Molecular Medicine 4: 715-723, 1998

Molecular Medicine
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Post-transcriptional Contribution of a cAMP-dependent Pathway to the Formation of α- and β/γ-Secretases-Derived Products of βAPP Maturation in Human Cells Expressing Wild-type and Swedish Mutated βAPP

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Communicated by P. Chambon. Accepted September 14, 1998.

Abstract

Background: The physiopathological maturation of the β-amyloid precursor protein can be modulated by effectors targeting a protein kinase C–dependent pathway. These agents increase the recovery of APPα, the physiological α-secretase-derived product of βAPP processing, and concomittantly lower the production of the pathogenic β/γ-secretase-derived Aβ fragment.

Methods: We set up stably transfected HEK293 cells expressing wild-type or Swedish mutated βAPP. By combined metabolic labeling and/or immunoprecipitation procedures, we assessed the effect of various cAMP effectors on the production of the βAPP maturation products Aβ40, Aβ42, APPα, and its C-terminal counterpart.

Results: We show here that the cAMP-dependent protein kinase (PKA) effectors, dibutyryl-cAMP (dBut-cAMP) and forskolin, but not the inactive analog dideoxyforskolin, enhance the secretion of APPα and the intracellular production of its C-terminal counterpart (p10) in stably transfected HEK293 cells. The above agonists also drastically increase both Aβ40 and Aβ42 secretions and intracellular Aβ recovery. The same influence was observed with HEK293 cells overexpressing the Swedish mutated βAPP. We attempted to delineate the relative contribution of transcriptional and post-transcriptional events in the cAMP-mediated response. We show here that the dBut-cAMP and forskolin-induced increase of APPα and Aβs secretions is not prevented by the transcription inhibitor actinomycin D.

Conclusion: Our data suggest a major contribution of post-transcriptional events in the cAMP-dependent effect on βAPP maturation. It appears likely that cAMP triggers the PKA-dependent phosphorylation of a protein involved in βAPP maturation and occuring upstream to α- and β/γ-secretase cleavages.

Introduction

Alzheimer's disease is characterized by the invasion of brain cortical areas by proteinaceous deposits called senile plaques (1). The main component of these extracellular aggregates is a poorly soluble 39–43 amino acid peptide called Aβ (2,3). This fragment derives from a larger precursor (βAPP, β amyloid precursor protein) through proteolytic attacks by β- and γ-secretase activities that release the N- and C-terminal moities of Aβ, respectively (4–6). Alternatively, another enzyme named α-secretase triggers the release of APPα, a physiologically secreted product that derives from the

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cleavage of βAPP at a peptide bond located inside the Aβ sequence (7,8). Several lines of evidence have indicated that the physiopathological maturation of βAPP is a highly regulated process under the control of phosphorylation events (for review see ref. 5). Thus, effectors of the protein kinase C (PKC) pathway trigger a drastic increase in APPα production and concomittantly lower the recovery of Aβ (9–12). This opposite effect on α- and β/γ-derived products appears mimicked by all agonists of the PKC pathway (for review see ref. 5).

We and others have recently shown that the APPα secretion could also be modulated by effectors of the protein kinase A pathway in intact cells (13,14) as well as in a cell-free system (14). Thus, treatment of HEK293 and PC12 cells with forskolin and cAMP analogs led to a drastic stimulation of the α-secretase-derived APPα secretion (13,14). The mechanism by which cAMP could affect α-secretase pathway remains to be established. Some lines of evidence suggest possible transcriptional events since βAPP synthesis can be stimulated by cAMP analogs (15–17) or by agonists of cAMP-coupled adrenergic receptors (18). On the other hand, cAMP response could be due to the involvement of PKA in the budding of constitutive secretory vesicles (14).

Nothing is known concerning the putative effect of PKA agonists on the β/γ-derived Aβ production. Furthermore, Aβ production appears to be affected by several mutations responsible for early-onset Alzheimer's disease (for review see ref. 5) and therefore could be differently affected by PKA agonists. We have taken advantage of the setting of stably transfected cell lines overexpressing wild-type βAPP (wtβAPP) and Swedish mutated βAPP (SwβAPP) to examine (*1*) the cAMP-dependent modulation of Aβ40 and Aβ42 as well as APPα formation in wtβAPP-expressing cells, (*2*) the effect of cAMP agonists on α- and β/γ-secretase-derived products generated by SwβAPP-expressing cells and (*3*) the respective contributions of transcriptional and post-transcriptional events in these cAMP-mediated responses.

We show here that PKA agonists elicit increased formations of both APPα and Aβs and that these cAMP responses are mainly due to actinomycin D-insensitive post-transcriptional events likely occuring upstream to the α- and β/γ-secretase cleavages taking place on βAPP.

Materials and Methods

Cell Culture

HEK293 cells and neuronal cells were grown in 5% CO_2 in F12/DMEM (vol/vol) and in Opti-MEM (GibcoBRL) respectively, supplemented with 10% fetal calf serum containing penicillin (100 units/ml), streptomycin (50 μg/ml), and geneticin (1 mg/ml).

Stable Transfections in HEK293 and Neuronal Cells

HEK293 cells were stably transfected by calcium phosphate precipitation with 1 μg of pcDNA3 containing either wild-type $βAPP_{751}$ or Sw-$βAPP_{751}$ and identified as described (19,20). TSM1 neocortical neuronal cell line (clone Q) (21) was transfected with $wtβAPP_{751}$ and positive clones were selected as above.

Cell Treatment with PKA Agonists and Detection of βAPP

Cells were incubated at 37°C for 7 hr in the presence or absence of the following PKA agonists: dideoxyforskolin (20 μM); forskolin (20 μM) or dibutyryl-cyclic AMP (1 mM). Cells were then collected, homogenized in a Tris-buffer (20 mM pH 7.4), and about 30 μg of protein was resuspended in the loading buffer, electrophoresed on a 8% SDS-PAGE and Western blotted for 3 hr at 100 V. Nitrocellulose sheets were incubated in skim milk [5% in Tris buffer saline (TBS) and exposed overnight to a 5000-fold dilution of mAb antibodies (WO2) (22)]. Nitrocellulose sheets were rinsed with TBS buffer, then incubated with goat anti-mouse IgGs coupled to peroxidase, revealed, and quantified by enhanced chemiluminescence as previously described (23).

Metabolic Labeling and Detection of Secreted Aβ40, Aβ42, and APPα

Cells were preincubated for 1 hr without or with the above concentrations of PKA agonists, then metabolically labeled for 6 hr in the presence of the agonists. Conditioned media were collected, diluted in a one-tenth volume of 10× RIPA buffer. Media were incubated overnight with a 350-fold dilution of FCA3542 (24) then further incubated for 5 hr with protein A-Sepharose. After centrifugation, a tenth of the resulting supernatant was incubated overnight with a 3000-fold dilution of 207 antibody (25) in the presence of pansorbin (20 μl, Cal-

biochem) while the remaining supernatant was exposed for 15 hr to a 350-fold dilution of FCA3340 and protein A-Sepharose as above. After centrifugation, pellets were resuspended with loading buffer, then submitted to a 16.5% Tris-tricine electrophoresis (Aβ40 and Aβ42) or to a 8% Tris-glycine (APPα). Gels were then radioautographed as previously described (20), and densitometric analyses were performed by phosphorImager (Fuji).

Detection of Total Intracellular Aβ and p10

Cells were treated and metabolically labeled as above, then cells were scraped, rinsed in PBS, and lysed in 1× RIPA. Cellular lysates were centrifuged, then the resulting supernatants were incubated overnight with a 350-fold dilution of FCA18 and protein A-Sepharose as above (total Aβ). After centrifugation, the supernatant was incubated for 15 hr with a 1000-fold dilution of B11.4 antibody (p10). Pellets were resuspended with loading buffer then submitted to a 16.5% Tris-tricine electrophoresis and analyzed as above.

Northern Blot Analysis

Total RNA was extracted with RNABle reagent (Eurobio) according to the manufacturer's recommendations. Ten micrograms of RNA was denatured in 50% formamide/17% formaldehyde by heating 15 min at 65°C and was electrophoresed on a 1% agarose/formaldehyde gel, then transfered to Hybond-N membrane (Amersham) by capillary blotting 15 hr in 20× SSC (1× SSC: 0.15 M NaCl, 0.015 M sodium citrate, pH 7.0). RNA was cross-linked by UV for 30 sec and the blot was prehybridized and hybridized with radiolabeled human cDNA probes of βAPP751 prepared by random primer labeling technique (Appligene). The blot was incubated overnight at 42°C with labeled probe in 4× SSC then was washed in 0.5–1× SSC, 0.1% SDS, at 42°C. The blot was finally exposed to PhosphorImager (Fuji).

Antibodies

FCA3340 and FCA3542 specifically recognize the C terminus of Aβ40 and Aβ42, respectively, and FCA18 interacts with the N terminus of Aβs (24). Antibody B11.4 is directed toward the C terminus of βAPP (26). The 207 antibody (Cephalon, West Chester, PA) interacts with the N terminus of βAPP and APPα. WO2 mainly interacts with the 5–8 sequence of Aβ (22).

Results

Figure 1 illustrates the effect of several agents targeting the cAMP pathway on the βAPP mRNA and protein expressions in stably transfected HEK293 cells overexpressing wtβAPP_{751}. The adenylate cyclase activator, forskolin, and the cAMP analog, dibutyryl-cAMP, both drastically increase the transcription of a 2.8 kb mRNA revealed with a βAPP-specific probe, whereas dideoxyforskolin (DDF), an inactive analog of forskolin, does not (Fig. 1B). Such an augmentation is also observed with the cAMP-coupled β-adrenergic receptor agonist isoproterenol (not shown). The expression of βAPP appeared similarly affected by the above agents (Fig. 1C). Thus, forskolin and dBut-cAMP elicit a 3- to 4-fold increase in both mature and immature βAPP immunoreactivities whereas DDF was ineffective (Fig. 1D).

We examined the influence of the above agents on the various products of βAPP maturation by wtβAPP_{751} HEK293 transfectants. By means of antibodies specific for the C-terminal ends of Aβ40 and Aβ42 (24), we showed that forskolin and dBut-cAMP drastically augment the recovery of both Aβ species whereas DDF is totally inactive (Fig. 2A, B). Quantitative analyses indicate that the stimulatory effect of forskolin and dBut-cAMP appears to be very similar for both Aβ species (Fig. 2C).

A low-molecular-weight doublet protein was also immunoprecipitated by the antibodies (Fig. 2A, B). These fragments are not interacting with an antibody (FCA18) that recognizes the Aβ N terminus (not shown) and therefore could correspond to the α/γ (for review see ref. 5) or α′/γ (27) secretase-derived products. Whatever their nature, these x-40 and x-42 fragments appear to increase as their Aβ counterparts with the above pharmacological treatment (Fig. 2A, B).

The fact that both p3s fragments and Aβs formations are similarly affected by agonists of the cAMP pathway suggests that both physiological and potentially pathogenic pathways of βAPP maturation are modulated by cAMP agonists in HEK293 cells. Accordingly, we showed that forskolin and dBut-cAMP affect the α-secretase-derived fragment APPα (Fig. 2D) and its C-terminal counterpart p10 (Fig. 2E) to a similar extent (Fig. 2F).

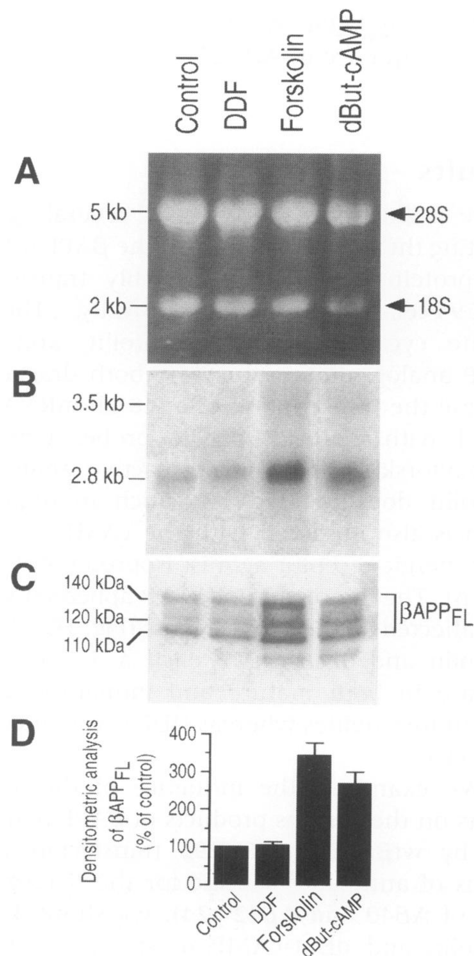

Fig. 1. Effect of cAMP effectors on the βAPP mRNA and protein expressions in HEK293 cells. Stably transfected HEK293 cells overexpressing wtβAPP751 were obtained, cultured, and treated for 7 hr at 37°C without (control) or with dideoxyforskolin (DDF, 20 μM), forskolin (20 μM), or dibutyryl cAMP (dBut-cAMP, 1 mM) as described in Materials and Methods. Total mRNA was extracted, denaturated, and electrophoresed as described in Materials and Methods. Ribosomal mRNA (A) were visualized by UV and βAPP mRNAs (B) were revealed by means of a radiolabeled specific cDNA probe. Expression of immature and mature full-length βAPP ($βAPP_{FL}$) was analyzed by Western blot with WO2 antibody (C) as described in Materials and Methods. Panel D corresponds to the densitometric analysis of the βAPP immature and mature 110 and 140 kDa immunoreactive bands. Values are expressed as the percent of the control densitometry obtained in untreated cells and the mean ± SEM of four independent determinations.

We examined whether the cAMP-dependent stimulation of βAPP expression could account for the overall effect observed on βAPP maturation products. In this context, we studied the effect of the various cAMP effectors on βAPP processing in the presence of the transcriptional inhibitor actinomycin D. As expected, actinomycin D totally blocks the cAMP-dependent increase in βAPP expression (Fig. 3A). However, actinomycin D does not prevent the increase in Aβ40, Aβ42, and their x-40/42-related products' recoveries triggered by the cAMP-pathway agonists (Fig. 3B–D). Furthermore, APPα secretion still remains stimulated by forskolin and dBut-cAMP in the presence of actinomycin D (Fig. 3E, F). Altogether, our data indicate that the formation of the α- and β/γ-secretase-derived products can be modulated by a cAMP-dependent and actinomycin D–insensitive pathway. It should be noted that the cAMP-dependent production of both APPα and Aβ-derived products is not affected by cycloheximide (not shown), ruling out the possibility that neosynthesis could account for the observed effect on the formation of the βAPP maturation products.

Several lines of evidence indicate that the maturation of FAD-linked βAPP is distinct from that of wtβAPP (for reviews see refs. 4,5). Thus, the production of Aβ by cells expressing the Swedish mutated βAPP (SwβAPP) appears to occur in an intracellular compartment distinct from that of wtβAPP (28). In this context, it was of interest to examine whether cAMP effectors also modulate SwβAPP maturation in HEK293 cells. Figure 4 demonstrates that forskolin, dBut-cAMP, but not DDF, stimulate the recovery of secreted Aβ40 and Aβ42 (Fig. 4A, B) to a similar extent (Fig. 4E). The same increase is observed for their x-40- and x-42-related species (Fig. 4A, B) and APPα (Fig. 4D). Here again, this cAMP-dependent stimulatory effect is not affected by actinomycin D (Fig. 4A, B, D–F).

We assessed whether intracellular Aβ was also affected by cAMP agonists, thus total Aβ (because intracellular Aβ42 is poorly detectable) was immunoprecipitated by FCA18. As expected, this antibody directed toward the Aβ N terminus does not immunoprecipitate x-40/42 species (Fig. 4C). Clearly, forskolin and dBut-cAMP but not DDF also increase the intracellular production of total Aβ in SwβAPP-expressing HEK293 cells (Fig. 4C).

Is the cAMP-dependent Aβ secretion cell specific? We took advantage of a recently neocortical cell line established by oncogenic retroviral infection (21) to examine the susceptibility of neuronal cells to cAMP effectors. Cells stably

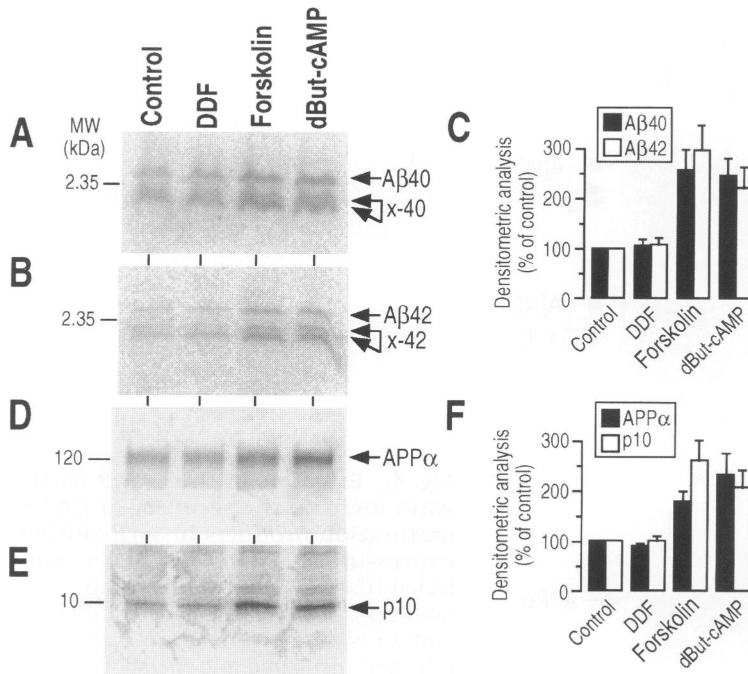

Fig. 2. Effect of cAMP effectors on the maturation of wtβAPP751 in HEK293 cells. wtβAPP751-expressing HEK293 cells were cultured, metabolically labeled, and incubated in the absence (control) or presence of cAMP agonists as in Figure 1. Media were collected, diluted in 10× RIPA, and incubated overnight with FCA3542 antibodies then for 5 hr with protein-A Sepharose. Of the resulting supernatant, 500 μl was then exposed to the 207 antibody while the remainder was incubated with FCA3340 as described in Materials and Methods. After centrifugation, pellets were resuspended with the loading buffer and electrophoresed on a 16.5% Tris-tricine [Aβ40 (A) and Aβ42 (B)] or on a 8% Tris-glycine [APPα (D)]. Intracellular p10 (E) was measured after immunoprecipitation of cell lysates with B11.4 and 16.5% Tris-tricine electrophoresis as in Materials and Methods. Densitometric analysis of gel radioautographies of Aβs (C) and APPα/p10 (F) are expressed as the percent of control densitometry obtained in the absence of effector and are the means of three to four independent experiments.

transfected with wtβAPP751 secrete mainly Aβ40, concomittantly with its x-40 related fragments (Fig. 5A), whereas Aβ42 is poorly recovered (not shown). Interestingly, Aβ40 secretion is enhanced by the treatment of neurons by forskolin and dBut-cAMP but not by DDF (Fig. 5A, B), indicating that the response to cAMP agonist does not seem to be cell specific.

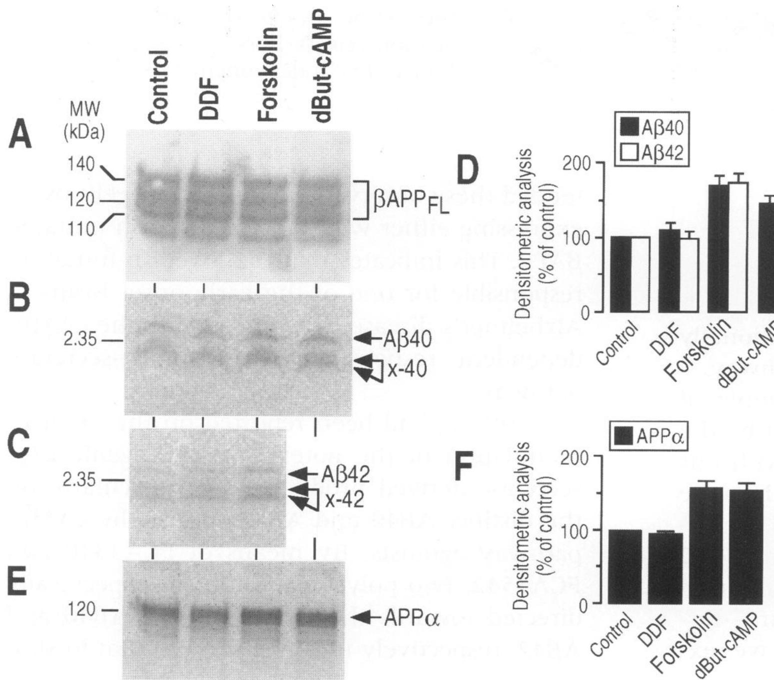

Fig. 3. cAMP-dependent stimulation of βAPP maturation products is not affected by actinomycin D in wtβAPP751-expressing HEK293 cells. Stably transfected HEK293 cells overexpressing wtβAPP751 were preincubated for 1 hr with actinomycin D (5 μg/ml) then incubated and metabolically labeled with actinomycin D in the absence (control) or presence of the indicated cAMP agonists. Intracellular βAPP immunoreactivity was revealed with WO2 (A) while Aβ40 (B), Aβ42 (C), and APPα (E) were immunoprecipitated as described in the Figure 2. Densitometric analysis of gels radioautographies of Aβs (D) and APPα (F) are expressed as the percent of control densitometry obtained in the absence of effector and are the means of three to four independent experiments. Densitometric analysis of the three bands of βAPP in panel A indicate a mean value (expressed as the percent of control) of (upper band) 110, 88.5 and 103.5; (middle band) 107, 88 and 101; (lower band) 117, 101, and 106 for DDF, forskolin and dBut-cAMP, respectively.

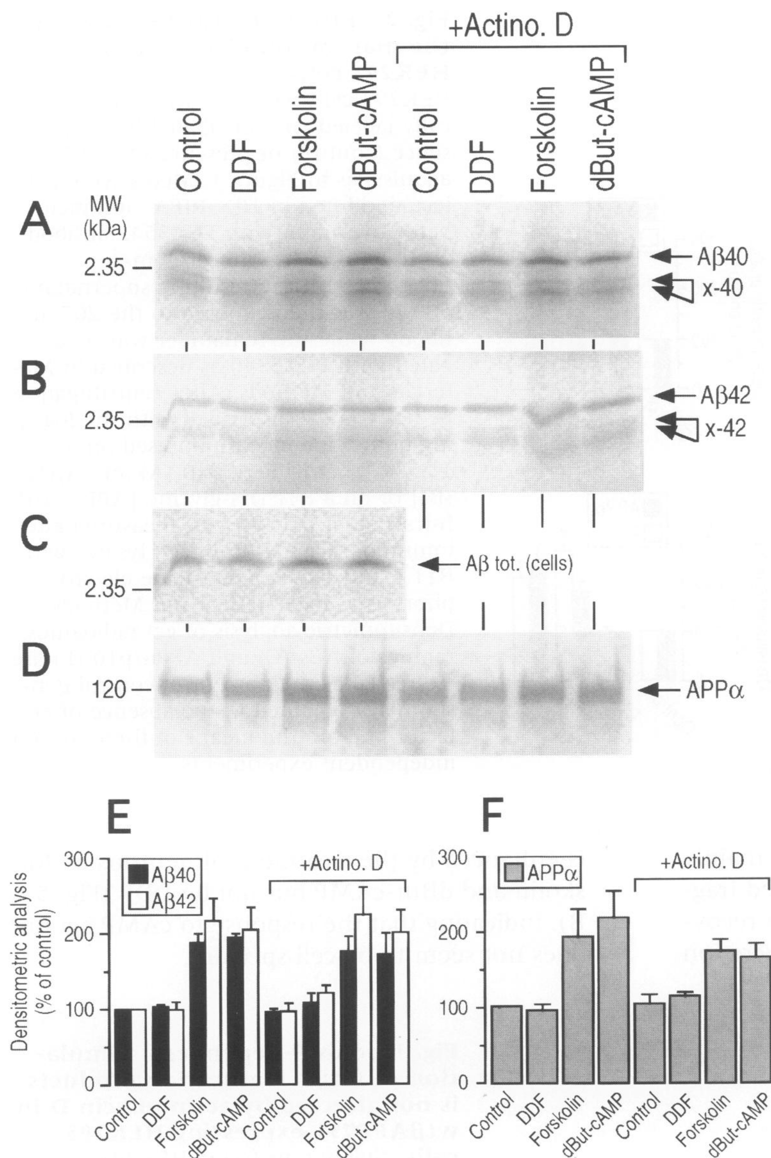

Fig. 4. Effectors of the cAMP pathways increase the recovery of βAPP maturation products in SwβAPP751-expressing HEK293 cells. Stably transfected HEK293 cells overexpressing SwβAPP751 were preincubated for 1 hr with or without actinomycin D (5 μg/ml) then incubated and metabolically labeled in the same conditions, in the absence (control) or presence of the indicated cAMP agonists. Secreted Aβ40 (A), Aβ42 (B) and APPα (D) were immunoprecipitated as described in Figure 2. Intracellular total Aβs (C) was immunoprecipitated with FCA18 as described in Materials and Methods. Densitometric analysis of gels radioautographies of secreted Aβ40 and Aβ42 (E) and APPα (F) are expressed as the percent of control densitometry obtained in the absence of effector with or without actinomycin D. Bars are the means of four to five independent experiments.

Discussion

Our study demonstrates that the α- and β/γ-secretase pathways taking place in HEK293 cells overexpressing either wild-type (wt) or Swedish (Sw) mutated βAPP can be post-transcriptionally modulated by effectors of the cAMP pathway.

The recovery of secreted APPα is stimulated by forskolin and dibutyryl-cAMP but not by the inactive analog DDF. This agrees well with our and other previous studies showing that the α-secretase pathway can be up-regulated by protein kinase A agonists in HEK293 (13) and PC12 (14) cells. We reinforced these data by showing a similar increase in the APPα intracellular C-terminal counterpart p10. Furthermore, we extended these observations to HEK293 cells overexpressing either wild-type or Swedish mutated βAPP. This indicates that the Swedish mutation responsible for one of the early-onset forms of Alzheimer's disease does not modify the cAMP-dependent responsiveness of the α-secretase pathway.

Nothing had been reported on the putative modulation of the potentially pathogenic β/γ-secretase-derived products, and particularly on the distinct Aβ40 and Aβ42 species, by cAMP-pathway agonists. By means of FCA3340 and FCA3542, two polyclonal antibodies specifically directed towards the C terminus of Aβ40 and Aβ42, respectively (24), we showed that forsko-

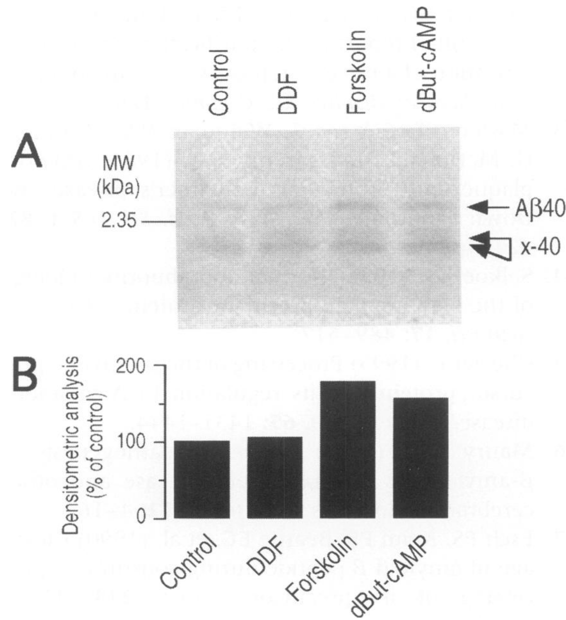

Fig. 5. Effect of cAMP effectors on the secretion of Aβ40 in wtβAPP751-expressing TSM1 neuronal cell line. TSM1 neocortical neuronal cell line expressing wtβAPP751 was obtained, cultured, and stably transfected as described in Materials and Methods. Cells were treated with cAMP effectors and metabolically labeled for 7 hr then secreted Aβ40 was immunoprecipitated with FCA3340 (A) as described in Materials and Methods. Densitometric analysis (B) was performed as above. Bars correspond to the mean values of two distinct experiments and are expressed as the percent of control obtained without agent.

lin and dBut-cAMP drastically enhance the recovery of both secreted Aβs by wtβAPP-expressing HEK293 cells. Interestingly, these antibodies allow the precipitation of Aβs and Aβ-related doublet protein truncated at their N terminus as they are not recognized by FCA18 (24). Although not definitely identified, these fragments likely correspond to previously described α/γ (Aβ17-40/42 also called p3) and α'/γ (Aβ11-40/42) secretase-derived products (24,27). Clearly, both productions appear to be responsive to cAMP agonists.

Altogether, our data indicate that cAMP-pathway effectors stimulate the production of both physiological α-secretase-derived products (APPα and p10) and potentially pathogenic fragments (Aβ40 and Aβ42) as well as Aβ17-40/42 and Aβ11-40/42 in wtβAPP-expressing HEK293 cells.

It has been previously described that the production of Aβ occurs in distinct cellular compartments in wtβAPP- and SwβAPP-expressing cells. Thus, most of the Aβ production seems to occur after internalization in wtβAPP-expressing cells whereas this event takes place earlier, in the late compartments of the Golgi, in SwβAPP-expressing cells (28). It was therefore interesting to assess whether cAMP-agonists also influence Aβ40 and Aβ42 secretion in SwβAPP-expressing HEK293 cells. We demonstrate that this is indeed the case. This indicates that the target of the cAMP-dependent modulator of βAPP maturation in HEK293 cells is likely located upstream to both α- and β/γ-secretases cleavages.

The classical cAMP pathway includes the protein kinase A that can phosphorylates either cellular proteins or cAMP-responsive transcription factors. To delineate the respective contribution of transcriptional and post-transcriptional events, we have examined the effect of the transcription blocker actinomycin D on the cAMP-dependent response of wtβAPP- and SwβAPP-expressing HEK293 cells. First we showed that forskolin and dBut-cAMP but not DDF increase, in an actinomycin-sensitive manner, the βAPP mRNA and protein expressions. This agrees well with a previous study showing that cAMP analogs (15–17) or agonists of the cAMP-coupled adrenergic receptors stimulate the synthesis of βAPP in astrocytes (18). However, we have established that actinomycin D does not prevent the stimulation of the various α- and β/γ-secretase-derived products triggered by cAMP-pathway agonists. This clearly demonstrates that the cAMP-dependent regulation of βAPP maturation in HEK293 cells is not due to endogenous βAPP or transgene activation but involves post-transcriptional events likely mediated by intermediate proteins targeted by protein kinase A.

Several lines of evidence suggest that protein kinase A could modulate the secretory processes (29) and more precisely, the budding of βAPP-containing vesicles from the *trans*-Golgi network (14). Whether PKA targetting leads to a nonspecific increase in the secretion of various proteins including Aβ40/42 and APPα remains to be established. However, it should be noted that in our recent study, we established that in non-stimulated basal conditions, PKA inhibitors prevent the secretion of Aβ40 and Aβ42 by HEK293 cells and cultured neurons, without affecting APPα secretion (30). This first indicates that PKA also controls the constitutive processing of βAPP. Furthermore, the fact that PKA inhibitors do not affect APPα secretion also argues in favor of a selective control of βAPP maturation rather than

a nonselective stimulation of general secretory processes.

The present study showing that cAMP effectors also increase the intracellular formation of total Aβ, suggests that the PKA target is located early along the βAPP secretory pathway, likely before the γ-secretase cleavage responsible for the generation of Aβ42, since this proteolytic event was recently shown to occur before the γ-cleavage leading to Aβ40 (31,32).

Although the nature of the phosphoprotein behaving as substrate of PKA remains to be identified, it appears clear that it is distinct from that targeted by protein kinase C, since, unlike PKA, PKC elicits opposite effects on APPα and Aβs production in various cell lines (5).

Our study further supports the view that βAPP maturation is a highly regulated process. We previously identified a multicatalytic proteinase complex called proteasome as a contributor of the βAPP maturation. This enzyme degrades FAD-linked presenilin1, thereby lowering the ratio of Aβ42 to total Aβ and we suggested activators of this enzyme as potential pharmacological blockers of Aβ42 production (33). Activation of the PKC can be another alternative to lower Aβ production. Savage et al. (34) recently reported on the decrease of Aβ production in vivo, with PDBu stimulation of endogenous PKC. Here, we identify another putative therapeutic target, PKA, whose inhibition could lower production of Aβ40 and its more aggregable and pathogenic form, Aβ42.

Acknowledgments

We sincerely thank Dr. Bart de Strooper (Leuven, Belgium) for the generous supply of B11.4. We are grateful to Dr. K. Beyreuther (Heidelberg, Germany) for providing us with WO2. We thank Drs. B. Greenberg and M. Savage for the kind supply of the 207 antibody. Dr. Chun and Allelix Biopharmaceutical Inc. (Missisauga, Canada) are thanked for providing the TSM1 cell line. We thank J. Kervella for secretarial assistance. This work was supported by the Centre National de la Recherche Scientifique and the Institut National de la Santé et de la Recherche Médicale.

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