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MODULATION OF ß-AMYLOID PRECURSOR PROTEIN SECRETION IN DIFFERENTIATED AND NONDIFFERENTIATED CELLS

Jürgen Löffler and Gerda Huber

Pharma Division, Preclinical Research, F. Hoffmann-La Roche Ltd, CH-4002 Basel, Switzerland

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The regulation of ß-amyloid precursor protein (ß-APP) secretion was compared in differentiated and nondifferentiated PC12 and B104 cells. Phorbol esters stimulated the release of ß-APP in all cells examined. However, differentiated PC12 cells were much more sensitive to phorbol ester treatment than nondifferentiated PC12 cells and their ß-APP release was also induced by the protein phosphatase inhibitor okadaic acid and the Ca^{++}-ionophore A23187. In contrast, ß-APP release from B104 cells was strongly stimulated by A23187 and to lesser degree by phorbol esters. This effect was most pronounced in nondifferentiated B104 cells, which might be due to a higher basic release of ß-APP from differentiated B104 cells. Thus, the regulation of ß-APP cleavage and release varies depending on the cell type and differentiation state of the cell.

The proteolytic processing of ß-APP is a key event in the pathogenesis of Alzheimer's disease (AD), giving rise to the ß/A4 amyloid peptide, a major component of the senile plaques in the brains of AD patients (1-3). C-terminally truncated soluble forms of the membrane-bound ß-APP are generated by a postulated "α-secretase" which cleaves within the ß/A4 sequence. An alternative proteolytic route must therefore exist in which the ß/A4 peptide is produced (4-6). A substantial amount of membrane-bound ß-APP is reinternalized from the cell surface and targeted to the lysosomes (7), where potentially amyloidogenic fragments of ß-APP might be generated (8,9). However, ß/A4 is also produced in the secretory pathway (10) and released from the cell (10-13). Regulatory cellular mechanisms may determine the fate of membrane-bound ß-APP, and activation of protein kinase C by phorbol esters was shown to stimulate the secretion of ß-APP in nondifferentiated PC12 cells (14).

In the present study nondifferentiated and differentiated PC12 and B104 cells were examined and compared with respect to modulation of ß-APP release by protein phosphorylation and Ca^{++}-influx.

Abbreviations:

ß-APP, ß-amyloid precursor protein; DMSO, dimethylsulfoxide; KPI, Kunitz proteinase inhibitor; PMA, phorbol-12-myristate-13-acetate; PDB, phorbol-12,13-dibutyrate; 4α-PDD, 4α-phorbol-12,13-didodecanate; SDS, sodium dodecylsulfate.

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Materials and Methods

Materials. Cell culture media and sera were obtained from Gibco-BRL. Dibutyryl-cAMP was purchased from Sigma, phorbol esters from Fluka (Switzerland), NGF and okadaic acid from Boehringer Mannheim. A23187 was obtained from Serva.

Cell cultures. PC12 stock cultures were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum and 5% horse serum. B104 cells were grown in DMEM containing 10% fetal calf serum. Differentiation of PC12 cells was induced by plating $2x10^6$ cells on collagen-coated dishes (diameter 60 mm, Corning) in DMEM containing 0.2% horse serum and either 1 mM db-cAMP or 100 ng/ml NGF. B104 cells (10^5 per well of a 24-well plate) were differentiated using 1 mM db-cAMP in serum-free DMEM. Experiments with PC12 cells were performed after four days in culture, with B104 cells after three days in culture.

Secretion assay. The cells were washed twice with serum-free medium and then incubated with serum-free medium containing vehicle (0.05% DMSO) alone, 1 µM 4α-PDD, 1 µM PMA, 1 µM PDB, 1 µM PDB combined with 1.2 µM okadaic acid, 1.2 µM okadaic acid, or 1 µM A23187. After 30 min the media were separated from the cells and placed on ice until sodium deoxycholate was added to give a final concentration of 0.2% to allow quantitative protein precipitation. 15 min later the proteins were precipitated with 10% trichloroacetic acid, washed with ethanol/2% sodium acetate and dissolved in a fixed volume of 2x SDS-sample buffer (15). Cell pellets were washed with phosphate-buffered saline and directly dissolved in 2x SDS-sample buffer. The protein concentration was determined using the detergent-compatible protein assay from Bio-Rad.

Electrophoresis and immunoblotting. Protein samples were separated in 7.5% polyacrylamide mini-gels (15; Bio-Rad system) and transferred to nitrocellulose (16). 25 µg of cell lysate proteins were loaded per lane. For secreted proteins one quarter of the protein precipitated from the medium of one culture dish (PC12 cells) or half of the protein from two pooled wells (B104 cells) was loaded per lane. Immunoreaction was performed either with the monoclonal antibody 22C11 (Boehringer Mannheim) which recognizes the N-terminal region of ß-APP (17), or with affinity-purified polyclonal anti-KPI or anti-ß(1-16) antibodies (18). Alkaline phosphatase-conjugated secondary antibodies were used for the immunostaining which was quantified using a video densitometer.

Results

ß-APP forms in cells and culture supernatants. In order to analyze Kunitz proteinase inhibitor domain-containing (KPI^+) and -lacking (KPI^-) forms of ß-APP in PC12 cells three different antibodies were used. On immunoblots of PC12 cell media, the anti-N-terminal 22C11 reacted with proteins of 145, 135 and 105 kDa (Fig. 1A, lane 1). The same proteins are recognized by the anti-ß(1-16) antibody (Fig. 1A, lane 3), indicating that they were cleaved at or close to amino acid 17 of the ß/A4 domain. The anti-KPI antibody immunostained only the 145 and 135 kDa bands (Fig. 1A, lane 2). In PC12 cell extracts, 22C11 immunoreacted with a protein doublet of 160 kDa, one protein of 130 kDa and one of 120 kDa (Fig. 1B, lane 1). The anti-KPI antibody reacted only with the 160 kDa doublet and the 120 kDa band (Fig. 1B, lane 2). All immunoreactive proteins were detected in both nondifferentiated and differentiated PC12 cells. The differential immunoreaction observed indicates that the 130 kDa cellular protein and the 105 kDa protein in cell media represent KPI^--forms of ß-APP. In B104 cells only KPI^+-ß-APP was found (not shown).

Stimulation of ß-APP secretion in PC12 cells. The proteolytic cleavage and secretion of ß-APP was studied by modulation of cellular signalling pathways. The protein kinase C-activating phorbol esters PMA and PDB stimulated the secretion of ß-APP (Fig. 2). The inactive phorbol ester 4α-PDD was used as a control and had no effect. PC12 cells differentiated with either db-cAMP or NGF (Fig. 2C, 2D) were both more sensitive to phorbol ester treatment than nondifferentiated cells (Fig. 2B). The phorbol ester-mediated increase of ß-APP secretion during

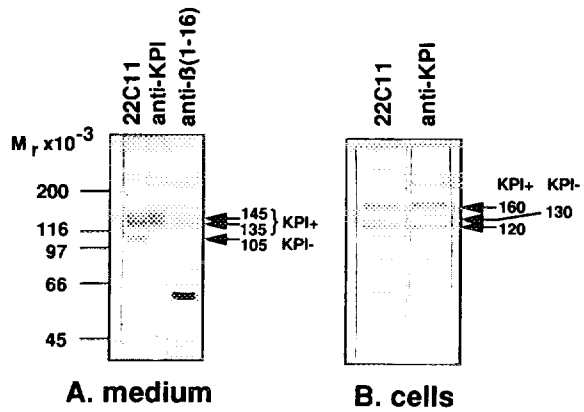

Figure 1. ß-APP forms in PC12 cells and cell media. Secreted proteins precipitated from cell media (**A**) and cellular proteins (**B**) of PC12 cells were immunostained with either monoclonal antibody 22C11, polyclonal anti-KPI antibody or polyclonal anti-ß(1-16) antibody. Molecular masses of standard proteins are indicated on the left, that of ß-APP bands as determined using a video densitometer on the right.

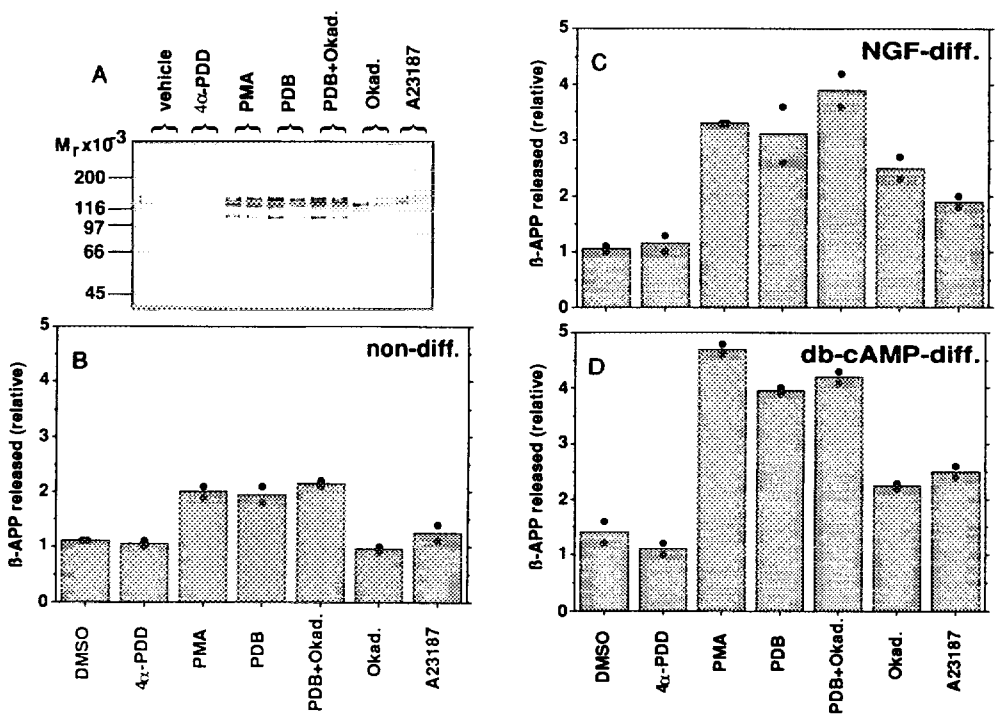

Figure 2. Stimulation of ß-APP secretion in PC12 cells. 22C11 immunostaining of the media of PC12 cells treated with vehicle (0.05% DMSO) alone, the inactive phorbol ester 4α-PDD, the actice phorbol esters PMA or PDB, PDB combined with the protein phosphatase inhibitor, okadaic acid (OKAD), okadaic acid alone, or the Ca^{++}-ionophore A23187. **A**, example shown for NGF-differentiated cells. Densitometric analyses of: **B**, nondifferentiated cells; **D**, db-cAMP-differentiated cells; **C**, NGF-differentiated cells. The relative values shown are the average (bars) and single data points (closed circles) of two experiments.

the 30 min incubation period was about 80% in nondifferentiated cells, and about 250% above background in differentiated cells. The protein phosphatase inhibitor okadaic acid and the Ca^{++}-ionophore A23187 stimulated ß-APP secretion to a lesser extent than phorbol esters and had only an effect in differentiated cells under our experimental conditions (Fig. 2). Okadaic acid was most effective in cells differentiated with NGF, where a 100% increase of ß-APP secretion was induced as compared to a 70% increase in db-cAMP-differentiated cells. A23187 caused an increase of ß-APP secretion by 70-80% in both types of differentiated PC12 cells. In all cases where a stimulation of ß-APP secretion was observed, both KPI^+- and KPI^--ß-APPs were affected. However, because the relatively weak KPI^--band alone was more difficult to quantify, the total amount of ß-APP immunostaining is shown in figure 2.

Stimulation of ß-APP secretion in B104 cells. PMA treatment also stimulated the secretion of ß-APP in B104 neuroblastoma cells. However, the effect of A23187 was much stronger and very striking (Fig. 3). In contrast to PC12 cells, differentiated B104 cells were less sensitive to PMA or A23187 treatment than nondifferentiated cells. This might be due to a higher basic release of ß-APP in differentiated B104 cells. KPI^--ß-APP was neither detectable in neat B104 cells nor in cells treated with a stimulatory agent.

Discussion

Proteolytic processing of ß-APP occurs in at least two different cellular pathways. Mature membrane-bound forms are directed to the plasma membrane, cleaved by an "α-secretase" within the ß/A4 domain (4-6), by a "ß-secretase" at its N-terminus (19), or further C-terminal to the α-site (20). The resulting soluble N-terminal fragments are released into the extracellular space. Alternatively, membrane-bound ß-APP is targeted to the lysosomes where different proteolytic processes occur and potentially amyloidogenic fragments are generated (7-9). At least a fraction of

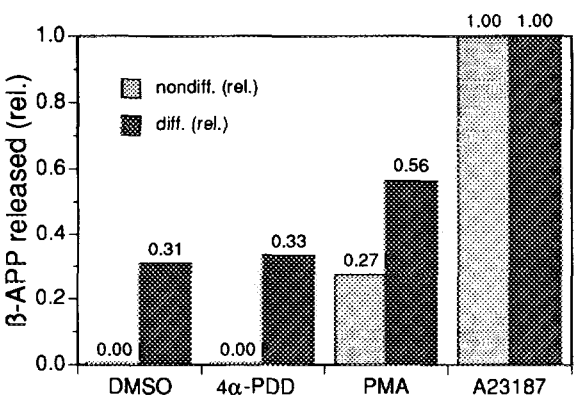

Figure 3. Stimulation of ß-APP secretion in B104 cells. Comparison of the relative amount of ß-APP released from differentiated and nondifferentiated B104 cells treated with the indicated agents. Data were obtained by densitometric analysis of anti-KPI reactive proteins. Values of A23187 treated cells were set to 1.0 in both series to allow a comparison of relative effects.

the lysosomal ß-APP seems to be derived from reinternalized cell surface-ß-APP which has escaped the secretory cleavage event (7).

It was shown that protein kinase C activation in nondifferentiated PC12 cells leads to an augmented release of truncated ß-APP into the medium (14). In the present study, regulation of ß-APP secretion in differentiated PC12 and B104 cells was compared to the nondifferentiated cells. In PC12 cells KPI^+- and KPI^--ß-APP secretion was similarly inducible, whereas only KPI^+-ß-APP was detected in B104 cells. Stimulation of ß-APP secretion by phorbol ester treatment was consistently observed. However, both NGF- and db-cAMP-differentiated PC12 cells were substantially more sensitive to protein kinase C activation than nondifferentiated PC12 cells. Similar results had been obtained with PC12-L cells, where, however, not the secretion of soluble ß-APP derivatives but the production of a C-terminal 15 kDa fragment was measured (21). Differentiated PC12 cells also reacted to the protein phosphatase inhibitor okadaic acid and the Ca^{++}-ionophore A23187, while nondifferentiated PC12 cells were insensitive within our detection range. Nevertheless, the stimulatory effect of okadaic acid on nondifferentiated PC12 cells described earlier (14) is consistent with our results considering the use of radiolabelling and a longer incubation period. The phosphatase-inhibition- and Ca^{++}-influx-dependent stimulation of ß-APP secretion in differentiated PC12 cells, however, was less pronounced than the effect induced by protein kinase C activation. In B104 neuroblastoma cells the strongest stimulation of ß-APP secretion was induced by the Ca^{++}-ionophore, yet phorbolesters were also effective. The stimulation by both A23187 and PMA was more pronounced in nondifferentiated than in differentiated B104 cells, which is in contrast to PC12 cells.

The effects of phorbolesters and A23187 suggest that ß-APP secretion can be modulated via various signalling pathways. In analogy, the cleavage of pro-TGFα at the cell surface is stimulated independently by both protein kinase C activation or Ca^{++}-influx (22). The different response of nondifferentiated and differentiated PC12 and B104 cells is particularly interesting with respect to the differentiation state of neuronal cells in the brain. Differentiated cells might imitate neuronal ß-APP processing in a better way than nondifferentiated cells. Furthermore, a single cell line may not reflect all the regulation possibilities of ß-APP cleavage and secretion that might be used in brain cells.

In the cell culture studies reported so far the "secretase" cleavage occurred between amino acids 15 and 17 of the ß/A4 domain (4,6). In the present study the anti-ß(1-16) immunostaining of the secreted ß-APP forms of PC12 cells is consistent with these results suggesting that the secretory cleavage investigated here is most probably due to this "α-secretase" and not to another proteinase.

Another open question is how protein kinase C-mediated phosphorylation induces proteolysis of ß-APP. One possibility is a direct activation of the "secretase". Alternatively, the substrate, ß-APP, could be phosphorylated inducing a conformational change and rendering the cleavage site accessible to the "secretase". Evidence supporting the second possibility is that C-terminal ß-APP peptides were phosphorylated in vitro by protein kinase C at Ser 655 (numbering according to the sequence of ß-APP_{695}) (23). This site in ß-APP is also phosphorylated by protein kinase C in a semi-intact cell system (24). However, phosphorylation of ß-APP might also prevent its reinternalization and/or targeting to lysosomes thereby favouring a secretory cleavage simply by

supplying the "secretase" with more substrate. This could also involve tyrosine phosphorylation of the endosomal/lysosomal targeting sequences of ß-APP (25). In any of these cases protein kinase C-mediated phosphorylation and perhaps additional regulatory mechanisms determine the amount of ß-APP to be degraded in the lysosomes thus modulating indirectly the production of ß/A4. In this context it is of interest that the activity of certain protein kinase C isozymes is reduced in AD brains (26).

In summary, our data demonstrate that phorbol esters stimulate the proteolytic secretion of ß-APP in various cell types. We have extended and compared the findings on modulated ß-APP secretion to differentiated PC12 cells which are much more sensitive to protein kinase C mediated secretion of ß-APP. In addition, we have examined differentiated and nondifferentiated B104 cells where ß-APP secretion was strongly stimulated by the Ca^{++}-ionophore A23187. The observed variability between different cell types and differentiation states suggests that multiple regulatory pathways may be involved in the regulation of secretory ß-APP processing in the brain.

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