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AMYLOID PRECURSOR PROTEIN SECRETION VIA MUSCARINIC RECEPTORS: REDUCED DESENSITIZATION USING THE M1-SELECTIVE AGONIST AF102B

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Summary: Secretion of amyloid precursor protein (APP) by cultured cells is coupled to several receptors, including m1 muscarinic (m1AChR), and is associated with decreased production of βA4 amyloid. Secreted and cell-associated APP levels were measured in m1AChR-transfected PC12 cells stimulated with the non-selective agonist carbachol or the M1-selective agonist, AF102B. Secreted APP levels following stimulation with AF102B (5-60 min) were about half compared with carbachol. Yet, following 24 h stimulation with carbachol or AF102B, cell-associated APP levels were similarly decreased. This may be associated with a smaller reduction in APP secretion following 24 h stimulation with AF102B as compared with carbachol. AF102B may therefore have an advantage over non-selective muscarinic ligands for sustained decrease of cell-associated APP.

Amyloid β protein (βA4) is the major component of the senile plaques, one of the distinct neuropathological lesions in the brains of Alzheimer's disease (AD) patients. Deposition of aggregated βA4 amyloid deposits in plaques, and the neurotoxicity that may be associated with these deposits, are implicated in the etiology of AD (1). The βA4 peptide is a proteolytic product of a larger membrane-associated glycoprotein, the amyloid precursor protein (APP), whose processing may result in secretion of non-amyloidogenic isoforms by numerous cell types. Such secreted APP isoforms may have neuro-protective properties (2). It has been shown that APP secretion involves cleavage by an as yet unidentified protease ("α-secretase") at residue 687 of the fully mature APP_{770} (3), which corresponds to residue 16 of the βA4, thus precluding amyloid generation. The "α-secretase" pathway was shown to involve activation of protein kinase C, which may proceed via several receptor-activated signals (4). In rat pheochromocytoma PC12 cells or

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chinese hamster ovary (CHO) fibroblasts stably transfected with the m1 muscarinic cholinergic receptor (m1AChR) subtype, the agonist carbachol stimulated the secretion of APP (5,6). These observations suggested a linkage between the well-documented cholinergic deficiencies and amyloid deposition in AD, and implied an additional possible benefit for cholinergic replacement therapy in AD.

AF102B is an M1-selective muscarinic agonist currently under development for treating AD (7-9). Recent studies employing m1AChR-transfected CHO cells indicate that AF102B exhibits a distinct signaling pattern, being not only M1-selective but also a signal-selective agonist: AF102B elevates intra-cellular Ca^{2+} similarly to carbachol, but is a weak partial agonist for stimulation of phosphoinositide (PI) hydrolysis or for arachidonic acid release, and an antagonist for carbachol-activated adenylyl cyclase (9). APP secretion may also proceed independently of protein kinase C activation, via elevation of intra-cellular Ca^{2+} (10). Therefore, stimulation of APP secretion by such signal-selective muscarinic agonists may depend on the distinct activated signals. The present studies explore these possibilities by comparing APP secretion and cell-associated APP levels following stimulation with the non-selective agonist carbachol or with the M1-selective, signal-selective agonist AF102B.

MATERIALS AND METHODS

Transfected cells: The preparation and culture conditions of PC12 cells stably transfected with the m1AChR (PC12M1 cells) is described in detail elsewhere (11). Cells were plated in either 6-well (APP studies) or 12-well (biochemical signals) plates (Corning, NY) in complete RPMI medium (Beth Haemek, Israel) and used at the age of 4 - 5 days after plating.

APP secretion studies: For studies of APP secretion, cells were washed twice in serum-free RPMI medium supplemented with 20 mM HEPES and 0.2 mg/ml BSA. Incubations with the muscarinic ligands were carried out in this medium at 37 °C for the indicated periods (typically 60 min), and terminated by collecting the conditioned medium samples into tubes containing protease inhibitor cocktail (0.1 mM PMSF; 5 ug/ml leupeptin and pepstatin; 5 units/ml aprotinin) and concentrating with Centricon tubes (Amicon, Beverly, MA). Following protein determination (Bio-Rad protein assay), equal protein amounts were loaded on 10% PAGE-SDS gels; gels were subsequently blotted to nitro-cellulose membranes (Hoefer Scientific Instruments, San Francisco, CA) and probed with mAb 22C11 (Boehringer Mannheim, FRG). Membranes were probed with a peroxidase-linked rabbit anti-mouse IgG (Jackson ImmunoResearch, West Grove, PA) followed by 4-chloro-1-naphtol staining. Densitometric scanning of the stained bands was performed by video-imaging densitometry (Biological Detection Systems, Pittsburgh, PA). Data of APP secretion are expressed as percentage of control stimulation employing 0.1 mM carbachol, included in every experiment. This enabled consolidation of data from separate experiments.

Cell-associated APP levels: Determination of cell-associated APP was done similarly to the APP secretion, with the exception that for 24 h stimulation with agonists, complete medium was employed to avoid apoptotic cell death which takes place when PC12 cells are grown without serum. Incubations were terminated by washing the cells three times in serum-free RPMI medium followed by scrapping in lysis buffer (20 mM TRIS, 150 mM NaCl, 5 mM EDTA, 1 % Triton-X-100, and protease inhibitor cocktail). Following 5 sec sonication, samples were centrifuged (5 min x 10,000 g) and equal amounts of supernatant proteins were loaded on SDS-PAGE and analyzed for cell-associated APP similarly to the secreted APP studies.

Biochemical signals: PI hydrolysis, arachidonic acid release and cyclic AMP levels were analyzed as described in detail elsewhere (9). Routine incubations with ligands were for 20 min at 37 °C in serum-free RPMI medium, supplemented with 20 mM HEPES and with 10 mM LiCl (for PI hydrolysis), 1 mg/ml BSA (for arachidonic acid release) or 0.1 mM IBMX (for cyclic AMP accumulation).

Materials: AF102B was synthesized as described (9). Tissue culture reagents and antibiotics were from Beth Haemek, Israel. [^{3}H]inositol, [^{3}H]arachidonic acid, cyclic[^{3}H]AMP and [^{3}H]methyl-scopolamine were from DuPont/NEN as described (9). Molecular weight standards were from Bio-Rad (Hercules, CA). All other chemicals were from Sigma (St. Louis, MO).

RESULTS AND DISCUSSION

Stimulation of PC12M1 cells with either carbachol or AF102B resulted in increased measured levels of APP in the conditioned media (Fig. 1A). Maximal APP secretion was observed with 10 μM carbachol (Fig. 1B); mean stimulated levels were 11 ± 3 fold over basal, as calculated from video-imaging densitometry. APP secretion observed in parallel experiments using AF102B (100 μM) yielded only $52\pm15\%$ of the maximal carbachol-mediated secretion (Fig. 1B). This increased secretion was blocked by 10 μM atropine (Fig. 1A), and was time-dependent (Fig. 2A). Stimulation with AF102B consistently yielded about half the response observed using carbachol (both at 0.1 mM) at any time point between 5 and 60 min (Fig. 2B).

When secreted APP levels were measured at longer incubation periods (2 - 24 h), we observed persistent basal APP secretion, whereas muscarinic agonist-stimulated APP secretion decreased with time. Thus, the difference between APP levels in media from control and

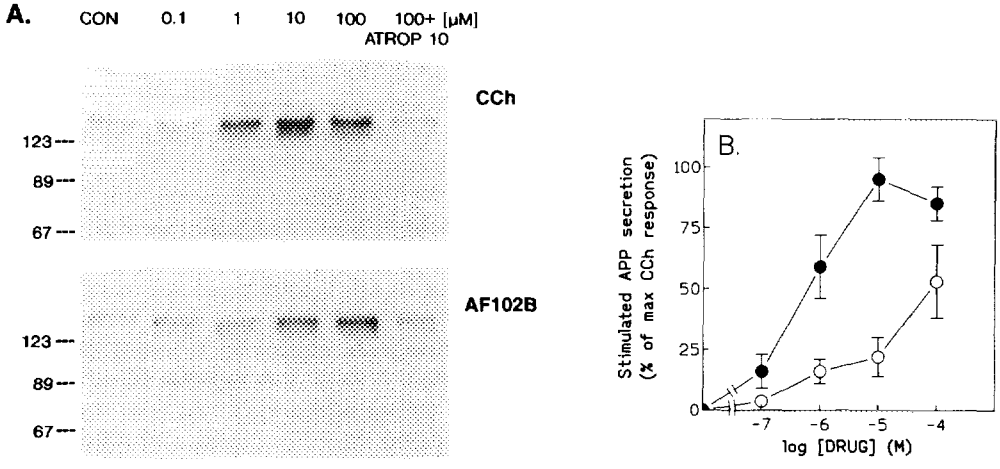

Figure 1. Concentration dependent m1AChR-stimulated APP secretion (1 h) from PC12M1 cells.
A. Representative immunoblot of secreted APP. The numbers and bars to the left side of the figure represent the values and position of the molecular weight markers used on the gels.
B. Video-imaging densitometry data (mean$\pm$SEM from 5 independent experiments) for APP secretion with carbachol (closed circles) and AF102B (open circles).

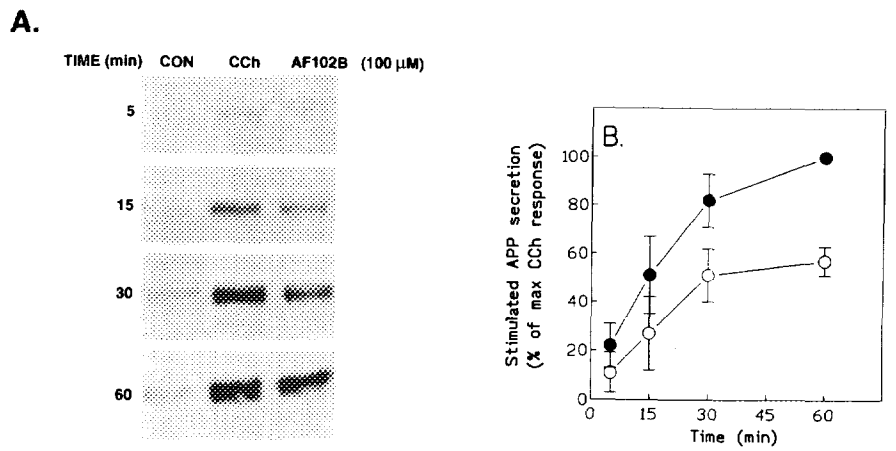

Figure 2. Time dependent m1AChR-stimulated APP secretion from PC12M1 cells. Carbachol and AF102B were used at 0.1 mM.
A. Representative immunoblot.
B. Video-imaging densitometry data (mean±SEM for 5 independent experiments) for APP secretion with carbachol (closed circles) and AF102B (open circles).

stimulated cells diminished (not shown). This may reflect increased removal of soluble APP from the medium (12), and also desensitization of m1AChR-mediated signals. Longer stimulation periods with agonists were therfore studied by measuring the levels of cell-associated APP, which were shown to decrease in association with increased APP secretion (4-6). Fig. 3A demonstrates that following stimulation for 24 h with either carbachol or AF102B (both at 0.1 mM), the levels of cell-associated APP were similarly decreased. This similar reduction was consistently observed in 7 independent experiments (mean video-imaging densitometry values of total APP-

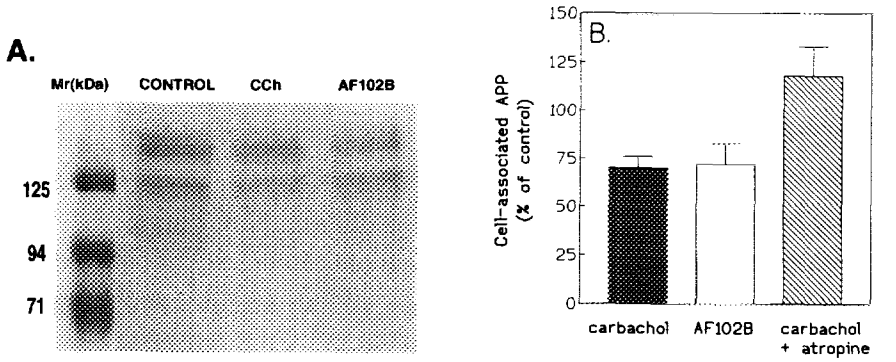

Figure 3. Cell-associated APP levels following 24 h incubations with carbachol or AF102B.
A. Representative immunoblot. Cells were stimulated for 24 h in complete medium with the indicated additions (0.1 mM).
B. Video densitometry data (mean±SEM for 7 independent experiments; carbachol and AF102B, 0.1 mM; atropine, 10 μM). Two-way ANOVA indicated that the reduction was significant ($p<0.001$) and that there was no significant difference between the reductions following carbachol or AF102B treatments ($p>0.1$).

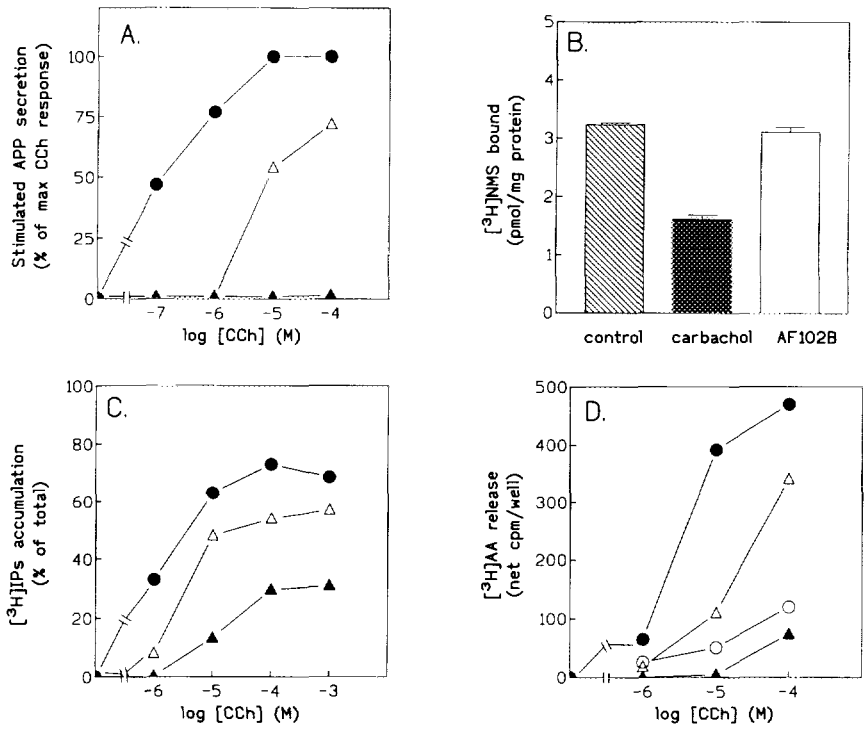

Figure 4. Diminished m1AChR-mediated APP secretion, m1AChR density and m1AChR-mediated PI hydrolysis and arachidonic acid release following 24 h incubations with muscarinic ligands.
A. Carbachol-stimulated (1 h) APP secretion in control cells (closed circles) or following 24 h incubations with 0.1 mM carbachol (closed triangles) or 0.1 mM AF102B (open triangles). Video-imaging densitometry data from a representative experiment are shown.
B. Specific [^{3}H]methyl-scopolamine binding (at a saturating concentration of 4 nM) to intact PC12M1 cells following 24 h incubations with 0.1 mM carbachol or 0.1 mM AF102B.
C. Carbachol-stimulated PI hydrolysis in control cells (closed circles) or following 24 h incubations with 0.1 mM carbachol (closed triangles) or 0.1 mM AF102B (open triangles).
D. Carbachol-stimulated arachidonic acid release in control cells (closed circles) or following 24 h incubations with 0.1 mM carbachol (closed triangles), 0.1 mM AF102B (open triangles), or 10 μM phorbol 12-myristate 13-acetate (open circles).

immunoreactive bands were 70±6% and 72±11% vs. control for carbachol and AF102B, respectively) and was completely blocked by atropine (Fig. 3B).

These observations allude to possible desensitization of the m1AChR-mediated APP secretion, which may be more pronounced following stimulation with the full agonist carbachol as compared with the partial agonist AF102B. Therefore, we incubated PC12M1 cells for 24 h with either carbachol or AF102B (both at 0.1 mM), followed by extensive washout of the agonist and subsequent measurements of carbachol-stimulated APP secretion. Fig. 4A demonstrates that, following 24 h incubation with carbachol, the carbachol-mediated APP secretion was completely lost, with no stimulation even at 0.1 mM carbachol. In contrast, following a similar incubation protocol with AF102B, the subsequent carbachol response was still evident, but was shifted to

higher drug concentrations. Measurement of [^{3}H]methyl-scopolamine binding indicated a 50% loss of m1AChR sites following 24 h incubation with carbachol, but only 5% loss after AF102B (Fig. 4B). In addition, following carbachol exposure we observed a diminished carbachol-stimulated PI hydrolysis signal (Fig. 4C) and a complete loss of the carbachol-stimulated arachidonic acid release signal (Fig. 4D). Similarly to APP secretion, these signals were less affected in PC12M1 cells following a 24 h AF102B exposure (Fig. 4A-D).

Our studies demonstrate similar decreases of cell-associated APP levels in PC12M1 cells stimulated for 24 h with carbachol or AF102B. This seems to contradict with the lower levels of stimulated APP secretion measured at short incubation periods with AF102B as compared with carbachol (Fig. 1-2). This contradiction may reflect reduced desensitization, which may be a consequence of both reduced receptor down-regulation (Fig. 4B) and diminished desensitization of APP-related signaling events (Fig. 4C-D) for the partial agonist AF102B as compared with the non-selective, full agonist carbachol. Early studies implied that stimulation of APP secretion proceeds predominantly via PI hydrolysis, yielding IP_3 which releases Ca^{2+} from intracellular stores, and diacylglycerol, which both in turn activate protein kinase C (4,5). Recent studies suggested that inhibitors of arachidonic acid release may attenuate APP secretion (13). It is therefore plausible that the observed similar desensitization profile of m1AChR-mediated APP secretion and arachidonic acid release (Fig. 4A and 4D) denotes a shared biochemical pathway. Notably, recent studies indicate that in addition to protein kinase C activation, APP secretion is governed by additional pathways, e.g., increase in intra-cellular Ca^{2+} (10,14).

AF102B and other M1-selective muscarinic agonists are being developed for cholinergic replacement therapy in AD patients (7-9). The present findings suggest that in addition to the obvious benefit for AD patients of reduced cholinergic side-effects, M1-selective muscarinic agonists may convey the additional advantage of reducing cell-associated APP levels over prolonged periods, thus limiting amyloid deposition and possibly delaying the disease progression.

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