Calpain and caspase: can you tell the difference?
Kevin K.W. Wang

Both necrotic and apoptotic neuronal death are observed in various neurological and neurodegenerative disorders. Calpain is activated in various necrotic and apoptotic conditions, while caspase 3 is only activated in neuronal apoptosis. Despite the difference in cleavage site specificity, an increasing number of cellular proteins are found to be dually susceptible to these cysteine proteases. These include α- and β-fodrin, calmodulin-dependent protein kinases, ADP-ribosyltransferase (ADPR/PARP) and tau. Intriguingly, calpastatin is susceptible to caspase-mediated fragmentation. Neurotoxic challenges such as hypoxia-hypoglycemia, excitotoxin treatment or metabolic inhibition of cultured neurons result in activation of both proteases. Calpain inhibitors can protect against necrotic neuronal death and, to a lesser extent, apoptotic death. Caspase inhibitors strongly suppress apoptotic neuronal death. Thus, both protease families might contribute to structural derangement and functional loss in neurons under degenerative conditions.


A POPTOSIS, or programmed cell death, is a form of physiological cell death characterized usually, but not always, by the presence of DNA condensation in the nuclei, DNA fragmentation at the nucleosome linkage regions, cell shrinkage and the ultimate formation of apoptotic bodies1. It is induced by the organism during development and at other stages to eliminate unwanted cells. Necrosis usually occurs when cells are injured by extreme physical stress or chemical challenges to the point where they are beyond repair, and is characterized by the presence of massive ion influx, mitochondrial swelling, cell swelling, cytoplasmic DNA breakage in the nuclei and, ultimately, plasma-membrane rupture1. Unscheduled apoptosis might also be triggered when cells face some certain physical (for example, hypoxia) or chemical challenges (for example, toxins). It is also conceivable that other forms of cell death exist but have not yet been identified.

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neuronal death has also been reported in various chronic neurodegenerative conditions, such as Huntington’s disease (HD), Alzheimer’s disease (AD), amyotrophic lateral sclerosis (ALS) and AIDS-associated dementia. Yet, other researchers have evidence that does not support apoptotic neuronal death as having a major role in models of cerebral ischemia and glutamate toxicity, and therefore apoptotic and necrotic cell death are both probably occurring in these disorders. Recent studies have confirmed that in cultured neurons, neurotoxic challenges, such as hypoxia or hypoglycemia, neurotoxin administration [excitotoxins, 1-methyl-4-phenylpyridinium (MPP+)], metabolic inhibition (for example, 3-nitropropionic acid), and β-amyloid peptide or NO treatment could also result in both neuronal necrosis and apoptosis.

**Calpain in necrotic death**

Over the past decade, the significant focus of research was on calpain-mediated proteolysis, and its contribution to necrotic neuronal death in ischemic and excitotoxic neuronal injury. The two ubiquitous calpains (m- and µ-calpain) exist as a pro-enzyme heterodimer (80 kDa–29 kDa) in resting cells but this is activated by Ca2+ and autolytic processing (to produce a heterodimer 78 kDa–18 kDa; Table 1). Physiologically, the activity of these calpains might also be regulated by the endogenous protein inhibitor calpastatin (Table 1). Calpains could become over-activated under extreme conditions that result in sustained elevation of cytosolic Ca2+ levels, which is generally associated with necrosis. For example, in Ca2+-ionophore-treated cells (such as A23187-treated Molt 4 cells), Ca2+ rushes into the cells through the pore-forming compound, A23187. In glutamate-treated rat cerebrocortical neurons, Ca2+ enters through the ionotropic glutamate receptors. Similarly, maitotoxin, a potent marine toxin that opens both voltage- and ligand-gated Ca2+ channels, also induces rapid and massive Ca2+ influx and subsequent calpain activation. The mode of cell death induced by hypoxia or ischemia has been investigated, but no DNA laddering or condensation has been found. General DNA degradation and dissolution, rather than condensation, were observed: properties that are associated with necrosis.

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 of target proteins are rather diverse (for example, Tyr, Glys, Arg) (Table 1). As expected, most proteins contain sites that are susceptible to the actions of calpain, yet only a small subset of cellular proteins have been reported to be vulnerable, while the majority of cellular proteins are resistant. In addition, unlike digestive proteases (such as trypsin), calpain substrates are cleaved into ‘limited fragments’ without further degradation (Table 1). Calpain substrates include cytoskeletal, plasma-membrane-associated proteins (such as epidermal growth-factor receptor, platelet-derived growth-factor receptor), and signal transduction and calmodulin-dependent proteins and transcription factors (for a review, see Refs 13,16).

TABLE 1. Comparing and contrasting the properties of caspase 3 and calpain

<table>
<thead>
<tr>
<th>Caspase 3</th>
<th>Calpain</th>
</tr>
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<tbody>
<tr>
<td>Protease class</td>
<td>Cysteine protease (IAP1, IAP2, NAIP)</td>
</tr>
<tr>
<td>Endogenous inhibitor(s)</td>
<td>Inactive pro-enzyme (32 kDa)</td>
</tr>
<tr>
<td>Raising mode</td>
<td></td>
</tr>
<tr>
<td>Activation mode</td>
<td>Proteolytic processing (to 17 kDa–15 kDa)</td>
</tr>
<tr>
<td>Preferred cleavage site (*)</td>
<td>Subset of cytoskeletal, cytosolic and nuclear proteins or protease-sensitive enzymes</td>
</tr>
<tr>
<td>Endogenous substrates</td>
<td>Produces limited proteolytic activation Most forms of apoptosis</td>
</tr>
<tr>
<td>Consequence of substrate proteolysis</td>
<td></td>
</tr>
<tr>
<td>Cell-death involvement</td>
<td>Inhibitors as neuroprotectants</td>
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**Caspases in apoptosis**

The discovery that a protein required for apoptosis in the nematode, Caenorhabditis elegans, CED-3 is homologous to the mammalian interleukin-1β-converting enzyme (ICE) led to the discovery of a large number of ICE-like proteases (renamed caspases) and their roles as mediators of apoptosis in a wide range of the cell types. Caspase 3 (previously called CPP32) is of particular interest as it appears to be a common downstream apoptosis effector. It exists as proenzyme (pro-caspase 3) in most cells, including neurons, and is processed and activated by caspase 9 or caspase 8 to the heterodimeric form (17 kDa–12 kDa) by two distinct pathways. First, in a mitochondria-dependent pathway, the complexing of mitochondrial release cytochrome C and apoptotic protease-activating factor 1 (APAF1) to caspase 9 leads to its dimerization and autolytic activation, which in turn processes and activates pro-caspase 3 (32 kDa). Second, through activation of death-domain containing receptors (such as tumor necrosis factor α receptor 1 (TNFR1), FAS), the receptors recruit adapter protein(s), which also contain death domains (FADD (FAS-associated death-domain protein) and TNFR1-associated death-domain protein (TRADD)). The TRADD-FADD complex or receptor-associated FADD then induces the autolytic activation of the associated caspase 8, which activates caspase 3 proteolytically. In resting cells, caspase 3 might be suppressed directly by inhibitor proteins, such as inhibitor of apoptosis proteins 1 and 2 (IAP1 and IAP2) or neuronal apoptosis inhibitory protein (NAIP) (Table 1).

**Abbreviations:** IAP, inhibitor of apoptosis protein; NAIP, neuronal apoptosis inhibitory protein.
Calpain and caspase: diagnostic markers for neuronal apoptosis versus necrosis

Non-erythroid α-spectrin (also called α-fodrin) is degraded to a 120 kDa fragment, spectrin breakdown product 120 (SBDP120) in apoptotic neurons but not in necrotic neurons 14. Yet, calpain-mediated α-spectrin breakdown to a 150 kDa and 145 kDa doublet is not only present in necrotic neuronal death, but also in most forms of neuronal apoptosis 15,23,24. These data were obtained primarily by detection of calpain autolysis and specific α-spectrin II fragments that are generated by calpain (calpain-specific SBDP150 and SBDP145; see Fig. 1). Calpain inhibitors (for example, calpain inhibitor 1 and PD150606) appear to inhibit apoptosis only in specific systems (cerebellar granule neurons, neutrophils, etc.) 15,23,24 (Table 1). Most recently, Knepper-Nicolai showed that α-actinin and actin degradation was mediated by calpain in constitutive apoptosis in aged human neutrophils, and that a calpain inhibitor (calpeptin), in combination with a proteasome inhibitor (lactocystatin), suppressed this form of apoptosis 15.

Distinct α-spectrin breakdown patterns generated by calpain and caspase: diagnostic markers for neuronal apoptosis versus necrosis

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cell death. In apoptosis, proteolysis is likely to be important in suspending cell function by disabling a number of enzymes involved in signal transduction, which disables the mechanisms that allow the cell to repair its DNA or to go through the cell cycle, and degrades its cytoskeleton network, causing membrane blebbing to occur and the subsequent phagocytosis of the apoptotic bodies by macrophages. In necrosis, although not necessarily by design, calpain-mediated proteolysis undoubtedly has a similar role in disabling signal transduction, membrane and cytoskeleton integrity, and the nuclear functioning of the cell. Furthermore, calpain might facilitate the apoptotic cell death by aiding caspases involved in the proteolysis of cellular proteins. It seems that calpain and caspase indeed share a number of dual substrates and a few related substrates (Table 2).

The nonerythroid β-spectrin, in addition to α-spectrin is also susceptible to both calpain and caspase 3 (Ref. 34). Again, adjacent calpain and caspase-3 cleavage sites lead to 110 kDa β-MDPS, while caspase cleavage at another major site produces an 85 kDa fragment. The simultaneous degradation of α- and β-spectrin in apoptosis (and necrosis) might compromise the cytoskeletal integrity, and possibly membrane permeability, significantly. Vimentin is an intermediate filament of the cytoskeleton. It was previously found to be degraded by calpain in lens-cell network and its cleavage sites have been mapped out. Recently, its degradation was also observed in tamoxifen-treated apoptotic skin fibroblasts and in ionizing-radiation-induced apoptosis in prostate epithelial tumor cells (Ref. 40). On the basis of its sensitivity observed in tamoxifen-treated apoptotic skin fibroblasts, PARP is degraded by caspase to a distinct 89 kDa fragment. During apoptosis, the 113 kDa ADPRT/poly(ADP) ribose polymerase (PARP) is the most-well-known substrate for both proteases provides the best protection. In vitro, PARP cleavage pattern obtained in necrotic cells (Ref. 50); when MTX is used, ADPRT/PARP is cleaved into 70 kDa and 40 kDa fragments that are sensitive to calpain-inhibitor I in necrosis, but the extent of calpain-mediated proteolysis in necrosis is much less than that observed by caspase-3-mediated apoptosis (Ref. 51). It has also been established that calmodulin-dependent protein kinase IV (CaMKIV) is fragmented by caspase 3 and calpain in staurosporine-induced apoptosis, but cleaved by calpain only in MTX-induced necrosis (Ref. 34). Here, caspase 3 produces an immunoreactive 35 kDa N-terminal fragment, while calpain produces a 38 kDa N-terminal fragment, in parallel with its proteolysis during apoptosis, a corresponding decrease in CaMK activity is seen in these cells. It has previously been shown that CaMKII is sensitive to calpain proteolysis (Ref. 35). In a recent study, CaMKII was also found to be sensitive to caspase-3-mediated proteolysis in apoptotic cells (Ref. 54). Tau is also susceptible to cleavage by both calpain (Ref. 36) and caspase 3, and similarly, actin, a major spectrin-binding protein, is degraded by both proteases. Actin, a major spectrin-binding protein, is degraded by both proteases and is also found to be in a calpain-inhibitor-I-sensitive state (Ref. 34). Inhibition of proteolysis by caspase-3-mediated apoptosis (Ref. 53).

Another recent study has described the purification of a non-caspase protease that cleaves bovine ADPRT/PARP (Ref. 52). This protease was identified as bovine m-calpain. The fragmentation of ADPRT/PARP by calpain in vitro again yields fragments that range from 70 kDa to 40 kDa in size, similar to the ADPRT/PARP cleavage pattern obtained in necrotic cells (Ref. 36), when MTX is used, ADPRT/PARP is cleaved into 70 kDa and 40 kDa fragments that are sensitive to calpain-inhibitor I in necrosis, but the extent of calpain-mediated proteolysis in necrosis is much less than that observed by caspase-3-mediated apoptosis (Ref. 36).

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Degradation

Pro-caspase 3
dependent protein kinase II; PKC; protein kinase C.

Abbreviations: ADPRT, ADP-ribosyltransferase (also known as PARP); CaMKII, Ca2+
and nuclear substrates, resulting in functional and structural destruction of the neuronal cell. Hence, caspase 3 and calpains further degrade a subset of important cytosolic, cytoskeletal
membrane permeability to Ca2+
caspase-8 pathway leads to processing and activation of caspase 3. The action of caspase 3
interactions.

Fig. 2. The roles of calpain and caspase in protein degradation in neuronal death and their
interactions. A pro-apoptotic signal, via the mitochondrial caspase-9 pathway or receptor-linked
caspase-8 pathway leads to processing and activation of caspase 3. The action of caspase 3
on cortical cytoskeletal and plasma membrane integral proteins probably compromises the
membrane permeability to Ca2+, leading to elevated intracellular Ca2+ levels. Caspase 3 also
degrades calpastatin, which, in combination with these events, facilitates calpain activation.
Hence, caspase 3 and calpains further degrade a subset of important cytoskeletal, cytoskeletal
and nuclear substrates, resulting in functional and structural destruction of the neuronal cell.
Abbreviations: ADPRT, ADP-ribosyltransferase (also known as PARP); CaMKIV, Ca2+/calmodulin-
dependent protein kinase IV; PKC, protein kinase C.

by caspase 3 at two sites in the extracellular N-terminal domain and at a third site near the C terminus. Again, the cleavage in the C-terminal region, although not at the exact β and γ cleavage sites, helps to facilitate the production of β-amyloid peptides.

The Ca2+-dependent α, β, and γ isoforms of protein kinase C (PKC) have been found to be degraded by calpain in vitro and under a variety of conditions in cell culture. In fact, calpain is suspected to have a role in the downregulation of PKC activity. Interestingly, two Ca2+-independent PKC isoforms (δ and ε) have now been shown to be degraded by caspase 3, but not the Ca2+-dependent isoforms.

The PDI-specific phospholipase Cβ3 is activated proteolytically by calpain, whereas phospholipase A2 (PLA2)β is activated proteolytically by caspase 3. However, Adam-Klages reported that cytosolic PLA2 was actually inactivated by caspase cleavage. Both phospholipid hydrolases are thought to be involved in transmembrane signal transduction. While the anti-apoptotic protein BCL2 is cleaved by caspases to produce a truncated form with pro-apoptotic properties, BAX is also found to be truncated by calpain (not caspase) in drug-induced apoptosis of HL-60 cells and in staurosporine-treated SH-SY5Y cells. It has been reported previously that calpastatin (CAST) is degraded by calpain in A23187-treated cells. More recently, the 105 kDa high-molecular-weight form of CAST was also found to be very sensitive to caspase proteolysis during early apoptosis (staurosporine-treated SY5Y cells, anti-FAS-treated Jurkat cells and TNFα-treated U937 monocytic leukemia cells) (Ref. 2) (Fig. 2). This proteolysis, which is thought to involve caspases 1 and 3, produces a major 75 kDa fragment of CAST and several low-molecular-weight fragments. One can envision that, in early apoptosis, caspase 3 begins to degrade CAST and compromise plasma-membrane and cortical cytoskeleton integrity simultaneously, which results in the elevation of intracellular Ca2+ levels. The overall effects form a trigger for increasing calpain activity. Hence, calpain furthers the proteolytic destruction initiated by caspase 3. While it might not be directly related to cell death, it is worth noting that caspase 1 (ICE) is the key enzyme that processes pro-interleukin 1β (Ref. 74), while calpain is a major protease for processing pro-interleukin-1α (Ref. 75, Table 2).

Most recently, pro-caspase 3 was found to be a calpain substrate. Instead of processing pro-caspase 3 (32 kDa) to its activated form (17 kDa–12 kDa), calpain truncated it in the N-terminal region to a 30 kDa, which appeared to be less vulnerable to being activated by caspase 8 or caspase 9. Thus, these findings add to the complex interaction between the two protease systems. Two proteases: two forms of neuronal death

In this article, the role of two cytosolic cysteine proteases (calpain and caspase 3) in two forms of neuronal death (necrosis and apoptosis) has been highlighted (Table 1). On the basis of genetic data and the elucidation of the apoptosis cascade, it is apparent that caspase has a central role in transducing the apoptosis signal. Caspase-3 activation is a unique feature of apoptosis. By contrast, necrotic cell death is almost without exception, associated with massive Na+ and Ca2+ influxes and, thus, calpain activation. Conversely, apoptosis might or might not be associated with Ca2+ overload. In anti-FAS-treated Jurkat T cells and other cells, little evidence of calpain activation is seen. The difference could be the rapid onset of apoptosis and the resultant cell death induced by anti-FAS (which takes 3–4 h). It has also been found that EGTA treatment (2 mM) leads to calpain-independent but caspase-3 dependent apoptosis. In this case, the presence of extracellular EGTA sequesters intracellular Ca2+ from the cells, and therefore prevents calpain activation. Yet, it has been demonstrated repeatedly that both caspase and calpain are activated in apoptotic neurons (as determined from the spectrin breakdown pattern). Given the importance of Ca2+/homeostasis and the abundance of calpains in neurons, calpain activation can be expected in these extraneous conditions. Thus, in the case of neuronal apoptosis, in addition to caspase 3, calpain appears to assist in the degradation of key cellular proteins and, thus, can be viewed as having an auxiliary and augmentative role in the transduction of neuronal apoptosis (Fig. 2).
The striking similarity between the identified sub-
states for calpain and caspase raise the possibility that both protease families are active participants in the irreversible destruction or functional alteration of a subset of important cellular enzymes or structural pro-
teins, and, thus, contribute to structural derangement and functional loss in neurons under acute neurode-
generative conditions (Table 1). A number of earlier and recent publications have illustrated that calpain and caspase inhibitors both have impressive neuropro-
tective effects in in vivo models of cerebral ischemia2,3,4 (Table 1). Interestingly, calpain and caspase inhibitors appear to have additive neuroprotective effects against neuronal injury, which is of great interest to explore the benefit of combination-therapy approach that involves both a calpain inhibitor and a caspase inhibitor, and in vivo models of neurodegenerative dis-
es where both neurosis and apoptosis have been implicated.

Selected references
Acknowledgements

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Retinal mosaics: new insights into an old concept
Jeremy E. Cook and Leo M. Chalupa

It has been known since the middle of the 19th century that different neuronal types are distributed across the retinal surface in non-random arrays: indeed, these arrays, called ‘mosaics’, have long been considered to be a fundamental feature of retinal organization. However, until recently, little was known about how such mosaics are established during development. In the hope of stimulating further research, this article reviews the current status of three very different approaches to this intriguing general problem. The first postulates arrays of molecular markers, which are produced by specific cell types shortly after their final mitotic divisions and could be influential in the differential of other cell types. The second involves the elimination of wrongly positioned cells through the third postulates arrays of molecular markers, which are produced by specific cell types shortly after their final mitotic divisions and could be influential in the differential of other cell types.

All progress in science depends on the recognition of patterns and relationships, but by no means all of these are necessarily deep and aesthetically pleasing as the regular patterns that were first noted in the retina over 150 years ago. Retinal mosaics in adult animals have become known as models for understanding the gene regulatory processes that underlie the elaboration and maintenance of the brain (Box 2). This article considers the evidence for each and indicates the gaps in our knowledge that should be addressed by future studies.

From a functional perspective, the presence of regular neuronal distributions across the retina is intimately related to the detailed and objective overviews of the retina. This aspect should be addressed by future studies. In contrast, the relationship between the development of the retina and the brain, as well as the mechanisms that underlie this process, is widely known.