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Possible Role of Calpain in Normal Processing of β-Amyloid Precursor Protein in Human Platelets

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Abnormal proteolytic processing of β-amyloid precursor protein (APP) underlies the formation of amyloid plaques in aging and Alzheimer's disease. The proteases involved in the process have not been identified. Here we found that spontaneous proteolysis of intact APP in detergent-lysed human platelets generated a N-terminal fragment that was immunologically indistinguishable from secreted APP, reminiscent of the action of a putative α-secretase. This proteolysis of APP was inhibited by EDTA, suggesting that a metal-dependent protease was involved. Among the several metals tested, calcium was the only one that enhanced APP proteolysis and the reaction was blocked by EGTA as well as by several calpain inhibitors. The APP fragments generated by spontaneous proteolysis in platelet lysates were identical to those produced by exposure of partially purified APP to exogenous calpain. Finally, the secretion of APP from intact platelets was inhibited by cell-permeable calpain inhibitors. Taken together, these results suggest that normal processing of APP in human platelets is mediated by a calcium-dependent protease that exhibits calpain-like properties. © 2000 Academic Press

Key Words: **Alzheimer; amyloid; calcium; calpain; aging.**

Alteration in the proteolytic processing of β-amyloid precursor protein (APP) leading to amyloid plaques is a prominent feature in patients with Alzheimer's disease (AD). Amyloid plaques predominantly contain β-amyloid peptide (Aβ), a 39–43-amino acid peptide derived from APP. Physiologically, APP is mostly cleaved within the Aβ domain at the Lys^{16}-Leu^{17} bond on the cell surface by an unidentified protease known as α-secretase, producing a secreted 100–120 kDa form of APP (APP_s). Alternatively, APP is cleaved by β- and γ-secretases to produce Aβ (1–4).

Considerable research efforts have been devoted to the identification of the foregoing secretases and a number of proteases have been proposed as potential candidates for putative α-secretase. These include multicatalytic protease, gelatinase A, metalloendopeptidase, proteosome, two yeast proteases (Yap3 and Mkc7), glycosyl-phosphatidylinositol-linked aspartyl proteases, and TNF-α-converting enzyme (5–11). In most cells, the major product of α-secretase, APP_s, is much more abundant than Aβ (4). This characteristic may allow α-secretase activity to be traced with less difficulty. Previous studies including our own have shown that platelets are the primary circulating repository for APP and Aβ (12–14). Inasmuch as APP processing in platelets is similar to that in neuronal cells, platelets offer an excellent model for the study of APP processing.

MATERIALS AND METHODS

Materials

Fresh platelets were obtained from the blood of healthy volunteers. Monoclonal antibodies 22C11 (to APP N-terminus) was purchased from Boehringer Mannheim (Indianapolis, IN), Dako (to Aβ8-17) from Dako Co. (Carpinteria, CA), and 4G8 (to Aβ17-24) from Senetek PLC (Maryland Heights, MO). Polyclonal antibody 369 to C-terminal residues 645–694 of APP_{695} was a gift from Dr. Samuel Gandy (Rockefeller University). The epitope-specificities of these antibodies are shown (Fig. 1). Peptides Aβ1-16 and Aβ17-28 were from QCB, Inc. (Hopkinton, MA). Trypsin and protease K were from Boehringer Mannheim (Indianapolis, IN). Calpastatin (recombinant) was from CalBiochem (San Diego, CA). Thrombin, iodoacetate, benzamidine, aprotinin, pepstatin A, calpeptin, E64d, E64c, A23187, calpain (80 kDa subunit of rabbit skeletal muscle *m*-calpain), calpain inhibitor I and II, and a calpastatin-derived peptide (27 amino acids corresponding to residues 162–188 of muscle calpastatin) were all from Sigma (St. Louis, MO).

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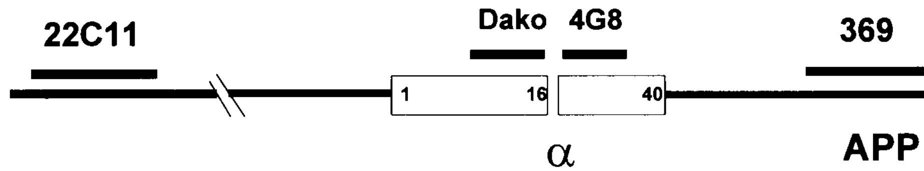

FIG. 1. The specificity of the antibodies used in this study. Aβ domain is depicted as open boxes with the α-secretase cleavage site indicated by "α." The specificity of the antibodies has been described in previous studies (19–22).

Methods

APP proteolysis in lysed platelets. Platelets were washed by differential centrifugation as previously described (14, 15) and lysed in HEPES-Triton buffer (1% Triton X-100, v/v, 140 mM NaCl, 5 mM KCl, 20 mM HEPES, pH 7.2) to yield platelet lysates (10^8 platelets/ml). To determine the effects of metal ions, the following metal salts were used: $ZnSO_4 \cdot 7H_2O$; $MgCl_2 \cdot 6H_2O$; $MnCl_2$; $CaCl_2 \cdot 2H_2O$; $CuSO_4 \cdot 5H_2O$; $FeCl_2$, and $Pb(NO_3)_2$. To examine the effects of varying pH, the buffers contained 1% Triton X-100, 140 mM NaCl, 5 mM KCL plus one of the following buffering systems: pH 5.2, 10 mM sodium acetate; pH 6.1, 20 mM MES; pH 7.2, 20 mM HEPES; pH 8.5, 20 mM Tris; and pH 9.2, 10 mM sodium carbonate.

SDS–PAGE and Western blotting. Pre-cast polyacrylamide gels were used in a Novex Mini-Cell electrophoresis system according to the manufacturer's protocol (Novex, San Diego, CA). The electrophoresis conditions and sample preparation were essentially as previously reported (16, 17). The immunoreactive proteins were visualized with an enhanced chemiluminescent (ECL) detection kit (Amersham, Arlington Heights, IL) and exposed to Kodak X-OMAT films.

Partial purification of platelet APP. Washed platelets were solubilized at 4°C in the HEPES-Triton buffer plus 10 mM EDTA, 10 mM EGTA, and 0.1 mg/ml leupeptin. The suspension was centrifuged and the supernatant was applied to a heparin-Sepharose column (Pharmacia, Piscataway, NJ) equilibrated in a Tris buffer (10 mM Tris–HCl, pH 7.4, 140 mM NaCl, 2.7 mM KCl, 10 mM EDTA, and 10 mM EGTA). Unbound materials were extensively washed with 10-column volumes of the buffer, and the bound proteins were eluted with a 0–0.6 M NaCl gradient in the same buffer plus 10 mM EGTA and 0.1 mg/ml leupeptin. The fractions were collected and screened by Western blotting with antibody 22C11 and the APP-containing fractions were pooled.

APP secretion from intact platelets. Fresh platelets were preincubated at a density of 5×10^7 platelets/ml with the protease inhibitors added at various concentrations for 20 min at 37°C. The preincubation buffer contained 20 mM Tris, pH 7.4, 150 mM NaCl, 5 mM KCl, 2 mM $MgCl_2$, and 1 mM $CaCl_2$. After preincubation, the platelets were washed (except for the experiment with EGTA) and resuspended in the same buffer. The platelets were activated by adding 0.1 mM A23187 (18). After incubating for additional 10 min at 37°C, the reaction suspension was centrifuged and analyzed.

RESULTS

In studies attempting to isolate platelet APP, we repeatedly observed that when fresh platelets lysed in a detergent-containing buffer in the absence of protease inhibitors, APP in the lysates underwent rapid proteolysis (i.e., from an apparent molecular weight of 120–140 kDa to 100–120 kDa) as evidenced by Western blotting using antibody 22C11 (Fig. 2A, lanes 1–2; also see Fig. 3). This APP proteolysis followed a time course identical to that we previously observed for the hydrolysis of talin and filamin, two platelet proteins known to be rapidly degraded by calpain (15).

Since antibody 22C11 is specific to the APP N-terminus (19), it was clear that the cleavage occurred at the C-terminal region of APP. The migration of the resulting 100–120 kDa band was similar to that observed for APP_s released from intact platelets (compare lanes 2 and 9), suggesting that this band could be truncated in the same manner as APP_s. To confirm this possibility, we tested the immunoreactivity of APP bands to a battery of epitope-specific antibodies. As illustrated in Fig. 2, the 120–140 kDa band was immunoreactive to antibody 369 against the APP C-terminus (20) (lane 3), whereas the 100–120 kDa band showed no reaction (lane 4). Moreover, the 120–140 kDa band was reactive to both Dako (21) and 4G8 (22) (lanes 5 and 7), whereas the 100–120 kDa only reacted to Dako, not to 4G8 (lanes 6 and 8). Preincubation of Dako and 4G8 with corresponding peptides Aβ1-16 and Aβ17-28, respectively, abolished their immunoreactivity (not shown). These characteristics of the APP bands were compatible with those in the previous reports (12, 13, 23–25) and suggested that the spontaneous proteolysis of intact APP (120–140 kDa)

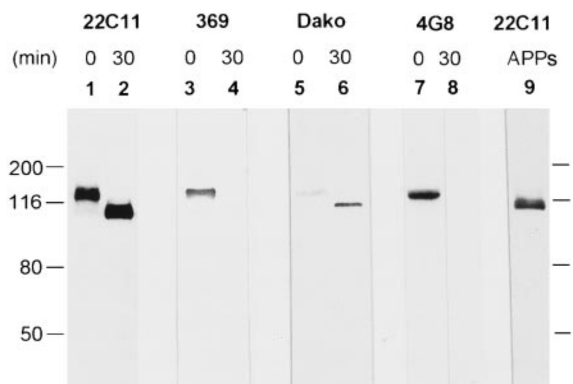

FIG. 2. Full-length, secreted APP, and C-terminal fragment found in lysed platelets. Platelets were lysed in HEPES-Triton buffer and analyzed either immediately (0 min, lane 1) or after 30 min of incubation (lane 2) at 22°C. Identical samples were used in panels 1–2 through panels 7–8 but probed with different antibodies as indicated. Lane 9, secreted APP (APPs) from the A23187-activated platelets was probed with 22C11 (also see Fig. 9). Western blotting was performed using 8% gels for all panels.

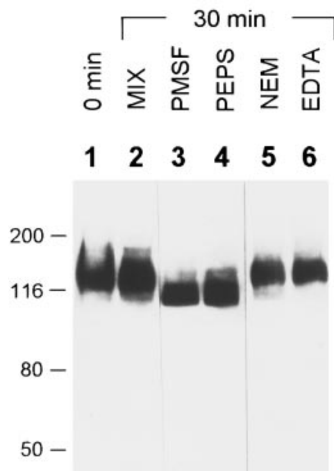

FIG. 3. Effects of the protease inhibitors in the proteolysis of APP. Platelets were lysed and incubated as described in Fig. 2. Lane 1, platelets boiled immediately in the absence of inhibitors (0 min); lane 2, in the presence of the protease inhibitor mixture (MIX); lane 3, PMSF (2 mM); lane 4, pepstatin A (PEPS)(2 mM); lane 5, NEM (5 mM); lane 6, EDTA (5 mM). Incubation was for 30 min at 22°C. Samples were analyzed by Western blotting with 22C11.

TABLE 1

Effects of Protease Inhibitors in APP Proteolysis*

Inhibitors	Concentration (mM)	APP cleavage (%)
Control	–	100 ± 12
PMSF	2	102 ± 16
aprotinin	2	85 ± 21
pepstatin A	2	92 ± 9
TPCK	1	90 ± 11
benzamidine	2	95 ± 12
leupeptin	1	23 ± 5
EGTA	2	17 ± 2
EDTA	2	15 ± 5
E64c	2	25 ± 6
NEM	5	12 ± 3
iodoacetate	2	18 ± 5
calpain inhibitor I	2	17 ± 4
calpain inhibitor II	2	25 ± 7
calpastatin	2	13 ± 3

*Platelet lysates were incubated for 30 minutes at 22°C. Samples were analyzed by Western blotting with 22C11. The inhibitory effects were expressed as remaining proteolytic activity relative to the control. The effect of each inhibitor was determined in comparison with the same solvent used for the inhibitor. Results are means ± S.D.M. of three separate experiments.

in the cell lysates occurred preferentially within the Aβ domain at or near Lys^{16}. This was reminiscent of the action of a putative α-secretase.

To characterize this protease, we tested the effects of a protease inhibitor cocktail comprising PMSF, NEM (n-ethylmaleimide), pepstatin A, and EDTA, i.e., inhibitors to four classes of proteases: serine-, cysteine-, aspartic-, and metallo-proteases, respectively. This cocktail effectively blocked the APP proteolysis in the lysates (Fig. 3, lane 2). To identify the active component(s) in the cocktail responsible for the inhibitory activity, the effects of individual inhibitors were examined. NEM and EDTA blocked the proteolysis, but PMSF and pepstatin A had no effect (Fig. 3, lanes 3–6), suggesting that the protease was both cysteine- and metal-dependent. This was confirmed using additional inhibitors. As shown (Table 1), other cysteine protease inhibitors such as E64c, iodoacetic acid, and leupeptin were also effective, whereas serine protease inhibitors such as aprotinin, TPCK, and benzamidine failed to show any inhibitory activity. Also, the APP proteolysis occurred over a wide pH range, but was maximal at pH 7.2 (Fig. 4).

To identify the specific metal ion(s) required for the protease activity, we examined the effects of a group of divalent metals. Results in Fig. 5 revealed that calcium was the only metal that accelerated the rate of proteolysis. Consistent with this, EGTA abolished the stimulating effect of calcium (Fig. 5; "Ca^{++} + EGTA"). Calcium stimulated the reaction in a time- (not shown) and concentration-dependent manner (Fig. 6). At 0.1 mM, it showed a moderate effect (lane 3), and at higher concentrations it progressively accelerated APP cleavage (lanes 4–5). Thus, calcium was required for the activity of this protease.

Because this protease was similar to calpain, we determined the effects of several calpain inhibitors (Table 1). APP proteolysis was also blocked by calpastatin, a highly calpain-specific inhibitor (26, 27). The effect of calpastatin was evident even in the presence of added calcium (Fig. 6, lane 6). To rule out the possibility that the inhibitory effect of calpastatin may have been caused by contaminants in the commercial calpastatin preparation, we determined the action and specificity of a synthetic peptide derived from calpastatin (CPS-P) (27, 28). The inhibitory activity of this peptide in the platelet lysates was as potent as that of calpastatin (lanes 7).

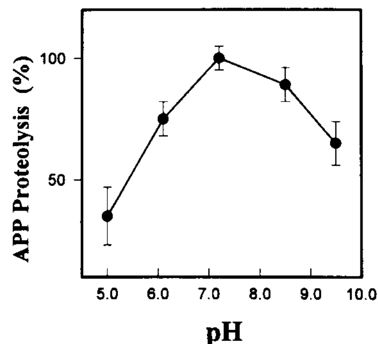

FIG. 4. pH dependence of APP proteolysis. Aliquots of platelet suspension were lysed in buffers of different pH. The ingredients of each buffer were described under Materials and Methods. The incubation was for 30 min at 22°C. Western blotting with 22C11. Results were shown as the means ± S.E.M. of three separate experiments.

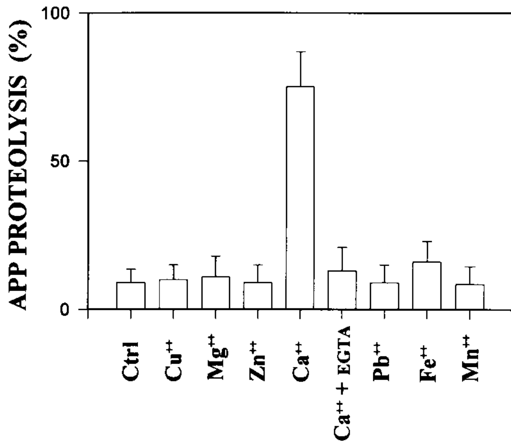

FIG. 5. Effects of various metal ions in the proteolysis of APP. Platelet lysates were incubated for 10 min at 22°C with the indicated metals at 2 mM each. The samples were analyzed by Western blotting with 22C11. The relative extent of APP proteolysis was measured against a defined scale using the intact 120–140 kDa band at time zero as 0% (minimum cleavage) and the complete disappearance of this band as 100% (maximum cleavage). "Ca^{++} + EGTA," 2 mM calcium added together with 5 mM of EGTA. "Ctrl," control.

Further studies showed that the inhibition of APP proteolysis took place gradually as the CPS-P concentration increased and there was an intermediate stage where part of the intact APP was degraded (Fig. 7A, lane 2). The peptide concentration giving 50 percent inhibition (IC_{50}) was 0.18 ± 0.1 mM (Fig. 7B). Notably, APP was completely protected from degradation by 2 mM CPS-P even when the incubation was extended to 16 h (Fig. 7A, lane 5). In contrast, APP in the control lysates was degraded beyond detection (lane 6). Interestingly, the hydrolysis of talin and filamin was also inhibited when APP proteolysis was inhibited by CPS-P (data not shown).

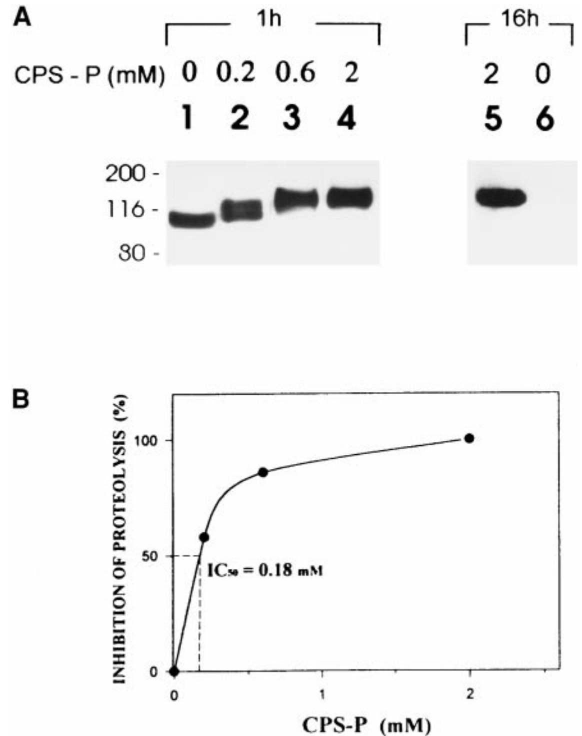

FIG. 7. Dose-dependent inhibition of APP proteolysis by calpastatin-derived peptide (CPS-P). (A), aliquots of platelet lysates were incubated for 1 h at 22°C in the absence (lane 1) or presence of CPS-P (lanes 2–4) at the concentrations indicated. Lanes 5–6, the incubation was extended to 16 h with or without the peptide. Samples were analyzed on a 10–20% gradient gel and Western blotting with 22C11. (B), a plot of the scanning data from (A, lanes 1–4) using lane 1 as 0% of inhibition and lane 4 as 100% inhibition.

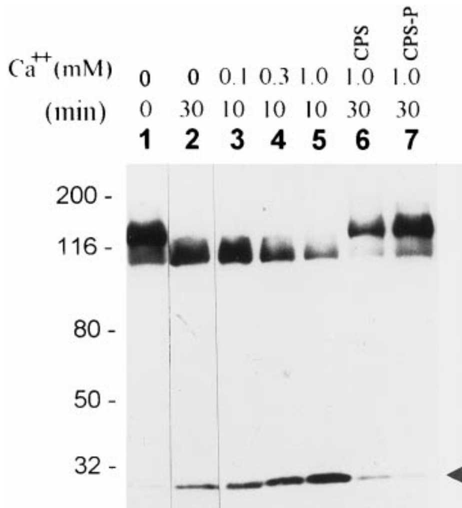

FIG. 6. Calcium concentration-dependence of APP proteolysis. Platelet lysates were incubated in the absence (lanes 1–2) or presence of calcium (lanes 3–5) at the concentrations and incubation time indicated. SDS–PAGE analysis was performed on 10% gel followed by Western blotting with 22C11. Lanes 6–7, 2 mM calpastatin (CPS) and 1 mM calpastatin-derived peptide (CPS-P), respectively, added prior to the addition of 1 mM calcium. Arrowhead, a 30 kDa fragment.

To further examine the potential relationship of this protease to calpain, we studied its cleavage specificity. In addition to APP_s, the spontaneous proteolysis of APP by this protease also generated a 30 kDa fragment whose appearance was in accordance with the decrease of APP_s (Fig. 6, arrowhead). This fragment was not held in 8% gels (see Fig. 2 and Fig. 3), but was seen at the bottom of 10% gels (Fig. 6). As shown in Fig. 8 (10–20% gradient gel), addition of calcium to the lysates accelerated the appearance of this fragment (lanes 2 and 3). In turn, partially purified APP incubated with exogenous *m*-calpain (29) generated the same APP fragments (lane 4) but at an even accelerated rate (5 min). After 10 min, APP_s was completely degraded to 30 kDa (lane 5). The cleavage of APP was not due to the platelet calpain contaminating the APP preparation because preincubation of the purified APP did not result in any degradation (lane 6). Notably, the

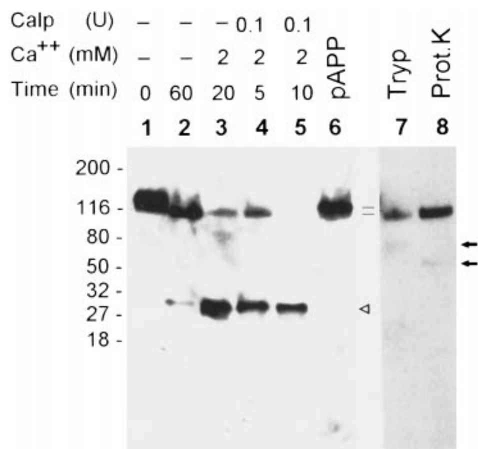

FIG. 8. Comparison of APP fragments generated by the platelet protease and by exogenous calpain. Platelet lysates were incubated in the absence (lanes 1–2) or presence (lane 3) of calcium for the time intervals indicated. Lanes 4–5, partially purified APP incubated with *m*-calpain (Calp; U, unit) plus calcium. Lane 6, partially purified APP (pAPP) incubated for 30 min without additions. Lanes 7–8, APP incubated with trypsin (Tryp) or protease K (Prot. K) for comparison. Two short lines indicate the position of APP before and after proteolysis. Arrows, two faint bands at 80 and 55 kDa, respectively. Arrowhead, 30 kDa fragment. All samples were analyzed on 10–20% Tris-tricine gel followed by Western blotting with 22C11.

fragments generated by calpain were different from those generated by trypsin or protease K. The latter enzymes clipped off a ~80 kDa and a ~55 kDa fragment from APP, respectively, but no 30 kDa band was observed (lanes 7–8).

Since the above experiments were conducted in a cell-free system, we also tested whether calpain mediated APP secretion in intact cells. As shown in Fig. 9, APP_s secretion was induced when platelets were stimulated by calcium ionophore A23187 (lane 0), and the effect was blocked by EGTA. The activation of calpain by A23187 was confirmed by the hydrolysis of talin and filamin (data not shown) (18). Under these conditions, APP_s secretion was reduced in a concentration-related manner by E64d and calpeptin (CPT), two cell-permeable calpain inhibitors (13, 18).

DISCUSSION

We report here that proteolysis of intact APP by a protease in the platelet lysates generates a N-terminal fragment that is immunologically indistinguishable from APP_s, suggesting that the cleavage occurs at or near Lys^{16}, thus may be the action of α-secretase. We also show that this α-like proteolysis is completely inhibited by calpastatin (Fig. 6; Table 1) or CPS-P after 16 h of incubation in the lysates (Fig. 7A). This suggests that the possibility of the proteolysis being mediated by other proteases than calpain is unlikely, since such proteases would have cleaved APP in the lysates in the presence of the calpain-specific inhibitor. Our finding that APP_s secretion from intact platelets is inhibited by EGTA and calpain inhibitors (Fig. 9) further supports the involvement of calpain, or a calpain-like protease, in APP normal processing.

If the observed APP proteolysis in the lysates is mediated by the putative α-secretase, then why can this protease preserve its cleavage specificity in a cell-free system? Many proteases loose their cleavage specificity when cells are lysed; yet, calpain may be an exception. It is well-known that calpain preserves its cleavage specificity after cells are lysed by detergents. Indeed, filamin, talin, spectrin and tau, the four best-known calpain-specific substrates (30), are cleaved by the enzyme at the same or similar sites either in intact cells or in lysed cells (15, 31, 32). While the reason for this is not fully understood, it must be noted that calpain is a dominant protease in platelets (15, 18) and, unlike many other proteases, its cleavage activity is highly site-specific (30).

APP normal secretion is regulated by signal transduction pathways (4, 20). Our review of the literature revealed that many agents which enhance APP_s secretion also activate Ca^{2+} signals, whereas other reagents which decrease APP_s secretion also inhibit Ca^{2+} action (33, 34). This indicates that APP_s cleavage/secretion is likely mediated by a Ca^{2+}-dependent protease. Our findings and those by Buxbaum *et al.* (35) and Jolly-Tornetta *et al.* (36) support this view.

Yamasaki *et al.* (37) and Zhang *et al.* (38) have further shown that several calpain inhibitors enhance the production of $A\beta$ in cultured cells. Frautschy *et al.* (39) and others (40) have found that infusion of leupeptin into rat brain leads to accumulation of $A\beta$ and $A\beta$-containing

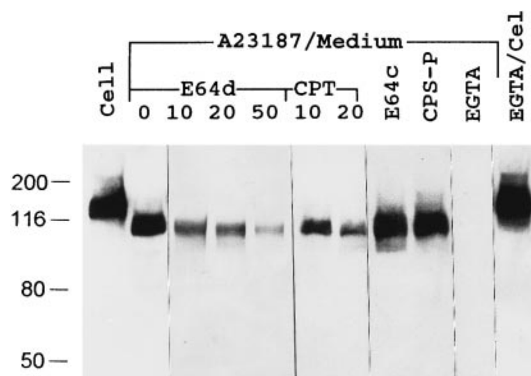

FIG. 9. Calpain inhibitors reduce APP_s secretion from intact platelets. Washed platelets were preincubated with the inhibitors at the concentration indicated (μM). After activation by A23187, the reaction suspension was centrifuged and supernatant (medium) and cells were analyzed by Western blotting with 22C11. Cell, platelets before activation. CPT (calpeptin) and CPS-P were at 50 μM each. Note that E64c and CPS-P, two cell-impermeable inhibitors, did not show the inhibitory effect. APP_s secretion did not occur in the presence of EGTA (3 mM), but preserved in the cells after incubation (lane "EGTA/Cel").

peptides. Additionally, leupeptin inhibits the degradation of such peptides in cultured cells (41, 42). These reports are consistent with the possibility that a calpain-like protease acts as α-secretase. Thus, inhibition of calpain would provide extra intact APP as substrates for the increased production of Aβ. Recent studies have established that α-secretase competes with β-/γ-secretases for the same APP pool (11, 38, 43).

Can calpain be a candidate for α-secretase? This requires calpain to penetrate the membrane and function on the cell surface. While calpain activity is mostly found in the cytosol and on the inner-side of membranes (30), McGowan *et al.* (44) and Schmaier *et al.* (45) have observed that calpain can also function on the platelet outside surface. It thus appears that calpain, or one of its subtypes (30), may be considered as a reasonable α-secretase candidate for further investigation.

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