Amyloid precursor protein metabolism in fibroblasts from individuals with one, two or three copies of the amyloid precursor protein (*APP*) gene

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Protein kinase C (PKC)-activated modulation of amyloid precursor protein (APP) metabolism has been investigated in natural models of altered APP expression due to the presence of one, two or three copies of the *APP* gene. We show that levels of APP present in human skin fibroblasts strongly influence the effect of PKC activation of soluble APP (sAPP) release. Thus fibroblasts derived from a patient with a deletion in chromosome 21 including the APP locus (Δ21) had lower levels of both APP mRNA and cell-associated APP, and showed an exaggerated phorbol-ester-induced sAPP release, when compared with fibroblasts from control individuals. In contrast, fibroblasts from chromosome 21 trisomic Down's syndrome patients failed to show a concentration-dependent response to phorbol ester treatment. These results suggest that the levels of APP expression can affect the degree of response to PKC-mediated modulation of the metabolism of this protein.

Key words: Alzheimer's disease, β-amyloid, chromosome 21, Down's syndrome, protein kinase C, α-secretase.

INTRODUCTION

A number of human pathologies are characterized by the abnormal deposition of proteins in a fibrillar form, causing disruption of cellular functions. In brain from an Alzheimer's disease patient, the extracellular amyloid deposits are composed of fibrils of the β-amyloid peptide (Aβ) [1]. Aβ is generated by the proteolytic processing of a ubiquitously-expressed integral membrane protein called the amyloid precursor protein (APP) [2–4]. The *APP* gene is located on chromosome 21 [5].

APP is processed by at least two different pathways, involving unidentified enzyme activities named α-, β- and γ-secretase. α-Secretase cleaves APP between amino acids 16 and 17 of the Aβ peptide [6–8], resulting in the release of the soluble N-terminal ectodomain of APP (sAPP) into the extracellular space, thereby preventing generation of full-length Aβ. An alternative pathway operates in parallel with α-secretase processing whereby β-secretase cleaves APP at the N-terminus of Aβ to generate a shortened form of sAPP, leaving a C-terminal fragment from which Aβ can be liberated by the action of γ-secretase and finally released into the extracellular space [9–11]. The relative amounts of APP committed to these pathways can be affected by activation of cell-surface receptors, which signal via a number of transduction pathways, including calcium, arachidonic acid metabolites and protein kinase C (PKC). PKC activation apparently increases the amount of APP processed via the α-secretase pathway, although the phosphorylation target responsible for mediating this effect is presently unknown [12,13].

In Down's syndrome, caused by trisomy of chromosome 21, APP expression is increased due to the presence of a third copy of the *APP* gene [14]. This condition is associated with the appearance of cortical amyloid plaques by the age of 30–40, very similar to those seen in Alzheimer's disease neuropathology [15–17]. Clinical studies also show that a considerable proportion, if not all, of Down's syndrome patients develop Alzheimer-type cognitive deficits, typically between the ages of 50 and 55 years [18–20]. There are also reports in the literature of chromosome 21 monosomy, but many of these individuals have been shown to carry instead extensive deletions or translocations of part of the chromosome. Some case studies have reported deletions which included the *APP* locus [21–24]. Fibroblasts derived from one of these subjects, referred to as Δ21, were used in the present study. This individual carries an extensive deletion of the paternal chromosome (21q11.1–21q22.1), which is associated with mild mental retardation, hypothyroidism and hyperopia [24]. This represents the largest chromosome 21 deletion described to date that was not associated with severe malformations or mental retardation.

In the present study, the modulation *in vitro* of APP secretion via PKC activation in fibroblast cell lines derived from the Δ21 patient, control subjects and Down's syndrome individuals has been compared. These cell lines represent natural models of altered APP expression due to the presence of one, two or three *APP* genes. The object of this study was to determine whether gene-dosage-related differences in APP expression, confirmed by APP mRNA quantification, affected basal or PKC-stimulated APP secretion, which was assessed by Western immunoblotting and immunocytochemical analyses.

EXPERIMENTAL

Materials

All culture media, supplements and fetal-calf serum were obtained from Gibco Life Technologies (Paisley, Scotland, U.K.). Electrophoresis reagents were obtained from Bio-Rad (Hercules,

Abbreviations used: APP, amyloid precursor protein; sAPP, soluble APP; PKC, protein kinase C; PdBu, phorbol-12,13-dibutyrate; Δ21, fibroblasts from an individual with a deletion of chromosome 21; Aβ, β-amyloid peptide; TNA, total nucleic acid.

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CA, U.S.A.). All other reagents were of the highest grade available and were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.), unless otherwise specified.

Cell culture

Fibroblast cell lines from Down's syndrome subjects ($n = 4$, mean age 45.5 ± 6.4 years) and fibroblasts from young healthy volunteers ($n = 4$, mean age 44.3 ± 14.7 years) were obtained from the fibroblast repository of the I.R.C.C.S. ('Centro S. Giovanni di Dio – FBF', Brescia, Italy) [25,26]. Fibroblasts were collected and cultured from the Δ21 patient as described previously [24]. Cells were cultured in Eagle's minimal essential medium supplemented with 10 % (v/v) fetal-calf serum, 100 units/ml penicillin, 100 μg/ml streptomycin, a 1:100 dilution of a stock solution of non-essential amino acids and Tricine buffer (20 mM, pH 7.4) at 37 °C in CO_2/air (1:19, v/v). Experiments were performed at 37 °C in serum-free Eagle's minimal essential medium on cells at an equivalent number of passages.

Treatment of cells with phorbol-12,13-dibutyrate

Confluent monolayers of cells were washed twice with PBS and once with serum-free culture medium before incubation for 2 h at 37 °C with phorbol-12,13-dibutyrate (PdBu). A stock solution of 5 mM PdBu in DMSO was prepared and controls, with vehicle alone, were included in all experiments.

Harvesting of cells and collection of conditioned media

Conditioned medium was collected after 2 h incubation and centrifuged at 13000 ***g*** for 5 min to remove detached cells. Proteins in the conditioned medium were precipitated quantitatively by the deoxycholate/trichloroacetic acid procedure as described previously [26,27]. For studies of full-length cell-associated APP, fibroblast monolayers were washed twice with ice-cold PBS and lysed on the tissue-culture dish by the addition of ice-cold lysis buffer [50 mM Tris/HCl, pH 7.5/150 mM NaCl/5 mM EDTA/1 % (v/v) Triton X-100] (500 μl/dish) and scraped from the dish with a rubber spatula. An aliquot of the cell lysate (normally 5–10 μl) was used for protein estimation using the Bio-Rad protein assay kit.

For APP mRNA studies, total nucleic acid (TNA) was prepared from the fibroblasts by two different methods and similar results, expressed as APP mRNA copies/pg DNA, were obtained regardless of the method used. Using the first method, cells from four 75-cm² flasks or six 100-mm dishes were harvested by treatment with trypsin followed by centrifugation and were stored at −70 °C until needed for TNA preparation. The cells were incubated with Proteinase K (200 μg/ml; IBI) and TNA was extracted with phenol/chloroform (1:1, w/v), as previously described [28]. Using the second method, fibroblasts were grown on a 100-mm plate (55 cm²), washed with ice-cold PBS and then 0.65 ml of RNA extraction buffer [0.14 M NaCl/1.5 mM $MgCl_2$/10 mM Tris/HCl, pH 8.6/0.5 % (v/v) Nonidet P-40/1 mM dithiothreitol] was added. Protein digestion buffer [0.3 M NaCl/0.2 M Tris/HCl, pH 8.0/25 mM EDTA/2 % (w/v) SDS] (0.65 ml) was added, the cells were scraped from the plate and transferred to a tube containing Proteinase K (200 μg/ml) for 30 min at 37 °C. TNA was extracted with phenol/chloroform (1:1, w/v) precipitated with ethanol and the TNA pellet was recovered by centrifugation.

Western immunoblotting of APP and sAPP

Normalization of protein loading on to each blot was obtained by application of an equal amount of cell-lysate protein or an equal volume of conditioned media standardized to total cell-lysate protein concentration. The samples were separated by SDS/PAGE (10 % gel) (250 mA for 1 h) and then transferred to a nitrocellulose membrane (Costar, Cambridge, MA, U.S.A.). Unoccupied sites on the membrane were blocked by incubation with 5 % (w/v) non-fat dried milk in 20 mM Tris/HCl, pH 7.5/500 mM NaCl/1 % (v/v) Tween 20 and the blots were incubated overnight at room temperature with 2 μg/ml of monoclonal antibody 22C11 (Boehringer Mannheim) in the same buffer. For detection of sAPP, the membrane was incubated with horseradish-peroxidase-conjugated goat anti-(mouse) IgG (Kirkegaard and Perry Labs., Gaithersburg, MD, U.S.A.) for 1 h. The blots were then washed extensively with 20 mM Tris/HCl, pH 7.5/500 mM NaCl/1 % (v/v) Tween 20 and the immunoreactivity was revealed using enhanced chemiluminescence (ECL®, Amersham).

Solution hybridization–RNase protection assay

APP mRNA was quantified as described in detail elsewhere [28]. TNA was prepared from fibroblasts as described above. The TNA pellet was dissolved in 0.2 × SET [1 × SET = 0.2 % (w/v) SDS/2 mM EDTA/4 mM Tris/HCl, pH 7.6] and stored at −70 °C. TNA and DNA concentrations were determined using spectrophotometry and fluorimetry (Hoechst 33258) respectively. RNA concentrations were calculated by subtracting the DNA from the TNA concentration. An ^{35}S-labelled CTP antisense RNA probe, designed to hybridize to human APP (at nt 99–207) was incubated with either increasing amounts of unlabelled sense RNA transcribed *in vitro*, which allowed construction of a standard curve, or a sample of TNA for 18 h at 68 °C in 25 % (w/v) formamide/0.75 mM dithiothreitol/0.6 M NaCl/20 mM Tris/4 mM EDTA (40 μl total volume) covered with paraffin oil. Following incubation with 1 ml of RNase T1 (2 μg/ml), RNase A (40 μg/ml) and salmon sperm DNA (100 μg/ml) (Sigma) at 37 °C for 45 min, ribonuclease-resistant hybrids were precipitated with 10 % (w/v) trichloroacetic acid, captured on Whatman GF/C filters and radioactivity in the samples was quantified by scintillation spectroscopy. The amount of specific mRNA present was calculated by comparison with the standard curve, converted to the number of mRNA copies and expressed as a function of either the DNA or total RNA content of the sample [28].

Densitometry and statistics

Western blots were analysed by calculating the relative density of the immunoreactive bands using a Nikon CCD video camera module and the Image 1.47 program (Dr Wayne Rasband, National Institutes of Health, Research Service Branch, N.I.M.H., Bethesda, MD, U.S.A.). The relative densities of the bands were expressed as arbitrary units and normalized with respect to data obtained from control samples run under the same conditions. Controls were processed in parallel with PdBu-treated cell samples and always included on the same blot. Preliminary experiments with serial dilutions of secreted proteins allowed determination of the optimal linear range of the chemiluminescence reaction. Data from the three study groups were analysed by unpaired Student's t test when comparing data of either Down's syndrome or Δ21 cells versus controls and by Dunnet's multiple comparison test when analysing the

concentration-response curves. In both cases a P value < 0.05 was considered significant.

Immunocytochemistry

Fibroblasts were plated (5000–10000 cells/well) on to 12-mm diam. glass coverslips coated with 0.5% (w/v) gelatine and cultured as described above. After 3–4 days, the cells were fixed for 15 min in 4% (w/v) paraformaldehyde in 0.1 M phosphate buffer, pH 7.4, followed by two rinses in PBS. Staining with 1G5 antibody (kindly provided by Athena Neurosciences, South San Francisco, CA, U.S.A.) at 5 μg/ml [29] was carried out overnight at 4 °C. Fluorescein-conjugated secondary antibody (Vector, D.B.A., Milan, Italy) at a 1:100 dilution was then added for 1 h at room temperature. Slides were examined by microscope (Axioskop, Zeiss) and photographed (exposure 1 min) on 400 ASA film (Kodak).

RESULTS

APP mRNA levels

The basal levels of APP mRNA in three of the fibroblast cell lines used as controls ranged between 18.4 and 29.7 copies/pg DNA (mean 24.3 ± 3.3 S.D.) (Table 1). A lower level of APP mRNA was present in the Δ21 cell line, with only 9.8 copies of APP mRNA/pg DNA. As expected from the increased gene dosage, the four Down's syndrome fibroblast lines showed elevated levels of expression, ranging from 67.7–74.1 copies of APP mRNA/pg DNA (mean 71.2 ± 2.7 S.D.), which however did not strictly follow the order of gene dosage and reached well above the expected 50% increase.

Analysis of levels of cell-associated APP holoprotein

The levels of full-length, cell-associated APP were studied using both immunocytochemical analysis of paraformaldehyde-fixed cells and a Western blot of cell lysates. Figure 1 shows the immunocytochemical staining of APP in Δ21 (Figure 1A), control (Figure 1B) and Down's syndrome (Figure 1C) fibroblasts. The Δ21 cell line stained faintly and the APP immunoreactivity detected was clearly lower than that of control cell lines, whereas consistently higher levels were present in the Down's syndrome fibroblasts. Western-blot analysis of APP in fibroblast lysates, shown as representative experiments in Figures 1(D), (E) and (F), also confirmed that the Δ21 cell line contained less APP than controls, which was consistent with the low levels of APP mRNA. Western blots of parallel lanes of four Down's syndrome cell lines, four control cell lines and triplicate samples of Δ21 were used for semiquantitative analysis. The results showed that the levels of cellular APP in Δ21 were 77.3 ± 8.3% ($P < 0.05$) of the mean control level and APP in Down's syndrome fibroblasts was 167.7 ± 3.5% of control levels ($P < 0.01$).

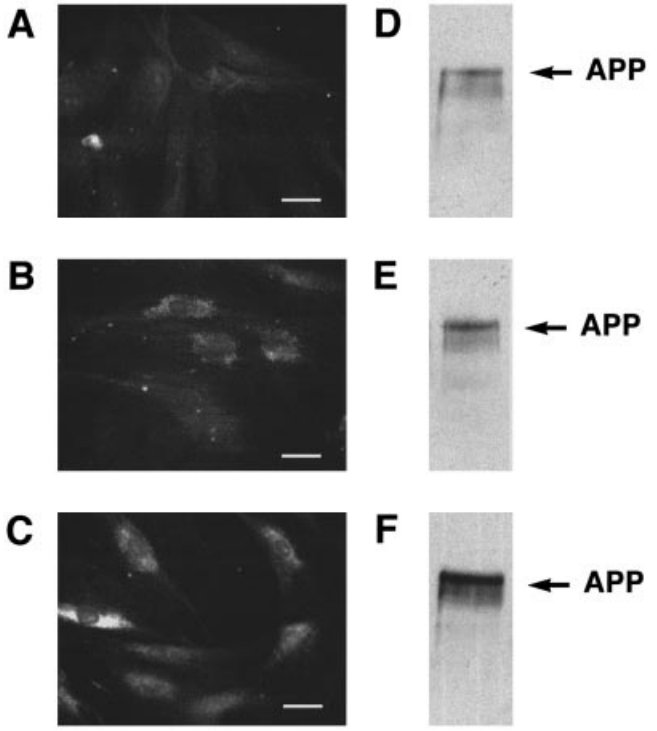

Figure 1 Immunocytochemistry of APP in cultured fibroblasts of Δ21 (A), control (B) and Down's syndrome (C) subjects

The staining of Δ21 is faint and less than that of controls, whereas Down's syndrome cells consistently express a higher total immunoreactivity compared with control cells. Western blots of cell-associated APP in lysates of Δ21 (**D**), control (**E**) and Down's syndrome (**F**) fibroblasts.

Table 1 Levels of APP mRNA expression in fibroblast cell lines

Total nucleic acid was extracted by method 1 for all cell lines except 21 and 67 where method 2 was used (see the Experimental section). APP mRNA was quantified by solution hybridization–RNase protection assay as described in the Experimental section. CBD, cortico-basal degeneration; DS, Down's syndrome. APP mRNA levels were lower in the Δ21 cell line and significantly increased in the Down's syndrome cell lines ($P \leqslant 0.0001$, unpaired Student's t test), compared with fibroblasts with a normal APP gene dosage.

Cell line	Clinical status	APP mRNA (copies/pg DNA)
603	Δ21	9.8
922	Control	18.4
9	CBD	24.9
21	Control	29.7
67	DS	74.1
66	DS	67.7
73	DS	70.9
79	DS	71.9

Basal and PdBu-induced sAPP levels

sAPP levels in cell culture medium conditioned by Δ21, control and Down's syndrome fibroblasts were determined under basal conditions and after direct PKC stimulation by PdBu. Densitometric analysis of Western blots for sAPP from conditioned medium from Down's syndrome cell lines, four control cell lines and triplicate samples of Δ21, revealed that medium conditioned by the Down's syndrome fibroblasts contained approx. 200% of control levels, whereas that from Δ21 was only 60%, if the level of sAPP in medium conditioned by control cell lines was taken as 100% (Figure 2). The sAPP levels thus reflected the levels of cell-associated APP detected in the cell lysates.

The sAPP response to PdBu activation of PKC differed in the Δ21, control and Down's syndrome fibroblasts. As shown in Table 2, sAPP levels in media conditioned by control fibroblasts

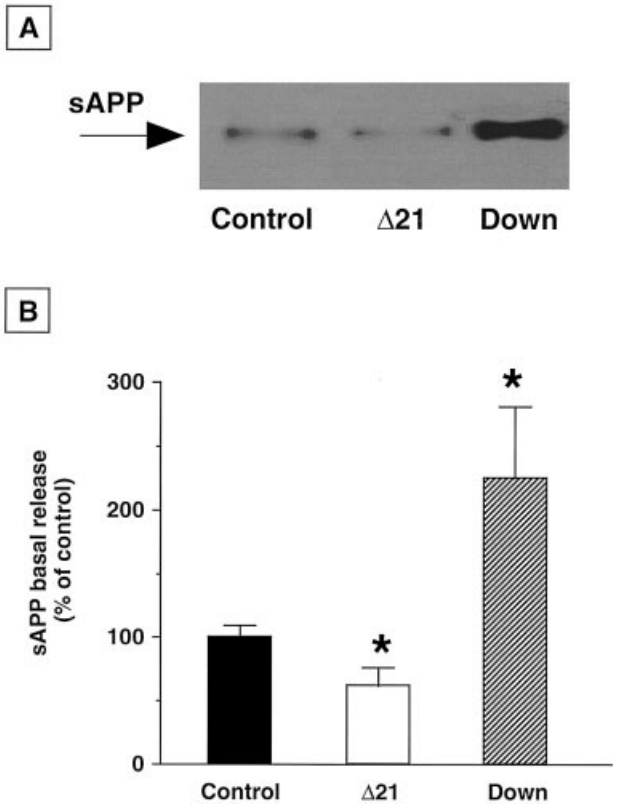

Figure 2 Basal release of sAPP from control, Δ21 and Down's syndrome fibroblasts

sAPP basal release was evaluated by incubating the cells in serum-free medium for 2 h, followed by Western blotting of proteins released into the conditioned medium. (**A**) Example of Western blot of conditioned media from control, Δ21 and Down's syndrome (Down) fibroblasts. (**B**) Semiquantitative evaluation of Western blots with data expressed as a percentage of the average basal release from control cells. Data are the means ± S.D. of four control, four Down's syndrome (Down) cell lines and of three replicate experiments for the Δ21 cell line. ($^{*}P < 0.05$, compared with basal sAPP release from control cells).

Table 2 Secretory response to phorbol ester treatment of control, Δ21 and Down's syndrome fibroblasts

sAPP release was measured semiquantitatively by Western blot and the data were expressed as a percentage of sAPP released compared with the respective basal level of each group of cells. Data are the means of single experiments performed on three different cell lines for control and Down's syndrome fibroblasts, and triplicate experiments performed with the Δ21 cell line. Samples from each cell group were run separately and all statistical comparisons were made with respect to the basal release of each cell line. Results were then averaged and subjected to multiple comparison Dunnet's test. $^{*}P < 0.05$, $^{**}P < 0.01$ (compared with basal sAPP release); n.s. not significant.

	sAPP release (% of respective basal release)		
Treatment	Control	Δ21	Down's syndrome
Vehicle	100	100	100
PdBu 9 nM	140 ± 10*	178 ± 14**	118 ± 8 n.s.
PdBu 18 nM	178 ± 12*	260 ± 28**	115 ± 7 n.s.
PdBu 150 nM	198 ± 20**	385 ± 20**	112 ± 10 n.s.

increased to 140, 178 and 198 % of basal levels following a 2 h exposure to 9, 18 or 150 nM PdBu respectively. The Δ21 fibroblasts showed an exaggerated response to PdBu, where

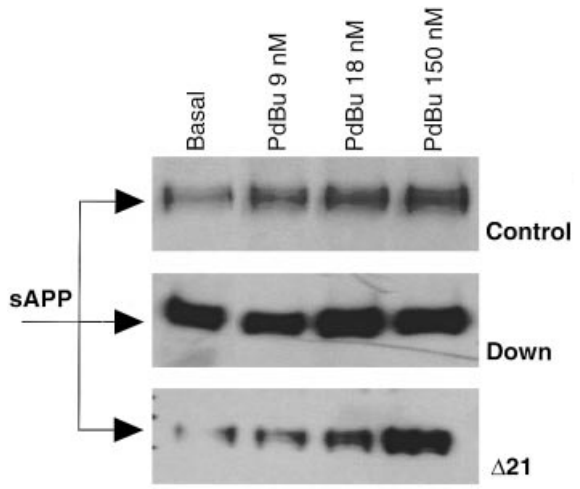

Figure 3 Comparison of phorbol-ester-stimulated sAPP release from control, Δ21 and Down's syndrome (Down) fibroblasts

Cells were incubated in serum-free medium for 2 h at 37 °C with 9, 18 or 150 nM PdBu. The Western blots shown are typical of the patterns of responsiveness to phorbol-ester treatment of the three cell types.

sAPP levels increased to 178, 260 and 385 % of basal levels in the presence of 9, 18 or 150 nM PdBu. By contrast, sAPP release from Down's syndrome fibroblasts did not show a concentration-dependent response to PdBu and failed to increase significantly above basal levels at any of the concentrations used (Figure 3).

DISCUSSION

This study shows that PKC-mediated modulation of APP processing via the non-amyloidogenic α-secretase pathway is strongly influenced by alterations in the levels of APP which result from abnormal gene dosage. These results were obtained in human skin fibroblasts derived from skin biopsies of either a patient with a chromosome 21 deletion (Δ21), normal control subjects or Down's syndrome patients. These primary cells are model systems which are free from exogenous genetic manipulation. We demonstrate that the percentage increase in sAPP release in response to PKC-activation by the phorbol ester, PdBu, is exaggerated in Δ21 and reduced in the Down's syndrome fibroblasts when compared with controls. The results obtained with Down's syndrome patients may be due to saturation of the PKC-responsive secretory pathway as a result of over-expression of the APP gene. Conversely, reduced expression of APP in the Δ21 cell line may allow the more dramatic PdBu-induced increases in sAPP release over the lower basal level of sAPP secretion. Previous studies of Down's syndrome have characterized the effect of gene dosage on the expression of genes mapping on chromosome 21. For example, the expression of the superoxide dismutase (*SOD-1*) gene [30] was reported to be increased by 50 %, as expected from a strict gene-dosage effect due to triplication of the gene. The levels of APP mRNA were increased in the Down's syndrome fibroblasts and decreased in Δ21 when compared with controls, but did not seem to follow a strict gene-dosage-dependent expression, since they were substantially above the 50 % predicted (+192 %) increase in Down's syndrome and lower than the 50 % predicted decrease (40 % of controls) in the Δ21 cell line. The alterations in APP mRNA levels were reflected qualitatively in altered levels of cellular

expression of the protein, although the striking differences observed in APP mRNA levels were somewhat attenuated at the protein level. Immunocytochemical and Western-blot analyses showed that the levels of cell-associated APP were reduced in Δ21 and increased in the Down's syndrome fibroblasts, although it is not possible to compare the levels of mRNA with those of the holoprotein directly, since the estimation of the latter relies on semi-quantitative methods. Analysis of sAPP secretion under basal conditions also demonstrated that the Δ21 cell line sAPP was secreted at levels approximately half those of controls, whereas, as expected, Down's syndrome cells secreted significantly more sAPP. This suggests that the secretory mechanisms involved in the regulation of APP metabolism are strongly influenced by the amount of precursor protein present.

In addition to constitutive processing and secretion, APP metabolism can also be regulated by a number of mechanisms, incuding activation of PKC. In many cellular systems, the activation of PKC by phorbol esters has been shown to increase sAPP release by increasing the rate of α secretase processing of APP in a concentration-dependent manner [12]. We have shown previously that exposure of human skin fibroblasts to increasing concentrations of PdBu elicits a concentration-dependent release of sAPP into the conditioned medium [31]. This PKC-dependent secretory mechanism has been shown to be defective in fibroblasts derived from individuals with sporadic Alzheimer's disease due to altered levels and activity of PKCα [31], one of the major PKC isoforms present in human skin fibroblasts [32]. The mechanism by which PKC activation effects the release of sAPP is at present unknown but it could conceivably be via direct activation of the presently unidentified α secretase enzyme(s) or by altering vesicular trafficking which brings the substrate protein to the site (either the plasma membrane or secretory vesicles) [33,34] where α secretase processing takes place.

In most cell types, the activation of sAPP secretion is often associated with a reduction in Aβ release. The detection of Aβ released from cultured skin fibroblasts requires incubation periods of more than 24 h [35]. It was, therefore, not possible to measure the release of Aβ from fibroblasts using the same experimental regime as that used for the detection of sAPP. The effect of altered gene expression on the levels of Aβ remains to be investigated, and is dependent on the design of an appropriate experimental approach.

Longer periods of exposure to phorbol esters (24 h) are also capable of increasing APP expression via the interaction of c-jun with the APP promoter activator protein 1 (AP1) site [36]. The sAPP response to short-term phorbol ester treatment, such as the one used in the present study, has been shown to occur rapidly (within 1 h of incubation) in fibroblasts and was not blocked by the presence of cycloheximide, a protein synthesis inhibitor [37]. This indicates that this response occurs at the level of protein processing and secretion and in the absence of changes in APP expression.

Overall, these data show that the levels of expression of APP can greatly influence the pattern of responsiveness of APP secretory mechanisms to pharmacological modulation. This is of particular interest in the study of long-term pharmacological treatments of Alzheimer's disease, in particular those that may directly or indirectly affect the levels of APP expression.

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