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Phorbol Esters but Not the Cholinergic Agonists Oxotremorine-M and Carbachol Increase Release of the Amyloid Precursor Protein in Cultured Rat Cortical Neurons

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Abstract: Conventional secretory processing of the amyloid precursor protein is nonamyloidogenic, releasing carboxyl-terminus-truncated amyloid precursor protein derivatives while cleaving the amyloid β-peptide within its sequence. Alternative processing routes are potentially amyloidogenic, yielding the amyloid β-peptide segment intact. In continuous cell lines, secretory processing of the amyloid precursor protein is regulated by both protein kinase C and muscarinic receptor stimulation. However, the first and second messenger systems that regulate amyloid precursor protein release in central neurons are still under investigation. In the present investigation, we examined whether or not first and second messengers of cholinergic neurotransmission increase production of soluble derivatives of the amyloid precursor protein in primary cultures of rat cortical neurons. Activation of protein kinase C by the phorbol esters phorbol 12,13-dibutyrate and phorbol 12-myristate 13-acetate increased production of the soluble form of the amyloid precursor protein dramatically. In contrast, activation of muscarinic receptors by oxotremorine-M or carbachol did not result in a significant increase in amyloid precursor protein release. Similarly, chemically induced depolarization using 35 m*M* KCl did not alter production of soluble amyloid precursor protein derivatives. Our data suggest that although protein kinase C stimulation plays an important role in regulating release of the amyloid precursor protein, cholinergic neurotransmission does not regulate its release in cultured rat cortical neurons. **Key Words:** Amyloid precursor protein—Amyloid β-peptide—Protein kinase C—Muscarinic receptors—Cortical neurons—Alzheimer's disease.
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The progressive deposition of the amyloid β-peptide (Aβ) in parenchymal brain tissues and the walls of the cerebral microvasculature is an invariant feature of Alzheimer's disease (AD) pathology (Hardy and Allsop, 1991). Aβ is a proteolytic derivative of a much larger membrane-spanning glycoprotein, termed the amyloid precursor protein (APP) (Kang et al., 1987). Mature APP can be processed by multiple metabolic pathways. The best characterized of these is via the activity of an enzyme known as α-secretase, which cleaves APP within the Aβ sequence (Sisodia et al., 1990; Anderson et al., 1991; Wang et al., 1991), releasing soluble carboxyl-terminus-truncated APP (APP_s) of 90–100 kDa (Weidemann et al., 1989). As this proteolytic event occurs within the Aβ segment, α-secretase cleavage precludes the generation and deposition of amyloid. Alternative processing routes permit the Aβ sequence to remain intact and therefore are potentially amyloidogenic. These include a secretory pathway resulting in the release of soluble Aβ fragments (Haass et al., 1992*b*; Shoji et al., 1992) and an endosomal–lysosomal pathway yielding Aβ containing carboxyl-terminal derivatives (Estus et al., 1992; Haass et al., 1992*a*).

Some early-onset autosomal dominant forms of AD are strongly linked to mutations in the APP gene (for review, see Hardy, 1992). Some of these mutations are associated with increased production of Aβ or an increased formation of more amyloidogenic forms of Aβ in vitro (Citron et al., 1992; Cai et al., 1993; Suzuki et al., 1994). Transgenic mice expressing one of these mutations in the APP gene exhibit many of the pathological signs of AD, including neuritic plaques with thioflavin-S-positive deposits of Aβ (Games et al., 1995). Decreased release of APP_s has been documented in the CSF of AD patients (Farlow et al., 1992; Van Nostrand et al., 1992). These and other data suggest that secreted levels of APP_s or Aβ may offer clues

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Abbreviations used: Aβ, amyloid β-peptide; AD, Alzheimer's disease; APLP, amyloid precursor-like protein; APP, amyloid precursor protein; APP_s, soluble carboxyl-terminus-truncated amyloid precursor protein; CDP-DAG, CDP-diacylglycerol; NMS, *N*-methylscopolamine; PDBu, phorbol 12,13-dibutyrate; PI, phosphoinositide; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; TTX, tetrodotoxin.

regarding mechanisms underlying the disease process (Selkoe, 1993). Accelerated metabolism of APP via the nonamyloidogenic α-secretory pathway as a means of decreasing Aβ production has been proposed as a potential area of therapeutic intervention (Buxbaum et al., 1993).

Studies on the cellular mechanisms that control APP metabolism have emphasized the role of protein kinase C (PKC) and neurotransmitter receptors linked to the phospholipase C–diacylglycerol signal transduction pathways (Nitsch and Growdon, 1994). Activation of PKC in continuous cell lines has been demonstrated to accelerate the processing and release of APP_s while coincidently inhibiting soluble Aβ production (Buxbaum et al., 1993; Gabuzda et al., 1993; Hung et al., 1993). Similarly, cholinergic agonists have caused an increase in the release of APP_s in cell lines (Buxbaum et al., 1992, 1994; Nitsch et al., 1992) and concomitant decrease in Aβ release (Hung et al., 1993; Buxbaum et al., 1994).

Although the PKC signal transduction pathway has been implicated in the regulation of APP_s release, few studies have tested this hypothesis in central neurons (Farber et al., 1995; Mori et al., 1995). In the present investigation, we examined whether direct or indirect stimulation of PKC increases the release of APP_s in primary cultures of rat cortical neurons. We show that stimulation of PKC by addition of phorbol esters does indeed increase the release of APP_s but that activation of a well-known PKC-coupled cell surface receptor did not significantly increase the release of APP_s.

MATERIALS AND METHODS

Cell culture

Timed pregnant Sprague–Dawley rats were anesthetized with halothane at 15–17 days of gestation, and the cerebral cortex was removed from the rat embryos (Soderback et al., 1989). Tissue was collected and stored in Hanks' buffer (4°C). Following the dissection, Hanks' buffer was aspirated, and the tissue pieces were dissociated by mild trituration in Dulbecco's modified minimum essential medium containing N1 supplements (Bottenstein et al., 1980) plus 10% fetal calf serum. A single-cell suspension was plated on 12-well tissue culture plates precoated with poly-D-lysine (0.1 mg/ml) at 1.1×10^6 cells per well. After 7 days in vitro, cells were exposed to cytosine arabinoside (10^{-5} *M*) in medium overnight to halt nonneuronal cell growth and enrich for cortical neurons: 50% of the cells stained positively for neuron-specific enolase. Medium replacement was carried out on a biweekly schedule, and cells were maintained at 37°C in 5% CO_2. After 15–16 days in vitro cultures were washed with HEPES-buffered saline and exposed to pharmacological agents—phorbol esters, oxotremorine-M, atropine, or KCl—in minimum essential medium containing 25 m*M* HEPES or carbachol in Dulbecco's modified minimum essential medium containing N1 supplements without fetal calf serum. Phorbol esters were diluted from concentrated (20 m*M*) stocks made up in dimethyl sulfoxide. Medium containing the drug vehicle was not different from medium alone as determined by two-tailed *t* test ($p > 0.05$; n = 6).

Quantification of APP and amyloid precursor-like protein (APLP)

Following exposure, phenylmethylsulfonyl fluoride was added (final concentration, 2 m*M*) to the medium, which was subsequently spun to remove cellular debris. The medium was then desalted, concentrated by centrifugation (using Millipore filters having a molecular weight cutoff of 30,000), and lyophilized. Cells were lysed in an extraction buffer containing 1% Nonidet P-40 and 1% sodium deoxycholate and centrifuged to remove detergent-insoluble material. Laemmli sodium dodecyl sulfate sample buffer was added to concentrated secreted proteins or lysates, and the samples were boiled. Secreted and cellular protein was quantified with the bicinchoninic acid assay (Pierce). Unless stated otherwise, reconstituted culture media proteins corresponding to 4 μg of total secreted protein and cellular protein corresponding to 4 μg of total cellular protein were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis on 10% gels. APP was detected by western blot analysis using a well-characterized anti-APP NH_2-terminal antibody [anti-PreA4 monoclonal antibody, clone 22C11 (Weidemann et al., 1989); purchased from Boehringer Mannheim] or the affinity-purified anti-GID polyclonal antibody (Cole et al., 1991), whereas APLP was detected using polyclonal antibody D2-I raised against mouse APLP2 (Thinakaran and Sisodia, 1994). The secondary horseradish peroxidase-linked antibodies were visualized by enhanced chemiluminescence (Amersham) using Hyperfilm. Immunoreactive bands were compared densitometrically with the use of a Molecular Dynamics image quantifier. Densitometric measurements were performed in the linear range as determined by standard dilution curves of secreted cellular proteins. For each trial, densitometric analysis of control APP_s secretion was arbitrarily defined as 1; measurement of pharmacological effects on APP_s secretion from the same western blot was then scaled to the control measurement. All comparisons within a single trial were made on matched sister cultures derived from a single plating. ANOVA followed by Fisher's post hoc analysis was used to determine the significance of observed differences.

Measurement of phosphoinositide (PI) turnover

Accumulation of [^{3}H]CDP-diacylglycerol formation ([^{3}H]CDP-DAG) (Godfrey, 1989; Hwang et al., 1990) was measured using cultures kept in vitro for 14–16 days according to the method of Murphy et al. (1992). In brief, cultures were prelabeled with 1.5 μCi/ml [^{3}H]cytidine (25 μCi/mmol; catalogue no. 32, 206-7; Sigma Chemical Co.) in a Hanks' balanced salt solution for 40 min. LiCl was added at 2.5 m*M* to all cultures in the presence of 1.5 μCi/ml [^{3}H]cytidine 20 min before drug treatment. Cells were incubated with cholinergic drugs in the presence of 2.5 m*M* LiCl and 1.5 μCi/ml [^{3}H]cytidine for 1 h. Incubations were terminated with addition of 1 ml of ice-cold chloroform/methanol (1:2 vol/vol). Phase separation was accomplished by adding 0.8 ml of chloroform and 0.8 ml of water and centrifuging at low speed. The organic phase was collected, washed once with 2.0 ml of 1 *M* HCl/methanol, and evaporated to dryness, and radioactivity was counted by liquid scintillation spectrophotometry.

Measurement of *N*-[^{3}H]methylscopolamine ([^{3}H]NMS) binding to intact cortical neurons

Measurement of ([^{3}H]NMS) binding to intact cortical neurons was performed using a modified assay of Eva et al.

(1990). In brief, cortical cultures were washed with phosphate-buffered saline$^+$ (with 90 m*M* $CaCl_2$ and 49 m*M* $MgCl_2$) and incubated in 1 n*M* [^{3}H]NMS in phosphate-buffered saline$^+$ for 1 h (37°C). Following this incubation period, cultures were washed with phosphate-buffered saline$^+$ and phosphate-buffered saline$^-$ (without $CaCl_2$ and $MgCl_2$) containing 1% sodium dodecyl sulfate and 1% Triton-X was added to lyse the cells. Radioactivity of the cell lysate was counted by liquid scintillation spectrophotometry. Cellular protein was quantified in sister wells with the bicinchoninic acid assay (Pierce).

RESULTS

Detection of APP_s in the culture medium of rat cortical neurons

Soluble proteins in the medium of rat cortical neurons were subjected to western blot analysis using the monoclonal antibody 22C11 raised against a full-length APP fusion protein. The immunoreactive proteins with apparent molecular masses ranging from 100 to 130 kDa are similar to those of secreted amino-terminal APP_s derivatives previously described in cell culture media and human CSF (Nitsch and Growdon, 1994).

Phorbol esters increase APP_s release

PKC stimulation by phorbol esters has been shown to increase dramatically the release of amino-terminal APP_s derivatives in a wide variety of cell lines (Buxbaum et al., 1992, 1993; Caporaso et al., 1992; Gabuzda et al., 1993; Hung et al., 1993). To determine if phorbol esters can regulate APP_s production in central neurons we examined primary cultures of rat cortical neurons. The levels of APP_s increased significantly when the cells were incubated with either the phorbol ester phorbol 12,13-dibutyrate (PDBu) or phorbol 12-myristate 13-acetate (PMA). PDBu markedly stimulated APP_s in a dose-dependent manner. Neuronal cultures incubated with 1 μM PDBu and 10 μM PDBu increased APP_s release by 210.3 ± 27.4 and 277.2 ± 31.4%, respectively ($n = 3$, $p < 0.05$; Fig. 1). Cortical cultures incubated with 1 μM PMA increased APP_s generation by 199.3 ± 34.0% ($n = 3$, $p < 0.05$; Fig. 1).

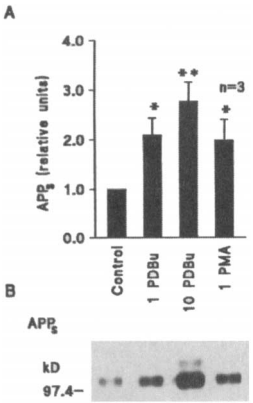

FIG. 1. PKC stimulation increases APP_s release. **A:** Densitometric analysis of the effect of PDBu and PMA on basal APP_s. Data are mean ± SEM (bars) values of three experiments performed in duplicate. *$p < 0.05$, different from control values; **$p < 0.05$, different from all other treatment groups. **B:** Representative western blot of APP_s fragments released by rat cortical neurons alone or in the presence of 1 and 10 μM PDBu or 1 μM PMA, as indicated above. Cortical APP_s fragments were detected with the monoclonal antibody anti-PreA4.

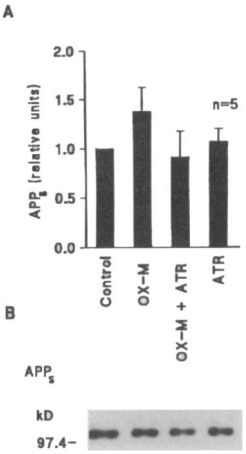

FIG. 2. Oxotremorine-M (OX-M) does not increase the formation of APP_s. **A:** Densitometric analysis of the effect of OX-M on basal APP_s. Data are mean ± SEM (bars) values of five experiments performed in duplicate. None of the treatments was significantly different from control values ($p > 0.05$). **B:** Representative western blot of APP fragments secreted by cultured rat cortical neurons alone or in the presence of 10 μM OX-M with or without 1 μM atropine (ATR) as indicated above. Cortical APP_s fragments were detected with the monoclonal antibody anti-PreA4.

Cholinergic receptor stimulation does not increase APP_s release

APP processing can be regulated in cell lines by the stimulation of cell surface receptors linked to activation of phospholipase C and PKC (Buxbaum et al., 1992, 1993; Nitsch et al., 1992; Hung et al., 1993). In particular, an increase in APP_s production has been demonstrated with drugs that stimulate the muscarinic acetylcholine receptor M_1 or M_3 (Nitsch et al., 1992; Hung et al., 1993). To determine whether or not muscarinic receptor stimulation can augment APP_s production in neuronal cultures we examined the effect of the muscarinic receptor agonist oxotremorine-M on basal levels of APP_s. We observed that APP_s production in culture wells incubated for 1 h with 10 μM oxotremorine-M was not statistically different from the control ($n = 5$, $p > 0.05$; Fig. 2). This did not appear to be caused by saturation of the receptor by endogenously released neurotransmitter, as APP_s release in culture wells incubated with the muscarinic receptor antagonist atropine was not different from control wells. Similar results were found for the cholinergic agonist carbachol (Fig. 3).

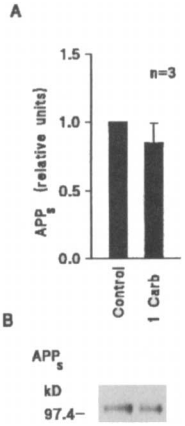

FIG. 3. Carbachol (Carb) does not increase the release of APP_s. **A:** Densitometric analysis of the effect of Carb on basal APP_s corresponding to 500 μg of cellular protein. Data are mean ± SEM (bars) values of three experiments. Sister cultures exposed to Carb were not different from controls ($p > 0.05$ by paired *t* test). **B:** Representative western blot of APP_s fragments secreted by cultured rat cortical neurons alone or in the presence of 1 m*M* Carb. Cortical APP_s fragments were detected with the monoclonal antibody anti-PreA4.

Cultured cortical neurons express functional muscarinic receptors

To test for expression of muscarinic receptors in our rat cortical cultures, we carried out binding studies using [^{3}H]NMS. In three experiments, [^{3}H]NMS binding was 71.86 ± 11.10 fmol/mg of protein (mean ± SEM). This level of expression is comparable to that of other rat cortical cultures (Eva et al., 1990). However, it is worth noting that the percentage of cultured cortical *neurons* expressing muscarinic receptors and the specific subtypes expressed are unknown.

To determine whether the muscarinic receptors in our cortical cultures were functionally coupled to the phospholipase C pathway, we measured PI turnover following exposure to muscarinic receptor agonists by assaying for stimulation of CDP-DAG accumulation. Although an indirect measure of PI turnover, CDP-DAG formation has been shown to be a particularly accurate measure of inositol phosphate formation for muscarinic receptor activation in the cortex and hippocampus (Heacock et al., 1993). Application of 10 μM oxotremorine-M to cortical neurons resulted in a 10-fold increase in PI turnover, and this effect was readily blocked by the muscarinic receptor antagonist atropine; 1 mM carbachol resulted in an even larger increase in PI turnover (Table 1). To determine whether these increases in PI turnover were reflective of PKC activity, PKC assays were carried out as described (Lanius et al., 1995). In brief, α- and β-PKC isoenzymes were immunoprecipitated from particulate or cytosolic fractions of cultured neurons, and PKC histone H1 phosphotransferase activity was subsequently measured. In preliminary experiments, we found that 10 μM PDBu dramatically increased translocation of PKC as well as evoked a 282% increase in particulate PKC activity, whereas 10 μM oxotremorine-M and 1 mM carbachol produced comparatively little PKC translocation and only a modest increase in cytosolic PKC activity (115 and 121%, respectively) (J. Mills, P. B. Reiner, H. Paddon, and S. Pelech, unpublished data). These data suggest that muscarinic receptor stimulation of the PKC pathway is not sufficient to alter APP_s release in cortical neuronal cultures.

TABLE 1. *PI turnover associated with cholinergic receptor stimulation*

Treatment	% of control [^{3}H]CDP-DAG level
Oxotremorine-M (10 μM)	1,189.32 ± 282.38
Oxotremorine-M (10 μM) + atropine (1 μM)	162.73 ± 72.38
Carbachol (1 mM)	3,156.15 ± 677.09

Results have been normalized to the control (cultures that were treated only with 2.5 mM LiCl). Oxotremorine-M at 10 μM and carbachol at 1 mM produced significant elevations in [^{3}H]CDP-DAG content as determined by two-tailed t test ($p < 0.05$). Data are mean ± SEM values (n = 3).

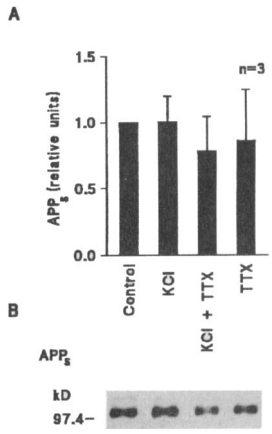

FIG. 4. Chemical depolarization does not increase the formation of APP_s. **A:** Densitometric analysis of the effect of KCl on basal APP_s release. Data are mean ± SEM (bars) values of three experiments performed in duplicate. None of the treatments was significantly different from control values ($p > 0.05$). **B:** Representative western blot of APP_s release from cortical neuronal cultures under basal conditions or in the presence of 35 mM KCl with or without 1 μM TTX. Cortical APP_s release was detected using the monoclonal antibody anti-PreA4.

Effect of chemical depolarization on APP_s

Regulation of APP processing by electrical depolarization has been demonstrated using rat hippocampal slices (Nitsch et al., 1993). To determine whether neuronal depolarization can also increase APP_s release in primary cultures, cortical neurons were exposed to 35 mM KCl for 1 h. Chemically induced depolarization did not significantly alter APP processing (n = 3, $p > 0.05$; Fig. 4). Because cortical cultures are spontaneously active, we considered the possibility that spontaneously released transmitter might be sufficient to activate phosphoinositol turnover (Murphy et al., 1992) and thereby tonically increase APP_s release. However, application of 1 μM tetrodotoxin (TTX), a treatment that blocks voltage-sensitive sodium channels necessary for the generation of action potentials, did not alter basal APP_s release (Fig. 4).

Effect of cholinergic receptor stimulation and PKC stimulation on APP_s and APLP release

During the course of these experiments it was discovered that the anti-PreA4 used in this study cross-reacts with members of the APLP family (Wasco et al., 1992, 1993; Slunt et al., 1994). As further confirmation that the pattern of protein release was representative of APP_s, additional trials were performed, and secreted APP_s was quantified using the affinity-purified anti-GID polyclonal antibody raised against peptide 175–186 of APP (Cole et al., 1991). The sequence of APLP shows poor homology in the APP176–186 region, and therefore the anti-GID antibody should not cross-react with APLP. As with APP_s release analyzed using the anti-PreA4 antibody, APP_s production in culture wells incubated with 1 μM PDBu was well above basal levels, whereas those incubated with 10 μM oxotremorine-M were not different from the control (n = 3; Fig. 5). The effect of these same drug treatments on APLP 2 release was also studied. APLP 2 is a protein within the APP family of membrane glycoproteins and is recognized by the anti-PreA4 antibody (Thinakaran and Sisodia, 1994). Parallel western blots were probed with the polyclonal antibody D2-I raised

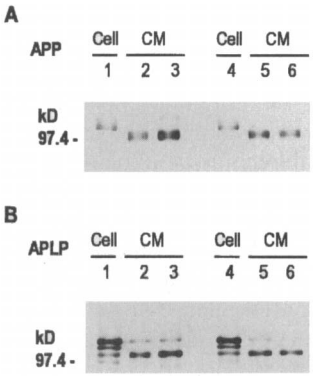

FIG. 5. PKC stimulation but not cholinergic receptor stimulation increases release of APP_s and APLP. Parallel western blots of cellular (cell) and secreted [conditioned media (CM)] proteins from cultured rat cortical neurons alone (lanes 1, 2, 4, and 5) or in the presence of either 1 μM PDBu (lane 3) or 10 μM oxotremorine-M (lane 6). **A:** APP was detected using the polyclonal antibody anti-GID. **B:** APLP was detected using the polyclonal antibody D2-I. Immunoblots are representative of three separate experiments performed in parallel.

against mouse APLP 2 (Thinakaran and Sisodia, 1994). D2-I is specific for APLP 2 and does not cross-react with APP or APLP 1 (Thinakaran and Sisodia, 1994). Like APP_s, APLP release in cortical cultures increased following exposure to 1 μM PDBu, whereas those exposed to 10 μM oxotremorine-M were similar to the control (n = 3; Fig. 5).

DISCUSSION

In the present study, we examined whether or not first and second messengers of cholinergic neurotransmission increase APP_s release. We found that whereas APP_s release can indeed be regulated by direct activation of PKC with phorbol esters, muscarinic receptor stimulation did not significantly increase APP_s release above basal levels.

Regulation of APP processing has been shown to be influenced by direct activation of PKC by phorbol esters in several continuous cell lines, including PC12 pheochromocytoma (Buxbaum et al., 1990; Caporaso et al., 1992), Chinese hamster ovary (Buxbaum et al., 1993), human glioma (Hs 683) (Buxbaum et al., 1993), COS (Gabuzda et al., 1993), and human embryonic kidney 293 (Gillespie et al., 1992) cells. Our data indicate that this phenomenon can be extended to include cortical neurons in culture and are in agreement with recent reports showing that phorbol esters increase APP_s release in primary hippocampal neuronal cultures (Lee et al., 1995). Therefore, the relevance of PKC-mediated regulation of APP_s in both cell lines and central neurons is firmly established.

Cell lines overexpressing muscarinic acetylcholine receptor subtype M_1 or M_3 show increased release of APP_s on addition of cholinergic agonists (Buxbaum et al., 1992, 1994; Nitsch et al., 1992; Slack et al., 1995), an effect that is antagonized by various protein kinase inhibitors (Nitsch et al., 1992; Slack et al., 1995). Similarily, cholinergic regulation of APP_s secretion has been shown to occur in cell lines expressing their normal complement of muscarinic receptors (Buxbaum et al., 1992; Wolf et al., 1995). In contrast, we were unable to demonstrate any statistically significant change in APP_s release from cortical neurons when their natural complement of muscarinic receptors was stimulated. These receptors were fully functional, as evidenced by increases in PI turnover that were comparable to those seen in cortical tissue taken from the adult rat brain (Godfrey, 1989). However, the observation that only modest PKC stimulation was evoked by muscarinic receptor activation may suggest that only those conditions that produce robust increases in PKC activity enhance APP_s secretion in central neurons.

The evidence that cholinergic agonists regulate APP_s release in central neurons is somewhat contradictory at present. In agreement with our findings, recent in vivo studies argue against positive regulation of APP_s by acetylcholine, as APP_s release was elevated following lesion of the basal forebrain cholinergic neurons (Wallace et al., 1995). Similarly, Farber et al. (1995) found that the nonspecific cholinergic agonist carbachol had no effect on APP_s release from rat hippocampal or cortical slices.

In contrast to these studies, other data indicate that cholinergic neurotransmission can alter APP_s release in the mammalian brain. Exposure of hippocampal slices to carbachol in the presence of the selective M_2 antagonist gallamine significantly increased APP_s production (Farber et al., 1995). Moreover, a more selective M_1 agonist, WAL 2014 (Ensinger et al., 1993), enhanced APP_s release from cortical slices at low concentrations while lacking this effect at higher concentrations. The biphasic nature of the WAL 2014 response and the effect of gallamine suggest that M_2 receptor activation inhibits APP_s production. Both cholinesterase inhibitors and muscarinic receptor stimulation with bethanechol enhanced APP_s release from cortical slices of the rat (Mori et al., 1995). If, as suggested above, the M_2 receptor is negatively coupled to APP_s production, this finding is surprising given that bethanechol is a full agonist at the M_2 receptor and only a partial agonist at muscarinic receptors positively coupled to phospholipase C (Richards, 1990). Finally, in vivo data from patients receiving lithium or antidepressants show that drugs possessing relatively nonspecific anticholinergic properties have lower CSF APP_s levels than controls (Clarke et al., 1993).

Taken together, these findings suggest that cholinergic receptor stimulation of APP_s production in central neurons is pharmacologically complex. Under the experimental conditions described in this study, muscarinic receptor stimulation was insufficient to regulate APP_s secretion. It remains possible that other conditions may be found in which muscarinic receptor stimulation does regulate APP_s secretion. The ultimate

question relates to transmitter regulation of APP_s secretion in the intact human brain, an issue worthy of further study.

Electrical depolarization of hippocampal slices has been shown to increase release of APP_s, presumably by release of endogenous transmitters (Nitsch et al., 1993). However, when we exposed cortical neuronal cultures to 35 m*M* KCl, a treatment that should result in marked depolarization accompanied by both calcium entry and massive neurotransmitter release, APP_s release was unchanged. This finding is puzzling as in both cortical cultures and hippocampal slices, the main neurotransmitter released by such treatment would be glutamate, which would be expected to activate PKC either via metabotropic glutamate receptor stimulation or by calcium entry through NMDA receptor-gated channels. Indeed, both depolarization-induced increases in intracellular Ca^{2+} levels (Buxbaum et al., 1994; Querfurth and Selkoe, 1994) and metabotropic glutamate receptor stimulation (Lee et al., 1995) would be expected to stimulate an increase in APP_s production. Nevertheless, discrepancies in the two preparations are worthy of mention. In the hippocampal slice preparation, the terminals of hippocampal afferents would be intact, and depolarization could result in release of a considerable array of neurotransmitter agents that would undoubtedly differ from those released in cortical cultures (for review, see Nicoll et al., 1990). In addition, in the hippocampal slice preparation, depolarization was induced electrically, whereas in cortical neuronal cultures depolarization was induced chemically. Finally, PKC isoenzyme expression and compartmentalization may differ between intact and dissociated neuronal cell preparations (Shimohama et al., 1991). One or more of these discrepancies may enable electrical stimulation to activate PKC in hippocamal slices sufficiently so as to result in the regulation of APP_s release.

From these observations we conclude that regulated APP_s release can occur in cortical neurons, as stimulation of PKC with phorbol esters reliably increased APP_s release. However, stimulation of the natural complement of muscarinic receptors (as expressed in cultured cortical neurons) did not result in a statistically significant change in APP_s. Two distinct possible explanations for this apparent discrepancy exist. First, PKC activation by phorbol esters is both more robust and more likely to result in PKC translocation than that evoked by muscarinic receptor stimulation. Regulation of APP_s release may be sensitive to the level, location, and duration of PKC activation (Newton, 1995). Therefore, activation of the normal complement of muscarinic receptors in cortical neurons may be insufficient to result in regulation of APP_s release. Second, phorbol esters may stimulate signaling pathways distinct from those stimulated by cholinergic agonists. Recent observations indicate that partially divergent signal transduction pathways exist for regulating APP_s release (Buxbaum et al., 1994; Slack et al., 1995). Convergence of these pathways may occur downstream of PKC activation at a tyrosine phosphorylation-dependent step (Slack et al., 1995). Critical assessment of APP_s regulation by the various cholinergic receptor subtypes and their corresponding second messenger pathways may explain these apparent discrepancies.

These findings are important when considering both the etiology of AD and therapeutic strategies for ameliorating its pathology (Haass and Selkoe, 1993). Because the increased release of APP_s is accompanied by a decrease in Aβ release (Buxbaum et al., 1993; Hung et al., 1993), it is generally assumed that activation of the α-secretase pathway is beneficial. Moreover, it has been implied that the well-documented loss of cholinergic innervation of the cerebral cortex in AD (Coyle et al., 1983) might reduce APP_s release, thereby contributing to the deposition of amyloid (Buxbaum et al., 1992; Lahiri et al., 1992; Nitsch and Growdon, 1994). Our data and those of others (Wallace et al., 1995) would tend to caution against hasty acceptance of the hypothesis that the loss of cholinergic neurons is a primary event altering APP_s levels in AD.

With respect to treatment strategies, cholinomimetic replacement therapy has met with little success (Reiner and Fibiger, 1995). Assuming that the deposition of amyloid is the primary pathological feature of AD (Hardy and Allsop, 1991), our data would predict that cholinomimetic replacement therapy would not mitigate amyloid deposition in the cerebral cortex. On the other hand, it is clear that APP_s release can be regulated in cortical neurons. Unraveling the molecular pathways responsible for such regulation will be an important challenge for future studies.

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