Conventional protein kinase C (PKC)-α and novel PKCε, but not -δ, increase the secretion of an N-terminal fragment of Alzheimer's disease amyloid precursor protein from PKC cDNA transfected 3Y1 fibroblasts

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Abstract **A large soluble N-terminal fragment of Alzheimer's disease amyloid precursor protein (secreted form of APP: APP_s) is produced by constitutive processing in the middle of the amyloid β-protein portion of APP. Recent studies indicate that the activation of endogenous protein kinase C (PKC) with phorbol ester raises the rate of secretion of APP_s. We constructed rat fibroblast 3Y1 cells that stably overexpress PKC isoenzymes α, δ, or ε, and analyzed the amount of APP_s released from these PKC transfectants. The levels of APP_s released from 3Y1 cells overexpressing PKCα and -ε were higher than those from PKCδ-transfected and control cells expressing vector only. These results suggest that specific isoforms of PKC regulate the secretion of APP_s through a signaling pathway.**

Key words: Alzheimer's disease; Amyroid precursor protein; Amyloid β-protein; Protein kinase C; Phorbol ester; Secretion

1. Introduction

Alzheimer's disease is characterized by the formation of amyloid plaques containing amyloid β-protein (Aβ) in brain regions important for intellectual function. The accumulation of Aβ results in neuronal death and progressive loss of cognitive function and memory. Aβ, a 39–43-amino acid peptide, is generated by proteolytic processing of APP, which has three major alternative spliced isoforms, APP695, APP751, and APP770 [5–10]. In normal brain, APP is degraded within the Aβ domain by α-secretase, and a 90–100 kDa soluble non-amyloidogenic NH_2-terminal fragment of APP (APP_s) is secreted from the cells [11–15]. Aβ is not generated from this process.

It has been shown that the metabolism of APP is highly regulated not only by extracellular signals, such as hormones, but also by intracellular second messengers [11,16,17]. Especially, the activation of PKC by treatment with phorbol ester can increase the secretion of APP_s [1–3] and reduce the production of amyloidogenic fragments containing Aβ and Aβ itself [18,19]. It should be noted that the phosphorylation of APP itself is not enhanced by PKC activation. Thus, it is posssible that a target protein for phosphorylation by PKC could be an APP processing enzyme such as α-secretase or coated proteins in the trafficking vesicle containing APP_s.

Three different groups of PKC (conventional PKC (cPKC), novel PKC (nPKC), and atypical PKC (aPKC)) are reported in mammalian tissues [20]. It is still unknown which PKC group(s) mediates the secretion of APP_s. Thus, in order to analyze which PKC species is responsible for the secretion of APP_s, we selected three PKC isoenzymes with different activation profiles by phorbol esters (e.g. 12-*O*-tetradecanoylphorbol 13-acetate (TPA), phorbol dibutylate (PDBu)) [21], and examined the secretion of APP_s from rat fibroblast 3Y1 cells after transfection with their full-length cDNA [4]. cPKC members show Ca^{2+}-dependency for activity [21], while the activation of nPKC and aPKC do not require Ca^{2+}. We chose cPKCα because it is widely distributed in most tissues, especially in brain [22]. nPKCδ was also selected because of its universal distribution among most tissues [23–25]. Finally we introduced brain-specific nPKCε cDNA into 3Y1 fibroblasts. We measured the level of APP_s in the medium of PKC-transfected 3Y1 cells in the presence and absence of TPA.

2. Experimental

2.1. Cell culture

Rat fibroblast 3Y1 cells were transfected with full-length cPKCα, nPKCδ, or nPKCε cDNAs with a neomycin resistance gene [4]. These cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Nissui) containing 10% (v/v) fetal bovine serum and 200 mg/ml G418 (Gibco) in an atmosphere of 5% CO_2/95% air at 37°C.

2.2. Antibodies

In order to analyze and detect the processing products of APP from 3Y1 cells, we raised several kinds of antibodies against a portion of APP. Antibodies GID and β1–16 were produced against synthetic peptides corresponding to amino acid residues 175–186 and 597–612 of APP695, respectively [26]. Anti-PN antibody was raised against purified APP_s from APP695 cDNA transfected COS-1 cells. We also used monoclonal anti-APP NH_2-terminal antibody 22C11 (Boehringer Mannheim) [12].

2.3. Measurement of APP_s release

PKC-overexpressing 3Y1 cell lines grown to subconfluence were incubated in serum-free DMEM with 100 nM TPA or 0.1% DMSO for 24 h. To analyse APP_s from control and PKC-overexpressing cell lines, culture media were collected and centrifuged at $10,000 \times g$ to remove cells and debris. The media (7.5 ml) were desalted and concentrated on a PD-10 column (modified from [16]). The column eluates were frozen in liquid N_2 and dried under vacuum, and then dissolved in SDS-sample buffer. The samples were boiled for 5 min, separated by SDS-PAGE [27], and electroblotted onto polyvinylidene difluoride membranes (Immobilon-P, Millipore). The volumes of the loaded samples were normalized for the total number of the cells. Western blots were probed with monoclonal antibody 22C11, rabbit antisera PN, or GID. Each

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antibody and antisera was used at 1:1000 dilution. Binding of antibodies was detected by chemiluminescence (ECL, Amersham). The bands were quantified using an Imagemaster (Pharmacia).

2.4. PDBu-binding assay

PKC cDNA transfected 3Y1 cells were grown to subconfluence in 60-mm dishes, the culture medium was removed, and the cells were washed in a total volume of 6 ml of binding solution (DMEM, 1 mg/ml of bovine serum albumin, 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid), pH 7.0). The 3Y1 cells were then treated with 2 ml of binding solution for 20 min in a CO_2 incubator. The binding solution was removed, and 1 ml of a solution including 30 μM tritium-labelled PDBu ([^{3}H]PDBu) was added to the dish and the cells were incubated for 30 min at 37°C until the [^{3}H]PDBu had bound to PKCs in the cells [28]. Three ml of cold phosphate buffered saline was added to the dish on ice to stop the reaction. The cells were washed twice, collected, and the radioactivity was measured by a liquid scintillation counter.

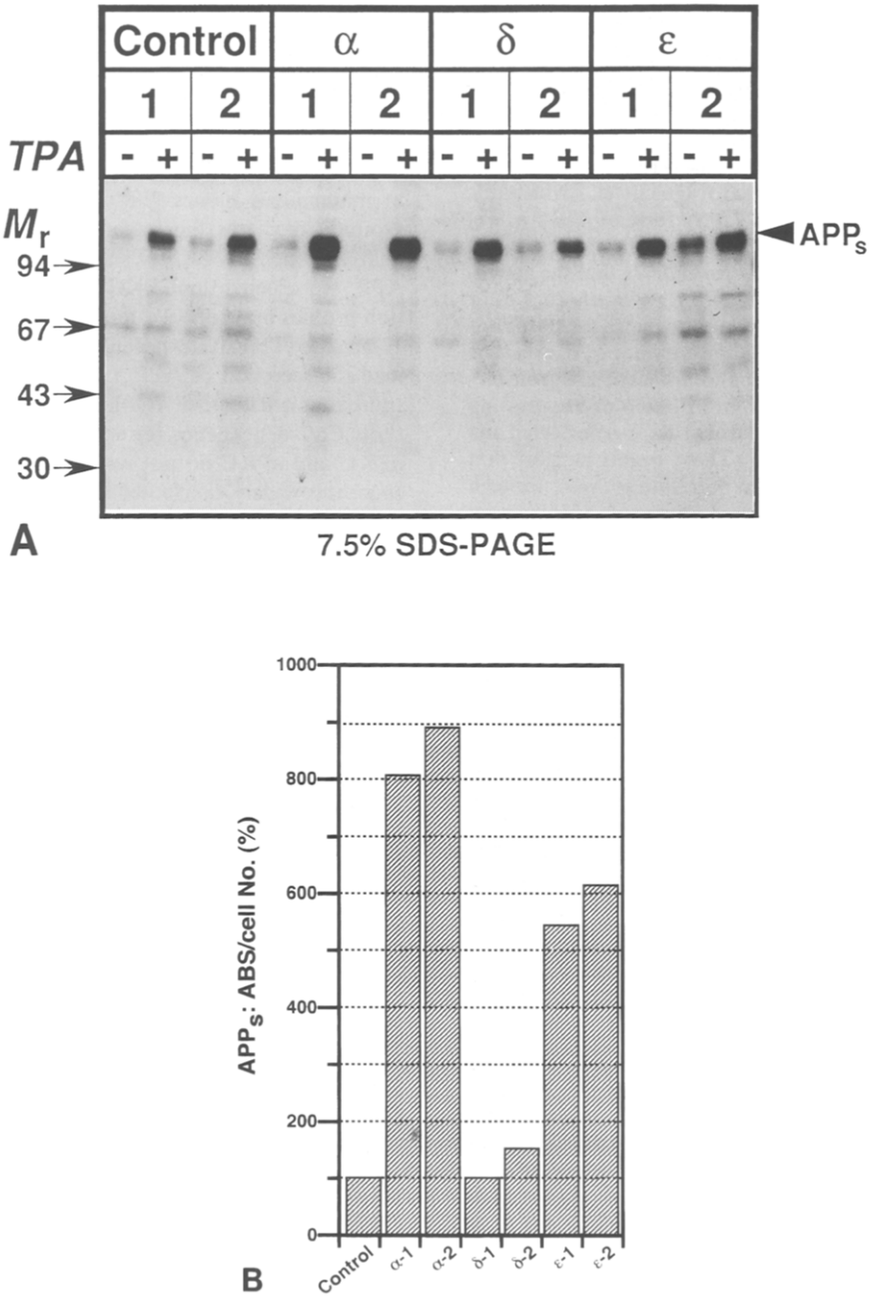

Fig. 1. APP_s is released into the medium when rat fibroblast 3Y1 cells are treated with TPA. (A) Desalted and freeze-dried media were analyzed after electrophoresis by Western blotting with antibodies against the N-terminal of APP,PN. Control and PKC-overexpressing 3Y1 cells were treated with 100 nM TPA (represented by +) or 0.1% DMSO (represented by −). 1 or 2 indicates PKC-transfected clone picked up independently. α, δ and ε, express the sample from PKCα, -δ and -ε-transfected cells. The volume of sample loaded in each lane was corrected for the number of cells per dish. The large arrow indicates APP_s. A major species of APP_s is a KPI containing type [37]. Size markers are shown on the right. (B) Levels of APP_s released into the medium. Control and PKC-overexpressing 3Y1 cells were incubated for 24 h in DMEM containing 100 nM TPA. Data are expressed as absorbance level (ABS) of APP_s from cells.

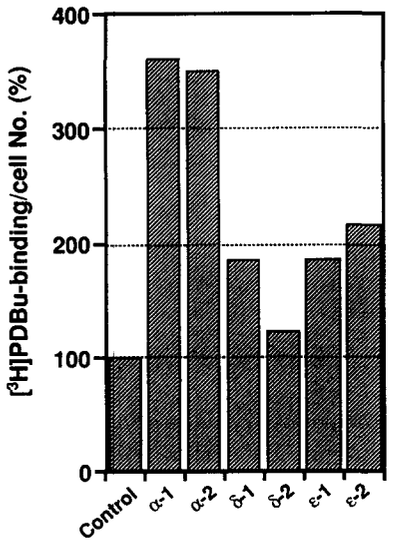

Fig. 2. Quantification of PKC expression in each transfectant by [³H]PDBu-binding assay. Data are expressed as the percent of radioactivity of [³H]PDBu that bound to control cells. The symbols refer to PKC-transfected clones; for example, α-1 means one PKCα-transfected cell.

3. Results and discussion

To examine which PKC isotypes mediate the secretion of APP_s, we constructed rat fibroblast 3Y1 cells that stably overexpressed three kinds of PKCs: cPKCα, nPKCδ and nPKCε [4]. We chose two PKC-transfected clones independently and measured the APP_s levels released into the medium treated with TPA or dimethylsulfoxide (DMSO) by Western blot analysis (Fig. 1A). Although the primary antibody used for Western blotting was anti-PN originally derived from human APP_s, anti-human APP antibodies could recognize rat APP_s as shown in Fig. 1A. When these cell lines were treated with TPA, the secretion of APP_s drastically increased. Quantification of secreted APP_s levels by densitometry showed that cPKCα and nPKCε-overexpressing cell lines experienced 8-fold and 6-fold higher increases in secreted APP_s levels, respectively, than control cells (Fig. 1B). When we treated nPKCδ-overexpressing cell lines with TPA or DMSO, the quantity of APP_s secreted was almost equal to the levels in control cells transfected with expression vector only. Although treatment with DMSO (i.e. −TPA) did not affect APP_s secretion in any transfectants, the addition of TPA raised the rate of secretion in cPKCα- and nPKCε-transfected cells. These results indicate that cPKCα and nPKCε, but not -δ, increase APP_s secretion in response to TPA.

However, it was not clear how much PKC was expressed in transfected 3Y1 cells. Therefore, the above results do not necessarily reflect the increase in activity of PKC for APP_s secretion. To analyze the expressed PKC level in transfected cells quantitatively,we performed PDBu-binding assays (Fig. 2) [28]. This assay is based on the fact that PKC binds PDBu stoichiometrically, and allows the simple estimatation of the rate of APP_s secretion per PKC molecule. Table 1 shows the specific activities of one PKC molecule for APP_s secretion. nPKCε had the highest specific activity for APP_s secretion among the three kinds of PKCs. The results strongly suggest that nPKCε, as well as cPKCα, is involved in APP_s secretion in the brain.

PKC takes part in cell signaling and occurs in many cellular responses. The cPKCα and nPKCδ used in this study are distributed in almost all tissues, while nPKCε is expressed mainly in the brain and secretory cells [20]. Since APP is expressed in almost every tissues [29] and APP_s is a kind of secretory molecule, the localized distribution of nPKCε in the brain strongly suggests its involvement in neuronal function.

In this paper, we report that cPKCα and nPKCε specifically increase the quantity of APP_s degraded by α-secretase, while nPKCδ does not change the level of APP_s. These results may indicate a difference in the intracellular localization of PKC isoforms. The reason we did not transfect an aPKC species into cells is that little is known about the cellular regulatory systems of aPKCs compared with those of cPKCs and nPKCs. In addition, aPKCs contain only one cysteine-rich zinc finger-like domain, making them independent of diacylglycerol and phorbol esters (exogenous PKC activator) [30,31]; thus we cannot control the activity of aPKCs in transfected cells with phorbol esters such as PDBu.

The next question involves the target(s) of PKC in APP_s secretion. First, it is expected that PKC directly phosphorylates the APP molecule in vivo. It has been reported that PKC directly phosphorylates APP in vitro and in semi-intact cells [32,33]; however, a recent report suggests that the phosphorylation of the C-terminal domain of APP is independent of the release of APP_s by phorbol ester treatment [34]. Almost all potential phosphorylation sites in APP by protein kinases such as PKC and casein kinase II exist on the N-terminal, and phosphorylation in the ecto-domain of APP may induce conformational change resulting in degradation by intracellular proteases. A second possibility is that PKC may control the activity of α-secretase by phosphorylation. However, the identity of the putative α-secretease remains to be determined.

Our data demonstrate that some isotypes of brain-specific PKC promote an increase in the release of APP_s, and the other isotypes are not involved in this process. We are interested in the involvement of nPKCε in the release of APP, because nPKCε, but not PKCδ, is activated by *cis*-unsaturated fatty acids [24,35]. When a cell expressing cPKCα and nPKCε tries to secrete APP_s more effectively, the phospholipases that produce the *cis*-forms of fatty acids must first be activated, and then activation of PKC subtype(s) may follow the increase in

Table 1
The difference in the ability of PKC species to enhance APP secretion

	APP_s: ABS/cell	PKC: [³H]PDBu/cell	APP_s/PKC
Control	100	100	1
α-1	695	261	2.66
α-2	777	253	3.07
δ-1	0	82	0
δ-2	52	21	2.47
ε-1	436	80	5.45
ε-2	507	117	4.33

Data for both APP_s: ABS/cell and PKC: [³H]PDBu/cell are expressed as the percents of control (n = 3). The values of both APP_s: ABS/cell and PKC: [³H]PDBu/cell are shown in Fig. 1B and Fig. 2, respectively. Therefore, in the APP_s/PKC lane, the specific activity of one cDNA-derived PKC molecule to secrete APP_s is estimated as 1 of the value of control. As a result, it was strongly suggested that nPKCε as well as cPKCα aggressively act as mediators to secrete APP_s.

the intracellular concentration of unsaturated fatty acids. Alternatively, nPKCε is directly activated by an unidentified signal, such as lipid derivatives like prostaglandins. Reports from other laboratories have suggested that transfection of cPKCα cDNA into Swiss 3T3 fibroblasts enhances APP_s secretion upon TPA treatment, indicating that at least cPKCα is necessary for TPA-induced APP_s release [36]. Our results suggest that not only cPKCα but also nPKCε is required for APP secretion, although the mechanism of enhancement is remains unknown. Interestingly, the introduction of nPKCε into 3Y1 cells also increased APP_s release without an external signal such as TPA. Therefore, nPKCε might be involved in basal APP_s release in neural cells.

Further work is necessary to elucidate the role of PKC subtypes in the secretion of APP_s molecules.

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References

[1] Caporaso, G.L., Gandy, S.E., Buxbaum, J.D., Ramabhadran, T.V. and Greengard, P. (1990) Proc. Natl. Acad. Sci. USA 89, 3055–3059.
[2] Gillespie, S.L., Golde, T.E. and Younkin, S.G. (1992) Biochem. Biophys. Res. Commun. 187, 1285–1290.
[3] Fukushima, D., Konishi, M., Maruyama, K., Miyamoto, T., Ishiura, S. and Suzuki, K. (1993) Biochem. Biophys. Res. Commun. 194, 202–207.
[4] Ohno, S., Mizuno, K., Adachi, Y., Hata, A., Akita, Y., Akimoto, K., Osada, S., Hirai, S. and Suzuki, K. (1994) J. Biol. Chem. 269, 17495–17501.
[5] Robakis, N.K., Ramakrishna, N., Wolfe, G. and Wisniewski, H.M. (1987) Proc. Natl. Acad. Sci. USA 84, 4190–4194.
[6] Kang, J., Lemaire, H.-G., Unterbeck, A., Salbaum, J.M., Masters, C.L., Grzeschik, K.H., Multhaup, G., Beyreuther, K. and Müller-Hill, B. (1987) Nature 325, 733–736.
[7] Goldegaber, D., Lerman, M.I., McBride, O.W., Saffiotti, V. and Gajdusek, D.C. (1987) Science 235, 877–880.
[8] Tanzi, R.E., Gusella, J.F., Watkins, P.C., Bruns, G.A., St. George-Hyslop, P., Van Keuren, M.L., Patterson, D., Pagan, S., Kurnit, D.M. and Neve, R.L. (1987) Science 235, 880–884.
[9] Tanzi, R.E., McClatchey, A.I., Lamperti, E.D., Villa, K.L., Gusella, J.F. and Neve, R.L. (1988) Nature 331, 528–530.
[10] Kitaguchi, N., Takahashi, Y., Tokushima, Y., Shiojiri, S. and Ito, H. (1988) Nature 331, 530–532.
[11] Schubert, D., Jin, L.-W., Saitoh, T. and Cole, G. (1989) Neuron 3, 689–694.
[12] Weidemann, A., Knig, G., Bunke, D., Fischer, P., Salbaum, J.M., Masters, C.L. and Beyreuther, K. (1989) Cell 57, 115–126.
[13] Sisodia, S.S., Koo, E.H., Beyreuther, K., Unterbeck, A. and Price, D.L. (1990) Science 248, 492–495.
[14] Esch, F.S., Keim, P.S., Beattie, E.C., Blacher, R.W., Culwell, A.R., Oltersdorf, T., McClure, D. and Ward, P.J. (1990) Science 248, 1122–1124.
[15] Wang, R., Meschia, J.F., Cotter, R.J. and Sisodia, S.S. (1991) J. Biol. Chem. 266, 16960–16964.
[16] Nitsch, R.M., Slack, B.E., Wurtman, R.J. and Growdon, J.H. (1992) Science 258, 304–307.
[17] Buxbaum, J.D., Oishi, M., Chen, H.I., Pinkas-Kramarski, R., Jaffe, E.A., Gandy, S.E. and Greengard, P. (1992) Proc. Natl. Acad. Sci. USA 89, 10075–10078.
[18] Buxbaum, J.D., Koo, E.H. and Greengard, P. (1993) Proc. Natl. Acad. Sci. USA 90, 9195–9198.
[19] Hung, A.Y., Haass, C., Nitsch, R.M., Qiu, W.Q., Citron, M., Wurtman, R.J., Growdon, J.H. and Selkoe, D.J. (1993) J. Biol. Chem. 268, 22959–22962.
[20] Nishizuka, Y. (1992) Science 258, 607–614.
[21] Nishizuka, Y. (1984) Nature 308, 693–698.
[22] Ohno. S., Kawasaki, H., Imajoh, S., Suzuki, K., Inagaki, M., Yokokura, H., Sakoh, T. and Hidaka, H. (1987) Nature 325, 161–166.
[23] Nishizuka, Y. (1988) Nature 334, 661–665.
[24] Ogita, K., Miyamoto, S., Yamaguchi, K., Koide, H., Fujisawa, N., Kikkawa, U., Sahara, S., Fukami, Y. and Nishizuka, Y. (1992) Proc. Natl. Acad. Sci. USA 89, 1592–1596.
[25] Liyanage, M., Frith, D., Livneh, E. and Stabel, S. (1992) Biochem. J. 283, 781–787.
[26] Tagawa, K., Yazaki, M., Kinouchi, T., Maruyama, K., Sorimachi, H., Tsuchiya, T., Suzuki, K. and Ishiura, S. (1993) Gerontology 39, 24–29.
[27] Laemmli, U.K. (1970) Nature 227, 680–685.
[28] Ohno, S., Akita, Y., Konno, Y., Imajoh, S. and Suzuki, K. (1988) Cell 53, 731–741.
[29] Tanaka, S., Nakamura, S., Ueda, K., Kameyama, M., Shiojiri, S., Takahashi, Y., Kitaguchi, N. and Itoh, H. (1988) Biochem. Biophys. Res. Commun. 157, 472–479.
[30] Ono, Y., Fujii, T., Ogita, K., Kikkawa, U., Igarashi, K. and Nishizuka, Y. (1989) Proc. Natl. Acad. Sci. USA 86, 3099–3103.
[31] Ways, D.K., Cook, P.P., Webster, C. and Parker, P.J. (1992) J. Biol. Chem. 267, 4799–4805.
[32] Gandy, S., Czernik, A.J., and Greengard, P. (1988) Proc. Natl. Acad. Sci. USA 85, 6218–6221.
[33] Suzuki, T., Nairn, A.C., Gandy, S.E. and Greengard, P. (1992) Neuroscience 48, 755–761.
[34] Jacobsen, J.S., Spruyt, M.A., Brown, A.M., Sahasrabudhe, S.R., Blume, A.J., Vitek, M.P., Muenkel, H.A. and Sonnenberg-Reines, J. (1994) J. Biol. Chem. 269, 8376–8382.
[35] Koide, H., Ogita, K., Kikkawa, U. and Nishizuka, Y. (1992) Proc. Natl. Acad. Sci. USA 89, 1149–1153.
[36] Slack, B.E., Nitsch, R.M., Livneh, E., Kunz Jr., G.M., Breu, J., Eldar, H. and Wurtman, R.J. (1993) J. Biol. Chem. 268, 21097–21101.
[37] Kinouchi, T., Ono, Y., Sorimachi, H., Ishiura, S. and Suzuki, K. (1995) Biochem. Biophys. Res. Commun. (in press).