Calcium Ionophore Increases Amyloid β Peptide Production by Cultured Cells†

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ABSTRACT: Amyloid β peptide (Aβ) is released into the media of a variety of cells in culture during normal metabolism. The discovery of several missense mutations within or flanking the Aβ region of the β amyloid precursor protein (βAPP) in familial Alzheimer's disease provides strong evidence for a role of altered processing of βAPP in the pathogenesis of this disorder. The cellular mechanisms that regulate the relative utilization of the secretory pathway, which causes βAPP to be cleaved within the Aβ domain, and the alternative proteolytic pathway, which produces intact Aβ, are unknown. It is hypothesized that a number of neurodegenerative diseases, including Alzheimer's disease, are characterized by abnormal calcium metabolism. We investigated the effect of disordered calcium homeostasis on Aβ production in human kidney 293 cells transfected with βAPP cDNA. Aβ immunoprecipitated from the conditioned media of cells was compared to immunoprecipitated full-length and secreted forms of βAPP in both metabolic labeling and pulse–chase labeling paradigms. The calcium ionophore A23187 consistently increased the production of Aβ approximately 3-fold. This effect was dependent on the presence of extracellular calcium in intact cells. Caffeine also increased Aβ production, possibly through release of calcium from intracellular stores. The increase in Aβ was cAMP-independent, and it was not mediated by a protein kinase C-dependent pathway, as treatment with phorbol esters decreased Aβ levels. The effects of the ionophore on βAPP maturation and phosphorylation were also established. We conclude that elevation of intracellular calcium levels has an important effect on βAPP maturation and proteolytic processing and substantially enhances the production and release of the amyloidogenic Aβ peptide.

A central role for the β amyloid precursor protein (βAPP) in the pathogenesis of at least some forms of Alzheimer's disease (AD) has been demonstrated by the discovery of several families in which the dominantly inherited phenotype (FAD) was linked to first one (Goate, 1991) and then several other point mutations in βAPP [reviewed in Hardy (1992)]. These missense mutations are found within or flanking the sequence encoding the 39–43-residue amyloid β peptide (Aβ), which accumulates extracellularly in meningocerebral vessels and myriad amyloid plaques in AD [reviewed in Selkoe (1993)]. The finding that one such mutation in a Swedish FAD pedigree (Mullan et al., 1992), when expressed in transfected human cells, increases Aβ secretion 6–8-fold directly implicates the abnormal production of this peptide in the FAD phenotype (Citron et al., 1992; Cai et al., 1993). These studies also established the relevance to AD pathogenesis of the cell culture model used in the present report. Evidence has also been presented that Aβ is neurotoxic *in vitro* [see, for example, Koh et al. (1990) and Yankner et al. (1990)]. Therefore, investigation of the physiological circumstances under which accelerated Aβ production may occur in cells expressing wild-type or mutant βAPP should lead to a greater understanding of both sporadic and familial AD.

Aβ is released during normal metabolism by βAPP-expressing cells and is detectable in normal cerebrospinal fluid and serum (Haass et al., 1992b; Seubert et al., 1992; Shoji et al., 1992; Busciglio et al., 1993). Little is currently known about the cellular processing pathways by which Aβ is generated. The first βAPP proteolytic processing event to be described involves cleavage by an as yet unknown protease (called α-secretase) at residue 687 [numbering according to Kitaguchi et al. (1988)] of fully mature, N'- and O'-glycosylated βAPP_{770} (residue 16 of the Aβ region) (Esch et al., 1990; Sisodia et al., 1990). The large, soluble ectodomain of the precursor (APP_s) is thereby released into the medium (Weidemann, 1989; Oltersdorf, 1990), and a 10 kD carboxyl terminal stub is retained in the membrane (Selkoe et al., 1988). This scission precludes formation of Aβ. An alternative processing route was shown to involve the coated pit-mediated reinternalization of βAPP from the cell surface and its trafficking to late endosomes/lysosomes, in which several Aβ-containing C-terminal βAPP fragments were detected (Golde et al., 1992; Haass et al., 1992a). However, treatment of cells with leupeptin to inhibit thiol proteases in lysosomes had no measurable effect on Aβ production (Shoji et al., 1992; Haass et al., 1993), and Aβ could not be detected in purified lysosomes of radiolabeled cells (C. Haass and D. Selkoe, unpublished data), suggesting that lysosomes per se might not be required for Aβ production. The importance of an acidic intracellular compartment for the basal production of Aβ was indicated by treating cells with NH_4Cl or monensin (Haass et al., 1993). βAPP degradation was also shown to be sensitive to chloroquine, supporting the requirement for an acidified vesicle (Caporaso et al., 1992). However, these agents are nonspecific in their effects on ionic balance, and they may also alter APP_s secretion. The effects that perturbations in cellular calcium homeostasis may have on Aβ production have not been reported, to our knowledge.

Altered calcium homeostasis may play a pivotal role in the selective neuronal degeneration that occurs during normal aging, in Alzheimer's disease and in related disorders (Gibson

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& Peterson, 1987; Siesjo et al., 1989; Mattson, 1992). Calcium influx into cultured human cortical neurons following treatment with the calcium ionophore A23187 causes microtubule disruption and neurofibrillary degeneration and a consequent increase in Alz 50 immunostaining, which indicates the presence of altered phosphorylated forms of the τ-protein (Mattson et al., 1991). These changes are reminiscent of the cytoskeletal abnormalities found in Alzheimer's disease (Kosik et al., 1986; Nukina & Ihara, 1986; Grundke-Iqbal, 1986a; Wolozin et al., 1986; Baudier & Cole, 1987; Lee et al., 1991).

Excitatory amino acid (EAA) neurotoxicity in susceptible neuronal populations such as are affected in cerebral ischemia or Huntington's disease is mediated in part by abnormal elevations of intracellular calcium levels (Choi, 1988; Greenamyre & Young, 1989; Miller et al., 1989). The possibility of a link between amyloid deposition in Alzheimer's disease and abnormal calcium homeostasis has been raised. In this regard, abnormal calcium fluxes could precede excess Aβ production and/or they could be a consequence of a cytotoxic effect of Aβ on surrounding cells. The latter situation, in which Aβ neurotoxicity in cortical neuronal cultures is mediated via a potentiation of calcium influx and increased vulnerability to excitotoxins, has been described (Koh et al., 1990; Mattson et al., 1992, 1993). In this report, we examine the first of these two possibilities: whether increases in intracellular calcium affect βAPP processing in a way that changes the regulation of Aβ production and release. The well-characterized calcium ionophore A23187 (Pressman, 1976) was used to stimulate calcium influx from media or mobilization from intracellular luminal stores [e.g., see Beckers et al. (1990)]. In addition, treatment with either inositol triphosphate (IP3) or caffeine was used to isolate the role of calcium mobilization from intracellular pools.

MATERIALS AND METHODS

Reagents and Stocks. Calcium ionophore A23187 (Sigma) was stored as a 5 mM stock solution in DMSO at −20 °C. Final concentrations of solvent (~0.1%) were uniform within any given experiment. Caffeine (Sigma) (0.15 M stock) was heated to 42 °C prior to use. Neuraminidase (Sigma) (3.8 milliunits/μL) was stored in 150 mM NaCl buffer at 4 °C. PMA (phorbol 12-myristate 13-acetate) (2 mM in DMSO) and PDBu (phorbol 12,13-dibutyrate) (10 mM in DMSO) (Sigma) were stored at −70 °C. D-Myoinositol triphosphate (IP3) (Sigma and Boehringer Mannheim) was reconstituted at 2 mM immediately prior to use. L-[^{35}S]Methionine was from New England Nuclear (Boston, MA), [^{32}P]orthophosphate from ICN Biochemicals (Irvine, CA), and dibutyryladenosine cyclic 3′,5′-monophosphate (cAMP) (10 mM) from Sigma Chemical (St. Louis, MO).

Cell Cultures. Human embryonic kidney 293 cells stably transfected with βAPP_{695} or βAPP_{751} cDNAs (Selkoe et al., 1988; Oltersdorf et al., 1989) were grown in DMEM (which contains 2 mM $CaCl_2$) plus 10% FCS to near-confluence in 60-mm dishes prior to being labeled. The semiadherent human megakaryocytoid cell line DAMI (gift of S. Greenberg and R. Handin) (Greenberg et al., 1988) was grown in 25 cm^2 flasks in either Iscoves medium/10% horse serum or DMEM/10% FCS. Approximately 5.0×10^6 DAMI cells were plated onto 60-mm dishes prior to being labeled. DAMI cells stably transfected with $\beta APP_{751 \text{ or } 695}$ cDNAs were obtained by G418 selection (1.1 mg/mL) after electroporation (Bio-rad) of ~14 $\times 10^6$ cells in 0.5 mL of serum-free DMEM (0.4-cm cuvette, 180 V, 960 μF, 27 °C) containing 25 μg of pCMV-$\beta APP_{751 \text{ or } 695}$ cDNA (gift of Athena Neurosciences, Inc.) linearized with *Hin*dIII and 3 μg of pCMV-neo as the selectable marker. Stable DAMI clones were chosen that showed similar levels of production of full-length βAPP and APP_s, as demonstrated by immunoblotting with affinity-purified antibody B5 to $\beta APP_{444-592}$ (βAPP_{695} numbering) (Oltersdorf et al., 1990).

To examine the effects of IP3 treatment, stably transfected 293 cells were permeabilized by either electroporation or suspension in a high-K^+/low-Ca^{2+} medium (described below), yielding identical results. Following trypsinization and a wash in PBS, 3 million cells in suspension were electroporated (250 V, 960 μF, 27 °C) in 500 μL of either methionine-free DMEM or calcium-deficient medium (described below). Under these electroporation conditions, 4–20% of 293 cells took up trypan blue, and an additional 25–40% took up 0.2% Erythrocin alone. The latter is a smaller molecular weight vital dye than trypan blue and was taken to indicate a subpopulation of permeable cells that was entirely viable. One hundred percent of 293 cells took up Erythrocin when permeabilized in 135 mM KCl, 1 mM $MgCl_2$, 1.2 mM KH_2PO_4, and 10 mM Hepes, pH 7.4 [formulation of Streb and Schultz (1983)]. "Calcium-deficient medium" is defined as that described by Mattson et al. (1992) and consists of calcium-free Hank's balanced saline solution (HBSS) with added 1 mM $MgCl_2$, 1 mM L-glutamine, 15 mM sodium bicarbonate, 1 mM pyruvate, 40 mM glucose, and 5 mM Hepes, pH 7.2. "Calcium-free medium" is defined as the calcium-deficient medium (above) with added EGTA (varying from 0.5 to 5 mM).

In order to produce oxidative damage and cellular death through formation of reactive oxygen species (ROS), ferrous sulfate and H_2O_2 (Fenton's reagents) were added during labeling in a metal-catalyzed Haber–Weiss reaction. In this system, EDTA was added optionally to complex with Fe in its reduced state (Dunford, 1987). We also utilized a less toxic system containing ferric chloride with ascorbate added to keep Fe in a reduced (and hence more catalytic) state. The latter system has been reported to increase protein degradation in erythrocytes (Davies, 1986).

Antibodies. R1280 is a polyclonal antibody raised to synthetic $A\beta_{1-40}$ and characterized previously (Tamaoka et al., 1992; Haass et al., 1992b). Specificity was demonstrated by the absence of protein bands when preabsorbed antibody or preimmune serum was used (Haass et al., 1992b). Polyclonal antibody C7 (Podlisny et al., 1991) is directed against the last 20 amino acids of the cytoplasmic tail of βAPP and precipitates N′- and (N′ + O′)-glycosylated full-length βAPP as well as a number of C-terminal fragments. Affinity-purified polyclonal antibody B5 was raised to a recombinantly expressed protein of $\beta APP_{444-592}$ [numbering of Kang et al. (1987)] and recognizes N′- and (N′ + O′)-glycosylated full-length βAPP as well as APP_s (Oltersdorf et al., 1990). Dilutions used for immunoprecipitation were as follows: R1280, 1:300; C7, 1:100; and B5, 1 μg/mL.

Metabolic Labeling and Immunoprecipitation. Metabolic labeling of cells was carried out as described by Haass et al. (1991). After culture medium was removed and the cells were washed in PBS, 1.5 mL of methionine-free DMEM containing 150 μCi of [^{35}S]methionine and the specific pharmacological agent cited (or its vehicle) was added to cells for a 12–14-h labeling period. The 1.5 mL of medium was then centrifuged at 10000*g* and carefully transferred to a fresh tube. Immunoprecipitation (ip) by the method of Weidemann et al. (1989) was used with modifications exactly as described by Haass et al. (1991). For analysis of labeled cellular proteins, total cell extracts were prepared according to Weidemann et al. (1989).

For pulse–chase experiments, label was added to cells in serum- and methionine-free medium for 1 h. Following a rapid wash, the cells were chased in 2 mL of complete medium (containing excess cold methionine) for up to 4 h in the presence or absence of agent. The collected medium was used for ip: 1.6 mL with R1280 and 0.4 mL with B5. To examine maturation of holo-βAPP in cell extracts, a 5-min pulse and varying chase times up to 90 min were used (Weidemann et al., 1989; Oltersdorf et al., 1990).

Proteins immunoprecipitated from media by R1280 were electrophoresed on 10–20% Tris–Tricine gradient polyacrylamide gels at 80 V. The gels were dried and exposed to film at −70 °C for 7 days. All other gel conditions are individually specified in the figure legends. The identities of the 4-kDa (Aβ) and the 3-kDa (p3) peptides precipitated by R1280 have been established previously by radiosequencing (Haass et al., 1992b). p3 is a fragment which begins at Leu_{17} of Aβ, immediately after the α-secretase cleavage site, and appears to derive from the 10-kDa carboxyl-terminal fragment of βAPP that is produced by this cleavage (Haass et al., 1993).

Phosphate Labeling. To study the effects of ionophore on βAPP phosphorylation, $βAPP_{751}$-transfected 293 cells were labeled with 1 mCi of [^{32}P]orthophosphate in 1.5 mL of phosphate-deficient medium containing 1% FCS (1 μg/mL final phosphate) for either 4 or 12 h. The cells were then washed with normal saline containing 50 mM β-glycerophosphate and 0.5 mM Na_3VO_4 as phosphatase inhibitors. Cell extracts were prepared in 1 mL of STEN buffer containing the phosphatase inhibitors, 1% NP40, 0.2% BSA, 20 mM PMSF, and 5 μg/mL leupeptin. The extract was cleared by layering over a 33% sucrose cushion and spinning at 12000*g* for 2 min prior to the usual wash steps of the ip protocol. Phospho-βAPP was quantitated by direct excision of the gel bands (visualized autoradiographically) and scintillation spectrometry (LKB 1212).

Deglycosylation. Neuraminidase was employed to remove sialic acids from the O′-linked sugars on βAPP and APP_s. Twenty-two milliunits of the enzyme was added to the pelleted protein-A–Sepharose/αC7–βAPP complex in 100 μL of 0.1 M sodium phosphate, pH 6.5, following the last wash step in STEN buffer of the ip protocol and incubated for 1 h at 37 °C [a modification of Oltersdorf et al. (1990)]. The beads were repelleted and suspended in 14 μL of reducing SDS sample buffer before electrophoresis.

Quantitation and Statistics. Aβ levels in media were quantitated by volume densitometric analysis of the R1280-precipitated 4-kDa band on gel fluorograms (Molecular Dynamics ImageQuant). Levels of full-length cellular βAPP from the corresponding labeled culture dish were determined by ip of total cell extracts with C7 antibody and direct excision of the N′- and (N′ + O′)-glycosylated βAPP polypeptides for scintillation spectrometry. Aβ levels were corrected for total cellular βAPP levels by establishing their ratio, expressed as densitometric units per cpm. The corrected Aβ level in the presence of drug was normalized to the level obtained from the control dish (i.e., vehicle alone) to determine the degree of change in Aβ attributed to that agent. The results of seven independent experiments were averaged and subjected to the paired Student's *t*-test.

RESULTS

Calcium Ionophore Increases Production of Aβ by Cultured 293 Cells. To determine the effects of calcium homeostasis on βAPP metabolism and amyloid β peptide (Aβ) production, we treated human 293 cells stably transfected with $βAPP_{751}$ cDNA (Haass et al., 1992b) with the calcium ionophore A23187. Radiolabeled Aβ and p3 were immunoprecipitated from the conditioned media of the cells either after 14-h metabolic labeling with [^{35}S]methionine in the absence or presence of A23187 (or other agents) or after a 1-h pulse-label in the absence of agent followed by a chase in medium containing excess cold methionine with or without agent. A23187 produced a dose-dependent increase in both Aβ and p3 production by metabolically labeled cells (Figure 1A). The increase in the release of Aβ and p3 into medium was maximal at a dose of 0.5 μM A23187 (Figure 1A,C). The mean rise in Aβ at the 0.5 μM dose was 3.7 (±1.5)-fold ($p < 0.005$, $n = 7$), as determined by densitometry of gel fluorograms. At higher ionophore concentrations (1.0–5.0 μM), generation of p3 fell substantially, whereas Aβ decreased less and still remained above basal levels (Figure 1A, lanes 4 and 5; and Figure 1C, lanes 5 and 6). When 293 cells transfected with $βAPP_{695}$ cDNA were treated with ionophore, a similar rise in Aβ and p3 was observed (Figure 1B). Thus, the presence of the Kunitz protease inhibitor region in $βAPP_{751}$ does not noticeably affect the calcium ionophore-induced elevation of Aβ production. Aβ peptide was not detected in the lysates of cells incubated with or without A23187 (results not shown).

The effect of A23187 on the generation of the peptides was markedly attenuated when calcium-deficient medium (see Materials and Methods) was used (Figure 1D, lanes 2 and 3). Addition of 2 mM calcium chloride to this medium (to approximate the levels in DMEM) restored the ionophore effect (not shown). In the presence of calcium-deficient medium containing 5 mM EGTA (defined as calcium-free medium; see Materials and Methods), A23187 produced no rise in Aβ and p3 (Figure 1E, lanes 3 and 4). Indeed, Aβ and p3 levels appeared to decrease below basal levels under these conditions. Therefore, the enhancement of Aβ production by ionophore in intact cells is dependent on extracellular calcium. Exposure of the 293 cells to A23187 (at 2 μM) during the 14-h metabolic labeling period resulted in no greater uptake of trypan blue than in untreated cells (untreated, 8.7 ± 2.8% of cells, $n = 5$; A23187, 5.4 ± 5.5% , $n = 4$; calcium-free medium, 10.5 ± 3.9%, $n = 4$; $p > 0.1$ for all comparisons). Moreover, ionophore-treated cells exhibited no unusual morphological features, suggesting that the ionophore at the concentrations used here was not noticeably toxic.

To establish the time course of Aβ augmentation, control for changes in precursor synthesis levels and control for any adverse effects of overnight incubation with ionophore, we carried out 1-h pulse-labeling in the absence of A23187 followed by a cold chase with or without the agent. Aβ secretion and p3 secretion were elevated above basal levels beginning 30–60 min after addition of A23187 (Figure 1F, lanes 7–10). Again, no rise was detected at 60 min or at 4 h in the absence of external calcium, i.e., with EGTA in calcium-deficient medium (lanes 11 and 12). These results confirm the findings shown in Figure 1A-E and validate the overnight labeling paradigm.

Effect of Ionophore on Total Cellular βAPP and APP_s. To address the possibility that the A23187-stimulated increase in Aβ and p3 production could result from a calcium-dependent induction of precursor protein synthesis, the effect of increasing concentrations of ionophore on holoβAPP in 293 cell extracts was examined by immunoprecipitation with antibody C7 (Figure 2A). An apparent small increase in total βAPP was observed that was maximal at 0.5 μM A23187. Quantitation of the excised mature plus immature βAPP bands by scintillation counting gave a mean 1.4 (±0.6)-fold increase

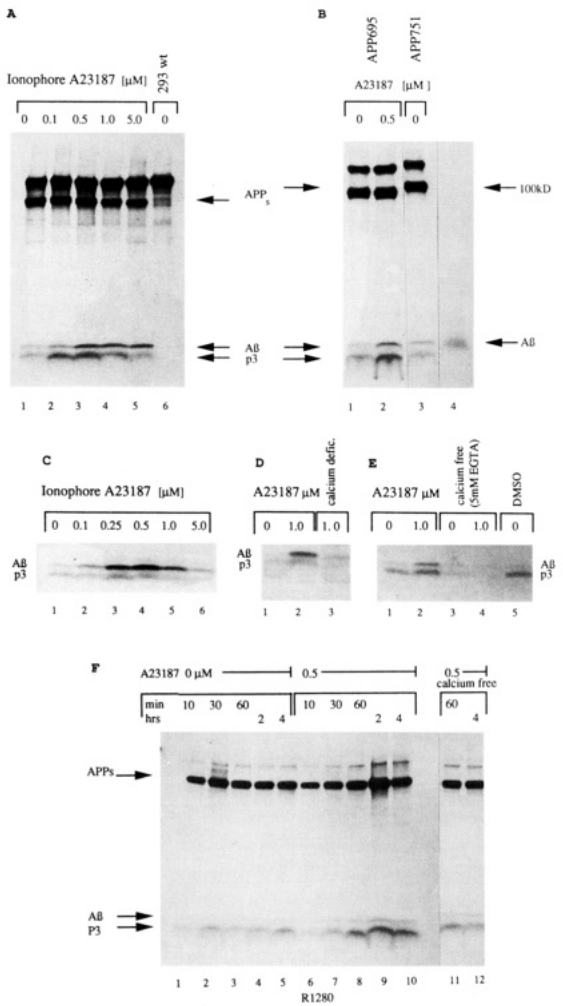

FIGURE 1: Calcium ionophore A23187 increases cellular release of Aβ and p3. (A) A23187 causes a dose-dependent increase in the secretion of the 4-kDa (Aβ) and 3-kDa (p3) peptides into the medium of metabolically labeled 293 cells stably transfected with βAPP_{751} cDNA. Lane 1, vehicle alone; lane 2, 0.1 μM; lane 3, 0.5 μM; lane 4, 1.0 μM; lane 5, 5.0 μM; lane 6, untransfected 293 cells (293 wt) produce levels of the peptides below the limits of detection of this assay, confirming the derivation of the 3- and 4-kDa peptides from the transfected βAPP. (B) Cells transfected with βAPP_{695} show a similar increase in Aβ and p3 after 0.5 μM A23187 (lane 2). Lane 3, untreated βAPP_{751}-transfected 293 cells; lane 4, ^{125}I-labeled synthetic $A\beta_{1-40}$. (C) Dose–response of Aβ and p3 to increasing concentrations of A23187. Note that the decline of the peptides to near-basal levels at doses >1 μM varies somewhat among independent experiments (compare lane 6 with Figure 1A, lane 5). (D) The ionophore effect is attenuated in the absence of external calcium. Lanes 1 and 2, normal medium without (1) or with (2) A23187 (1.0 μM); lane 3, calcium-deficient medium (see Materials and Methods) with A23187 (1.0 μM). Aβ remains at slightly above control levels in calcium-deficient medium. (E) The addition of EGTA (5 mM) with A23187 to calcium-deficient medium (lanes 3 and 4) completely suppresses the ionophore-induced increases in Aβ and p3 seen in normal medium (lanes 1 and 2). Lane 5 shows the lack of effect of the vehicle (0.1% DMSO) alone on Aβ and p3 production (compare to lane 1). (F) Pulse–chase experiments using 1-h labeling with [^{35}S]-methionine in serum-free medium and chasing in excess cold methionine for the times indicated either in the absence (lanes 1–5) or in the presence (lanes 6–10) of 0.5 μM A23187. The A23187 effect on Aβ production is noticeable at 30 min. Under calcium-free conditions (i.e., calcium-deficient medium with 0.5 mM EGTA), no effect of ionophore treatment is seen (lanes 11 and 12). All panels are gel fluorograms of immunoprecipitations with antibody R1280 of 1.5 mL of medium. R1280 precipitates only a small and variable minority of APP_s molecules (upper arrow), so that the amounts of APP_s band shown are not meaningful quantitatively. The uppermost bands above APP_s (MW ~218K) are nonspecific, high molecular weight immunoprecipitated material and can be seen with secondary antibody alone (Haass et al., 1992b).

over control levels ($0.05 < p < 0.10, n = 7$). This small change cannot explain the 3–4-fold increase in Aβ production which ionophore consistently produces. Whereas calcium-deficient medium prevents the ionophore-mediated rise in Aβ and p3 production, the small increase in total βAPP remains (Figure 2A, lane 6). Pulse–chase experiments using treatment with A23187 at 0.5 μM similarly showed no appreciable increase in total cellular βAPP (Figure 2D).

The mean 3.7-fold rise in Aβ levels produced by kidney 293 cells after 0.5 μM A23187 treatment was corrected for the mean 1.4-fold increase in total βAPP. This yielded a net 2.9 (±1.2)-fold increase in Aβ production corrected for the modest induction of βAPP synthesis ($p < 0.005$ compared to vehicle alone, $n = 7$; Figure 2B). Under calcium-deficient conditions, the ionophore effect was absent [1.0 (±0.5) × control, $n = 3$, NS)]. In calcium-free medium, Aβ production was significantly reduced below control levels, regardless of the presence of ionophore [0.30 (±0.05) × control, $n = 4$, $p < 0.001$)] (Figure 2B). HoloβAPP levels remained unchanged in calcium-free medium (not shown).

Ionophore treatment did produce a consistent qualitative change in the βAPP electrophoretic pattern. At 0.5 μM and above, the agent caused a decrease in the apparent molecular weight of the mature (N′ + O′-glycosylated) holoprotein, so that the two major full-length forms migrated closer together (Figure 2A, lanes 1–5). This change was accompanied by a corresponding dose-dependent decrease in the apparent molecular weight of the major secreted derivative (APP_s) in the conditioned medium (Figure 2C, lanes 1–5; see also Figure 2E). To determine whether these effects represented a change in the maturation of the βAPP holoprotein, we conducted pulse–chase experiments in the presence or absence of 0.5 μM A23187 (Figure 2D, lanes 1–10). The rapid appearance of an intermediate C7-precipitable protein (asterisk in Figure 2D) was observed beginning at 10 min of chase with ionophore, accompanied by a decrease in intensity of the fully mature, (N′ + O′)-glycosylated species (Figure 2D, lanes 7–10). These results suggest a partial inhibition of O′-glycosylation of βAPP by A23187, presumably in a pre-Golgi or early Golgi compartment prior to vesicular transport to the cell surface (Sambamurti et al., 1992). The ionophore-induced appearance of the intermediate βAPP species occurred independently of external calcium presence (Figure 2A, lane 6), in contrast to the enhancement of Aβ and p3 secretion (Figure 1D,E). Treatment with caffeine, an agent which enhances calcium release from intracellular stores, similarly led to the appearance of an intermediate species migrating just ahead of the (N′ + O′)-glycosylated holoprotein (Figure 2D, lanes 11–13). Precipitation of APP_s by αB5 from an aliquot of the chase medium corresponding to Figure 1F revealed no appreciable difference in the amount of APP_s in the presence and absence of ionophore (0.5 μM); the slightly smaller species of immature APP_s (asterisk) was again seen (Figure 2E).

A23187 Interferes with βAPP O′-Glycosylation. To determine whether the intermediate βAPP and APP_s species induced by A23187 represent molecules which have not undergone full O′-glycosylation, we compared their migration to those of full-length βAPP or secreted APP_s treated with neuraminidase to remove sialic acids on O′-linked sugars (Figure 3A,B). Incubation of immunoprecipitated βAPP with neuraminidase followed by washing and denaturation showed that this partially deglycosylated holoprotein comigrated with the intermediate form induced by ionophore (Figure 3A, lanes 2 and 3). Similarly, immunoprecipitated APP_s treated with neuraminidase comigrated with the APP_s form induced by

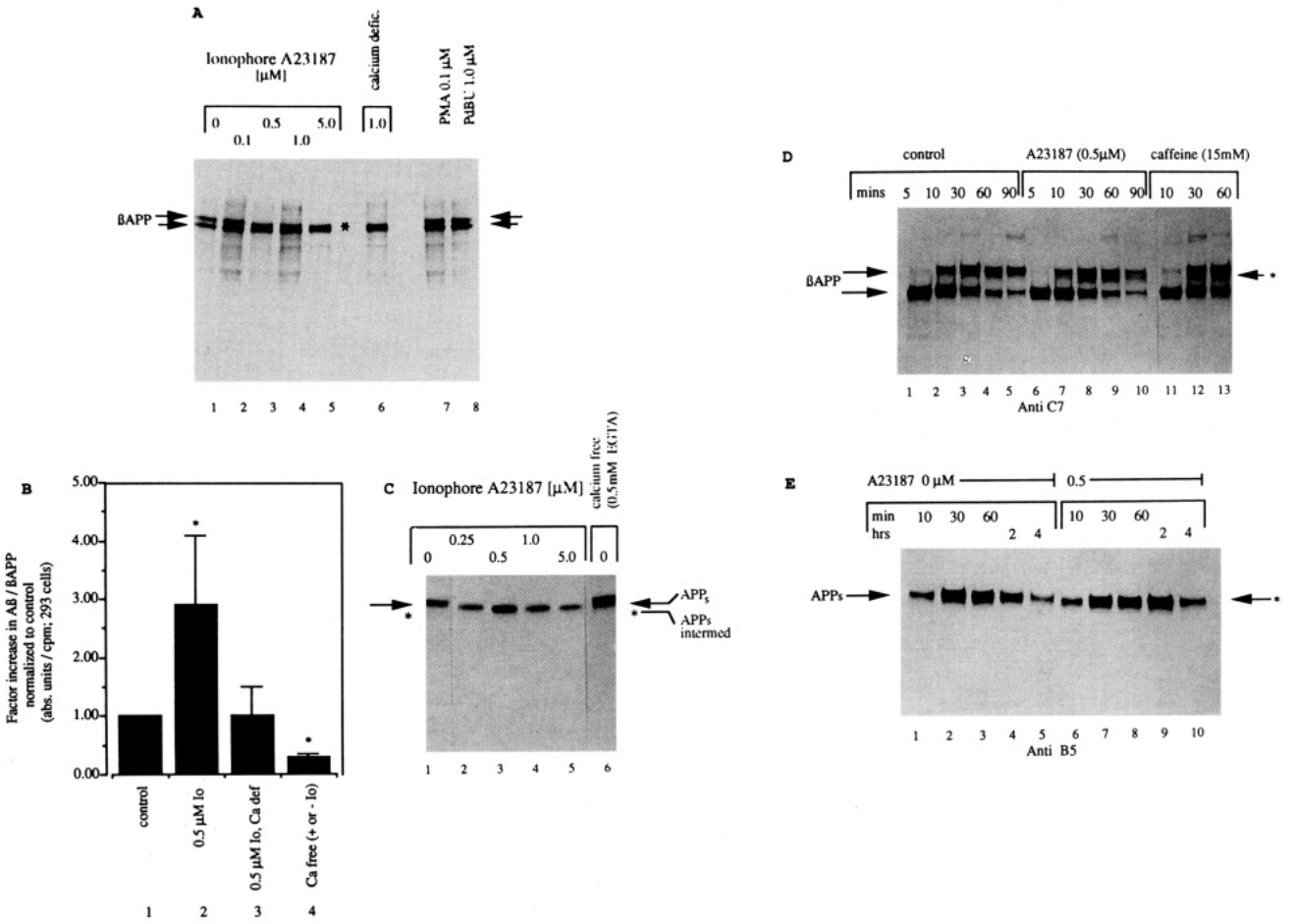

FIGURE 2: Effect of A23187 on βAPP and APP_s. (A) Immunoprecipitation of full-length βAPP from lysates of $βAPP_{751}$-transfected 293 cells metabolically labeled with [^{35}S]methionine (antibody C7). The upper arrow represents mature N′ + O′-glycosylated βAPP; the lower arrow indicates the immature N′-glycosylated species. Lanes 1–5, increasing concentrations of A23187, as indicated, produce a dose-dependent increase in the migration of the (N′ + O′)-glycosylated holoprotein, leading to an intermediate species (asterisk). Lane 6, the change in βAPP migration induced by ionophore persists in calcium-deficient medium. Lanes 7, 8, phorbol esters PMA and PDBu do not alter βAPP migration. (B) Increase in the production of Aβ peptide by A23187 corrected for total cellular βAPP levels. Experimental procedure was identical to Figures 1 and 2A, with A23187 used at 0.5 μM. Bars represent mean plus standard deviation. Immunoprecipitable Aβ from media was corrected for total βAPP precipitated from the corresponding cell extracts and was expressed as the ratio of densitometric units per cpm. This corrected Aβ value in the presence of A23187 (Io) was normalized to that obtained for the paired untreated dish [the latter was set at 1.00 (column 1)]. A23187 increases net Aβ secretion from overnight labeled 293 cells (2.9 ± 1.25)-fold (column 2; $n = 7$, $p < 0.005$). Column 3, the effect is abolished in calcium-deficient medium. Column 4, mean value obtained in calcium-free medium (see Materials and Methods); here, corrected Aβ levels are decreased below base line either in the presence or in the absence of ionophore. (C) Immunoprecipitation of APP_s from media of the metabolically labeled cells shown in Figure 1C (antibody B5). Lanes 1–5, A23187 induces a dose-dependent increase in electrophoretic migration of APP_s to yield an intermediate species (asterisk). Lane 6, same as lane 1 (vehicle alone) but in calcium-free medium. (D) Pulse–chase experiment involving pulse-labeling for 5 min in serum-free/calcium-deficient medium and chasing in calcium-deficient medium, for the times indicated, followed by immunoprecipitation with αC7. Lanes 1–5, control; lanes 6–10, A23187 (0.5 μM) induces the intermediate βAPP species (asterisk) by 30 min; lanes 11–13, caffeine (15 mM) also induces a similar migrating form. Note the delay in appearance of the (N′ + O′)-glycosylated species at 10 min in the presence of either A23187 or caffeine. (E) Media from a pulse–chase experiment (same media shown in Figure 1F) were immunoprecipitated with αB5. A23187 (0.5 μM) results in slightly increased migration of APP_s (asterisk).

A23187 (Figure 3B, lanes 2 and 3). The partial inhibition of O′-glycosylation by A23187 did not prevent secretory cleavage and secretion of βAPP, as shown by the expected level of APP_s release (Figure 2C, lanes 1 vs 3; Figure 2E).

We next examined βAPP maturation as well as Aβ and holoβAPP levels in a second cell type, the megakaryocytoid cell line DAMI (Greenberg et al., 1988). Wild-type (wt) DAMI cells produce little detectable holoβAPP (Figure 3C, lane 1), nor was there an induction of precursor synthesis by A23187 (lane 2). This polyploid cell line contains the human βAPP gene by Southern analysis (data not shown). DAMI cells stably transfected with $βAPP_{770}$ cDNA responded to A23187 with a large increase in βAPP production (Figure 3C, lanes 3 and 4). APP_s levels in the DAMI cells were correspondingly augmented after ionophore treatment (Figure 3D, lanes 1 and 2), in contradistinction to untransfected and $βAPP_{751}$-transfected 293 cells (Figure 3D, lanes 3 through 6; see also Figure 2C). An immature form of holoβAPP and APP_s predominates after A23187 treatment in DAMI cells (Figure 3C, lane 4; Figure 3D, lane 2), in accord with the effects of ionophore on βAPP maturation in 293 cells. Despite the large increase in synthesis of βAPP and secretion of APP_s by transfected DAMI cells following ionophore treatment, little or no Aβ production can be detected by densitometry (Figure 3E, lanes 1 and 2), as compared with transfected 293 cells expressing comparable levels of βAPP (lanes 5 and 6).

Increased Release of Aβ Is Not a General Consequence of Cell Stress. We questioned whether enhanced Aβ release could result from other mechanisms producing cell stress which do not directly require calcium, such as injury mediated by oxygen radicals. The addition of ferrous sulfate (100 μM) and H_2O_2 (1 mM), either in the absence or in the presence of EDTA, to 293_{751} cells during a 14-h labeling with [^{35}S]-methionine produced approximately 30% cell death (detached

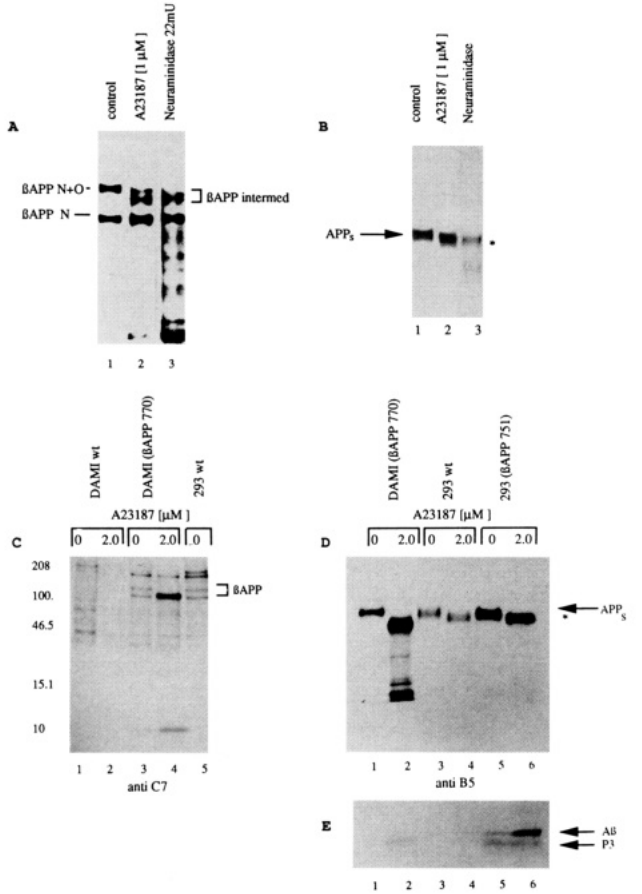

FIGURE 3: (A, B) A23187 interferes with βAPP O′-glycosylation. (A) Lane 1, cellular βAPP immunoprecipiated from lysates of βAPP_{751}-transfected 293 cells with αC7. Mature (N′ plus O′) and immature (N′) glycosylated species are indicated. Lane 2, A23187 (1 μM) treatment of the cells induces a species of intermediate molecular weight; lane 3, treatment of the immunoprecipitated cellular βAPP with neuraminidase (see Materials and Methods) produces a similar downward shift in migration of βAPP. The enzymatic treatment resulted in a considerable loss of signal; therefore, lanes 3 in both panel A and panel B are obtained from slightly longer exposures of autoradiograms. (B) APP_s immunoprecipitated from media of cells shown in (A) with αB5 also shows an intermediate comigrating species produced by both A23187 (lane 2) and neuraminidase (lane 3); 6% Tris–glycine gels. (C) Immunoprecipitation of various cell lysates with αC7 after overnight metabolic labeling in the presence or absence of 2 μM A23187. Lanes 1 and 2, the human megakaryocytoid cell line, DAMI, produces no endogenous βAPP. Lanes 3 and 4, DAMI cells stably transfected with βAPP_{770} and treated with A23187 show a large increase in the immature βAPP species. Lane 5, untransfected (wt) 293 cells. (D) Immunoprecipitation by αB5 of the ionophore-induced intermediate APP_s (asterisk) from media of βAPP_{770}-transfected DAMI cells (lanes 1 and 2), wt 293 cells (lanes 3 and 4), and βAPP_{751}-transfected 293 cells (lanes 5 and 6). (E) Immunoprecipitation by R1280 of 3- and 4-kDa peptides from media of the same cells used for panel D (labeling of lanes as in panel D). Note that DAMI cells stably transfected with βAPP_{770} do not produce significant amounts of Aβ (lanes 1 and 2). DAMI cells stably transfected with βAPP_{695} or βAPP_{751} similarly produce very low amounts of Aβ (not shown). Gels in panels C and E, 10–20% polyacrylamide Tris–Tricine–SDS; gel in panel D, 5% SDS–PAGE. Autoradiograms were exposed for 28 h, 12 h, or 5 days, respectively.

cell counts). Treatment with ferric chloride (100 μM) plus ascorbate (5 μM) resulted in ~10% cell death. Under either condition, no increase in Aβ and p3 formation was observed; indeed, levels of the peptides declined (Figure 4, lanes 2–4). Therefore, enhanced release of Aβ does not automatically follow conditions such as those induced by free radical damage, which produce death of some cells and presumed stress of the remaining majority of cultured cells.

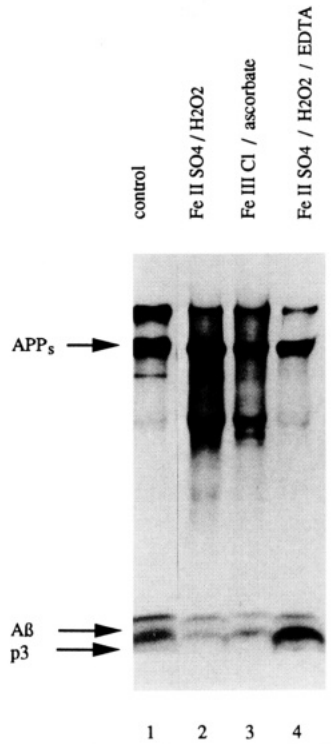

FIGURE 4: Generation of reactive oxygen species does not increase cellular production of Aβ. 293_{751} cells were exposed to conditions favoring oxygen free radical formation and partial cell death during overnight metabolic labeling. Media were immunoprecipitated with R1280 and electrophoresed on 10–20% polyacrylamide–Tris–Tricine–SDS gels; autoradiography, 8 days. Lane 1, untreated; lane 2, $FeSO_4$ (100 μM) plus H_2O_2 (1 mM); lane 3, $FeCl_3$ (100 μM) plus ascorbate (5 mM); lane 4, as in lane 2 plus EDTA (200 μM).

Neither PKC Activation nor Enhanced βAPP Phosphorylation Mediates the Effect of Ionophore on Aβ Production. We examined the possibility that the calcium ionophore effect on Aβ and p3 generation could result from the activation of the calcium-dependent protein kinase C (PKC) and/or the enhanced phosphorylation of βAPP, thereby regulating its proteolysis. The addition of the PKC activators PMA (0.1 μM) or PDBu (1 μM) during a 14-h metabolic labeling period did not increase Aβ release, as would have been expected if ionophore action were due to calcium-mediated PKC activation (Figure 5A, lanes 2 and 3). Instead, Aβ production fell substantially, in accord with results recently reported elsewhere (Hung et al., 1993; Buxbaum et al., 1993). p3 production increased with phorbol ester treatment (Figure 5A), as expected from the enhanced secretion of APP_s that this agent produces (Caporaso et al., 1992; Hung et al., 1993). Identical results were obtained from a 1-h pulse–2-h cold chase experiment using 1 μM PDBu (not shown). HoloβAPP levels and maturation were not noticeably affected by the phorbol esters (Figure 2A, lanes 7 and 8). The addition of A23187 together with PMA did not further stimulate p3 levels (Figure 5B, lane 2). Furthermore, treatment of cells with both PMA and A23187 resulted in no diminution of the enhancement of Aβ level by the ionophore, suggesting that these agents modify the processing of βAPP via independent pathways.

To ascertain directly any effects of A23187 treatment on βAPP phosphorylation, we labeled 293 cells with [^{32}P]-orthophosphate for 4 h and precipitated the holoprotein with αC7. A single phosphorylated band is observed, in accord with the experiments of Suzuki et al. (1992). A23187 resulted in a marked decrease in phosphate incorporation (Figure 5C, lanes 2 and 3), perhaps due to the inhibition of βAPP maturation by the ionophore (see Figure 2). Because the

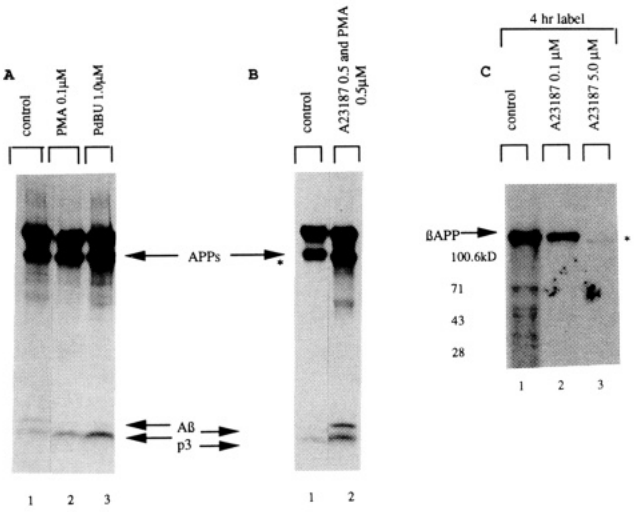

FIGURE 5: Protein kinase C activation and enhanced βAPP phosphorylation do not mediate the effects of A23187. (A) Phorbol ester activation of protein kinase C does not increase Aβ production. Lane 1, untreated; lane 2, phorbol 12-myristate 13-acetate (PMA) at 0.1 μM; lane 3, phorbol 12,13-dibutyrate (PDBu) at 1.0 μM. Note that there is no detectable induction of an intermediate βAPP species (asterisk), as also shown in Figure 2A, lanes 7 and 8. (B) Cotreatment with PMA and A23187. Lane 1, untreated; lane 2, A23187 (0.5 μM) plus PMA (0.5 μM). Gels in panels A and B show immunoprecipitation of media with R1280 and electrophoresis on 10–20% polyacrylamide–Tris–Tricine–SDS gels (autoradiography, 7 days). (C) A23187 does not increase phosphorylation of cellular βAPP. Immunoprecipitation of cell lysates with αC7 after a 4-h labeling with 1 mCi of ^{32}P. Metabolic labeling for 12 h gave identical results. Lane 1, untreated; lane 2, 0.1 μM A23187; lane 3, 5.0 μM A23187. 10–20% polyacrylamide–Tris–Tricine–SDS gels (autoradiography, 21 h).

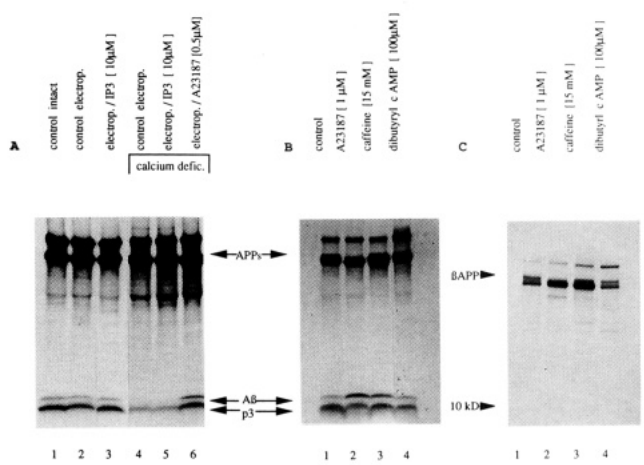

FIGURE 6: Effects of modulation of intracellular calcium on Aβ production and βAPP maturation. (A) Lanes 1–3, 293_{751} cells were permeabilized by electroporation (see Materials and Methods) and treated as indicated with D-myoinositol 1,4,5-triphosphate (IP3) at 10 μM for 14 h during [^{35}S]methionine labeling. Lanes 4–5, same as lanes 2 and 3 but in calcium-deficient medium. Very similar results were produced when IP3-treated cells were permeabilized in low-Ca^{2+}/high-K^+ buffer (see Materials and Methods) instead of by electroporation (not shown). Lane 6, A23187 (0.5 μM) treatment in calcium-deficient medium after permeabilization by electroporation. A similar result was obtained when A23187-treated cells were permeabilized in low-Ca^{2+}/high-K^+ buffer (not shown). (B) Exposure of intact 293_{751} cells under normal media conditions to (lane 2) A23187 (1.0 μM), (lane 3) caffeine (15 mM), and (lane 4) dibutyryl-cAMP (100 μM); 10–20% Polyacrylamide–Tris–Tricine–SDS gels. (C) Immunoprecipitation of lysates of the cells shown in (B) with αC7. Note that the change in migration of (N′ + O′)-glycosylated βAPP induced by caffeine is less marked than that induced by A23187; no such change is induced by dibutyryl-cAMP.

intermediate glycosylated species of βAPP induced by ionophore is associated with increased Aβ formation, an increase in phosphorylation of βAPP is apparently not required for genesis of Aβ [see also Hung and Selkoe (1994)]. Indeed, our results raise the possibility that the ionophore-induced increase in Aβ formation could be associated with a decrease in βAPP phosphorylation.

Calcium Release from Intracellular Stores May Also Affect Aβ Production. The possibility that intracellular stores of calcium, when mobilized, could reproduce the ionophore effect on Aβ generation was addressed by treating cells with IP3 or caffeine to stimulate receptor-mediated calcium release from distinct endoplasmic reticulum domains (Berridge, 1993). Use of the second-messenger IP3 in whole cultured cells requires prior permeabilization (Streb et al., 1983; Brass & Joseph, 1985; Israels et al., 1985; Saluja et al., 1992). Permeabilized 293 cells were incubated in the presence of maximal concentrations [10 μM; see Hofer and Machen (1993)] of IP3. No increase in Aβ over basal levels was observed after a 14-h exposure during metabolic labeling, whether done in normal or calcium-deficient medium (Figure 6A, lanes 3 and 5). This result was obtained whether cells were permeabilized by electroporation (Figure 6A) or by low-calcium/high-potassium permeabilization medium (not shown). IP3 (10 μM) used in a pulse–chase experiment (as in Figure 2D, except using permeabilized 293 cells in calcium-deficient medium) produced no inhibition of βAPP maturation (not shown). Unexpectedly, treatment of permeabilized cells with A23187 in calcium-deficient medium increased Aβ production (Figure 6A, lane 6). The latter result suggests that intracellular, IP3-independent calcium stores may exist which are not normally accessible to A23187 in intact cells. This result also makes it unlikely that IP3 did not produce an effect on permeabilized 293 cells in normal or calcium-deficient medium due to depletion of intracellular calcium stores. Indeed, permeabilized gastric cells in buffer containing <1 μM $CaCl_2$ are known to remain responsive to IP3 (Hofer & Machen, 1993).

When intact cells were exposed to caffeine at high concentrations (10–15 mM), an increase in Aβ production was observed, although caffeine was less effective than A23187 (Figure 6B, lanes 1–3). Furthermore, caffeine produced a shift in the apparent molecular weight of mature βAPP to a faster migrating form similar but not identical to that induced by A23187 (Figure 2D, lanes 11–13; Figure 6C, lanes 2–3). A caffeine-responsive store of intracellular calcium may therefore participate in calcium-mediated Aβ production and release. The possibility that the caffeine effect is mediated by increases in cAMP levels was addressed by adding dibutyryl-cAMP (100 μM) to intact 293 cells during metabolic labeling (Figure 6B,C, lanes 4). This produced little or no effect on both Aβ and holoβAPP levels. Forskolin, which is known to enhance cAMP levels in various cell types (Feinstein et al., 1983; Knight et al., 1984; Moos & Goldberg, 1988), also did not increase Aβ levels (not shown). These experiments suggest that the caffeine response is likely to involve intracellular calcium mobilization and that cAMP does not directly regulate Aβ production.

DISCUSSION

Our experiments demonstrate that elevations in intracellular calcium increase the production of the amyloidogenic Aβ peptide in βAPP-expressing cultured cells. A23187 raises both Aβ and p3 levels in the media of cells in a dose-dependent manner, with an apparent maximal effect at 0.5 μM. A variable decrease in both peptides, more noticeable for p3, occurs at ionophore concentrations ≥1.0 μM. The effect requires the presence of extracellular calcium, because calcium-deficient medium markedly attenuates or eliminates

the response. Residual free calcium concentrations in such nominally "calcium deficient" solutions have been reported to be on the order of 3–5 μM (Streb & Schulz, 1983; Streb et al., 1983; Brass & Joseph, 1985) or as low as approximately 1 μM (M. Mattson, personal communication); this amount is still an order of magnitude greater than intracellular calcium levels, which are approximately 0.1–0.4 μM (Streb & Schulz, 1983; Purdon et al., 1984). The addition of 0.5 mM EGTA to calcium-deficient medium essentially removes all traces of free calcium (Mattson et al., 1991; Blinks et al., 1982). The increase in the peptides is observed with a ~12-h exposure of cells to A23187 during metabolic labeling and after 30 min of treatment with A23187 in pulse–chase experiments. A23187, at the concentrations used for these experiments, produced an expected dose-dependent increase in intracellular calcium levels in our 293 cells, as evidenced by a change in the 405/485 nM fluorescence ratio after loading with the dye INDO-1 in a FACS analysis (H. W. Querfurth, unpublished data). Our studies with A23187 do not address any role that transient influxes or net redistributions of calcium among its various intraluminal stores and functional cytoplasmic sites of action might play in either basal or dynamic Aβ production. This is in part due to the likelihood of nonspecific equilibration of calcium pools between most compartments over the incubation period with A23187 (Beckers et al., 1990). Because very small amounts of Aβ are released into culture medium (Seubert et al., 1992), our assay precludes detecting changes in Aβ concentrations on the time scale of seconds customarily attributed to rapid calcium-mediated events [e.g., see Massini and Luscher (1974) and Sage and Rink (1987)]. Some calcium ionophore-induced events are known to occur on a time scale of hours (Ben-Av et al., 1988).

The rise of both Aβ and p3 after a brief chase in A23187 and the lack of a significant rise in full-length βAPP in the overnight labeling experiment suggest that the increases in the peptides arise from an acceleration of their proteolytic generation from βAPP rather than a greater availability of the precursor (see Figure 2A,D). The α-secretase-mediated cleavage of βAPP generates APP_s and a membrane-associated 10-kDa (88 amino acid) carboxyl-terminal fragment (Oltersdorf et al., 1990; Weidemann et al., 1989). It is believed that a precursor–product relationship exists between the 10-kDa peptide and p3 (Haass et al., 1992b). Simultaneous Aβ and p3 generation rates in shorter pulse–chase experiments do not support a precursor–product relationship *between* these two peptides (Haass et al., 1993), and we have confirmed this in the current work. The increase in p3 level, seen when either α-secretase cleavage [e.g., Hung et al. (1993)] or calcium-regulated pathways (this work) are activated, suggests to us that production of this fragment can increase through stimulation of either its N-terminal (α-secretase) or its C-terminal cleavage sites within βAPP, respectively. The rise in p3 after ionophore treatment in particular cannot be explained by increased α-secretase cleavage, because our experiments show no significant rise in APP_s production after A23187 (Figure 2C,E). Our data do not rule out the possibility that both p3 and Aβ could arise from a common precursor such as a 100 amino acid long C-terminal fragment or from a long Aβ-bearing form of APP_s [see Anderson et al. (1992)]. Once formed, Aβ and p3 are very stable in conditioned medium (C. Haass and D. Selkoe, unpublished data), making a direct ionophore effect which increases their half-life in medium unlikely, particularly in view of the rise in their levels seen after only a brief (30–60 min) treatment. The influx of calcium which A23187 mediates has several effects on βAPP metabolism (discussed below) that ultimately must lead to an enhancement of cleavage at the N- and/or C-termini of the Aβ fragment.

Transfection of the megakaryocytoid cell line DAMI with a βAPP cDNA resulted in the biosynthesis of βAPP and, upon ionophore treatment, the release of abundant APP_s but virtually no Aβ. This is consistent with our conclusion that the substantial rise in Aβ secretion observed in the 293 cells is not attributable to any increase in βAPP induced by A23187. The DAMI cells may have very low levels or activity of the N-terminal protease ("β-secretase") which cleaves Aβ from βAPP; α-secretase cleavage and secretion of APP_s still occur, and p3 generation is seen. The possibility that the inhibition of βAPP maturation by A23187 in DAMI cells precludes it from serving as substrate for Aβ is unlikely, because the same effect on βAPP maturation in 293 cells is associated with robust Aβ production. We favor the explanation that A23187 in the presence of extracellular calcium activates proteolytic events in 293 cells which generate both Aβ and p3. Enhancement of calcium-dependent exocytosis could play an additional role in secretion of these peptides once they are formed.

The increased production of Aβ and p3 we observed is unlikely to be a nonspecific consequence of ionophore/calcium-mediated membrane damage or cell death. Hypothetically, cellular stress could accelerate Aβ production through (1) nonspecific release from damaged membranes (e.g., via lipid peroxidation) and discharge of vesicle contents, or (2) endosomal/lysosomal breakdown and accelerated βAPP proteolysis. However, membrane disruption in the presence of ionophore has been reported not to occur at less than 2 μM concentrations of A23187 in platelets (Massini & Luscher, 1974). The concentration of ionophore we used was not associated with detectable cytotoxicity, as determined by lack of morphological changes, no increase in vital dye uptake and maintenance of normal levels of βAPP biosynthesis and APP_s secretion. Lipids and proteins in plasma membranes are known targets of iron-catalyzed reactive oxygen species (ROS), via lipid peroxidation and oxidative damage with increased proteolysis, respectively [see, e.g., Richards et al. (1988) and Jamieson (1989)]; calcium can exacerbate these processes (Braughler & Hall, 1989). However, using two ROS-generating systems that resulted in partial death of 293 cells, we found that Aβ and p3 production actually decreased. It remains to be explored whether lower doses of these agents, such as those that produce measurable lipid hydroperoxides but no cellular death, have an effect on Aβ production.

Calcium ionophore interfered with the full maturation of βAPP and led to the appearance of an intermediate molecular weight isoform that comigrates with enzymatically desialated βAPP, a species described previously (Oltersdorf et al., 1990). The intermediate βAPP species (as well as its corresponding APP_s derivative) obtained after ionophore treatment is an immature form of βAPP which apparently fails to undergo full O′-glycosylation. Monensin and NH_4Cl have been found to produce similar changes in βAPP maturation, but they decrease Aβ and p3 levels (Caporaso et al., 1992; Haass et al., 1993). Therefore, the partial inhibition of βAPP maturation caused by ionophore is not necessarily directly associated with the rise in Aβ. Neuraminidase treatment has previously been used to establish that the higher molecular weight form of the βAPP doublet seen on Western blots of cell extracts is produced by the addition of glucosamine residues to serine and threonine sites of N′-glycosylated βAPP (Oltersdorf et al., 1990). This modification probably occurs in the

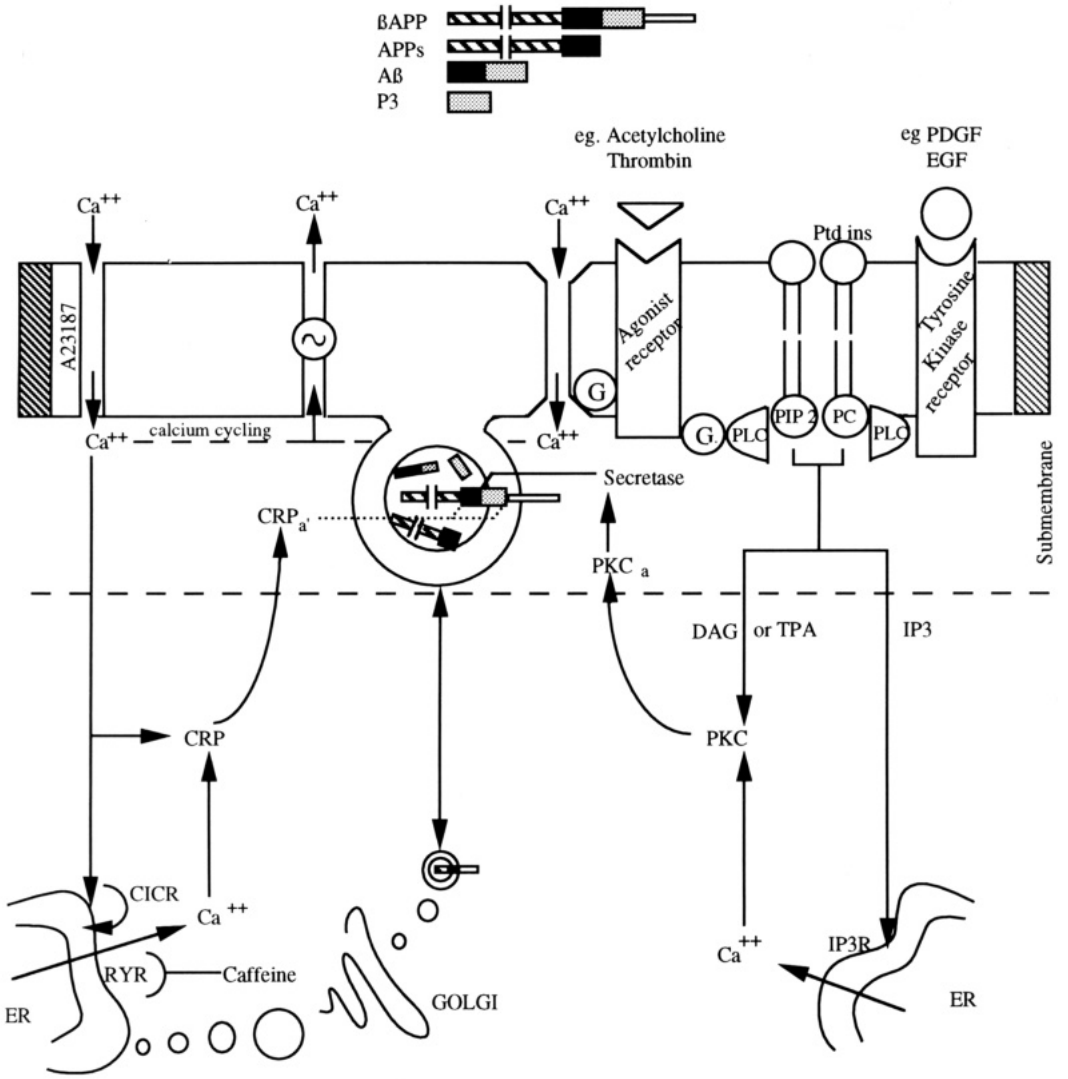

FIGURE 7: Hypothetical model for some aspects of the regulation of Aβ production. In this model, Aβ production and p3 peptide production occur in the same submembranous vesicle which, as depicted, either could be in the process of fusion with the plasma membrane to discharge its contents or could be engaged in the reinternalization of βAPP. The α-secretase cleavage of βAPP within the Aβ domain is known to be stimulated via the PKC-mediated pathway (right half of figure) and is responsible for the generation of APP_s, the 10-kDa C-terminal fragment and thus the N terminus of p3; Aβ formation is precluded. The initial event is an agonist–G-protein or agonist–tyrosine kinase receptor-induced hydrolysis of PIP2 to generate DAG and IP3 which directly and indirectly activate calcium–phospholipid–dependent PKC (Streb & Schulz, 1983; Nishizuka, 1984; Berridge, 1993). The membrane-bound PKC can be sustained in the activated state by (1) further hydrolysis of membrane lipids (Ptdins and PC) which serve to maintain DAG levels after calcium levels fall to base line (Rink et al., 1983; Nishizuka, 1992) and (2) receptor-linked calcium channel opening and calcium cycling (Rasmussen, 1986, 1989). The N-terminus of the Aβ peptide is hypothesized to be created by cleavage of βAPP by a protease which directly or indirectly requires calcium (CRP) (left half of figure). The distinct protease creating the C-terminus of Aβ and p3 may similarly be calcium-requiring. Abnormal calcium transients or sustained calcium fluxing, such as through the additional release of calcium from caffeine-stimulated RYR's or the effect of ionophores such as A23187, could activate a CRP (e.g., a membrane-bound calpain). Voltage-dependent and excitotoxic amino acid gated calcium channels are not shown. Abbreviations: G, GTP binding protein; PLC, phospholipase C; PIP2, phosphatidylinositol 4,5-bisphosphate; Ptdins and PC, other phosphatidylinositols and phosphatidylcholine, respectively; DAG, diacylglycerol; PKC, protein kinase C; IP3, inositol 1,4,5-triphosphate; ER, endoplasmic reticulum; RYR, ryanodine receptor; CICR, calcium- and caffeine-induced calcium release phenomenon; wavy line, calcium pump; TPA, phorbol ester; a, calcium-independent activated state; a′, calcium-requiring activated state.

Golgi apparatus (Weidemann et al., 1989). An early and rapid step of protein transfer from the ER to the cis-Golgi is known to be sensitive to local calcium concentrations and inhibited by A23187 (Beckers et al., 1990), and this occurs prior to late posttranslational events such as O′-glycosylation (Sheng et al., 1990). The inhibition of O′-glycosylation by ionophore did not decrease APP_s secretion and therefore does not appear to impair transport of βAPP or APP_s to the cell surface. Secretion of some other proteins such as human protein C also does not depend on complete Golgi processing (McClure et al., 1992).

Calcium influx could activate calcium/phospholipid-dependent protein kinase (PKC). PKC is responsible for the phosphorylation of certain membrane-bound receptors and other proteins, including the EGF receptor and myosin, thereby influencing their proteolysis (Hunter et al., 1984; Nishizuka, 1992). It is therefore possible that calcium ionophore could increase βAPP phosphorylation through activation of PKC (or another kinase) and render it a better substrate for the cleavage events that generate Aβ and p3. However, calcium ionophore resulted in a decrease in βAPP phosphorylation in our experiments. Moreover, direct activation of PKC with phorbol esters decreased Aβ levels, in contrast to the ionophore effect, and augmented p3 production to a greater degree, in agreement with recent studies (Hung et al., 1993; Buxbaum et al., 1993). Our data are consistent with the work of Nitsch and colleagues (Nitsch et al., 1992), who found no effect of ionophore on the βAPP secretory (α-secretase) pathway.

The inhibitory effect of A23187 on βAPP maturation was independent of the presence of external calcium, in contrast to the enhancement of Aβ and p3 formation in intact 293 cells. This result provides evidence that ionophore may also affect βAPP metabolism by release of calcium from intracellular stores. The fact that A23187 did not increase Aβ levels in calcium-deficient medium suggests that calcium mobilization from intracellular stores by the ionophore is of less importance than its effect on calcium entry as regards the regulation of Aβ formation. However, the observation that permeabilized cells can increase Aβ production when exposed to A23187 in calcium-deficient medium provides evidence that mobilization of calcium from intracellular stores could enhance amyloidogenic cleavage of βAPP under some circumstances.

It is generally accepted that the endoplasmic reticulum (ER) serves as the major reservoir of mobilizable Ca^{2+} in the cell (Berridge & Irvine, 1989). The second messenger inositol 1,4,5-triphosphate (IP3) produces a brief (1 min) release of Ca^{2+} upon binding to its receptor on the ER surface. However, IP3 had no effect on either Aβ production or βAPP maturation in intact 293 cells in our experiments, in accord with its defined role in the PKC pathway (summarized in Figure 7, right). A second mechanism for intracellular calcium release is mediated by the ryanodine receptor (Berridge, 1993). It is usually activated by cell-surface, voltage-gated calcium channel currents and a "calcium induced calcium release (CICR)" process acting on ER pools which may or may not be distinct from the IP3-responsive calcium pools (Walton et al., 1991; Zacchetti et al., 1991) (Figure 7, left). Caffeine is a potent stimulator of repetitive calcium release via the ryanodine receptor (Endo, 1977). As documented previously in other cell types (Giannini et al., 1992; Stein et al., 1992), our 293 cells contain caffeine-sensitive ryanodine receptors (J. Geiger, personal communication). In our experiments, high levels of caffeine produced both a cAMP-independent increase in Aβ and an inhibition of βAPP maturation. Intracellular calcium release mechanisms have been shown to substitute, albeit to a lesser extent, for external calcium entry in some *in vitro* situations (Feinman & Detwiler, 1974; Rink, 1983). The caffeine effect is in contrast to the absence of an IP3 effect and may be explained by differences in the utilization of one mechanism over the other in cellular processes requiring discrete calcium transients, as we hypothesize for βAPP in Figure 7 [see also Malgaroli et al. (1990)]. Caffeine may even inhibit IP3-sensitive calcium channel opening (Brown et al., 1992; Parker & Ivorra, 1991). The possibility remains that IP3 did not, for unclear or artifactual reasons, produce the expected rise in cytosolic calcium. Addressing this possibility will require the application of techniques that directly measure such changes in permeabilized, calcium clamped cells [see Hofer and Machen (1993)].

One explanation for our finding that enhanced intracellular calcium levels lead to increased Aβ production is that the N-terminal and/or C-terminal cleavage events necessary for the generation of Aβ are catalyzed by a calcium-requiring protease(s). The responsible enzyme(s) has (have) not yet been identified. Among numerous possible candidates for the N-terminal protease that have been proposed, one is a calcium-activated cathepsin G-like serine protease present in adult human and monkey brain (Abraham et al., 1991; Razzaboni et al., 1992). The nonlysosomal cytosolic and membrane-bound neutral cysteine protease calpain is another hypothetical candidate, and it has an absolute requirement for calcium ions (Mellgren, 1987; Suzuki, 1987). Calpain I has been reported to be activated in particulate fractions of postmortem Alzheimer disease brains (Saitoh, 1993), and its endogenous inhibitor calpastatin is reduced in the same brain regions (Nixon et al., 1992). Activation of calpain proceeds by calcium-mediated autolysis and submembrane association, which also serves to increase the enzyme's calcium sensitivity into the micromolar range. Calpains can effect the limited cleavage of membrane glycoproteins and cytoskeletal anchoring proteins into large fragments (Suzuki, 1987; Siman & Noszek, 1988; Siman et al., 1989; Mellgren, 1987). However, how a subplasmalemmal protease could attack the N-terminal cleavage site of Aβ, which is believed to be located in the lumen of the vesicle (Figure 7), is unclear.

Various aspects of the calcium-mediated events discussed herein that could lead to Aβ production are summarized in Figure 7 (left). Elevations in intracellular calcium concentrations could result from influx via ionophore(s) or voltage/ligand-gated calcium channels (e.g., the NMDA receptor) or by release from an appropriate ER store (e.g., the ryanodine receptor channel). Subsequently, a calcium-requiring protease could be activated and catalyze the Met–Asp cleavage of membrane-associated βAPP, creating the N-terminus of Aβ, and/or catalyze the Aβ C-terminal cleavage. Alternatively, the protease in question could act indirectly, i.e., on another substrate which then serves to enhance βAPP proteolysis. Calcium cycling known to occur within the submembrane region could maintain such a protease in an activated state after calcium transients return to base-line levels. Our finding that elevations in cellular calcium consistently increase Aβ production provides one specific example relevant to the hypothetical connection between altered calcium homeostasis and Alzheimer's disease. On the basis of these findings, inhibition of calcium-requiring proteases or blockage of agonist-operated calcium channels could represent possible therapeutic targets to decrease Aβ release and deposition in Alzheimer's disease.

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REFERENCES

Abraham, C. R., Driscoll, J., Potter, H., Van Nostrand, W., & Tempst, P. (1991) *Biochem. Biophys. Res. Commun. 174*, 790–796.

Anderson, J. P., Chen, Y., Kim, K. S., Robakis, N. K. (1992) *J. Neurochem. 59* , 2328–2331.

Baudier, J., & Cole, R. D. (1987) *J. Biol. Chem. 262*, 17577–17583.

Beckers, C. J. M., Plutner, H., Davidson, H. W., & Balch, W. E. (1990) *J. Biol. Chem. 265*, 18298–18310.

Ben-Av, P., Rubinstein, S., & Breitbart, H. (1988) *Biochim. Biophys. Acta 939*, 214–222.

Berridge, M. J. (1993) *Nature 361*, 315–325.

Berridge, M. J., & Irvine, R. F. (1989) *Nature 341*, 197–205.

Blinks, J. R., Wier, W. G., Hess, P., & Prendergast, F. G. (1982) *Prog. Biophys. Mol. Biol. 40*, 1–114.

Brass, L. F., & Joseph, S. K. (1985) *J. Biol. Chem. 260*, 15172–15179.

Braughler, J. M., & Hall, E. D. (1989) *Free Radical Biol. Med. 6*, 289–301.

Brown, G. R., Sayers, L. G., Kirk, C. J., Michell, R. H., & Michelangeli, F. (1992) *Biochem. J. 282*, 309–312.

Busciglio, J., Gabuzda, D. H., Matsudaira, P., & Yankner, B. A. (1993) *Proc. Natl. Acad. Sci. U.S.A. 90*, 2092–2096.

Buxbaum, J. D., Oishi, M., Chen, H. I., Pinkas-Kramarski, R., Jaffee, E. A., Gandy, S. E., & Greengard, P. (1992) *Proc. Natl. Acad. Sci. U.S.A. 89*, 3055–3059.
Buxbaum, J. D., Koo, E. H., & Greengard, P. (1993) *Proc. Natl. Acad. Sci. U.S.A.* 90, 9195–9198.
Cai, X.-D., Golde, T. E., & Younkin, G. S. (1993) *Science 259*, 514–516.
Caporaso, G. L., Gandy, S. E., Buxbaum, J. D., & Greengard, P. (1992) *Proc. Natl. Acad. Sci. U.S.A. 89*, 2252–2256.
Choi, D. W. (1988) *Neuron 1*, 623–634.
Citron, M., Oltersdorf, T., Haass, C., McConlogue, L., Hung, A. Y., Seubert, P., Vigo-Pelfrey, C., Lieberburg, I., & Selkoe, D. J. (1992) *Nature 360*, 672–674.
Davies, K. J. A. (1986) *J. Free Radicals Biol. Med. 2*, 155–173.
Dunford, H. B. (1987) *Free Radical Biol. Med. 3*, 405–421.
Endo, M. (1977) *Physiol. Rev. 57*, 71–108.
Esch, F. S., Keim, P. S., Beattie, E. C., Blacher, R. W., Culwell, A. R., Oltersdorf, T., McClure, D., & Ward, P. J. (1990) *Science 248*, 1122–1124.
Feinman, R. D., & Detwiler, T. C. (1974) *Nature 249*, 172–173.
Feinstein, M. B., Egan, J. J., Sha'afi, R. I., & White, J. (1983) *Biochem. Biophys. Res. Commun. 113*, 598–604.
Giannini, G., Clementi, E., Ceci, R., Marziali, G., & Sorrentino, V. (1992) *Science 257*, 91–94.
Gibson, G. E., & Peterson, C. (1987) *Neurobiol. Aging 8*, 329–343.
Goate, A., Chartier-Harlin, M.-C., Mullan, M., Brown, J., Crawford, F., Fidani, L., Giuffra, L., Haynes, A., Irving, N., James, L., Mant, R., Newton, P., Rooke, K., Roques, P., Talbot, C., Pericak-Vance, M., Roses, A., Williamson, R., Rossor, M., Owen, M., & Hardy, J. (1991) *Nature 349*, 704–706.
Golde, T. E., Estus, S., Younkin, L. H., Selkoe, D. J., & Younkin, S. G. (1992) *Science 255*, 728–730.
Greenamyre, J. T., & Young, A. B. (1989) *Neurobiol. Aging 10*, 593–602.
Greenberg, S. M., Rosenthal, D. S., Greeley, T. A., Tantravahi, R., & Handin, R. I. (1988) *Blood 72*, 1968–1977.
Grundke-Iqbal, I., Iqbal, K., Quinlan, M., Tung, Y.-C., Zaidi, M. S., & Wisniewski, H. M. (1986a) *J. Biol. Chem 261*, 6084–6089.
Haass, C., Hung, A. Y., & Selkoe, D. J. (1991) *J. Neurosci. 11*, 3783–3793.
Haass, C., Koo, E. H., Mellon, A., Hung, A. Y., & Selkoe, D. J. (1992a) *Nature 357*, 500–503.
Haass, C., Schlossmacher, M. G., Hung, A. Y., Vigo-Pelfrey, C., Mellon, A., Ostaszewski, B. L., Lieberburg, I., Koo, E. H., Schenk, D., Teplow, D. B., & Selkoe, D. J. (1992b) *Nature 359*, 322–325.
Haass, C., Hung, A. Y., Schlossmacher, M. G., Teplow, D. B., & Selkoe, D. J. (1993) *J. Biol. Chem. 268*, 3021–3024.
Hardy, J. (1992) *Nature Genet. 1*, 233–234.
Hofer, A. M., & Machen, T. E. (1993) *Proc. Natl. Acad. Sci. U.S.A. 90*, 2598–2602.
Hung, A. Y., & Selkoe, D. J. (1994) *EMBO J. 13*, 534–542.
Hung, A. Y., Haass, C., Nitsch, R. M., Qiu, W. Q., Citron, M., Wurtman, R. J., Growdon, J. H., & Selkoe, D. J. (1993) *J. Biol. Chem. 268*, 22959–22962.
Hunter, T., Ling, N., & Cooper, J. A. (1984) *Nature 311*, 480–483.
Israels, S. J., Robinson, P., Docherty, J. C., & Gerrard, J. M. (1985) *Thromb. Res. 40*, 499–509.
Jamieson, D. (1989) *Free Radical Biol. Med. 7*, 87–108.
Kang, J., Lemaire, H., Unterbeck, A., Salbaum, J. M., Masters, C. L., Grzeschik, K., Multhaup, G., Beyreuther, K., & Muller-Hill, B. (1987) *Nature 325*, 733–736.
Kitaguchi, N., Takahashi, Y., Tokushima, Y., Shiojiri, S., & Ito, H. (1988) *Nature 331*, 530–532.
Knight, D. E., Niggli, V., & Scrutton, M. C. (1984) *Eur. J. Biochem. 143*, 437–446.
Koh, J.-Y., Yang, L. L., & Cotman, C. W. (1990) *Brain Res. 533*, 315–320.
Kosik, K. S., Joachim, C. L., & Selkoe, D. J. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 4044–4048.
Lee, V. M.-Y., Balin, B. J., Otvos, L., & Trojanowski, J. Q. (1991) *Science 251*, 675–678.
Malgaroli, A., Fesce, R., & Meldolesi, J. (1990) *J. Biol. Chem. 265*, 3005–3008.
Massini, P., & Luscher, E. F. (1974) *Biochim. Biophys. Acta 372*, 109–121.
Mattson, M. P. (1992) *Exp. Gerontol. 27*, 29–49.
Mattson, M. P., Engle, M. G., & Rychlik, B. (1991) *Mol. Chem. Neuropathol. 15*, 117–142.
Mattson, M. P., Cheng, B., Davis, D., Bryant, K., Lieberburg, I., & Rydel, R. E. (1992) *J. Neurosci. 12*, 379–389.
Mattson, M. P., Barger, S. W., Cheng, B., Lieberburg, I., Smith-Swintosky, V. L., & Rydel, R. E. (1993) *Trends Neurosci 16*, 409–414.
McClure, D. B., Walls, J. D., & Grinnell, B. W. (1992) *J. Biol. Chem. 267*, 19710–19717.
Mellgren, R. L. (1987) *FASEB J. 1*, 110–115.
Miller, R. J., Murphy, S. N., & Glaum, S. R. (1989) *Ann. N.Y. Acad. Sci. 568*, 149–156.
Moos, J. M., & Goldberg, N. D. (1988) *Second Messengers Phosphoproteins 12*, 163–170.
Mullan, M., Crawford, F., Houlden, H., Axelman, K., Lilius, L., Winblad, B., & Lannfelt, L. (1992) *Nature Genet. 1*, 345–347.
Nishizuka, Y. (1984) *Nature 308*, 693–698.
Nishizuka, Y. (1992) *Science 258*, 607–613.
Nitsch, R. M., Slack, B. E., Wurtman, R. J., & Growdon, J. H. (1992) *Science 258*, 304–307.
Nixon, R. A., Saito, K.-I., Cataldo, A. M., Hamos, J., Hamilton, D., Honda, T., & Pope, A. (1992) *Soc. Neurosci. 18*, 198 [93.8].
Nukina, N., & Ihara, Y. (1986) *J. Biochem. 99*, 1541–1544.
Oltersdorf, T., Fritz, L. C., Schenk, D. B., Lieberburg, I., Johnson-Wood, K. L., Beattie, E. C., Ward, P. J., Blacher, R. W., Dovey, H. F., & Sinha, S. (1989) *Nature 341*, 144–147.
Oltersdorf, T., Ward, P. J., Henriksson, T., Beattie, E. C., Neve, R., Lieberburg, I., & Fritz, L. C. (1990) *J. Biol. Chem. 265*, 4492–4497.
Parker, I., Ivorra, I. (1991) *J. Physiol. 433*, 229–240.
Podlisny, M. B., Tolan, D., & Selkoe, D. J. (1991) *Am. J. Pathol. 138*, 1423–1435.
Pressman, B. C. (1976) *Annu. Rev. Biochem. 45*, 501–530.
Purdon, A. D., Danield, J. L., Stewart, G. J., & Holmsen, H. (1984) *Biochim. Biophys. Acta 1984*, 178–187.
Rasmussen, H. (1986) *N. Engl. J. Med. 314*, 1094–1170.
Rasmussen, H., Barrett, P., Zawalich, W., Isales, C., Stein, P., Smallwood, J., McCarthy, R., & Bollag, W. (1989) *Ann. N.Y. Acad. Sci. 568*, 73–80.
Razzaboni, B. L., Papastoitsis, G., Koo, E. H., & Abraham, C. R. (1992) *Brain Res. 589*, 207–216.
Richards, D. M. C., Dean, R. T., & Jessup, W. (1988) *Biochim. Biophys. Acta 946*, 281–288.
Rink, T. J., Sanchez, A., & Hallam, T. J. (1983) *Nature 305*, 317–319.
Sage, S. O., & Rink, T. J. (1987) *J. Biol. Chem. 262*, 16364–16369.
Saitoh, T. (1993) *J. Cell Biol.* (in press).
Saluja, A. K., Dawra, R. K., Lerch, M. M., & Steer, M. L. (1992) *J. Biol. Chem. 267*, 11202–11207.
Sambamurti, K., Shioi, J., Anderson, J. A., Pappolla, M. A., & Robakis, N. K. (1992) *Neurobiol. Aging 13*, S79 no. 312.
Selkoe, D. J. (1993) *Trends Neurosci. 16*, 403–409.
Selkoe, D. J., Podlisny, M. B., Joachim, C. L., Vickers, E. A., Lee, G., Fritz, L. C., & Oltersdorf, T. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 7341–7345.
Seubert, P., Vigo-Pelfrey, C., Esch, F., Lee, M., Dovey, H., Davis, D., Sinha, S., Schlossmacher, M. G., Whaley, J., Swindlehurst, C., McCormack, R., Wolfert, R., Selkoe, D. J., Lieberburg, I., & Schenk, D. (1992) *Nature 359*, 325–327.

Sheng, Z., Hull, S. R., & Carraway, K. L. (1990) *J. Biol. Chem. 265*, 8505–8510.
Shoji, M., Golde, T. E., Ghiso, J., Cheung, T. T., Estus, S., Shaffer, L. M., Cai, X., McKay, D. M., Tintner, R., Frangione, B., & Younkin, S. G. (1992) *Science 258*, 126–129.
Siesjo, B. O., Bengtsson, F., & Grampp, W. (1989) *Ann. N.Y. Acad. Sci. 568*, 234–290.
Siman, R., & Noszek, J. C. (1988) *Neuron 1*, 279–287.
Siman, R., Noszek, J. C., & Kegerise, C. (1989) *J. Neurosci. 9*, 1579–1590.
Sisodia, S. S., Koo, E. H., Beyreuther, K., Unterbeck, A., & Price, D. L. (1990) *Science 248*, 492–495.
Stein, M. B., Padua, R. A., Nagy, J. I., & Geiger, J. D. (1992) *Brain Res. 585*, 349-354.
Streb, H., & Schulz, I. (1983) *Am. J. Physiol. 245*, G347-G357.
Streb, H., Irvine, R. F., Berridge, M. J., & Schulz, I. (1983) *Nature 306*, 67–69.
Suzuki, K. (1987) *Trends Biochem. Sci. 12*, 103–105.
Suzuki, T., Nairn, A. C., Gandy, S. E., & Greengard, P. (1992) *J. Neurosci. 48*, 755–761.
Tamaoka, A., Kalaria, R. N., Lieberburg, I., & Selkoe, D. J. (1992) *Proc. Natl. Acad. Sci. U.S.A. 89*, 1345–1349.
Walton, P. D., Airey, J. A., Sutko, J. L., Beck, C. F., Mignery, G. A., Sudhof, T. C., Deerinck, T. J., & Ellisman, M. H. (1991) *J. Cell Biol. 113*, 1145–1157.
Weidemann, A., Konig, G., Bunke, D., Fischer, P., Salbaum, J. M., Masters, C. L., & Beyreuther, K. (1989) *Cell 57*, 115–126.
Wolozin, B. L., Pruchniche, A., Dickson, D. W., & Davies, P. (1986) *Science 232*, 648–650.
Yankner, B. A., Duffy, L. K., & Kirschner, D. A. (1990) *Science 250*, 279–282.
Zacchetti, D., Clementi, E., Fasolato, C., Lorenzon, P., Zottini, M., Grohovaz, F., Fumagalli, G., Pozzan, T., & Meldolesi, J. (1991) *J. Biol. Chem. 266*, 20152–20158.