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Amyloid precursor protein secretion and βA4 amyloid generation are not mutually exclusive

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Abstract

The cellular factors regulating the generation of βA4 from the amyloid precursor protein (APP) are unknown. Protein phosphorylation by protein kinase C (PKC) has been found to influence the processing and metabolism of APP. In this report, we show that in the human neuroblastoma cell line SY5Y, βA4 generation from full-length APP is not changed by PKC activation whereas production of the non-amyloidogenic secretory fragment (APPsec) and of the C-terminal fragment of βA4 (p3) are stimulated. In addition, βA4 generation from the membrane inserted C-terminal 100 residues (SPA4CT) of APP is stimulated by PKC activation. Accordingly attempts to divert APP processing from the amyloidogenic, βA4-generating, to the non-amyloidogenic, secretory, pathway, have to address the nature and regulation of the two pathways and/or of the process leading to the cleavage of APP at the C-terminus of the βA4 domain. The data reported here suggest that these mechanisms are cell-type specific.

Key words: APP; A4CT; βA4; Porcessing; SY5Y; Alzheimer's disease

1. Introduction

The β-amyloid protein (βA4) is a cleavage product of the amyloid precursor protein (APP) and accumulates at high levels in the brains of Alzheimer's disease (AD) patients. While a large proportion of normal secretory processing of APP involves cleavage within the β-amyloid domain and thus precludes βA4 generation [1,2,3], recent reports have shown that β-amyloid related peptides are also normally secreted by cultured cells and can be detected in human cerebrospinal fluid [4,5,6,7]. In addition to the 4-kDa β-amyloid peptide (βA4) a 3-kDa peptide (p3) corresponding to a truncated fragment of βA4 at residue 16 by APP secretase is also produced [8]. It has been reported that activation of PKC in some non-neuronal cell culture systems increased the generation of secreted forms of APP (APPsec) and p3 but markedly decrease βA4 release from these cell lines [9,10].

To analyse further the regulatory mechanisms involved in βA4 generation, especially the relationship between the two protease activities generating the N- and C-terminus of βA4 (termed protease 1 = β-secretase and protease 2 = γ-secretase, respectively) and the βA4 cleaving activity (termed APP secretase; α-secretase), we have expressed, in addition to full-length APP695, also the C-terminal 100 residues of APP, fused to the APP signal sequence (termed SPA4CT), in the human neuroblastoma cell line SY5Y. Since βA4 release from SPA4CT-type APP fragments which carry the βA4 sequence at the N-terminus, depends only on the protease activity generating the C-terminus of βA4 (i.e. protease 2, γ-secretase), and was shown not to be influenced by APP-secretase [11], we were able to analyse βA4 generation independent of the processes generating the N-terminus of βA4 or of APP secretion.

2. Experimental procedures

2.1. Cloning procedures

Preparation of plasmid DNA, restriction enzyme digestion, agarose gel electrophoresis of DNA, DNA ligation and bacterial transformation were carried out as described by Sambrook et al. [12].

2.2. Plasmid construction

For construction of CEP/SPA4CT the *SmaI*/*Hin*dIII fragment from SP65/SPA4CT [11] was cloned into pCEP4, digested with *Pvu*II/*Hin*dIII. The resulting plasmid CEP/SPA4CT encodes the APP signal sequence and two additional residues from APP695 in frame with the amyloid βA4 sequence and the entire C-terminal domain of the APP. CEP/APP695 was obtained by cloning the *SmaI*/*Hin*dIII fragment into pCEP, digested with *Pvu*II/*Hin*dIII.

2.3. Tissue culture and transfection

The subclone neuroblastoma cell line SH-SY5Y was grown in equal parts of Minimum Essential Medium (MEM, with Earle's Salts and L-glutamine) and Ham's F-12 supplemented with non-essential amino acids (Eagle's formulation), penicillin (50 U/ml), streptomycin (40 μg/ml) and 10% (v/v) fetal calf serum (FCS) (all Gibco/BRL). For stable transfection 5×10^5 cells were seeded onto 60-mm plates, and incubated overnight at 37°C. After reaching ca. 80% confluence, cells were washed three times with Opti-MEM I (Gibco) prior to addition of DNA/Lipofectin mixture. The DNA/Lipofectin mixture was produced by combining the DNA solution (10–20 μg DNA in 1.5 ml Opti-MEM) with the Lipofectin solution (30–50 μg Lipofectin (BRL, Eggenstein, Germany) in 1.5 ml Opti-MEM). After incubation for 8 h at 37°C, cells were grown for a further 24–48 h in cell-culture medium and stable lines were selected by exposure to hygromycin B (300 μg/ml).

2.4. Pulse-chase labelling of tissue culture cells

After removal of the culture medium, the cells were treated with 1.5 ml of MEM lacking methionine for 40 min. Then 500 μCi of [^{35}S]methionine were added, and cells were labelled for 60 min at 37°C. Cells were then washed once with MEM (37°C) and chased in methion-

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ine-enriched medium (MEM; 0.3 mg/ml L-methionine, 37°C) for the times indicated in results. After the chase period, the medium was harvested, and the cells were washed once with MEM and scraped from the surface of the dish. For lysis, the cells were resuspended in 0.6 ml of SOL (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 2 mM EDTA, 1% Triton X-100) supplemented with 2 mM penylmethylsulfonyl fluoride (PMSF) and placed on ice. After 20 min, samples were centrifuged at 10,000 × *g* for 5 min, and the supernatants (cell lysates) were stored at −20°C for subsequent analysis.

2.5. Immunoprecipitation [13]

For immunoprecipitation the conditioned medium (of one 6-cm cell culture dish) was first adjusted to 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 2 mM EDTA, 1% Triton X-100 and 2 mM PMSF and then medium and cell lysate were incubated for 30 min with 10 μl preimmune serum and 30 μl (3 mg) protein A-Sepharose (Pharmacia). The samples were centrifuged briefly and the supernatants (1.5 ml) were incubated with antiserum (5 μl anti-APP or 20 μl anti-2–43) for 90 min at room temperature. Following the incubation, 30 μl (3 mg) protein A-Sepharose was added for an additional 90 min at room temperature. The insoluble complexes were washed three times with wash A (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Triton X-100, 2 mM EDTA), two times with wash B (10 mM Tris-HCl, pH 7.5, 500 mM NaCl, 0.2% Triton X-100, 2 mM EDTA) and once with wash C (10 mM Tris-HCl pH 7.5) before pellets were resuspended in 2 × Laemmli sample buffer. After boiling for 5 min at 100°C, labelled proteins were seperated on Tris-tricine gels (16.5% or 8–20%) and fluorographed with EN^{3}HANCE (DuPont).

3. Results

3.1. Stimulation of βA4 generation from SPA4CT in SY5Y cells

To examine the role of protein kinase C in the regulation of βA4 production by C-terminal cleavage independent of the processes generating the N-terminus of βA4 (protease 1, γ-secretase) or the C-terminus of secretory APP (APP-secretase, α-secretase), we used SY5Y cells stably transfected with the C-terminal 100 residues of APP, fused to the APP signal sequence (termed SPA4CT). Expression of this APP-fragment in SY5Y cells resulted in a 12 kDa protein associated with the cell lysate and a βA4-like peptide which is immediately secreted into the medium as previously shown [11]. Peptide p3, which is generated from full-length APP by the activity of βA4-protease 2 and APP-secretase, is not generated from SPA4CT because we showed that APP secretase does not cleave this C-terminal APP-fragment.

SY5Y cells transfected with SPA4CT were pulse-labelled with [^{35}S]methionine and then chased in the absence or presence of compounds which activate PKC directly or indirectly by activation of the G-protein-coupled muscarinic receptor. Treatment of the transfected cells with carbachol (1 mM) or PMA (phorbol 12-myristate 13-acetate) (1 μM) stimulates βA4 generation by 1.6-and 2-fold, respectively, compared with control cells (Fig. 1A and B). The effect of 1 mM carbachol was blocked by the muscarinic antagonist atropine (1 μM).

This carbachol-stimulated βA4 secretion was mediated by the muscarinic M_3 receptor, as shown by the effects of selective muscarinic-receptor antagonists

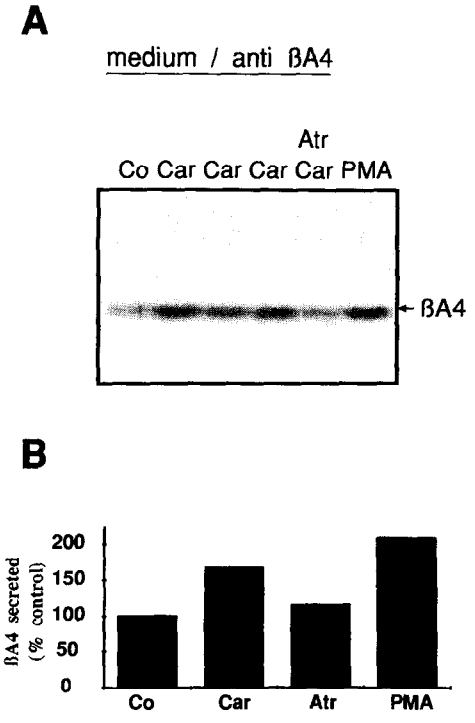

Fig. 1. Stimulation of βA4 generation from SPA4CT in SY5Y cells SY5Y cells stably transfected with CEP/SPA4CT were labelled for 60 min with [^{35}S]methionine and chased in methionine-enriched medium for 3.5 h in the presence or absence of the compounds indicated. The conditioned medium was analysed by immunoprecipitation with anti-2–43. Standardisation of the quantity applied in each lane was done by measuring the total radioactivity incorporated into protein. Co = control; Car = 1 mM carbachol; Atr = preincubation with 1 μM atropin for 15 min; PMA = 1 μM phorbolester. (A) Tris-tricine PAGE (16.5%). B: the autoradiography was quantified by densitometric scanning of the βA4 band of the autoradiogram (Amersham RAS System). The data represent the signal-intensity in percent of the 4 kDa signal of control cells.

(Fig. 2, lane M3). Preincubation of cells with 1 μM 4-DAMP (M_3-receptor antagonist) abolished carbachol-induced stimulation of βA4 secretion, whereas preincubation with 1 μM Telenzepine (M_1-receptor antagonist) had no effect (Fig. 2, lane M1).

3.2. Regulation of APP metabolism by PMA and carbachol

To confirm the role of phosphorylation in the regulation of βA4 generation also from full-length APP, the effects of the cholinergic agonist carbachol and the phorbol ester PMA were studied in SY5Y cells that had been transfected with APP695.

When these transfected cells were incubated with carbachol (1 μM) or PMA (1 μM), there was a 1.5-fold or 2.3-fold increase in the levels of secreted APP respectively, during a 60 min chase period compared with control cells (Fig. 3). Again the effect of 1 mM carbachol was blocked by the muscarinic antagonist atropine (1 μM).

To analyse further the effect of these compounds on

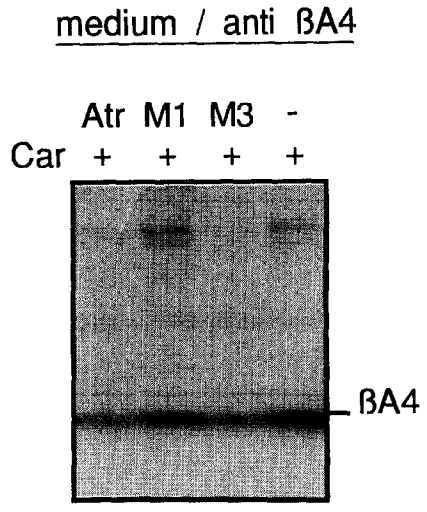

Fig. 2. Effect of muscarinic-receptor antagonists on carbachol-induced βA4 secretion SY5Y cells stably transfected with CEP/SPA4CT were labelled for 30 min with [^{35}S]methionine in the presence or absence of 1 μM concentrations of the muscarinic-receptor antagonists atropine (Atr), telenzepine (M1) or 4-DAMP (M3) as indicated. Thereafter 1 mM carbachol (Car) was added and the cells were chased with methionine-enriched medium for 3 h. The proteins were analysed by 16% Tris-tricine-PAGE after immunoprecipitation. Standardisation of the quantity applied in each lane was done by measuring the total radioactivity incorporated into protein.

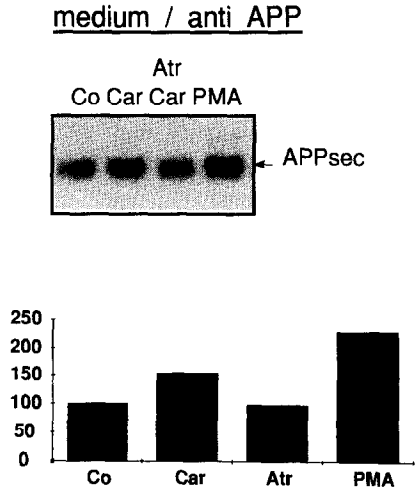

Fig. 3. Release of APPsec upon PKC stimulation SY5Y cells stably transfected with CEP/695 were labelled for 60 min with [^{35}S]methionine and chased in methionine-enriched medium for 60 min in the presence or absence of the compounds indicated. The conditioned medium was analysed by immunoprecipitation with anti APP. Co = control; Car = 1 mM carbachol; Atr = preincubation with 1 μM atropine; PMA = 1 μM phorbolester. Standardisation of the quantity applied in each lane was done by measuring the total radioactivity incorporated into protein. (Top) Tris-tricine PAGE (8–20%). (Bottom) The autoradiography was quantified by densitometric scanning of the APP band of the autoradiogram (Amersham RAS System). The data represent the signal-intensity in percent of the APP signal of control cells.

βA4 generation, the chase time was extended to 3.5 h to ensure processing and release of all labelled APP fragments. Under these conditions, analysis of conditioned medium revealed only a 1.2-fold stimulation of APPsecretion with carbachol and 1.7-fold with PMA (Fig.4A and Fig. 5). This decline of the effect on APP secretion in the 3.5 h chase experiment in comparison to the 60 min chase time period is due to the short half life time of APPsec [1].

In parallel to elevated APP levels in the media, secretion of a 3-kDa truncated βA4 species (p3), the amount of which has been shown to parallel that of APPsec released from cells [4,8], was elevated 1.6-fold by carbachol and 3.4-fold by PMA (Figs. 4A and 5). In contrast to the elevated levels of APPsec and p3, the level of βA4 peptide production was unchanged by PKC activation. This is surprisingly since an increase of secretory APP as a consequence of cleavage within the βA4 domain of APP is expected to reduce the pool of βA4 precursors accordingly and thus lower the amount of βA4 molecules released from the cells.

4. Discussion

It is now well established that transmembrane APP can either undergo secretion as APPsec, be processed into βA4-like peptides or degraded in the lysosomal compartment. In this regard, protein kinase C activation by phorbol esters has been shown to stimulate secretion of APPsec and to increase the amounts of membrane-retained C-terminal fragments in PC12 pheochromocytoma cells [14,15], as well as in human embryonic kidney 293 cells [16]. In addition, cells transfected with muscarinic acetylcholine receptor subtypes m1 or m3 show increased secretion of APPsec upon addition of the cholin-

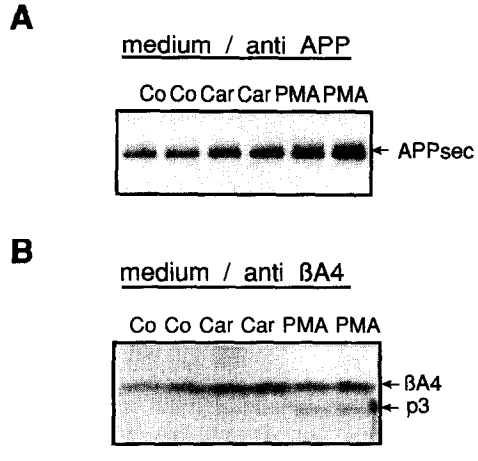

Fig. 4. Release of APPsec, p3 and βA4 upon PKC stimulation. SY5Y cells transfected with CEP/695 were labelled for 60 min with [^{35}S]methionine and chased in methionine-enriched medium for 3.5 h in the presence or absence of the compounds indicated. The conditioned medium was analysed by immunoprecipitation with anti-APP (A) or anti-2–43 (B) and subsequent Tris-tricine-PAGE (16.5%). Standardisation of the quantity applied in each lane was done by measuring the total radioactivity incorporated into protein.

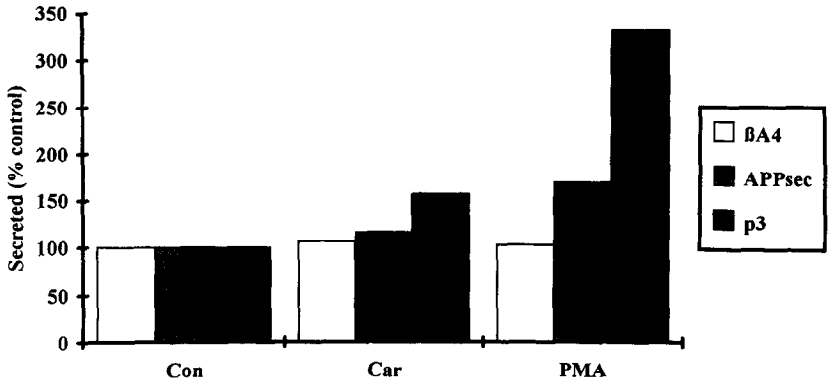

Fig. 5. Quantification of secreted APP-fragments upon PKC stimulation. APP, p3 and βA4 were quantified by cutting out the precipitated proteins from the dried gel (Fig. 4) and measurement of radioactivity in the slices in 5 ml of scintillation fluid. Each column represents the mean of two independent experiments. Co = control; Car = 1 mM carbachol; PMA = 1 μM phorbolester.

ergic agonist carbachol, a process which is blocked by the PKC inhibitor staurosporine [17,18]. Moreover, some groups have recently reported that activation of PKC, either directly by phorbol esters or indirectly by cholinergic agonists, results in a marked inhibition of βA4 release [9,19].

To examine further the role of protein kinase C in the regulation of βA4 production, we used human neuroblastoma cells stably transfected with both APP and the C-terminal 100 residues of APP (SPA4CT). The expression strategy allowed membrane insertion and signal peptide removal of SPA4CT and thus mimics the natural situation of this C-terminal APP fragment. Using these stable cell lines permits a more detailed analysis of the regulation of the three cleavages involved in βA4-generation and APP secretion. Analysis of cell lysates and conditioned medium of the stable cell lines showed that both SPA4CT and APP are processed into βA4-like peptides which are immediately released into the medium [11]. Using the stable SY5Y cells expressing SPA4CT we have been able to demonstrate for the first time that βA4 generation from the C-terminal 100 residues of APP, which may be an intermediate in the process of βA4 generation [20,21,22], is stimulated by PKC activation. In SY5Y cells the carbachol-stimulated βA4 secretion was mediated by the muscarinic M_3 receptor as demonstrated by inhibition with the selective M3-receptor antagonist 4-DAMP.

By analysing the effect of PKC stimulation on the metabolism of full-length APP in SY5Y cells we have shown that whereas activation of PKC increased the generation of APPsec and P3 it did not influence βA4 generation, an observation in contrast to other results obtained with other cell types. These experiments demonstrate for the first time that in some cellular systems, production of βA4 is not inhibited by PKC. Because in our cell system stimulation of APPsec did not lead to an inhibition of βA4 production, we conclude that in this system these two APP processing events, APP secretion and βA4 generation, are not mutually exclusive. Our results would suggest the existence of a surplus of full-length APP which either gives rise to APPsec, βA4, or lysosomal degradation products, depending on the enzymatic activities and regulatory mechanisms present. Thus, in SY5Y cells the simultaneous activation of APP secretase (4-fold), and protease 2 (2-fold), as indicated by experiments with the APP- and SPA4CT-transfected cells, in total results in two-fold increase in APP secretion and no change in the quantity of βA4 generation. Accordingly the consequences of PKC stimulation in different tissue – of cellular systems depends on the kinds of enzymatic activities and regulatory mechanisms present. While in SY5Y cells both βA4 protease 2 and APP-secretase appear to be stimulated by PKC activation, other cell types may contain different regulatory mechanisms for these two proteolytic activities. This is in agreement with the observation that in some cell types, inhibition of βA4 production can occur without increased secretion of APPsec [10].

The conclusion of our results is that different cell types seem to exercise different fine-tuning of the APP processing machinery. Accordingly, results concerning APP regulation and βA4 generation in special cell culture systems, and therapeutic strategies which are based on these results, have to be treated with caution.

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