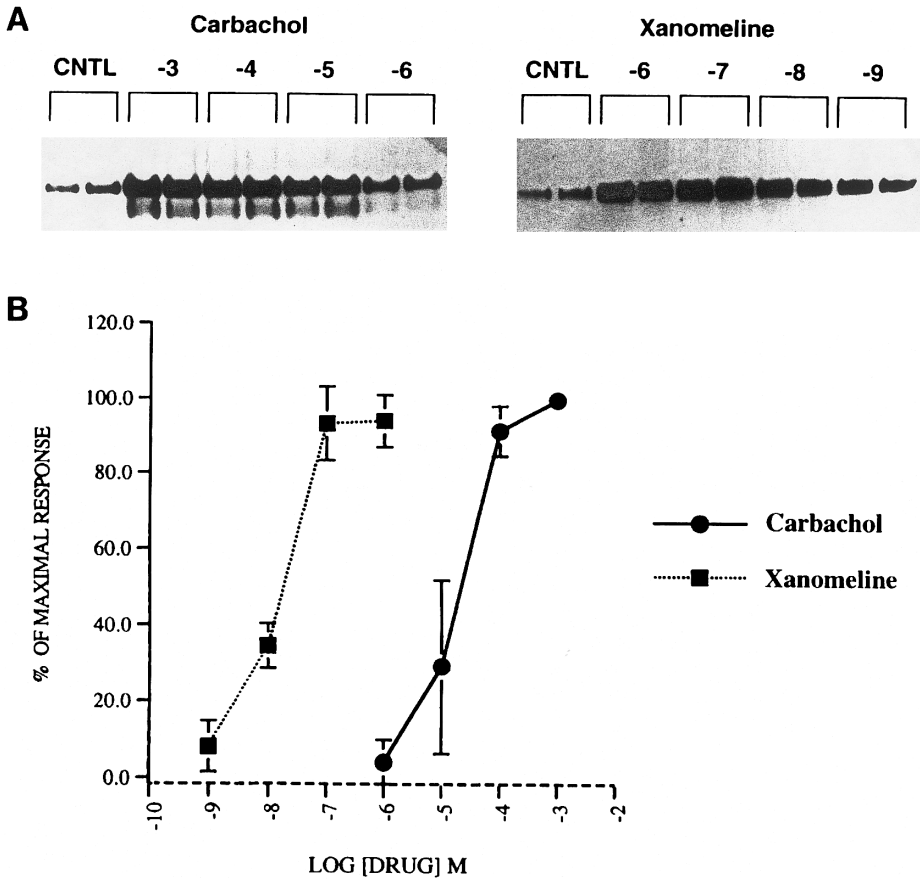

FIG. 2. Dose-response for stimulation of APPs release by carbachol and xanomeline. Flasks of cells were incubated in duplicate in the presence of medium alone or with increasing concentrations of the muscarinic agonists. Following a 30 minute incubation period media was collected and analyzed for APPs by western blot analysis with 6E10 as described in the methods section. (A) Western blot of dose response experiment. (B) Dose response curves for carbachol and xanomeline compiled from three western blot experiments. Numbers are the Log [agonist] M. Clone #25 was used.

the medium was collected into tubes containing protease inhibitor (Complete, Boehringer Mannheim, Indianapolis, IN). Medium was centrifuged to remove detached cells and was then immunoprecipitated with a combination of 6E10 and 4G8 antibodies using GammaBind G Sepharose (Pharmacia). Immunoprecipitates were electrophoresed on 10% MES-SDS NuPage gels (Novex) and the proteins were transferred to PVDF membrane and were subjected to autoradiography (BioMax Transcreen, Kodak).

RESULTS AND DISCUSSION

Clones expressing βAPP_{SM} were initially identified by resistance to hygromycin and were then further examined for secretion of APPs into the medium. Selected clones secreting APPs were identified by western blotting with monoclonal antibody 6E10 (Fig. 1A). Note that no secreted APPs was detectable in CHO m1 cells that had not been transfected with βAPP_{SM} (Fig. 1A, lane CHO m1). Extracts of non-transfected CHO m1 cells also showed no detectable APP with 6E10 (data not shown). Activation of m1 receptors on clones expressing human βAPP_{SM} by the muscarinic agonist carbachol caused large increases in release of APPs (Fig. 1B). Carbachol-stimulated release of APPs was blocked by the muscarinic antagonist atropine (data not shown).

Typical western blots showing dose response data for stimulation of APPs release by carbachol and xanomeline are illustrated in Figure 2A. Figure 2B shows the dose response data quantified from three experiments each for xanomeline and carbachol and illustrates the 1000-fold greater potency of xanomeline for stimulating APPs release from these cells.

Effects of carbachol and xanomeline on release of the Aβ peptide into cell culture medium were examined in pulse-chase experiments utilizing ^{35}S-methionine labeling of cellular proteins followed by immunoprecipitation with a combination of 6E10 and 4G8 antibodies. Incubation of prelabeled cells for a three hour time interval in the absence or presence of agonist concentrations that caused maximal effects on APPs release (i.e. 100 μM carbachol and 100 nM xanomeline, Fig. 3) resulted in significant reductions in a 4 Kd peptide identified by gel electrophoresis of immunoprecipitated

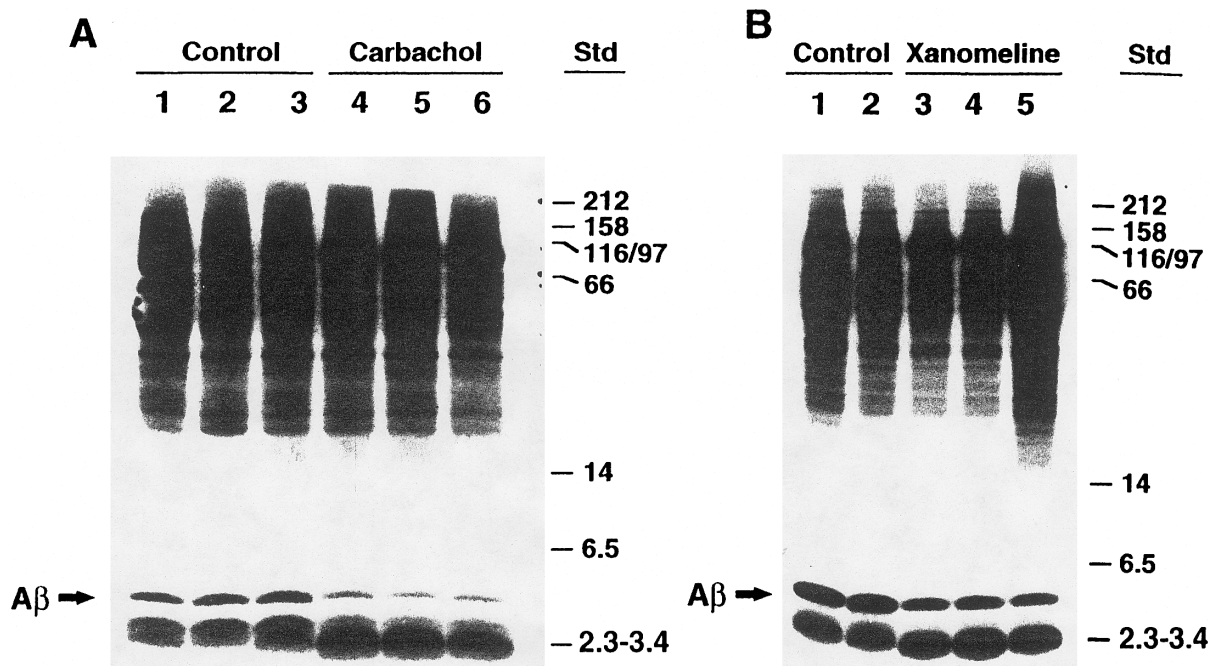

FIG. 3. Effect of carbachol and xanomeline on release of Aβ from CHO m1 cells transfected with βAPP$_{SM}$. Flasks of cells were labeled with ^{35}S-methionine for a period of four hours and were then chased for three hours in medium with an excess of unlabeled methionine in the presence or absence of muscarinic agonist. After the three hour chase period medium was collected and immunoprecipitated with a combination of 6E10 and 4G8 antibodies. Immunoprecipitates were subjected to SDS-PAGE, were transferred to PVDF membrane, and membranes were autoradiographed as described in MATERIALS AND METHODS. (A) Control media in lanes 1-3 and 100 μM carbachol in lanes 4-6. (B) Control media in lanes 1 and 2 and 100 nM xanomeline in rows 3-5. Each lane represents medium immunoprecipitated from a single flask of cells. The position of the 4 Kd Aβ bands are indicated. Clone #25 was used.

conditioned medium. Along with the reduction in Aβ release induced by each agonist, there was a corresponding increase in a lower molecular weight peptide, presumed to be the P3 peptide previously shown to be increased in culture media from cells under conditions which increase α-secretase activity (11). Table 1 shows the results of quantifying the Aβ and P3 bands by densitometry and illustrates that carbachol and xanomeline caused significant reductions in Aβ release along with significant increases in P3.

TABLE 1

Quantification of Agonist-Stimulated Effects on βA4 and P3 Release from Methionine-Labeled CHO m1-APPs$_M$ Cells[a]

Agonist	BA4	P3
Carbachol		
Control	19.44 ± 0.62	12.93 ± 0.60
Treated	13.90 ± 0.29	20.41 ± 0.99
	(28% decrease)	(58% increase)
	$p < 0.005$	$p < 0.005$
Xanomeline		
Control	27.58 ± 0.11	16.1 ± 0.22
Treated	14.95 ± 0.48	22.6 ± 1.63
	(46% decrease)	(40% increase)
	$p < 0.001$	$p = 0.05$

[a] Numbers are arbitrary units obtained from densitometry analysis of the data shown in Fig. 3. The data for carbachol and xanomeline were obtained in separate experiments. p values were obtained by analysis of variance.

Previously published data have shown increased APPs release from transfected cells and brain slices stimulated with the m1 selective agonists xanomeline, AF102B, WAL2014, and PD142505 (15, 18–21). These studies, however, have utilized the monoclonal antibody 22C11 which recognizes an amino terminal epitope on βAPP and cannot distinguish between amyloidogenic forms of APP and the non-amyloidogenic APLP proteins (22). WAL2014 activation of muscarinic receptors in brain slices was found to result in increased release of a soluble derivative of APLP2 (20). Thus it is uncertain from such studies to what extent APLP proteins may be contributing to muscarinic receptor-stimulated APPs release.

In the present study we utilized cells transfected with human Swedish mutation βAPP and measured APPs release using the monoclonal antibody 6E10. Monoclonal antibody 6E10 developed by Kim et. al. (23) recognizes the amino acid sequence 1-17 of human Aβ which differs from rodent Aβ in three amino acid residues (24). We observed no detectable release of APPs using 6E10 from CHO m1 cells not transfected with human βAPP (Fig. 1A), and have found that 6E10 does not recognize APP in extracts from CHO cells not con-

taining human APP (data not shown). Thus the APPs detected in this study was derived from the human amyloidogenic βAPP_{SM} transfected into our CHO m1 cells. This was further confirmed by the observation of reduced release of Aβ from these cells due to m1 receptor activation as determined by immunoprecipitation of conditioned medium with 6E10 in combination with monoclonal antibody 4G8 (also developed by Kim et. al. (25)) which recognizes amino acid residues 17-24 of the Aβ sequence. This study thus extends previous observations with m1-selective agonists and shows for the first time a reduction in Aβ release via activation of m1 receptors with an m1-selective agonist (xanomeline). These data extend and support previous studies indicating that m1 agonists may be useful for altering the progression of AD.

ACKNOWLEDGMENTS

We thank Bruce Glover for the synthesis and purification of oligonucleotides, Stanley Burgett and Pamela Rockey for DNA sequencing, and Rick Ludwig for his interest and support.

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