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THE ALZHEIMER'S PLAQUES, TANGLES AND MEMORY DEFICITS MAY HAVE A COMMON ORIGIN - PART IV: CAN CALPAIN ACT AS α-SECRETASE?

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1. ABSTRACT

Abnormality of protease activities and imbalance of intracellular calcium are two most salient aberrant events in Alzheimer's disease (AD). As such, calcium-dependent proteases such as calpain, as a critical link between these two events, must play a key role in the pathogenesis of AD, particularly in the abnormal processing of ß-amyloid precursor protein. Because α-secretase in this process appears to be a calcium-dependent protease and its enzymatic characteristics are impressively similar to those of calpain, a challenging possibility arises: Calpain might act as α-secretase *in vivo*. However, as the experimental evidence both for and against this possibility is compelling, the issue currently remains as a theoretical dilemma in which a central question is whether calpain, a cytosolic enzyme, can somehow reach the cell surface. This difficult issue needs to be addressed now. As a first attempt to explore the issue, we propose a working model for the membrane orientation of calpain and suggest several experiments that will critically test this model. The quest to this dilemma will not only impact our understanding of AD, but may also expand the current knowledge about Ca^{2+} signal transduction pathway. Finally, we discuss several competing models and the potential role of presenilins as "regulators" of α-secretase. It is of interest to note that some of our previous theoretical predictions have been experimentally observed.

2. INTRODUCTION

Alzheimer' disease (AD) is characterized by cerebral deposition of ß-amyloid protein (Aß) as senile plaques and accumulation of tau protein as neurofibrillary tangles (1-4). Aß is derived from its much larger precursor, ß-amyloid precursor protein (APP). Processing of APP *in vivo* occurs by two major pathways. APP can either be cleaved within its Aß domain at the Lys^{16} site by a putative α-secretase. In AD patients, APP is somehow excessively cleaved by ß/γ-secretases leading to overproduction of Aß (1,2). While the mechanisms underlying this abnormality are not well understood, two possibilities appear to be most relevant: either α-secretase is "inactivated", or ß/γ-secretases are "overactivated" in AD. Despite considerable experimental efforts devoted to this area, the identity of the secretases remains unknown (for reviews, see 3-6). This issue is of primary importance because Aß accumulation is among the earliest detectable histological lesions in the aging brain preceding the clinical manifestations of AD by decades. Although some other issues around amyloid lesions are also of interest (such as the mechanisms of Aß fibrillogenesis and its cell-damaging effects), it is apparent that these other events are secondary to Aß overproduction in AD progression.

In addition to protease abnormalities, an imbalance of intracellular calcium homeostasis is another salient defect in AD pathogenesis (though the direction of the imbalance is debatable)(8,9). Therefore, calcium-dependent proteases such as calpain should be expected to play a key role in the abnormal APP processing in AD by serving as a critical link between the protease abnormality and calcium imbalance. However, although the implication of calpain in long-term potentiation and tau degradation has begun to be elucidated (10,11), virtually nothing is

definitively known about the roles of this protease in APP processing. In this context, it is necessary to further explore this important issue.

3. DISCUSSION

3.1. Calcium-dependent α-secretase

Sensitive responses of APP processing to a wide variety of reagents in cultured cells suggest that the non-amyloidogenic cleavage/secretion of APP is a highly "regulated" process (3-6). However, current proposals have not fully explained this unique feature of the enzyme. During the course of our studies, we undertook a systematic analysis of the literature and recently proposed that the putative α-secretase is a calcium-dependent protease (7). This contention, though in contrast to current beliefs (5,6), is arisen from an unusually large number of highly consistent reports (7)(we listed 22 of them; in fact, the effects of some reagents, e.g. phorbol esters, have been repeatedly reported by many laboratories; for examples, see 4,12,13). Since these reagents belong to a wide diversity of the functional groups (growth factors, cytokines, toxins, etc.) but they all exert the same effect on APP processing, it is reasonable to assume that their cellular actions may converge into a common pathway underlying the α-secretase activity.

It is important to note that our contention parallels with, but does not contradict to, a widely reported observation that APP cleavage/secretion correlates with protein kinase C (PKC) activation (3,12,13). Since PKC is also a well-known calcium-activated enzyme (14) and mobilized Ca^{2+} in the cell will activate many downstream processes (proteases, phosphatases, kinases, etc.), it is likely that a multiplicity of data interpretations would be plausible depending on the specific process being examined. But, it appears to us that the attribution of the calcium effects to a "protease", rather than a "kinase", would explain more directly the actions of these reagents on the proteolytic "cleavage" of APP (to our knowledge, a PKC-regulated protease has not been reported thus far).

Of particular importance is that despite this difference, these two interpretations agree well on the ground that non-amyloidogenic processing of APP is under the control of the phospholipase C/Ca^{2+}/PKC signal pathway and Ca^{2+}/cation channels. Therefore, they together would argue against the suggestions that Aß production instead is a calcium-dependent process under the physiological condition (7), or that α-secretase could be an "unregulated" protease (i.e., not controlled by a signal transduction pathway)(5,15,15a). It is our opinion that any proposed proteases as α-secretase would need to explain the actions of various reagents in *a comprehensive* manner (not only one or a few of them), particularly the well-established correlation between APP secretion and PKC activation. And perhaps, any suggestions should also be judged by their explanatory potentials to other invariable AD features (16-18), as well as by the experimental outcome of their logical *predictions*.

Our contention predicts that as α-secretase is calcium-dependent, APP cleavage/secretion would *always* correlate with the intracellular Ca^{2+} levels. Thus, any confirmed calcium agonists (e.g., some hormones, growth factors and excitatory neurotransmitters) will enhance the release of soluble APP (APP_S) in cultured cells (16), whereas calcium antagonists (e.g., nimodipine, nifedipine and EGTA)(19) will display opposite effects. And also, APP_S secretion should fluctuate as a function of the reagent concentrations. Indeed, since the publication of our initial paper (7), at least 6 additional calcium agonists have been reported to enhance APP_S secretion. They are NGF, EGF, nicotine, vasopressin, kainate and AMPA (12,13,20-22); and their effects on calcium mobilization are known (19,22-25). The growth of such corroborating reports are expected to continue. Furthermore, several other reagents have also been reported to promote APP secretion (ACPD, CCG-1, quisqualate, xanomeline, AF102B, WAL2014 and PD142505)(22,26-28). Our proposal predicts that whatever their best known actions are, the cellular effects of these reagents must include, among other things, mobilization of intracellular Ca^{2+}. Altogether, these experiments should provide further evidence towards definitively establishing the regulatory mechanism of α-secretase.

Our analysis of several basic AD features has also suggested that the proposed regulatory mechanism of this enzyme, if correct, would have a far-reaching impact in the understanding of the overall state of Ca^{2+} in AD (16-18). In this context, the proposed mechanism should be subject to perhaps the most rigorous tests. As a matter of fact, our contention would be proved to be *incorrect* if it can be shown: (i) another unifying factor(s) than Ca^{2+} can be identified within the cellular actions of these reagents and this other factor(s) can better explain their effects on APP processing; or (ii) cogent arguments can be made on the basis of the established biochemical principles showing that such a unifying factor is unnecessary and the actions of the reagents can be explained individually; or (iii) the known calcium agonists (or antagonists) consistently affect APP_S secretion in a way that contradicts our predictions. However, the effects of the agents that also influence APP metabolism by other possible mechanisms (e.g., APP gene expression)(7,28a) may not be cogent to contest the contention (i.e., APP metabolism is not *only* affected by Ca^{2+}).

3.2. "Profile" of ß- and γ-secretases

The regulatory mechanism of α-secretase may also shed light on the potential roles played by ß- and γ-secretases. Since Ca^{2+} is the only known second messenger that can directly "regulate" proteases (29,30), it is logical to assume that ß/γ-secretases would belong to the group of "unregulated" proteases. This novel view coincides with the roles of ß/γ-secretases which are secondary to those of α-secretase in the phenotype development of the APP missense mutations, as we proposed based on a systematic analysis of genetic data

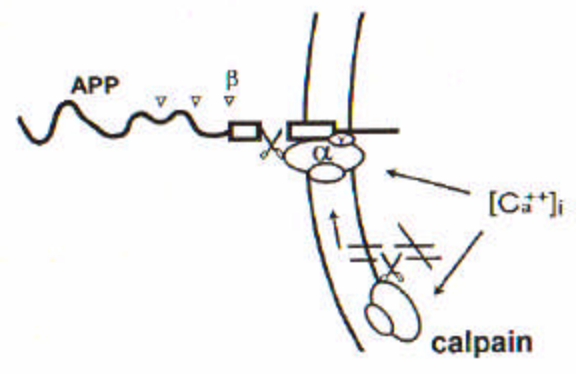

Figure 1. A proposed model for membrane orientation of calpain. The model postulates that: (i) calpain upon activation may possess a hydrophobic exterior, which would allow itself to be associated with the inner surface of the membrane where it cleaves cystoskeletal proteins; and (ii) such a hydrophobic exterior of calpain may also allow itself to bind to the hydrophobic domain of APP and to its own hydrophobic small subunit to form a large "hydrophobic complex". In this manner, calpain might be able to penetrate the membrane and reach to the Lys^{16} site of the Aß domain at the cell surface [or at the inner side of the membranes of the subcellular organelles, which is equivalent to the cell surface (29); not shown]. The locations of ß-like and γ-secretases are also shown. Large and small circles represent the catalytic (80 kDa) and regulatory (30 kDa) subunits of calpain, respectively.

showing that the severity of the phenotypes correlates only with the distances of the mutations relative to the α-secretase cleavage site (7). This view is also in line with the consideration that ß-secretase is located in the extracellular milieu (ECM) whereas γ-secretases is membrane-associated (7). At these distinct locations, it would be difficult to image that any cellular signal could overactivate both of them at the same time (or sequentially), a requirement for the two enzymes (if they were the regulated proteases) to account for the progressive increase of Aß in AD.

As unregulated proteases, the rate of the reactions catalyzed by ß/γ-secretases (Aß genesis) would be first-order, i.e., depending on the availability of their substrate, the intact APP. This view is corroborated by the observed inactivation of α-secretase in the AD patients (31,32), which would lead to an excessive availability of intact APP, a conceptual prerequisite for overproduction of either Aß40 or Aß42 (i.e., for every extra Aß40/Aß42 produced, there should be an extra APP available prior to it). In fact, α-secretase activity predominates over those of its ß/γ-counterparts (5,6); thus if α-secretase were normal in AD, then most APP would be α-processed perhaps before ß/γ-secretases could have a chance to overproduce Aß (unless the two latter enzymes were regulated by unknown factors that are even more sensitive than Ca^{2+} signaling, a remote possibility)(16,29).

If these considerations are reasonable, then they would imply that the identity of ß/γ-secretases would be difficult to ascertain since the strategies for their identification would be largely based on their cleavage specificities. As the cleavage specificities of most proteases are not entirely strict, and many are even overlapping (29,30), this would be an arduous endeavor (though a given protease can be shown to cleave a specific site on the substrate *in vitro*, it is extremely difficult to exclude the possible involvement of other proteases at the same site *in vivo*). Such attempts would be further complicated by the fact that the activities of ß/γ-secretases are minor in cells (Aß is much more difficult to detect than APP_S) and selective inhibitors and other information are unavailable for most minor proteases. Moreover, the so-called "ß-secretase" activity is apparently contributed by a group of proteases. This is predicted by its ECM location (where many soluble proteases can act on APP)(figure 1) and is confirmed by the "ragged" N-termini of the actual Aß proteins isolated from either AD brains or cultured cells (33-35). These considerations together would encourage a view that Aß is merely an alternative (passive) degradation intermediate of APP when the latter is somehow unprocessed by α-secretase.

Furthermore, even if the identity of ß/γ-secretases can be ascertained, their unregulated nature and secondary roles in APP processing would render them of little use as therapeutic targets, because it would be difficult to specifically and simultaneously modify both of them in order to reduce the Aß levels (let alone the multiple ß-like secretases). Finally, inhibition of ß/γ-secretases, or any one of them, although reducing Aß in concept, would lead to an accumulation of APP or its Aß-containing fragments if the inactivated α-secretase remains unmodified. These fragments, similar to the accumulation of tau, amylin, $ß_2$-microglobulin (6) or cholesterol, would be harmful to the body (accumulation of proteins, etc. is a common threat in aging)(36).

For all these reasons, we believe that the attempts to reduce Aß in AD should target the α-secretase dysfunction by stimulating the potency of Ca^{2+} signaling (this should also slowdown the processes of tau accumulation and memory reduction)(16-18). This view, though controversial for the time being, is strongly supported by the rapidly growing body of evidence emerged from various research areas showing that other Ca^{2+}-dependent activities such as calcineurin, PKC and neurotransmission are also reduced in AD (37-39).

3.3. α-Secretase and calpain

The foregoing analysis indicates that it is the catalytic state of α-secretase, but not of its ß/γ-counterparts, that governs the outcomes of APP processing *in vivo*. As such, elucidation of the identity of α-secretase would be of key importance for an in-depth understanding of APP metabolism. Although this also is a difficult task, α-secretase has several unique advantages which can largely narrow the search area.

In addition to its regulatory mechanism, α-secretase is known to be membrane-associated (40) and hence it is likely a phospholipid-binding enzyme (upon activation). As membrane-associated, its cleavage on APP would be highly "distance-specific" (due to double membrane anchorage)(7,40)(figure 1). This unique feature is consistent with the sequencing data of the C-terminus of APP_S from a variety of cells (although minor cleavages around Lys^{16} have also been detected, the Lys^{16}-specific cleavage is overwhelming)(7). This indicates that the α-secretase activity most likely is, or dominated by, the action of a *single*, membrane-associated enzyme (in contrast to ß-like secretases). This view, though departed from some of the current data interpretations (6), is consistent with a basic observation that intact Aß, once being generated and released into the ECM, is difficult to be degraded there (it accumulates throughout the normal aging process) despite the large amounts of active proteases present in the ECM. These proteases *in vivo* are apparently unable to act on the released Aß, or excessively released Aß for unknown reasons (though some *in vitro* studies suggest otherwise). This would imply that normal "Aß clearance" perhaps only occurs through α-secretase cleavage while the Aß domain is still membrane-associated; and if this enzyme is somehow inactivated, Aß will accumulate.

α-Secretase has been preserved in evolution since as early as in yeast (41). Moreover, the abundant occurrence and ready determination of its major product, APP_S , in a wide variety of cells (7) suggest that it is not only ubiquitous, but perhaps also a major protease activity in cells. These features together can help to significantly facilitate the quest. For example, the ubiquity of the enzyme implies that its identification can be carried out not necessarily in the fragile neurons but also in many other cells such as platelet, where calcium-dependent protease activity is overwhelming (42) [we and others have shown that platelets are the primary source for both APP and Aß in the circulation (43,44), indicating that all three APP secretases are present in platelets as they are in neurons]. The search for α-secretase might also be carried out in yeast, where protease systems may be simpler to dissect than in mammalian cells. Most importantly however, as a major protease activity in cells, it is even possible that α-secretase may be a known protease in the current enzyme repertoire.

Since calpain, one of the best characterized enzymes, is a major calcium-dependent protease in most if not all cells (10,45) and, to our knowledge, there is no other protease in the current enzyme repertoire whose features fit better with those of α-secretase than calpain (29,30; see below), it is reasonable for us at the present time to consider calpain as a primary candidate for α-secretase.

Calpain is a key mediator in Ca^{2+} signaling pathways (45). The enzyme contains a cysteine-protease domain and a calmodulin-like (calcium-binding) domain within the same polypeptide (46). This feature would render it extremely, perhaps the most, sensitive to Ca^{2+}, compared to many other calcium-dependent enzymes which require either calmodulin as a mediator (29) or cleavage by calpain for their activation (e.g., PKC). This feature is consistent with the essential roles of calpain in neurotransmission and memory formation (10,11), the most sensitively regulated activities of the brain, and may also be relevant to why accumulation of Aß (and tau) is the *earliest* detectable lesion accompanying the initial memory reduction in the aging brain (further discussed elsewhere).

There are two major subtypes of calpain, *m*- and m-calpains, depending on the concentration of calcium required for their activation as tested *in vitro*. *In vivo*, the calcium concentration required for calpain activation is believed to be dramatically reduced to *n*M range by its binding to phospholipids (45). In the calpain family, there are eight currently known isoforms occurring through gene splicing (45). However, the ubiquitous or tissue-specific feature as well as the presence or absence of calmodulin-like domain, may allow, in our opinion, exclusion of most of them, leaving the two ubiquitous subtypes (*m*- and m-calpains) as the most reasonable candidates for α-secretase.

3.4. Evidence in favor of calpain as α-secretase

First, a number of reagents that enhances α-secretase activity also mobilizes intracellular calcium (7); hence these reagents would be expected to activate calpain as well. Indeed, some of these reagents are well-known calpain activators such as phorbol esters (which are not only PKC activators), calcium ionophore A23187 and thrombin, as we and other have previously shown (47-49). The remaining agents in this group may have similar effects on calpain as well, though yet to be confirmed. Second, agents such as cAMP under some conditions can suppress α-secretase activity (7). In accordance with this, the calpain system in certain cell types is known to be down-regulated by cAMP (47,49,50). Third, the α-secretase cleavage site (the Lys-Leu bond of Aß) is identical to one of the actual cleavage sites in PKC by calpain (51). Fourth, α-secretase activity is vulnerable to oxidative stress (6), suggesting that it could be a cysteine protease, a class of proteases to which calpain also belongs (36). Fifth, calpain is co-localized with APP *in situ* in almost all structures where APP is found, including various types of neurons, reactive astroglia, senile plaques, synapse endings and neurofibrillary tangles (52-55).

Importantly however, as shown by Saido *et al.* (56), calpain is *not* co-localized with the N-terminus of the Aß domain in APP (i.e., the ß-secretase binding site, recognized by a strict epitope-specific antibody) in postischemic brain. This indicates that calpain cannot act as *ß-secretase*, a conclusion that is consistent with our theoretical analysis (7).

Additionally, we have observed that APP in platelet lysates is cleaved by an endogenous protease within its Aß domain at or near the Lys^{16} site and this protease in several aspects is indistinguishable from calpain (unpublished data). Finally, in sharp contrast to the widely held notions (52,57-59), Yamazaki *et al.* (60) have recently

demonstrated that several calpain inhibitors can *enhance* Aß secretion (both Aß40 and Aß42) in cultured kidney cells. Taken together, these studies substantially support the scenario that calpain is involved in the α-cleavage of APP *in vivo*; thus, inhibition of this enzyme would overcharge the amyloidogenic pathway leading to an increase in the release of Aß.

3.5. Evidence against calpain as α-secretase

It must be pointed out that none of the above listed results is stringent enough to allow for the conclusion that calpain is the single enzyme responsible for the non-amyloidogenic processing of APP. This is because the inhibitors and activators used are not absolutely selective for calpain and their precise actions in intact cells are not yet clear. Thus, the involvement of another protease(s), though calpain-like, cannot be ruled out.

A more serious theoretical challenge for calpain to act as α-secretase is that there is no large hydrophobic segment in its primary sequence to allow it to directly traverse the membrane (46). In fact, calpain, as a cytosolic enzyme, is currently thought to be only associated with the inner surface of the plasma membrane when it is activated (45). As such, it can diffuse laterally within the membrane, but may not be able to reach to the Lys^{16} site located at the outside surface of the cell by vertical diffusion (flip-flop).

3.6. Can calpain act as α-secretase?

While the above considerations have contested the possibility of calpain to function across the membrane, it is, however, intriguing to note that several lines of important evidence have also indicated that calpain can play some unexpected roles in the cell. For example, McGowan *et al.* (42) and Li *et al.* (47) have demonstrated that calpain *in vivo* can proteolytically modify glycoprotein Ib (GP-Ib), a platelet membrane surface receptor, leading to the release of glycocalicin, the extracellular domain of the receptor. The cleavage site by calpain has been found to be in the hydrophilic region of GP-Ib (61). Such a cleavage would be impossible if calpain were only functioning in the cytosol.

Moreover, Li *et al.* (47) have reported that calpain is involved in the cleavage of APP and a 22 kDa C-terminal fragment of APP in intact platelets. Since the latter fragment contains the entire Aß domain, it should be expected to preserve its original membrane orientation. Thus, its cleavage by calpain might occur on the cell surface, since the resulting product, a 17 kDa C-terminal fragment, might still contain part of the Aß sequence (if the cleavage occurred further downstream from the Aß domain, then the resulting C-terminal fragment would be smaller in size)(47). But, it is not yet clear whether the cleavage occurs precisely at Lys^{16} of Aß.

Further evidence in support of these findings can also be found in the literature. For example, it is indeed well-known that in addition to APP and GP-Ib cleavages, calpain is actively involved in the cleavages of many other membrane surface proteins including EGF receptor, integrin-ß4, Ca^{2+}-ATPase, N-CAM, glutamate receptor, thrombin receptor, PDGF receptor and others, leading to the release of their respective extracellular domains (45,62-64).

More importantly, an unidentified membrane-bound protease is known to release the ectodomains of still many other membrane-anchored proteins. These proteins include: proTNF-α, proTGF-α, CSF-1, kit ligand, CSF-1 receptor, IL-6 receptor and L-selectin, and they are cleaved on the cell surface in much the same way as APP (65). Although the responsible protease has not yet been explicitly attributed to calpain, it is well observed that their cleavage is a "regulated" process and can be potently stimulated by "phorbol esters and calcium ionophores" (65,66). This points to its significant similarity to calpain. Finally, it is worth noting that this protease also appears to be responsible for α-cleavage of APP (66).

Altogether, these observations argue that the cleavage of the ectodomains of a large number of membrane-anchored proteins by a calpain-like protease(s) is a common phenomenon in a diversity of cells. It must be noted that these observations, like α-processing of APP, cannot be explained by the current mechanism of action of either calpain or any other calcium-dependent proteases which are supposed to function only in the cytosol. Thus, these findings collectively raise a challenging possibility that calpain, or another calcium-dependent protease(s), can somehow reach the cell surface *in vivo*. In a broader sense, this further implies that Ca^{2+} signaling, a strictly intracellular event as currently thought (29), may somehow expand its boundary to cell surface by clipping off the extracellular domains of APP and other membrane proteins (in addition to the known Ca^{2+}-triggered cellular exocytosis)(29). Viewed from this perspective, the various released protein ectodomains, which must have important physiological functions (e.g., APP_S)(6,65), can all be considered as the consequences of Ca^{2+} signal transduction (figure 1; other membrane proteins not shown).

3.7. A theoretical model for calpain action

This serious paradox between the current theory and experimental observations is profound and may represent a major obstacle in understanding the full physiological function of calpain. Hence, it should be explicitly addressed now. As a first attempt to probe this difficult issue, we put forward a tentative model for the membrane orientation of calpain for discussion and testing. It is proposed that the enzyme might act on the cell surface through an as-yet-unknown mechanism. For example, calpain (or one of its subtypes) might be able to fold itself in such a way that its hydrophobic residues are facing outside of the molecule to create a hydrophobic "shield". Such a shield, if possible, would allow its catalytic domain to penetrate the membrane, or to form a large "hydrophobic complex" through its binding to the hydrophobic domain of APP, and to its own hydrophobic small subunit (63)(figure 1). In this manner, its catalytic domain might be able to penetrate the lipid bilayer, while the calcium-binding

domain does not lose the touch with cytosol. This model also implies that upon activation, calpain undergoes a conformational change from hydrophilic to hydrophobic, and functions at both inner and outer surfaces of the membrane (to be compatible with its known roles in the degradation of cytoskeleton proteins)(45)(figure 1).

Can such a membrane orientation of calpain take place *in vivo*? It is noteworthy that the catalytic and calcium-binding domains of calpain are located at both termini of its primary sequence (catalytic cysteine is at residue 108; and calcium-binding domain is ranging in residues 589-695)(46). Such a layout of the functional domains parallels with the potential orientation of calpain across the compartments. The existence of a hydrophobic shield in calpain is probably suggested by its binding to the membrane inner surface, a structure that is also hydrophobic. If calpain can assume such a folding, then given its relatively large size (catalytic subunit 705 amino acids, which may allow itself to assume a multiple-fold conformation across the thickness of the membrane, i.e., 20-30 amino acids)(29), it may not be impossible that the activated calpain might reach to the immediate surface of the membrane under certain circumstances. Through a similar scheme, calpain might also be involved in the cleavage of other membrane-anchored proteins.

Nevertheless, our considerations do not entirely preclude a possibility that there is another calcium-dependent protease(s) which can directly traverse the membrane. Alternatively, a more sophisticated model might be that α-secretase is a membrane surface protease but regulated by another calcium-binding protein which is in touch with the cytosol. However, if this protease is involved in the cleavage of APP and many other receptors, it would be almost certainly a major protease activity in cells. As a major protease activity in cells, it is highly unlikely that it has remained as an uncharacterized protease thus far.

3.8. Experimental testing of the model

It is usually difficult to unambiguously assign a known proteolytic event (e.g., the Lys^{16} cleavage of APP) to a specific protease because of the multitude and complexity of the protease systems *in vivo*. At present, a number of proteolytic events have been suggested to be calpain-mediated, but few has been convincingly proved (63). To this end, Saido *et al.* (67) and Croall and Demartino (63) have recommended several criteria for documenting a given proteolytic event as calpain-mediated *in vivo*. These criteria include: (i) concomitant activation of the proteolytic event in question with a known calpain-mediated process [e.g., degradation of talin, filamin and spectrin (45,63)]; (ii) the proteolytic event should be inhibited in cell extract by calpastatin and its related peptides (the most specific calpain inhibitors currently available); and, (iii) the inhibition of the reaction should be demonstrated in the living cells by membrane-permeable and calpain-selective inhibitors. Since most of the available inhibitors do not meet these criteria, the *in vivo* inhibition should be conducted by microinjection of the inhibitors or calpain-neutralizing antibodies, or by anti-sense strategy (63,67). But, it should be noted that gene knock-out paradigm, an effective means for pinpointing the roles of many other proteins *in vivo*, may not be feasible for calpain because calpain, as an indispensable mediator in Ca^{2+} signaling, is essential for life (67).

However, to prove that calpain acts as α-secretase, it seems necessary, in our opinion, to demonstrate additional parameters. For example, (a) the cleavage of APP by calpain *in vitro* should occur precisely at Lys^{16} of the Aß domain; (b) calpain on the surface of activated cells should be able to cleave the exogenous APP or Aß, and the reaction should be inhibited at cell surface by selectively blocking the single active site cysteine of calpain (figure 1); (c) antibodies raised against the sequence around the active site of calpain should label the enzyme on the cell surface (figure 1); (d) there should be a transient binding of calpain to APP; (e) molecular modeling and crystallography of the three-dimensional structure of calpain should reveal a hydrophobic folding; (f) calpain-selective and cell-permeable inhibitors (or oligonucleotides antisense to calpain mRNA) should give rise to the anticipated decrease of APP_S secretion with a concomitant increase of Aß in cultured cells; (g) repetitive and sufficiently prolonged infusion of such inhibitors into the brain of experimental animals should induce Aß overproduction (and perhaps tau accumulation as well; see 16-18); and finally, (h) an α-secretase-like protease has been found in yeast; our model predicts that this protease should be somewhat activated by Ca^{2+}/PKC signal pathway (i.e., by phorbol esters and calcium ionophores); and if isolated, this protease might even have some sequence similarity to calpain (Ca^{2+} signaling system, as an essential part of life, should exist in the primitive cells). Conversely, negative outcomes of such experiments could serve as evidence to disprove the role of calpain as α-secretase.

If α-secretase turns out to be another protease, then our profile of α-secretase would predict that this other protease should be sensitive to calcium, ubiquitous, membrane-associated, and inhibited indiscriminately by many calpain inhibitors (47,60). In turn, this would imply that α-secretase is a calpain-like protease, and probably difficult to distinguish from true calpain by conventional biochemical parameters.

A recent report suggested that α-secretase is a metalloprotease, TNF-α-converting enzyme (TACE)(68). However, it is known that conventional metalloproteases (not including calpain) typically require a metal ion as their catalytic center (not as an activity regulator), but they are not usually considered as "regulated" proteases because their activity does not fluctuate strictly as a function of the metal concentrations (but calpain does). Thus, if TACE is α-secretase *in vivo* but not regulated by Ca^{2+}, then it remains to be explained how TACE can be stimulated not only by phorbol esters but also by Ca^{2+} ionophore, growth factors, Ca^{2+}/cation channel activators, etc. (7,22), and specifically by Ca^{2+} signal in a "PKC-independent

manner" (4). And how TACE/α-secretase in cells can be inhibited by many calpain inhibitors (47,60).

3.9. Can presenilins regulate calpain?

Gene muations of presenilins (PS-1, PS-2)(PSs) account for most of the early onset cases of familial AD. Hence the physiological function of PSs holds a key to the understanding of AD pathogenesis and has been in the center of the intensive studies. While the roles of PSs remain obscure, we have predicted that PSs most likely act as Ca^{2+}/cation channels that supply the Ca^{2+} ions in proximity for α-secretase (16,17). Now, if calpain acts as α-secretase, then it should be expected that calpain would bind to PSs *in vivo*. An interesting episode is that such a binding of calpain to PS-2 has been observed recently by Shinozaki *et al.* (69), and the binding site on calpain is in the C-terminal region, where its Ca^{2+}-binding domain resides (46). Yet, it has also been observed that PSs bind to filamin (70) and tau (71), two known *in vivo* substrates of calpain (see above), further supporting the spatial proximity between PSs and calpain. In addition to these reports, PSs have been found to bind to a Ca^{2+}-binding protein ("calsenilin")(72) and ß-catenin (73), both of which are believed to be the mediators in the Ca^{2+}-related signal transduction processes (72,74). Thus, these observations altogether corroborate a central role of PSs in these processes as "Ca^{2+} suppliers". It remains however to be directly determined whether PSs themselves *are* Ca^{2+}/cation channels and whether their pathological mutations will *up*- or *down*-regulate the channeling function.

If PSs are Ca^{2+}/cation channels, then it would also be expected that insertion of a mutant PS gene into cells would give rise to extra (though defected) copies of a channel. As a result, such cells, when challenged with calcium agonists, would certainly exhibit a greater extent of Ca^{2+} mobilization than wild-type cells. Of interest is that this effect has been reported (75). But, it is obvious that such "mutant gene-inserted" cells (i.e., a normal plus a defect gene) do not represent the condition in the PS mutant human carriers (where only a defect gene exists).

Our model has further predicted that the mutations of PSs primarily down-regulate α-secretase activity (otherwise the APP source for the overly produced Aß42 would not be explained), but also affect the γ-secretase cleavage specificity by their steric effects (17). Notably, a direct regulation of α-secretase by PSs has recently been reported (76,77). On the other hand, another recent study has suggested instead that PS-1 regulates only γ-secretase activity (78). This contradicts our premise and it would be important for future investigations to clarify the discrepancy. In order to further test the role of PSs in AD, here we propose another direct experiment: In the PS gene knocked-out animals (surviving embryos)(79) where, according to our model, α-secretase should be severely inhibited (a Ca^{2+} channel eliminated), it is predicted that there should be an accumulation of APP or its Aß-containing fragments particularly on the walls of the blood vessels in the brain (similar to the severe disruption of the α-secretase functional integrity in the Dutch-type APP mutations as we proposed)(7).

4. CONCLUSIONS

The identity of α-secretase remains to be unambiguously defined but its *regulated* nature points to a narrow area of search, and the largely homogeneous C-terminus of APP_S suggests a single protease being responsible for APP α-processing. This would justify the identification of α-secreatse as a feasible project. Our theoretical analysis indicates that it is the state of α-secretase, or more precisely, it is the state of the regulatory factor behind this protease, Ca^{2+}, that governs the outcomes of APP processing *in vivo*. The enzymatic characteristics of α-secretase are significantly similar to those of calpain, and calpain is known to be critically implicated in memory process and in tau degradation. It is therefore reasonable to conclude that the roles played by calpain (and Ca^{2+}) are of *paramount* importance for unraveling the AD mystery. The issue of whether calpain can act as α-secretase currently remains a theoretical dilemma. This dilemma, as well as other competing models, should be attractive in the future investigations. The issue is also crucial for an in-depth understanding of the structure-functional relationships of calpain and the mechanisms whereby many membrane-anchored proteins regulate a large number of biological processes. The quest may impact our current knowledge about the boundary of Ca^{2+} signaling by addressing the question of whether, and if so how, a calcium-dependent protease can function across the cell membrane.

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