

Review

Spectrin and calpain: a ‘target’ and a ‘sniper’ in the pathology of neuronal cells

A. Czogalla^a and A. F. Sikorski^{a, b, *}

^a Institute of Biochemistry and Molecular Biology, University of Wrocław, ul. Przybyszewskiego 63/77, 51-148 Wrocław (Poland)

^b Academic Centre for Biotechnology of Lipid Aggregates, ul. Przybyszewskiego 63/77, 51-148 Wrocław (Poland), Fax: +48 71 3756 208, e-mail: afsbc@ibmb.uni.wroc.pl

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Abstract. It is well documented that activation of calpain, a calcium-sensitive cysteine protease, marks the pathology of naturally and experimentally occurring neurodegenerative conditions. Calpain-mediated proteolysis of major membrane-skeletal protein, α II-spectrin, results in the appearance of two unique and highly stable breakdown products, which is an early event in neural cell pathology.

This review focuses on spectrin degradation by calpain within neurons induced by diverse conditions, emphasizing a current picture of multi-pattern neuronal death and a recent success in the development of spectrin-based biomarkers. The issue is presented in the context of the major structural and functional properties of the two proteins.

Key words. Spectrin; calpain; calcium; proteolysis; neurodegeneration; biomarker.

Introduction

Over the past two decades, a significant focus of research has been on molecular processes leading to cell death. On the basis of numerous studies, a rise in the concentration of intracellular calcium has been identified as a primary trigger for pathology caused by various tissue-damaging factors. Such a condition is typical of neurons undergoing either ischemia, trauma or action of excitotoxins. Disturbances in calcium homeostasis are also characteristic of the pathology of several other tissues (e.g. muscle, kidney, lens); furthermore, these occur in tissues from aged human individuals either with or without associated age-related diseases. For that reason calcium-dependent processes which seem to be convergence points among various degenerative mechanisms undoubtedly play

a key role in cell pathology, and are potential generators of biomarkers applicable in diagnosis and clinical research.

One of the major components associating accumulation of Ca^{2+} ions in the cytosol with cell damage is a family of Ca^{2+} -dependent proteases called calpains. These belong to the family of cysteine proteases, which occur widely in animal cells. Calpain substrates include, among others, spectrin, which when digested yields specific fragments characterized by their high stability both *in vitro* and *in vivo*. Rapid appearance of spectrin degradation products, their abundance and high stability has been observed in many experimentally induced types of cell pathology as well as in clinical studies. It will become evident in this review that assays based on detection of calpain-induced α II-spectrin proteolytic fragments may be of great importance in describing the connection between calpain activation and the earliest stages of neuronal cell degeneration, and in designing optimal therapies.

* Corresponding author.

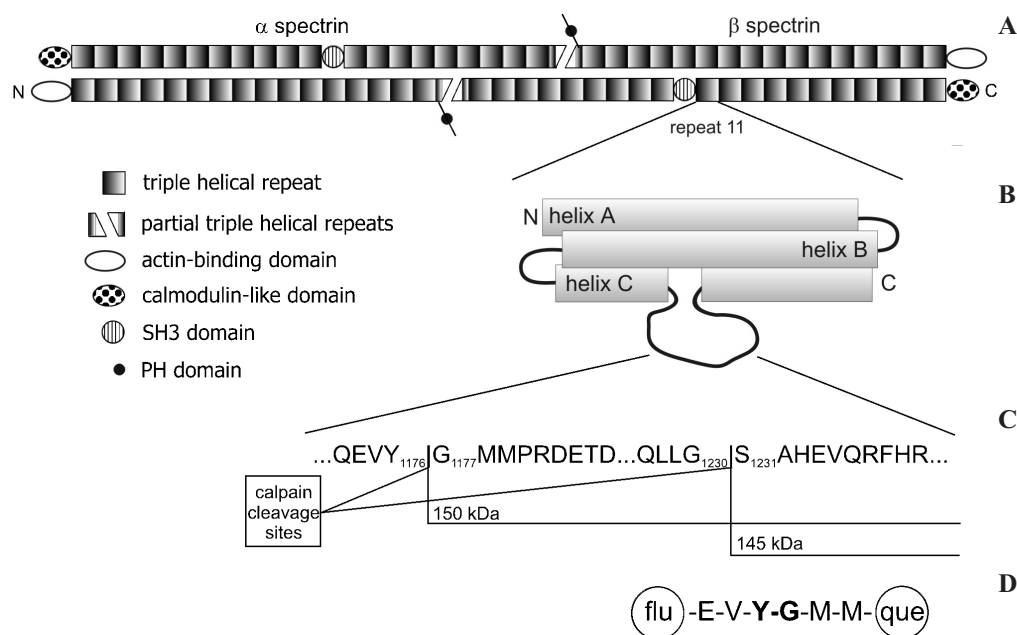


Figure 1. Structural features of spectrin and its calpain-recognition sites. (A) The α - and β -spectrin subunits differ from each other by several unique domains. Spectrin tetramer is formed by head-to-head interaction of two laterally associated $\alpha\beta$ -dimers [4, 7]. (B) The 11th repeat domain of vertebrate α II-spectrin contains a 35-residue exposed loop within helix C with calmodulin binding domain and calpain-cleavage sites [29]. (C) Calpain cleaves α II-spectrin between Y and G, producing a 150-kDa (molecular weight predicted by ProtParam tool (<http://us.expasy.org/tools/protparam.html>) (PMW): 148.8 kDa) fragment, and subsequently cleaves the region again, between G and S, producing a slightly smaller 145-kDa (PMW: 142,5 kDa) fragment [21]. (D) Construction of an internally quenched substrate derived from the α II-spectrin sequence for specific calpain activity measurements based on the FRET technique [28], flu fluorophore; que, quencher; cleavage site in boldface type.

Spectrin: the essential component of the membrane-skeleton

Spectrin is the main protein component of the cell membrane skeleton. It was first discovered in human erythrocytes [1]. The unique arrangement of spectrin, F-actin, protein 4.1 and ankyrin, with direct and indirect connections to the membrane, creates a filamentous network crucial for maintaining red-blood-cell shape and elasticity. Further investigations confirmed the presence of spectrin in mammalian nonerythroid cells [2] and in the majority of other eucaryotic cells.

Spectrin in its simplest form is an antiparallel heterodimer composed of a 280-kDa α subunit and a 247–460-kDa β subunit. However, within the skeleton it is a tetramer formed by head-to-head interaction of two heterodimers [3]. Generally, spectrin and related cytoskeletal proteins (e.g. α -actinin, dystrophin) share three main structural and functional elements, namely, EF-hands (Protein Data Bank (<http://www.rcsb.org/pdb/>) identifier (PDB ID): 1H8B), an actin-binding domain comprising CH domains (PDB ID: 1BKR) and an \sim 106-amino acid residue repeat segment (PDB ID: 1AJ3 and 1U5P) [4] (see fig. 1A). The conformation of the repeat unit is a triple helical coiled-coil bundle, and the helices are marked as A, B and C, of which A and C are parallel and B is antiparallel [5]. Subunit α contains 21 such segments, of which segment α 10

differs from the others by an \sim 60-residue-long insertion forming an SH3 domain (PDB ID: 1U06), a structural and functional motif present in many proteins participating in signal transduction. Both the SH3 domain and EF-hand motifs appear exclusively within the α chain of spectrin. On the other hand, subunit β contains 17 repeat segments in addition to an N-terminal actin-binding domain. Its C-terminus of variable length (50–250 aa residues) also differs from a typical repeat segment and contains multiple phosphorylation sites, but certain isoforms of β spectrin contain a PH domain (PDB ID: 1MPH), characteristic of proteins involved in signaling processes. Interestingly, spectrin repeats have recently been demonstrated to reversibly unfold and refold when subjected to forces up to 20 pN [6]. Therefore proteins with triple helical domains have the potential to function as nanosprings which can store energy and reduce deformations resulting from mechanical stress.

So far, two genes encoding α subunits of spectrin (α I and α II) and five genes for β subunits (β I–V), including a β heavy chain subunit (β V) have been described in humans. Additional diversity among spectrins is provided by alternative splicing. Taken together, the huge assortment of spectrin isoforms, characterized by specific localization in various cells and even in specific cell compartments, provides a wide range of biological membrane attributes [7]. The most extensively studied are isoforms: α I β I Σ 2,

which in neurons have been located in dendrites and soma; α II β III Σ 1 (also known as brain spectrin), which is located mainly in the axonal membrane and plays a crucial role in synaptic transmission [8, 9]; finally, the first discovered, is the erythrocyte spectrin α II β III Σ 1 – the main component of this cell skeleton, which is also expressed in a subset of neurons in the central nervous system [10]. As the most essential component of the membrane skeleton, spectrin is thought to be responsible for membrane stability, cell shape regulation and limitation of the lateral diffusion of membrane integral proteins. The multi-domain structure of spectrin molecules provides anchors for a group of proteins such as actin, protein 4.1, ankyrin, adducin and synapsin [7, 11], as well as for lipids [12, 13]. This feature – together with the domains responsible for binding of regulatory proteins (e.g. calmodulin) or homologous domains in signal-transducing proteins (as mentioned above) – may suggest participation of spectrin in signal transduction and propagation and regulation of many cellular processes.

Calpain: the multi-faceted protease

Calpain (EC 3.4.22.17), (calcium-dependent protease with papain-like activity) is a family of cytoplasmic cysteine proteases activated by calcium ions, widely expressed with both ubiquitous and tissue-specific isoforms in higher organisms. Homologues of calpain catalytic subunits are also present in other groups of organisms, including invertebrates, plants and fungi. The two ubiquitously expressed isoforms of the calpain superfamily, μ -calpain and m-calpain, are so far the best-characterized members of the superfamily and are called conventional calpains. They differ in calcium sensitivity; μ -calpain requires micromolar while m-calpain requires millimolar Ca^{2+} concentrations for activation. Both of them are heterodimers composed of a large ~80-kDa catalytic subunit (encoded by the *capn1* and *capn2* genes, respectively) (PDB ID: 1DF0) and a common ~30-kDa regulatory subunit (encoded by the *capn4* gene; PDB ID: 1NP8) [14]. The large subunit can be divided into four (I–IV) and the small into two (V and VI) domains. The protease domain II is composed of two subdomains (IIa and IIb) which contain the active sites Cys-105 and His-262/Asn-268, respectively, indicating that the inactive state of the protease in the absence of Ca^{2+} is caused by separation of the catalytic triad. This inactive conformation is stabilized by interaction of domain I with domain VI and interaction of IIb with domain III. Both domains IV and VI contain five tandem calmodulin-like EF-hand motifs. The last EF-hand motifs of each domain interact with one another to form a heterodimer [14].

Calpain exists in the cytosol in its latent form and translocates to membranes in response to influx of Ca^{2+} . Then the protease is activated by autolytic processing of domain I

and the following dissociation of the small subunit from the larger one [15]. Calcium-induced structural changes are prerequisite for activation to form a functional catalytic center. In the first stage, binding of Ca^{2+} to domains IV, VI and III (via the acidic loop region of the latter) leads to dissociation of the dimer. The second stage consists in rearrangement of the active site cleft caused by binding of two Ca^{2+} ions, one to the IIa and the other to the IIb subdomains. Calpains are inhibited by substances reacting with the cysteine in the catalytic triad in a nonspecific manner [16]. That residue is also sensitive to oxidants [17]. More specific are agents which inhibit calpain by interacting with the calmodulin domain. Activation of calpain in vivo remains under the control of its endogenous peptidic inhibitor, calpastatin. An additional checkpoint in calpain activity is its phosphorylation by a protein kinase A leading to restriction of domain III movement and arresting the enzyme in an inactive form [18].

To date, at least 14 mammalian calpains have been identified, together with 2 calpain small subunits. Various members of the calpain family, however, have structures that are rather divergent, probably because of their specialized physiological functions [19]. The EF-hand subfamily comprises the two above-mentioned conventional calpains, skeletal muscle-specific calpain 3 (its deficiency leads to a kind of muscular dystrophy), stomach smooth-muscle-specific calpain 8, calpain 9 (which plays a tumor-suppressing role in the mammalian digestive track) and the little known calpains 11 and 12. In the non-EF-hand subfamily one can include calpain 5, calpain 6 (which lacks the active-site catalytic cysteine), calpain 10 (identified as a type 2 diabetes susceptibility gene) and the most divergent members – calpains 7 and 13.

Calpain cleaves preferentially at Val, Leu or Ile residues in the P2 position of its target proteins, whereas the amino acids at the P1 site are less determined. Thus, sites susceptible to calpain activity should be found in many proteins, but only a small subset of intracellular proteins have been reported to undergo proteolysis, and most of them remain resistant. Furthermore, calpain cleaves its substrates into limited fragments resistant to further degradation. Calpain substrates include cytoskeletal proteins (e.g. spectrin, neurofilament proteins), membrane-associated proteins (e.g. ion channels and pumps, epidermal growth factor (EGF) receptors), enzymes involved in signal transduction, transcription factors and others [20, 21]. Thus, while operating, calpain has the capability to dramatically influence cell physiology or even directly participate in degenerative processes.

Spectrin versus calpain

Spectrin as a substrate for calpain has attracted a number of researchers interested in the physiology and pathology

of brain function. This protein was the first described to undergo calpain-mediated proteolysis in neuronal cells, and is particularly highly sensitive to it [22, 23], with increasing susceptibility in the presence of calmodulin [24] (see fig. 1B). Much attention has been given to digestion of the α II-spectrin subunit by calpains, as the process is involved in the spectrin-skeleton regulation in neuronal cells. In that case, one peptide bond is cleaved between Tyr 1176 and Gly 1177 of the 11th spectrin repeat unit, near the calmodulin-binding domain [25], resulting in two proteolytic fragments of nearly equal electrophoretic mobility (~150 kDa). Subsequently, calpain cleaves the region once more, producing a slightly smaller fragment (~145 kDa) (see fig. 1C) [21]. These fragments are the exclusive result of calpain action and can be detected by antibodies against α II-spectrin. Also, antibodies specific against calpain-mediated spectrin degradation products, which facilitate their localization within cells and tissues, were obtained [26, 27]. Identification of the calpain cleavage site within the molecule and relative resistance of spectrin to enzymatic cleavage by other proteases [26] helped to develop a fluorescence resonance energy transfer (FRET)-based probe for the specific detection of calpain activity applicable for model studies of neuronal disorders [28] (see fig. 1D).

Molecular modeling of the 11th repeat segment provided evidence that the calpain cleavage site (with the critical Tyr1176–Gly1177 bond) with its flanking calmodulin binding domain forms a highly exposed loop within helix C (see fig. 1B). Moreover, from site-directed mutagenesis experiments it was concluded that substitution of Val in the P2 position by Gly, Pro or Asp caused α II-spectrin to become resistant to calpain [29]. As those mutations subtly alter the conformation of the mentioned site, it appears that calpain recognizes specific secondary and tertiary conformational features of the site of hydrolysis rather than its amino acid sequence. Recent reports indicate that α II-spectrin is tyrosine-phosphorylated both in vitro and in vivo, and phosphorylation of Tyr1176 reduces its susceptibility to cleavage by μ -calpain [30, 31]. A reasonable conclusion is that spectrin may be a key point of signal convergence between tyrosine kinase/phosphatase and Ca^{2+} -mediated signal cascades.

Also, the β chain of spectrin exhibits a certain susceptibility to calpain [32], and it requires the presence of calmodulin [33]. But it has minor significance in studies of neuronal pathology. The recognition site in the β II Σ 1 is located between Ala2066 and Ala2067, while in the β II Σ 2 this site is located between Thr and Ala in the corresponding region, and surprisingly, in the β II Σ 1 it is located downwards of the putative cleavage site – between Trp2061 and Ala2062. It has been suggested [33] that calmodulin, coordinately with calpain, regulates the association of α II β II spectrin with actin filaments – as long as the β II chain is intact, the linkage exists. Since subcellular

localization of spectrin is different in neuronal cells (as mentioned above) and α I spectrin does not have a calpain-recognized site [25], it is possible that the effect of calpain-mediated proteolysis is different in the axon compared with the dendrites and soma.

Calpain activation at physiological conditions

At normal, physiological conditions, when Ca^{2+} concentration oscillates at 100 nM, slight proteolytic modification of some calpain substrates is associated with normal cell functioning. This may transduce a cellular signal, trigger membrane fusion or cell spreading. It is therefore not surprising that calpain-mediated proteolysis of α II-spectrin is a characteristic feature of the normal adult postmortem brain [34]. As that phenomenon is not correlated with either postmortem intervals and other clinical features or hypoxic/ischemic changes, it is probably a permanent process in the brain throughout life. The relationship between calpain-mediated proteolysis of spectrin and synaptogenesis or neurite extensions is evident [21, 35]. Creation of new synaptic contacts and formation of axonal growth cones [36, 37] during early development of the brain is facilitated by calpain, which tends to associate with membranes [38]. Physiological *N*-methyl-*D*-aspartate (NMDA) receptor stimulation induces the appearance of characteristic products of α II-spectrin degradation which are the effect of calpain-catalysed hydrolysis. The process is connected to synaptic changes that result in long-term potentiation and memory formation [39, 40]. By using the antibodies specific for degradation products, the calpain activity was located primarily in postsynaptic membranes of hippocampal pyramidal neurones and the process was sensitive to calpain inhibitors. Prolonged treatment of cultured hippocampal slices with selected ampakines – positive modulators of α -amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA) receptors, which enhance synaptic response and facilitate long term potentiation – also leads to calpain-mediated spectrin degradation [41]. It should be noted that within peripheral nerves, the persistent presence of a low concentration of calpain-induced spectrin degradation products can also be observed [42]. The fundamental role of calpains in biologically important processes has been demonstrated by genetic disruption of the regulatory subunit of murine calpain, as the *capn4*-deficient embryos died at midgestation displaying multiple defects [43]. On the other hand, μ -calpain-null mice have no apparent embryological defects and are viable and fertile [44]. This may suggest compensation of μ -calpain activity by m-calpain. However, the effect of μ -calpain disruption on neural tissue still remains to be examined. The latter, together with generation of m-calpain-deficient organisms, may considerably add to the current data presented here.

Calpain in neuronal death

Necrosis and apoptosis are two distinct forms of cell death and have different implications for the surrounding tissue [45]. Necrosis usually occurs when cells are physically or chemically injured to the point where they are beyond repair, and is indicated by massive ion influx, cell swelling, nonspecific DNA breakage and, eventually, spillage of the intracellular contents into the extracellular milieu, provoking damage to the whole tissue. Apoptosis, in contrast, does not induce inflammation or damage to tissue and is essential for the organism during development and later elimination of unwanted cells [46]. It is programmed, physiological cell death, usually characterized by its shrinkage, DNA condensation with further fragmentation and formation of apoptotic bodies. Necrotic cell death is, almost without exception, linked with massive Ca^{2+} influx and, consequently, calpain activation. Although another cysteine protease, but not Ca^{2+} -dependent caspases, plays a major role in apoptosis, substantial evidence indicates participation of calpains also in that process. As caspase 3 cleaves α II-spectrin in repeat 13, producing the apoptotic-specific ~120-kDa fragment [21], involvement of the two proteases in neuronal cell death can be determined from the spectrin breakdown pattern. It has been shown that activation of calpain is, in many circumstances, an early and contributing event in the development of cell death and pathology, with an increase in α II-spectrin degradation products as an early marker of its action [47].

Excitotoxicity and effects of toxins

Increased spectrin proteolysis accompanies intensive stimulation of NMDA receptors of hippocampal neurons [48, 49], which allow calcium ions to enter the cell and activate calpain. The process of extensive stimulation of excitatory amino acid receptors leading to neuron necrotic death is called excitotoxicity. Experiments carried out on cultured hippocampal neurons revealed that spectrin degradation products appeared as soon as 2 min after NMDA exposure [27], and their amount was proportional to the number of stimulated receptors [40, 50]. On the other hand, early symptoms of cell degeneration (tracked by propidium iodide staining) are observed much later, 30 min after the beginning of exposure to NMDA [51]. In vivo histological degenerative changes take place in the hippocampus as late as a few hours or even days following NMDA administration [49]. On the basis of in vitro studies with long-term hippocampal slices, it was shown that a 15-min NMDA infusion caused persistent spectrin degradation, whereas a 5-min infusion resulted in transient spectrin degradation [27]. Both spectrin proteolysis and cell degeneration processes could be inhibited by NMDA-receptor antagonists [27, 49, 50, 52], calpain messenger RNA (mRNA) antisense nucleotide [53] or calpain in-

hibitors [54]. The effect of NMDA on neurons depends on its dose [49] and the number of functional NMDA receptors. That is why hippocampal field CA1, with its high NMDA receptor concentration, manifests such intense spectrin breakdown products staining following excitotoxicity [53]. It should be noted that calpain-mediated cleavage of the NR2B subunit of NMDA receptors occurs in neurons and gives rise to active forms of these receptors after excitotoxic glutamatergic stimulation [55]. Moreover, this cleavage correlates spatially and temporally with spectrin breakdown in the hippocampus of epileptic rats [56]. It should be emphasized that while Ca^{2+} influx and calpain activation initiate excitotoxicity in neurons, other processes may contribute to the destructive pathways [48, 49]. Indeed, studies with cultured cerebellar granule cells showed that spectrin proteolysis induced by calpain and glutamate-mediated neurotoxicity are not causally related events [57]. Therefore, one may speculate that calpain-dependent cleavage of α II-spectrin may rather be an event directing a nerve cell to a reversible state of higher sensitivity. Experiments with cultures of rat hippocampal neurons confirmed that calpain was fully blocked by its inhibitors; however, the latter did not mitigate the evoked excitotoxicity [58]. This finding is in full agreement with the above speculation, making it likely a more common condition, yet in contradiction to the majority of other studies. The issue is even more complicated, as most of the observations are correlative, and as will be discussed below, the lack of calpain inhibitors of absolute specificity impedes efforts to prove causality. Although calpain-mediated spectrin proteolysis may not be a critical event in the pathway to neuronal death, it is undoubtedly one of the hallmarks of its initial stage.

Recently, AMPA receptor-mediated toxicity was described as an apoptotic process with a crucial role of calpain and caspase 3 in it, as indicated by α II-spectrin cleavage into characteristic products [59]. Early activation of calpain following stimulation of AMPA receptors as well as compromised neuronal survival were indicated in cultured hippocampal neurons [60]. Breakdown products of spectrin were detected as early as 15 min after exposure to kainic acid and calpain, but no caspase inhibitors showed a protective effect in cultured cells. In vivo, levels of spectrin degradation products were highest in the CA3 region and, to a lesser extent, in the CA2 and CA1 regions of the hippocampus after kainate injection, which is in accordance with the distribution of adequate receptors. The first proteolysis symptoms were detected 3–16 h following kainic acid injection, reaching a maximum at 24 h and persisting for 9 days, and were dose dependent [49, 61, 62]. Corticosterone appeared to be an indirect amplifier of kainic-induced and calpain-mediated spectrin proteolysis [63], whereas some polyamines, the concentration of which increases after kainate treatment, stimulate calpain activity more directly [61, 64].

There are other mechanisms of pathological calpain activation which do not directly engage glutamatergic systems. Depolarization of neurons in culture with KCl results in calpain activation in an extracellular calcium-dependent mode, and calpain inhibitors appear to be neuroprotective [65]. Maitotoxin, as a calcium channel opener, is an effective activator of cellular calpain and necrosis. A characteristic 150-kDa spectrin breakdown product was found to be released into cell-conditioned media from neuroblastoma cells treated with maitotoxin, and the increase in product was related to cell death in a time-dependent manner [66]. Rat cerebellar granule neurons treated with either NMDA or kainate behaved similarly. Once again, a cysteine-protease inhibitor attenuated neuronal cell death and reduced expression of the specific calpain-mediated α II-spectrin breakdown products after maitotoxin injury [67]. Intrahippocampal injections of colchicine, an axonal transport-blocking alkaloid, caused spectrin degradation within 24 h, to the largest extent in the dentate gyrus [68]. Neurotoxicity of pesticides such as trimethyltin revealed similar symptoms [69]; these were, however reducible via positive modulation by ampakines [70]. Although andamide is an endo cannabinoid that acts on NMDA receptors, it appeared to induce neurotoxicity insensitive to these receptor antagonists; moreover, even though apoptosis was manifested during the process, calpain, but not caspase, was activated [71]. Mitochondrial inhibition by 3-nitropropionic acid may lead to initiation of oxidative stress and calpain-mediated spectrin proteolysis [72], but administration of calpain inhibitors shifted the cell death morphology from necrosis towards apoptosis [73], which is in agreement with the calpain-caspase cross-talk hypothesis (see below). Enhanced calpain activity combined with increased cleavage of α II-spectrin was also observed in alcoholic neurodegeneration [74] and methylmercury treatment of cortical neurons [75], with a possible role of NMDA receptors in the latter. With respect to the data presented, potential artifactual calpain activation should be considered. In many cases control samples showed significant basal calpain activity and α II-spectrin degradation, which may not necessarily result from physiological activity. Although, the differences in magnitude may be useful in terms of comparative studies, they are still not quantitatively meaningful.

Ischemic and traumatic neuronal death

In neurological events associated with ischemia, calpain appeared to be one of the major contributors to injury [76–78]. Remarkably, casein zymography and changes in expression levels of calpain mRNA implicated involvement of both conventional calpains in ischemic neurodegeneration [78]. Extensive research efforts have focused on cerebral ischemia, which as a result of strokes and cardiac arrest, usually leads to death and is the main cause of

disability. Brain vulnerability to ischemia was elucidated in experiments with transient carotid artery occlusion, in which the CA1 region of the hippocampus was the most sensitive [26]. Considering the fact that within this region NMDA receptors are most abundant and that antagonists of these receptors ameliorate ischemic injury [79, 80], NMDA receptor mediation in ischemia seems to be obvious. It was shown that a 10-min ischemia induces rapid α II-spectrin proteolysis by calpain; however, maximum spectrin degradation was found in the CA1 hippocampal region within 15 min after reperfusion, and after 4 days this region experienced a second, more intense wave of proteolysis [81]. The latter, drastic and persistent phase, which can be observed even earlier – 4–24 h after the incident [82] – should be considered to be a more direct cause of the degeneration of neurons. A bimodal pattern of calpain-mediated α II-spectrin degradation in the hippocampus, cortex and striatum was observed, with an initial increase after 1 h and calpain activity localized to dendritic fields followed by a more prominent secondary increase 36 h after reperfusion with calpain activation in the neural soma and subsequent neuronal degeneration [83]. Owing to studies using antibodies against calpain-generated α II-spectrin proteolytic fragments [26, 82], correlations between spectrin proteolysis by calpain and a progressively compromised neural cell structure and function in vulnerable brain areas became evident. However, in some cases it may be manifested by a continuing, steady – rather than biphasic – accumulation of spectrin breakdown products [84]. Moreover, calpain activity must persist beyond the initial phase to irreversibly destroy neurons. In vitro studies with hippocampal slices showed induction of spectrin proteolysis as early as after 5 min of hypoxia [85]. Thus, initial spectrin proteolysis appears to be unrelated to reperfusion, but rather seems to be a response to hypoxia. In contrast to most studies, one reports that while cultured hippocampal neurons subjected to ischemia were marked by calpain-induced spectrin proteolysis followed by plasma membrane integrity loss, a number of them degenerated without pronounced spectrin cleavage [86], calling into question the role of calpain in degenerative processes. Nevertheless, within some subpopulations of neurons early calpain activity on other cytoskeletal proteins could render the cell more vulnerable to ischemic damage which might be executed by some other proteolytic cascades. Calpain activation, in combination with caspase-3 activation, may contribute to apoptotic cell death observed in ischemia both in vitro [87] and in vivo [88], with respect to spectrin breakdown product appearance. Currently, it becomes more and more evident that postischemic neuronal death may involve a combination of necrotic and apoptotic processes even at the level of the individual cell [89, 90], as calpain can function upstream of caspases, and subsequently caspase-3 mediates calpastatin degradation, thus indirectly activating calpain (see fig. 2).

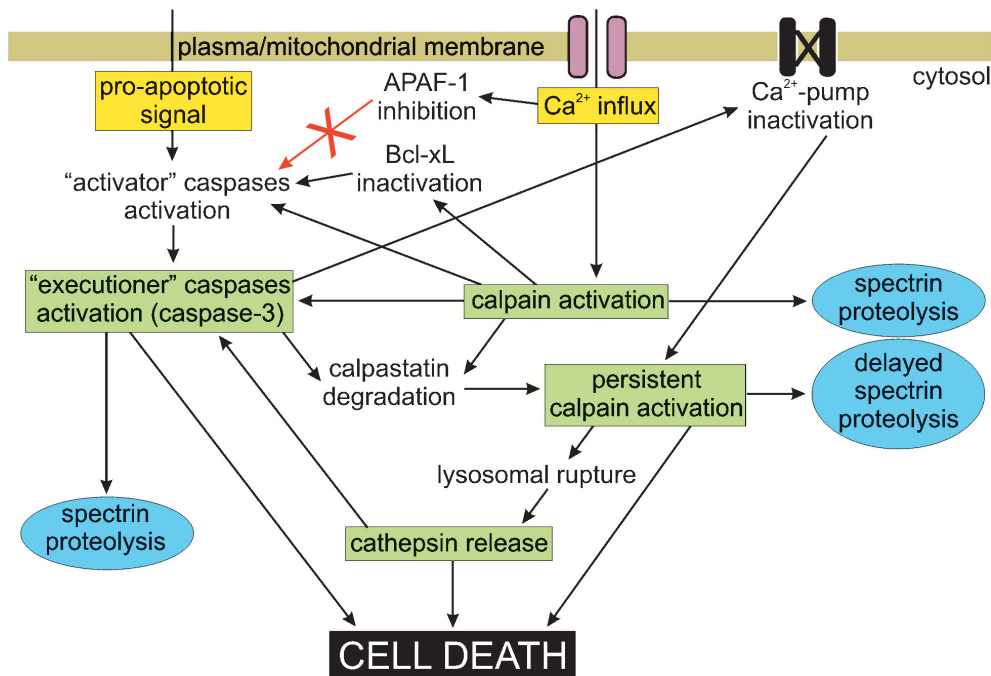


Figure 2. Major interactions between calpain and caspase in neuronal injury. A mixed profile of neuronal degeneration (necrotic and apoptotic) can be observed in many examples of insult [103], and a greater or lesser proportion of apoptotic degeneration may depend upon the time course over which the insult initially develops [91]. An elevated intracellular Ca^{2+} level activates calpain and initiates a calpain-cathepsin cascade leading to necrotic neuronal death [77]. Ca^{2+} influx may also inhibit caspase activation by triggering calpain-independent degradation of APAF-1 [92]. However, calpain can directly activate both 'executioner' caspase-3 [47] and 'activator' caspase-12 by cleaving them, as well as indirectly by cleaving Bcl-xL and making it proapoptotic [90]. Cathepsins have also been reported to activate caspase-3 [77]. On the other hand, caspase-3 also contributes to neuronal necrosis by cleavage and inactivation of Ca^{2+} pumps [76], thus producing sustained Ca^{2+} elevation. Calpastatin degradation either by caspase-3 or by calpain may lead in certain situations to persistent calpain over-activation, which is most probably connected with biphasic calpain-mediated spectrin degradation in ischemic and traumatic neuronal injury [90,114]. Caspase-3 also contributes to spectrin proteolysis, producing characteristic 150-kDa (PMW: 147,7 kDa) and 120-kDa (PMW: 114 kDa) fragments [21]. Finally, all the three proteases, caspase-3, calpain and cathepsin, participate in multiple proteolytic events leading to cell death.

This mechanism may explain why, in nearly all cases, calpain activation persists long after transient Ca^{2+} levels increase at the beginning of ischemia. The sustained calpain activity contributes to lysosomal membrane disruption and leakage of lysosomal enzymes, including cathepsins, which execute neuronal necrosis [77].

It has been shown that calpain-induced α II-spectrin breakdown products appeared and could be quantified from cell-conditioned media [66]. Further reports revealed that calpain- and caspase-3-mediated spectrin breakdown products may serve as a biochemical markers of central nervous system ischemia [93]. In cerebrospinal fluid after middle cerebral artery occlusion followed by reperfusion, spectrin-derived products of calpain activity were particularly abundant, which may be a useful diagnostic indicator of cerebral infarction in a rodent model of a transient focal stroke in rats. According to the definition, biomarkers should appear in accessible biological material (blood or cerebrospinal fluid), make it possible to predict the magnitude of injury, possess high sensitivity and selectivity, and provide information on the mechanism of injury (as distinct from surrogate markers of injury).

Numerous proteins have been considered as potential biochemical markers of ischemic [94] and traumatic [95, 96] brain injury. Immunoblots of α II-spectrin provide concurrent information about calpain [97] and caspase-3 [98] activation, the two important regulators of cell death following traumatic brain injury, thus offering insight into the pathological mechanism of the trauma. On the basis of traumatic spinal cord injury studies, it was suggested that calpain produced by astrocytes may participate in spectrin proteolysis and further axon degeneration after injury [99]. It is necessary to emphasize that similarly to previously mentioned pathological conditions, increases in calcium ions via voltage- and receptor-gated calcium channels have been reported in central neuronal system trauma in vivo [100, 101]. Following cortical impact traumatic brain injury in rodents, intact α II-spectrin was diminished in brain tissue and increased in cerebrospinal fluid from 24 to 72 h after injury, whereas calpain-specific spectrin breakdown products increased in both brain and cerebrospinal fluid after injury [102]. According to the pattern of spectrin proteolysis, activation of both caspases and calpains is evident, highlight-

ing the heterogeneity of the pathological and molecular responses to traumatic brain injury [103]. However, calpain seems a more important effector of cell death than caspase-3 in traumatic brain. Recently it was shown that the level of spectrin breakdown products in the ipsilateral cortex and cerebrospinal fluid increases with the magnitude of injury. Moreover, its increased levels in cerebrospinal fluid after 24 h of traumatic brain injury indicate the presence of a lesion [104]. These findings highlight the cerebrospinal fluid spectrin breakdown product level as a promising biomarker of injury and provide a foundation for future assessment of the utility of this marker in human brain injury.

Development of specific biomarkers provides researchers and physicians with powerful tools for diagnosing injuries and their further monitoring, as well as guiding appropriate administration of therapeutic compounds. Calpain inhibitors have been shown to have significant neuroprotective properties in models of both ischemia [78, 105–109] and traumatic injury [110–113]. However, there is no mechanistic explanation for the effects of calpain inhibitors, as they do not reveal sufficient specificity and may act on cathepsins or on proteasomes. One of the exceptions might be an α -mercaptoacrylic derivative which turned out to be a selective, cell-permeable calpain inhibitor and made neurons more resistant to hypoxic/hypoglycemic challenge [108]. On the other hand, it appeared recently that calpain-specific inhibitor MDL 28170 affects caspase-3 as well [109]. Biphasic calpain activation in ischemic (see above) and traumatic [114] neuronal injuries implies a window of opportunity for therapeutical efficacious inhibition of calpain that may extend as long as 6 h after the insult [106]. Nevertheless, the therapeutic window may be shorter after focal injuries compared with diffuse traumatic brain injuries due to the observed differences in the time course of α II-spectrin degradation and brain neurodegeneration [115]. Combined use of both calpain and caspase inhibitors provided additive neuroprotection, which is consistent with calpain-caspase cross-talk in neuronal cell death [116] (see fig. 2). Calpain (and caspase) inhibition not only increases the window of therapeutic opportunity, as opposed to blocking receptor activation (see fig. 2), but it might also be less detrimental to normal cell function than intervention at the receptor level.

Aging and age-related diseases

Consistent with observations of increased calcium ion influx and its intracellular concentration rise in aging tissues, calpain is additionally activated during aging [47, 117]. In the brain, aging is synchronised to progressively increase of calpain-mediated spectrin proteolysis [118]. However, the magnitude of the latter rises with age (3–30 months) in the telencephalon but not in other parts of the

mouse brain. Recently, α II-spectrin fragmentation by calpain was found in myelin and microglia in the white matter of the aged rhesus monkey [119], suggesting that these represent a major source of the increase in activated calpain in the aging brain and implicating activated microglia in its pathology. Studies of the *klotho* gene, which encodes a membrane protein that is thought to be a calcium and phosphorus homeostasis regulator, helped to determine whether calpain activation is a cause or effect of the aging processes. In a *klotho*-deficient mouse that showed phenotypes resembling human aging, abnormal calpain activation, calpastatin depletion and degradation of α II-spectrin was observed even before the appearance of aging symptoms [120]. However, restoring serum concentrations of calcium and phosphorus improves the aging phenotype [121].

In Alzheimer's disease, chronic toxicity of deposited β -amyloid peptides in parallel with buildup of the glutamate level may lead to persistent intracellular calcium elevation in susceptible neurons and thus calpain activation, which in turn may contribute to multiple aspects of disease development [47, 122]. Calpain is involved in generation of a pathological, hyperphosphorylated form of tau by activating cyclin-dependent kinase 5 (cdk5) and mitogen-activated protein kinase (Erk/MAPK) [116, 123], and most probably in processing the precursor β -amyloid protein [47]. Calpain-specific α II-spectrin degradation appeared in the frontal cortex in an animal model of cholinergic degeneration [124]. This supports the idea that spectrin degradation is related to the neuronal degeneration encountered in Alzheimer's disease and that calpain activation develops at an early stage in the disease process. Additionally, high levels of calpain-induced spectrin breakdown products appear in the cerebrospinal fluid of patients with Alzheimer's and Pick's disease [47]. Spectrin breakdown suggesting calpain activation is also connected with the loss of nigral dopamine neurons in cases of Parkinson's disease [125]. However, it is not known which phase of the disease calpain activation represents.

Concluding remarks

Increased calpain activation is an early event in heterogenic pathologic and molecular responses to injury of various cells, which is especially well documented in the case of neural tissue, though the exact mechanism of neurodegeneration remains to be elucidated. Spectrin, in particular its α II-subunit, is a preferential endogenous calpain substrate; therefore, breakdown products of spectrin may serve as an early marker of calcium-activated proteolysis. Observations of progress and the range of calpain-mediated α II-spectrin breakdown products provide not only information about the magnitude and consequences

of the insult, but may also elucidate its mechanisms. Moreover, as the characteristic spectrin-derived products also appear in readily accessible material, this may facilitate better monitoring of the progression of the damage, response to medical intervention and prediction of its outcome. Advancements in antibody-based specific identification technologies will undoubtedly facilitate development of fast, sensitive, and easy-to-operate systems for research and clinical usage.

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