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ACTIVATION OF THE SECRETORY PATHWAY LEADS TO A DECREASE IN THE INTRACELLULAR AMYLOIDOGENIC FRAGMENTS GENERATED FROM THE AMYLOID PROTEIN PRECURSOR

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SUMMARY: The activation of the secretory pathway for the amyloid protein precursor (APP) by phorbol 12-myristate 13-acetate in human glioblastoma A-172 cells leads to a decrease in intracellular ß-amyloidogenic fragments derived from APP . The result suggests that a metabolic switch of APP from the endosomal/lysosomal pathway to a secretion route occurs after PMA treatment.

The abnormal metabolism of amyloid ß-protein precursor (APP) could be pivotal in the production of characteristic amyloid deposition in Alzheimer's disease (AD) (1-3). Although the ß/A4 peptide (ßP) has been found in normal cerebrospinal fluid (4,5), the hypothesis that a malfunction in the secretory pathway might lead to the over-production of ßP and subsequently cause the insoluble amyloid deposition is still attractive, because increased in vitro-production of soluble ßP has been observed after cDNA transfection of the mutant APP found in familial AD (6,7).

It has been demonstrated that the activation of protein kinase C (PKC) by phorbol ester (8), muscarinic acetylcholine receptor agonist (9), interleukin 1 (10), and fibroblast growth factor (11) accelerates the secretion of APP resulting in a decrease in the amounts of membrane-bound mature protein (8,9). If the endosomal/lysosomal digestion of APP is the major source of amyloidogenic fragments and ßP itself, then stimulation of the secretory pathway by the activation of PKC might change the ratios in intracellular processes thus producing the amyloidogenic fragments.

To test this hypothesis , we examined the correlation between the amounts of the secreted form of APP (sAPP) and intracellular amyloidogenic fragments

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when PKC is activated. We report here that phorbol ester enhances the amount of sAPP and suppresses the amount of intracellular amyloidogenic fragments derived from APP in human glioblastoma A-172 cells .

MATERIALS AND METHODS

Materials

Human glioblastoma A-172 (CRL-1620), T98G (CRL-1690), neuroblastoma IMR-32 (CCL-127), and astroblastoma CCF-STTG1 (CRL-1718) cells were purchased from Dainippon Pharmaceuticals and grown in the media described in the commercial instructions. Leupeptin and pepstatin were purchased from Peptide Institute Inc. (Osaka, Japan). ßP1-40 was obtained from Bachem (CA, USA). Protein kinase inhibitors were from Biomol (PA, USA). Phorbol 12-myristate 13-acetate (PMA), staurosporin, and other chemicals were obtained from Sigma. Pre-cast sodium dodecylsulfate -polyacrylamide gels (SDS-PAGE) were from Tefco (Nagano,Japan). N-Glycanase was obtained from Genzyme (MA, US).

Methods

All cells were grown in medium containing 10% fetal bovine serum (FBS, Gibco). Chemicals dissolved in dimethyl sulfoxide (DMSO) were diluted in serum free medium (final DMSO was 0.1% in each case.) and the confluent cells were exposed to this medium for 1 to 4 hours. To analyze sAPP, the culture medium was concentrated with a PD-10 column (Pharmacia) as described (9). Cells were harvested in phosphate buffered saline (PBS) containing 5 mM EDTA and dissolved directly in Laemmli's buffer containing 2-mercaptoethanol (12). Samples corresponding to 2 X 10^5 cells were applied to 16% tris/tricine SDS-PAGE. The separated proteins were electophoretically transferred onto nitrocellulose membranes (13). Rabbit polyclonal antibodies were raised against the secretory form of APP695 (anti-PN), C-terminal region: Gly^{756}-Asn^{770} (anti-C), and ßP region: Asp^{597}-Gln^{612} (anti-ßP) (14). Anti-N and anti-C were used at 1:1000 dilution (in PBS containing 3% bovine serum albumin (BSA) and 0.05% Tween 20) and the affinity purified anti-ß against ßP1-40 (15) was used for immunoprecipitation. Detection was by a Vectastain Elite system (Vector Labs) and ECL system (Amersham) according to the manufacturer's instructions. Results were quantitated densitometrically with an LKB Ultroscan laser scanner. Metabolic labelling with ^{35}S-methionine and immunoprecipitation with purified anti-ß antibody were performed as reported (6). Soluble APP was purified from the conditioned medium of A-172 cells according to the reported method (16) .

RESULTS

A-172 human glioblastoma cells were selected for the general analyses because of their relatively high expression of APP and sensitive secretory response compared to other cell lines (data not shown). Membrane-bound full-length APPs were found at 140 kD and 115 kD in the cell-lysates of A-172 (Fig.1A, lane 3). These seem to be relatively higher molecular sizes than for APP695 and 770, which are transiently expressed in cDNA-transfected COS-

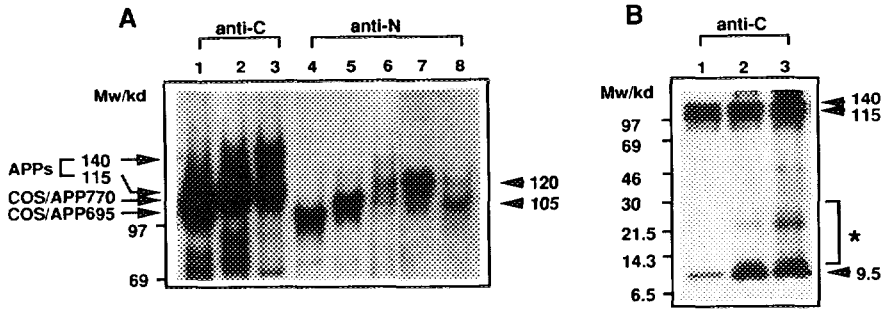

FIG.1. Identification of APPs in A-172 cells. Panel A, Immunoblotting from 4-12% SDS-PAGE; Lanes 1,4, APP695 expressed in COS-7 cells; lanes 2,5, APP770 expressed in COS-7 cells; lanes 3,6, APP expressed in A-172 (protein from COS-7 cells was 1/30 of A-172.); lane 7, soluble APP from A-172; lane 8, N-glycanase treated sAPP from A-172. Panel B, Immunoblotting of total cell proteins from A-172 after 4 hr incubation in serum-free Dulbecco's modified essential medium (DMEM) ,16% tris-tricine SDS-PAGE, Lane 1, control; lane 2, 20μg/ml of leupeptin; lane 3, 20μg/ml of leupeptin and pepstatin (4 hrs). Antibodies against N-terminal (anti-N) and C-terminal (anti-C) fragments were used in 1: 1000 dilution.

7 cells (lanes 1 and 2)(14). A unique soluble fragment with a molecular size of 120 kD was secreted from A-172 (lane 7). The position of this band was shifted to 105 kD by the N-glycanase treatment (lane 8). A preliminary polymerase chain reaction (PCR) study indicated that A-172 cells express three different forms of mRNA, APP695, 751, and 770, as reported in other glioblastoma cells (17). However, the dominant message was APP751 (data not shown). Anti-C also reacted with the 9.5 kD C-terminal fragment generated in the normal secretory pathway (Fig. 1B, lane 1) (1-3). In the presence of leupeptin and/or pepstatin more longer amyloidogenic C-terminal fragments were observed in the cells after 4 hr incubation (lane 2 and 3, asterisk) (18).

After treatment with 1 μM PMA, the cells showed an increased production of sAPP after one hour, and differences in the content of sAPP were distinct after 4 hours (19). The secretion of sAPP (Fig. 2A) was increased by 4.4±0.8 fold (n=8) over the control in the presence of 1μM PMA (Fig.2A, lanes 1 and 2), and this enhancement was completely supressed in the presence of 1 μM staurosporin (Fig.2A, lanes 3). When the protease inhibitor leupeptin (100 μM) was added to the system, the ratio of the increase in sAPP caused by PMA treatment remained unchanged, although the amount of sAPP generally increased (Fig.2A, lanes 4-6). On the other hand, multiple bands in the range of 10-25 kD were observed intracellularly after leupeptin treatment (Fig. 2B, lanes 4-6, asterisk). The recognition of these bands by anti-C was successfully cancelled by 100 μM of the peptide antigen corresponding to

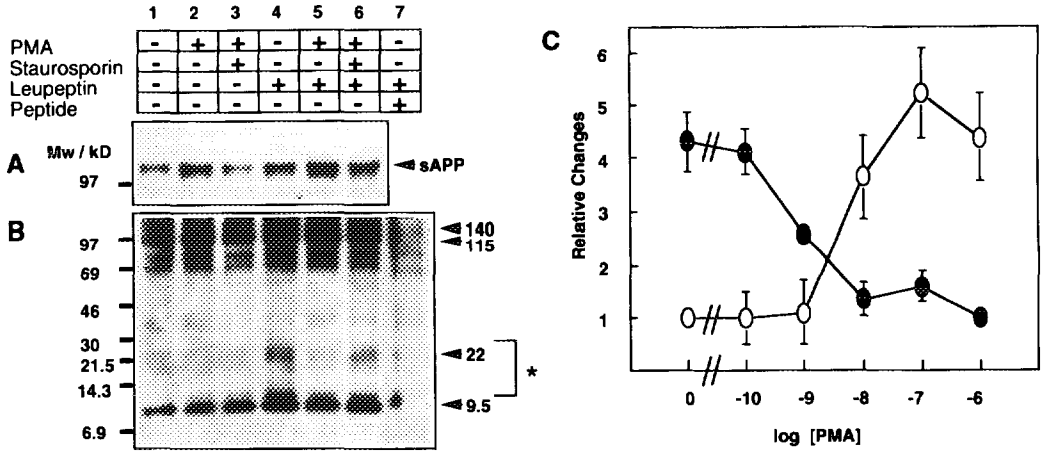

FIG. 2. Changes in sAPP and intracellular fragments in A-172 cells. *Columns 1-7 show the conditions in cultures containing* PMA (1μM), staurosporin (1μM) and leupeptin (100μM) during 4 hrs incubation in serum free DMEM. The blotted filter from lane7 was incubated with anti-C containing100μM of peptide antigen corresponding to Gly^{756}-Asn^{770}. Panel A: Immunoblotting of sAPP detected with anti-N. Panel B: Immunoblotting of intracellular proteins detected with anti-C. Both blottings were obtained from 16% tris/tricine SDS-PAGE. Panel C: Changes in sAPP (open circles) and 22kD intermediate (closed circles) quantified with a laser densitometer (n=3). Data for sAPP and 22kD were normalized to the negative and PMA controls (1μM), respectively.

Gly^{756}-Asn^{770} (Fig.2B, lane 7). The amounts of these amyloidogenic bands decreased in the presence of PMA (lane 5) and were restored by the addition of staurosporin (lane 6). The correlation between the increase in sAPP and the decrease in intracellular amyloidogenic fragments, e.g. 22 kD as an intermediate (20), showed a mirror image when the ratio of the control in the absence and presence of 1 μM of PMA was plotted against the concentration of PMA (Fig.2C).

Protein kinase inhibitors, staurosporin (5-1000 nM), H-7, A-3, and ML-9 (10-50 μM each) were examined for their ability to inhibit APP secretion. Although a relatively lower dose of staurosporin (5 nM) still effectively inhibited the APP secretion caused by 50 nM of PMA, other inhibitors could not be used because of the cytotoxicity of their effective concentrations (> 20 μM) (data not shown). Next, we tried to immunoprecipitate intracellular ßP to determine whether the change in the 22kD intermediate is directly related to the production of ßP. However, even with affinity purified anti-ßP , we could not detect any ßP in this cell line (data not shown).

DISCUSSION

A-172 cells secrete mainly glycosylated amyloid precursor protein woth a molecular size of 120 kD, the mobility of this protein in SDS gels changes to

105 kD upon N-glycanase treatment (Fig.1A). Human neuroblastoma cells, IMR-32, secrete two proteins of 120 and 105 kD, the smaller showing an almost identical size as the minor isoform of sAPP from A-172 (data not shown). As reported for PC-12 (11), these proteins might correspond to those derived from APP751 and 695, respectively.

Potential amyloidogenic C-terminal fragments were observed in the presence of leupeptin (Fig. 1B) (1-3, 18). The main fragment observed at 22 kD possibly corresponds to the reported intermediate (20) based on the anti-C specific recognition and fragment size.

In this report, we have shown that the activation of the secretory pathway by the stimulation of PKC leads to a decrease in amounts of intracellular processing products, the 22kD intermediate providing a typical case. The decrease in the mature protein (8,9) and the transient increase in the amounts of C-terminal fragments upon APP secretion have been reported (8,19). The balanced change in intracellular metabolites and the soluble secreted protein clearly indicate that amyloidogenic metabolites are generated in the endosomal/lysosomal process as a counterpart to the secretory pathway (1,19). The half-effective dose of PMA for the secretion of sAPP and the decrease in the amounts of intracellular C-terminal fragments, e.g. 22 kD, were 5 and 1 nM, respectively, similar to the requirement for the conversion of mature APP in PC12 cells (21). This means that these events are controlled by the same factor, most likely PKC. Although we could not show the direct participation of PKC, the preserved inhibitory activity of staurosporin at low concentration (5 nM) suggests the essential factor is the activation of PKC (22). It is still not clear whether the amyloidogenic C-terminal fragments described here are converted to soluble ßP in lysosomes. Our previous results suggesting that lysosomal proteases are involved in the digestion of APP (23,24), suggest the importance of lysosomes not only in the metabolism of APP but also in the production of amyloidogenic fragments from full-length APP. The failed immunoprecipitation reported here suggests that ßP might be generated by a process different from normal intracellular lysosomal digestion. This assumption agrees with a recent report that there is little correlation between sAPP and soluble ßP in the cDNA transfectants of Swedish mutant (6,7).

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