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Rapid stimulation of amyloid precursor protein release by epidermal growth factor: role of protein kinase C

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The amyloid precursor protein (APP) of Alzheimer's disease is a transmembrane protein that is cleaved by an uncharacterized enzyme known as α-secretase within its extracellular/intraluminal domain after the activation of guanine nucleotide-binding protein-coupled receptors linked to phosphoinositide hydrolysis. The secretory process results in the release of large soluble derivatives of APP (APPs), and, when elicited by muscarinic receptor activation, exhibits both protein kinase C (PKC)-dependent and tyrosine phosphorylation-dependent components [Slack, Breu, Petryniak, Srivastava and Wurtman (1995) J. Biol. Chem. **270**, 8337–8344]. In this report we examine the regulation of the release of APPs by epidermal growth factor (EGF) receptors, which possess intrinsic tyrosine kinase activity, and are coupled to a variety of effectors including phosphoinositide-specific phospholipase Cγ. In A431 cells, EGF caused time-dependent and dose-dependent increases in the formation of inositol phosphates in cultures prelabelled with *myo*-[^{3}H]inositol, and in the release of APPs into the culture medium; the two responses exhibited similar time courses and EC_{50} values for EGF. Concomitant with these effects, there were concentration-dependent (3–300 ng/ml) increases in the phosphorylation of tyrosine residues in several proteins, including the EGF receptor itself. The specific PKC antagonist GF 109203X decreased the effect of EGF by approx. 35% at a concentration that abolished the stimulation of the release of APPs by the PKC activator PMA. Tyrphostin AG 1478, an inhibitor of EGF receptor tyrosine kinase, abolished the EGF-induced release of APPs. These results demonstrate that in A431 cells, activation of the EGF receptor stimulates α-secretase activity by a mechanism that is partly dependent on PKC activity.

INTRODUCTION

The amyloid plaques found in the brains of patients with Alzheimer's disease are composed of proteins (Aβ peptides) derived from a family of larger molecules known collectively as the amyloid precursor protein (APP). A number of putative degradative pathways metabolize APP, giving rise to soluble Aβ fragments that can, under certain conditions, aggregate to form amyloid. Alternatively, APP can undergo secretory processing, an event stimulated by the binding of neurotransmitters to receptors coupled to phosphoinositide hydrolysis via guanine nucleotide-binding proteins (G-proteins) [1,2]. This hydrolytic event cleaves APP within the Aβ domain (and hence is non-amyloidogenic) and releases a large soluble fragment (APPs) into the extracellular space. Both full-length APP and APPs exhibit a variety of trophic and neuroprotective properties [3–8], suggesting that the maintenance of adequate levels of this protein in the external milieu might be important for neuronal viability.

A variety of G-protein-coupled receptors, including muscarinic acetylcholine [1,2,9–11], bradykinin [12], metabotropic glutamate [13], serotonin [14] and thrombin [15] receptors, are positively coupled to the release of APPs; typically, the response is observed within minutes of receptor activation. Ligands of receptors with intrinsic tyrosine kinase activity, including nerve growth factor, fibroblast growth factor and epidermal growth factor (EGF), also increase APP secretion [16–18]. However, the reported effects of these factors were assessed after 24 h or more; they might therefore reflect alterations in expression rather than acute stimulation of the secretory processing pathway. In this report we demonstrate that in A431 cells, a human epidermoid carcinoma cell line that endogenously expresses high levels of EGF receptors [19], as well as APP, EGF results in a rapid, concentration-dependent increase in the release of APPs that is mediated in part by the activation of protein kinase C (PKC).

EXPERIMENTAL

Materials

EGF and EGTA were purchased from Sigma (St. Louis, MO, U.S.A.), tyrphostin AG 1478 from Alexis Corporation (San Diego, CA, U.S.A.), bisindolyl maleimide I (GF 109203X) and wortmannin from LC Laboratories (Woburn, MA, U.S.A.), PMA from Calbiochem (San Diego, CA, U.S.A.) and *myo*[2-^{3}H]-inositol from DuPont–NEN (Boston, MA, U.S.A.). The inhibitors were dissolved in DMSO and kept at −20 °C. Stocks were diluted in serum-free medium before experiments. Final concentrations of DMSO did not exceed 0.2%; control media contained equivalent concentrations of DMSO alone.

Cell culture

A431 cells were grown in Dulbecco's modified Eagle's medium/Ham's F12 bicarbonate medium supplemented with 10% (v/v) fetal calf serum and maintained in an air/CO_2 (19:1) atmosphere. Cells were subcultured on plastic tissue culture dishes before experiments and grown to confluency. Cultures were incubated overnight in serum-free medium before an experiment. All subsequent pharmacological treatments were performed in serum-free medium.

Measurement of release of APPs

Secretory processing of endogenously expressed APP was measured in A431 cells by Western blot, as previously described [9]. Media were collected, centrifuged, desalted and freeze–dried.

Abbreviations used: Aβ, amyloid beta protein; APP, amyloid precursor protein; APPs, secreted APP derivatives; EGF, epidermal growth factor; PKC, protein kinase C.

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Cells were lysed in an extraction buffer containing 2% (v/v) Triton X-100 and 2% (v/v) Nonidet P40 [20]. Aliquots of the lysates were analysed for protein content with the bicinchoninic acid method. All samples were diluted 1:1 in extraction buffer and gel loading buffer, boiled and size-fractionated on 10% or 12% (w/v) polyacrylamide mini-gels (Bio-Rad, Hercules, CA, U.S.A.). The sample volumes loaded were corrected for protein content of the cell lysates. Proteins were transferred to PVDF membranes (Millipore, Bedford, MA, U.S.A.), blocked with 5% (w/v) powdered milk in Tris-buffered saline containing 0.15% Tween-20 (TBST) and immunoblotted with 6E10 (Senetek, Maryland Heights, MO, U.S.A.) monoclonal antibodies. 6E10 antibodies recognize residues 1–17 of the Aβ domain of APP [21] and can therefore be used to detect APPs derived from α-secretase cleavage. After being washed, membranes were incubated with sheep anti-mouse peroxidase-linked secondary antibody (Amersham Corporation, Arlington Heights, IL, U.S.A.). Bands were detected by a chemiluminescence method (DuPont–NEN) and quantified by laser scanning densitometry.

Immunoprecipitation and determination of tyrosine phosphorylation

Cells were rinsed in PBS containing 1 mM sodium orthovanadate (an inhibitor of tyrosine phosphatases) and collected in 1 ml of a lysis buffer containing 1% (v/v) Triton X-100 [9]. Lysates were centrifuged and incubated overnight with immunoprecipitating antibodies, usually at concentrations of 4–5 μg per 500 μg of protein, with Protein A–Sepharose (Pharmacia, Piscataway, NJ, U.S.A.) and Protein G–agarose (Oncogene Science, Cambridge, MA, U.S.A.) (each at 1.5 mg per sample), centrifuged and washed three times, then analysed by immunoblotting, as previously described [9]. Tyrosine-phosphorylated proteins were immunoprecipitated with anti-phosphotyrosine antibodies (clone PY 20, Transduction Laboratories) and detected on immunoblots with recombinant peroxidase-linked antiphosphotyrosine antibodies (RC20; Transduction Laboratories). The EGF receptor was immunoprecipitated with sheep anti-EGF receptor antibodies (Upstate Biotechnology, Lake Placid, NY, U.S.A.).

Inositol phosphate formation

Cells were labelled overnight in serum-free medium with 1.25 μCi/ml *myo*-[2-^{3}H]inositol. Total [^{3}H]inositol phosphate formation was assessed as previously described [22]. Briefly, labelled cells were washed twice with Hanks balanced salt solution (HBSS), then preincubated in HBSS containing 10 mM LiCl. Incubations were performed in HBSS containing Li^+ ions, with or without EGF. Cultures were washed and extracted, and the aqueous phase was separated on anion-exchange columns (AG 1-X8, formate form; Bio-Rad). Inositol phosphates were eluted in 1 M ammonium formate/0.1 M formic acid. Radioactivity was normalized for protein content.

Protein analysis

Proteins were measured with the bicinchoninic acid reagent (Sigma).

Statistical analysis

Statistical comparisons between groups were made by analysis of variance. Significance testing was performed with Fisher's least-significant-difference test. Differences were taken to be statistically significant at $P < 0.05$.

RESULTS

Stimulation of phosphoinositide hydrolysis and release of APPs by EGF

Basal release of APPs from A431 cells was detectable within 5 min and increased gradually over a period of 60 min. In the presence of EGF (100 ng/ml), release was significantly increased at the 30 and 60 min time points (Figure 1A). In cells prelabelled with [^{3}H]inositol, formation of inositol phosphates was also significantly increased 30 and 60 min after the addition of EGF (Figure 1B). EGF caused concentration-dependent increases in the release of APPs and the hydrolysis of phosphoinositides, with estimated EC_{50} values of 20 and 30 ng/ml respectively (Figure 2). Significant increases in the release of APPs were observed at lower concentrations of EGF than those required to elevate inositol phosphate formation.

Inhibition of EGF receptor tyrosine kinase activity blocks release of APPs by EGF

Like a number of other growth factor receptors, the EGF receptor possesses intrinsic tyrosine kinase activity [23,24]. Once bound by ligand, EGF receptor molecules dimerize and auto-

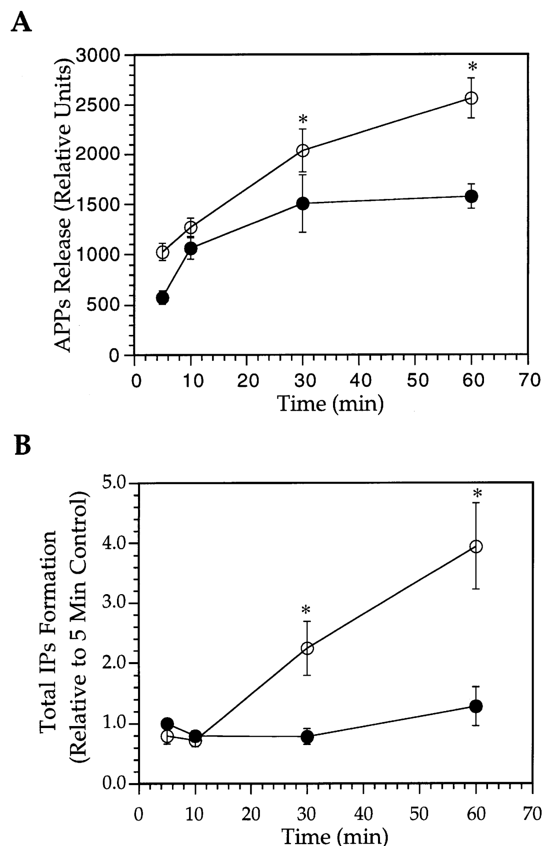

Figure 1 Time course of EGF-induced release of APPs and formation of inositol phosphates (IPs)

(**A**) Cells were incubated overnight in serum-free medium then exposed for various periods to fresh serum-free medium with (○) or without (●) 100 ng/ml EGF. APPs released into the medium was measured by immunoblot analysis with 6E10 antibodies. (**B**) Cells were labelled overnight in serum-free medium containing 1.25 μCi/ml *myo*-[2-^{3}H]inositol, washed and incubated for various periods in the presence (○) or absence (●) of 100 ng/ml EGF. EGF-induced increases in total inositol phosphates are expressed relative to the values observed at 5 min in control cultures. Results are expressed as means ± S.E.M. for three or four separate experiments. * Significant difference from corresponding control group ($P < 0.05$).

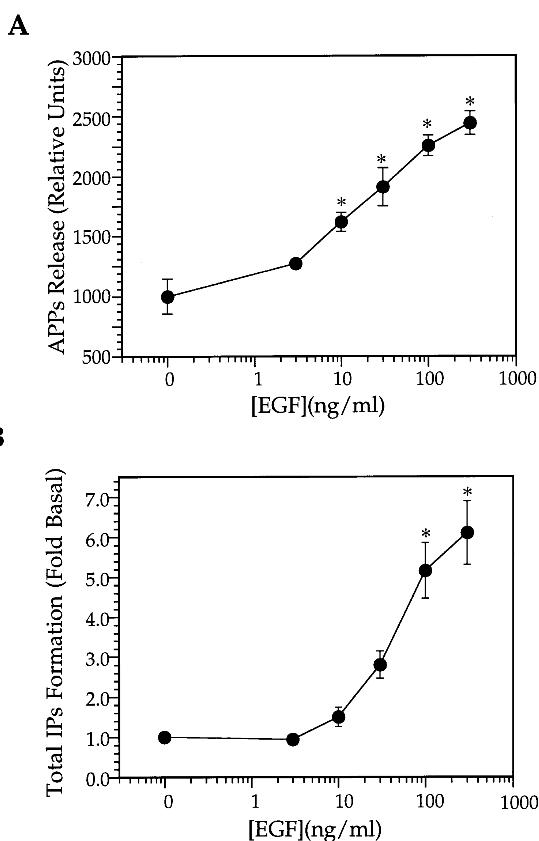

Figure 2 Concentration dependence of EGF-induced increases in release of APPs and formation of inositol phosphates (IPs)

(**A**) Cells were incubated for 1 h in serum-free medium containing the indicated concentrations of EGF. APPs in the medium was measured by immunoblot analysis with 6E10 antibodies. (**B**) Cells were labelled overnight in serum-free medium containing 1.25 μCi/ml *myo*-[2-^{3}H]inositol, washed and incubated for 1 h in medium containg 0–300 ng/ml EGF. EGF-induced increases in inositol phosphate formation are expressed relative to control values. Results represent means $\pm$ S.E.M. for three (**A**) or four (**B**) separate experiments. * Significant difference from control ($P < 0.05$).

phosphorylate. The phosphorylated tyrosine residues provide docking sites for other signalling molecules such as the non-receptor tyrosine kinase c-Src and phosphatidylinositol 3-kinase [25,26]. Tyrosine phosphorylation of several proteins was increased in a concentration-dependent manner after the addition of EGF to the cells (Figure 3A), and remained elevated for up to 30 min (Figure 3B). The most prominent phosphorylated protein band, with a molecular mass of approx. 170 kDa, was presumed to be the EGF receptor itself; a protein of equivalent size was found in immunoprecipitates prepared with antibodies against the EGF receptor and showed increased phosphorylation within 1 min of the addition of EGF to cell cultures (Figure 3C). The potent and selective EGF receptor tyrosine kinase inhibitor tyrphostin AG 1478 [27], at a concentration of 250 nM, inhibited the increase in tyrosine phosphorylation of the EGF receptor elicited by EGF (Figure 3D), and caused a decrease of approx. 80 % in the EGF-induced release of APPs (Table 1).

Release of APPs evoked by EGF is partly dependent on activation of PKC

The stimulation of phosphoinositide hydrolysis by EGF suggested that the activation of PKC might constitute an intermediate step in the regulation of the release of APPs. This possibility was tested with the selective PKC inhibitor GF 109203X [28]. The inhibitor, at a concentration of 5 μM, abolished the response to PMA and decreased the EGF-evoked release of APPs by approx. 35 % (Figure 4 and Table 2). It has been previously shown that GF 109203X has no effect on EGF-induced tyrosine phosphorylation of the EGF receptor [29].

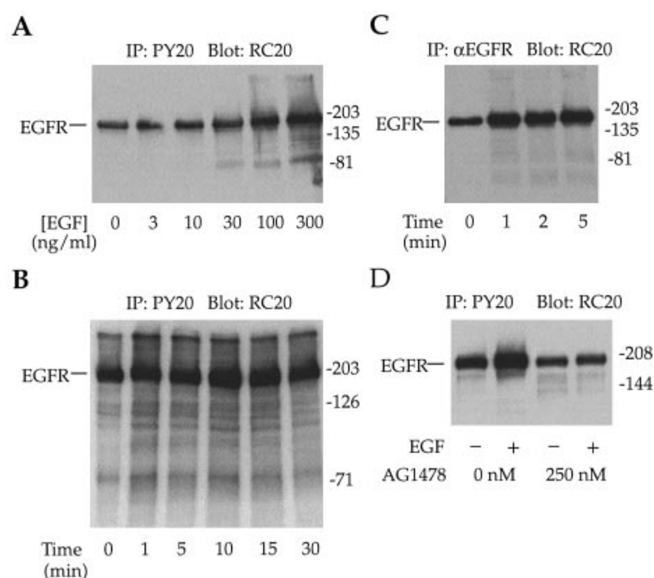

Figure 3 Time and concentration dependence of EGF-induced tyrosine phosphorylation

(**A**) Cells were treated for 5 min with various concentrations (0–300 ng/ml) of EGF. Cells were lysed and tyrosine phosphorylated proteins were immunoprecipitated with anti-phosphotyrosine antibodies (PY20), size-fractionated on polyacrylamide gels and immunoblotted with horseradish peroxidase-linked antiphosphotyrosine antibodies (RC20). (**B**) Cells were treated for various periods with EGF (100 ng/ml), then lysed and subjected to immunoprecipitation and immunoblotting with anti-phosphotyrosine antibodies as in (**A**). (**C**) Cells were treated with 100 ng/ml EGF for 0 to 5 min and lysed, and the EGF receptor was immunoprecipitated. Immunoprecipitates were size-fractionated on polyacrylamide gels and probed with anti-phosphotyrosine (RC20) antibodies. (**D**) Cells were pretreated with AG 1478 (250 nM) or DMSO for 15 min, then incubated for 10 min with EGF (100 ng/ml) in the presence or absence of AG 1478, and analysed for tyrosine phosphorylation as in (**A**). Abbreviation: EGFR, EGF receptor.

Table 1 Stimulation of release of APPs by EGF is blocked by the EGF receptor tyrosine kinase inhibitor AG 1478

A431 cells were pretreated for 15 min in medium containing 250 nM AG 1478 or DMSO, then incubated for 1 h in fresh medium containing DMSO or AG 1478 with or without 100 ng/ml EGF. APPs released into the medium was measured by immunoblotting with 6E10 antibodies. Results are expressed as means $\pm$ S.E.M. for four separate experiments. * Significant difference from control; † significant difference from EGF-treated cultures, $P < 0.05$.

	APPs release (relative units)	
Treatment	No inhibitor	AG 1478
Control	592 ± 128	636 ± 88
EGF (100 ng/ml)	1667 ± 118*	862 ± 165†

EGF-evoked release of APPs is not inhibited by EGTA or wortmannin

In addition to stimulating PKC activity and tyrosine phosphorylation, EGF receptor activation elevates cytosolic Ca^{2+} ion levels [30,31] and activates phosphatidylinositol 3-kinase [32]. However, these signalling events did not seem to mediate the

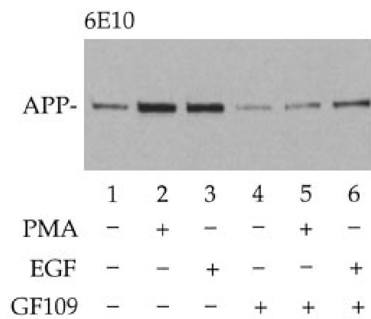

Figure 4 EGF-induced release of APPs exhibits partial dependence on PKC

A431 cells were pretreated for 15 min with 5 μM GF 109203X or DMSO, then incubated for 1 h in medium containing DMSO or GF 109203X with or without PMA (1 μM) or EGF (100 ng/ml). APPs released into the medium was measured by immunoblotting with 6E10 antibodies.

Table 2 EGF-mediated release of APPs is decreased by the PKC inhibitor GF 109203X

A431 cells were pretreated for 15 min in medium containing 5 μM GF 109203X or DMSO, then incubated for 1 h in fresh medium containing DMSO or GF 109203X, with or without PMA (1 μM) or EGF (100 ng/ml). APPs released into the medium was measured by immunoblotting with 6E10 antibodies. Results are expressed as means ± S.E.M. for three to five separate experiments (number indicated in parentheses). * Significant difference from control; † significant difference from EGF-treated cultures; ‡ significant difference from PMA-treated cultures; $P < 0.05$.

	APPs release (relative units)	
Treatment	No inhibitor	GF 109203X
Control	498 ± 109 (5)	427 ± 86 (4)
EGF (100 ng/ml)	1778 ± 165 (5)*	1240 ± 108 (5)*†
PMA (1 μM)	1980 ± 149 (3)*	552 ± 110 (3)‡

Table 3 EGF-mediated release of APPs is not affected by wortmannin

A431 cells were pretreated for 15 min in medium containing 100 nM wortmannin or DMSO, then incubated for 1 h in fresh medium containing DMSO or wortmannin with or without EGF (100 ng/ml). APPs released into the medium were measured by immunoblotting with 6E10 antibodies. Results are expressed as means ± S.E.M. for four separate experiments. * Significant difference from corresponding control cultures; $P < 0.05$.

	APPs release (relative units)	
Treatment	No inhibitor	Wortmannin
Control	1003 ± 259	969 ± 158
EGF (100 ng/ml)	2019 ± 194*	2060 ± 126*

release of APPs because neither the phosphatidylinositol 3-kinase inhibitor wortmannin (Table 3) nor the Ca^{2+} chelator EGTA (Table 4) caused any attenuation of the response to EGF.

DISCUSSION

It has been firmly established that secretory cleavage of APP is increased by the activation of G-protein-coupled receptors belonging to the seven-transmembrane-domain superfamily [1,2,9,13,14]. Although there have been a number of reports that

Table 4 EGF-mediated release of APPs is not affected by EGTA

A431 cells were pretreated for 15 min in medium containing 5 mM EGTA or the equivalent amount of water (vehicle control), then incubated for 1 h in fresh medium containing water or EGTA, with or without EGF (100 ng/ml). APPs released into the medium was measured by immunoblotting with 6E10 antibodies. Results are expressed as means ± S.E.M. for three separate experiments. * Significant difference from corresponding control cultures; $P < 0.05$.

	APPs release (relative units)	
Treatment	No inhibitor	EGTA
Control	582 ± 163	1000 ± 100
EGF (100 ng/ml)	1636 ± 81*	1789 ± 68*

the release of APPs from cells is also increased by ligands of receptors with intrinsic tyrosine kinase activity (e.g. nerve growth factor, fibroblast growth factor and EGF) [16–18], these determinations were made after prolonged treatment periods, at which time elevations in APP transcription or mRNA levels are also apparent [33–35]. Evidence presented here shows that the release of APPs is increased within 30 min of exposure to EGF, indicating that the secretory cleavage pathway is rapidly activated by this receptor. Like muscarinic receptors, EGF receptors are coupled to phosphoinositide hydrolysis [30,36–39]; however, whereas G-protein-coupled receptors activate phospholipase Cβ, the signal from activated EGF receptors is transmitted via tyrosine phosphorylation of phospholipase Cγ [40–42]. In both cases PtdIns(4,5)P_2 is hydrolysed to form diacylglycerol and Ins(1,4,5)P_3; the latter releases Ca^{2+} ions from internal stores [43], which, together with diacylglycerol, is required for the activation of conventional PKC isoforms [44]. The involvement of PKC in the stimulation of the release of APPs by EGF is indicated by the effect of the specific PKC inhibitor GF 109203X, which decreased the response by approx. 35%. GF 109203X and other bisindolylmaleimides potently inhibit PKC isoforms of the conventional and new classes but do not affect atypical PKCs [28,29,45]. However, the results clearly show that mechanisms in addition to PKC activation must contribute to the response.

The possibility was considered that PKCζ, an atypical PKC isoform expressed by A431 cells [46], might also have a role in the EGF-induced release of APPs. This isoform can be activated *in vitro* by PtdIns(3,4,5)P_3 [47], a lipid whose formation is catalysed by the enzyme phosphatidylinositol 3-kinase [48] after EGF receptor stimulation in A431 cells [32]. However, the phosphatidylinositol 3-kinase inhibitor wortmannin had no discernible effect on the EGF-mediated release of APPs (Table 3), suggesting that PKCζ is not a factor in the EGF-induced processing of APP.

One of the earliest effects of EGF on A431 cells is an increase in intracellular Ca^{2+} ions that results from the activation of voltage-sensitive Ca^{2+} ion channels as well as its release from internal stores [30,31]. However, the Ca^{2+} ion chelator EGTA (5 mM) did not attentuate the stimulation of the release of APPs relative to that elicited by EGF alone (Table 4), suggesting that Ca^{2+} ion influx was not involved in the response. Although release from intracellular stores might have a role in the EGF-mediated release of APPs, this possibility remains to be explored.

The mechanism by which PKC stimulates the release of APPs is not understood. Direct phosphorylation of APP by PKC is not required because deletion of the cytoplasmic tail of APP did not inhibit the increase in the release of APPs elicited by phorbol esters [49,50]. Recent evidence indicates that PKC increases the formation of APP-containing secretory vesicles from the *trans*-

Golgi network [51], an effect that might be a result of the ability of PKC to promote the binding of coat proteins to Golgi membranes [52] and hence the budding of the membrane to form vesicles [53,54]. Another potential target of PKC is α-secretase itself but testing of this hypothesis awaits the purification of the enzyme.

In summary, our results demonstrate that PKC has a role in the regulation of the release of APPs by a member of the tyrosine kinase receptor family, as well as by G-protein-coupled receptors, although in both cases it seems to mediate only part of the response. The EGF receptor is normally expressed by hippocampal and cerebral cortical neurons in human brain [55]; in patients with Alzheimer's disease it is found additionally in neurofibrillary tangles, in neuritic plaques and on vascular endothelial cells of brain and other tissues ([56], and references therein). Although it is not known whether increased EGF expression in AD contributes to the pathological process or represents an attempt at repair, our results, together with evidence supporting a neurotrophic, neuroprotective role for APP [3,5–7], are consistent with the second possibility. A complete elucidation of the mechanisms regulating the formation of APPs will contribute to our understanding of its role in both physiological and pathological processes.

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