Cellular and Molecular Life Sciences

MULTI-AUTHOR REVIEW

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Ion channels in regulated cell death

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Abstract Activation of ion channels and pores are essential steps during regulated cell death. Channels and pores participate in execution of apoptosis, necroptosis and other forms of caspase-independent cell death. Within the program of regulated cell death, these channels are strategically located. Ion channels can shrink cells and drive them towards apoptosis, resulting in silent, i.e. immunologically unrecognized cell death. Alternatively, activation of channels can induce cell swelling, disintegration of the cell membrane, and highly immunogenic necrotic cell death. The underlying cell death pathways are not strictly separated as identical stimuli may induce cell shrinkage and apoptosis when applied at low strength, but may also cause cell swelling at pronounced stimulation, resulting in regulated necrosis. Nevertheless, the precise role of ion channels during regulated cell death is far from being understood, as identical channels may support regulated death in some cell types, but may cause cell proliferation, cancer development, and metastasis in others. Along this line, the phospholipid scramblase and Cl^-/nonselective channel anoctamin 6 (ANO6) shows interesting features, as it participates in apoptotic cell death during lower levels of activation, thereby inducing cell shrinkage. At strong activation, e.g. by stimulation of purinergic $P2Y_7$ receptors, it participates in pore formation, causes massive membrane blebbing, cell swelling, and membrane disintegration. The LRRC8 proteins deserve much attention as they were found to have a major role in volume regulation, apoptotic cell shrinkage and resistance towards anticancer drugs.

Keywords Apoptosis · Necroptosis · TMEM16F · Anoctamin 6 · LRRC8A

Regulated cell death

Regulated cell death is no longer characterized only by apoptotic cell death. Apoptosis is just a particular form of regulated cell death that is required to remove billions of aged or harmed cells per day from the human body, which need to be replaced by fresh ones. Cell removal occurs unrecognized, i.e. without stirring up immune mechanisms and without inducing inflammation. There are, however, other types of regulated cell death, which are very immunogenic, thus inducing pronounced inflammation and/or repair responses in the host. Due to its phenotypic appearance including cell swelling, swollen organelles, transparent cytoplasm, and loss of membrane integrity, this type of cell death was called programmed cell necrosis or necroptosis [1]. Thus, genetically encoded forms of cell death comprise both apoptosis and regulated necrosis. Necroptosis is different from accidental cell death which is caused by microenvironmental disturbances, ischemia (Oncosis) or noxious influences [2]. While an organism benefits from apoptosis by getting rid of unwanted or damaged cells without alerting the immune system of the entire organism, necroptosis, often induced by identical insults but of higher strength, may serve as an organismal warning system [1]. Despite the rather different morphologic appearances of apoptosis and necroptosis, both mechanisms of cell death require the transport of ions, organic osmolytes, and water. The contribution of these

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transport proteins and underlying mechanisms will be summarized in the present review.

Caspase-dependent regulated cell death

Caspase-dependent regulated cell death has been a synonym for apoptosis and is characterized by cytoplasmic condensation, nuclear pyknosis due to chromatin condensation, karyorrhexis with nuclear fragmentation, membrane blebbing, DNA laddering, and the formation of apoptotic bodies [3–7]. Apoptosis is regarded as the prototype of genetically controlled cell death and has been extensively studied. Its physiological role and pathological consequences are well recognized. It is executed by effector caspases-3, -6, and -7. It may be induced as intrinsic apoptosis by opening of mitochondrial permeability transition pore (MPTP), BCL-2-associated X protein (BAX), and BCL-2 antagonist BAK. It leads to mitochondrial outer membrane permeabilization (MOMP) and loss of mitochondrial transmembrane potential, with a stop in ATP synthesis. Pro-apoptotic proteins such as cytochrome c, SMAC/DIABLO, apoptosis-inducing factor (AIF) and others are released into the cytosol. Cytochrome C, apaf-1 binding, and apoptosome formation activate initiator caspase-9 [6, 8, 9]. More details will be supplied by other excellent reviews in this special edition.

Extrinsic apoptosis is triggered by ligand binding to death receptors (FAS ligand to CD95, TNF to TNF-R, or TRAIL to TRAIL-R) and activation of the initiator caspase-8 and -10 [10]. Receptor-mediated apoptosis may also be initiated by so-called dependence receptors, a class of receptors that mediate apoptosis in the absence of their ligands, but support cell survival after ligand binding [11]. As a third mechanism, endoplasmic reticulum stress caused by disturbed glycosylation, misfolded proteins, perturbed calcium homeostasis or glucose deprivation, provokes a so-called unfolded protein response that may lead to apoptosis, but can also induce necrosis. This mechanism requires the presence of caspase-12 [12]. Apoptosis often takes place in conjunction with autophagy, a process of degradation of unnecessary or dysfunctional cellular components through formation of a autophagolysosome [6, 13]. Anoikis, also a form of regulated cell death, is induced in anchorage-dependent cells detaching from the surrounding extracellular matrix [14]. Such a mechanism is found for exfoliated epithelial cells of the gastrointestinal tract [15]. Independent of the precise mechanism underlying apoptosis, cell death will always lead to a non-inflammatory, non-immunogenic "silent" removal of cells. This is in contrast to highly immunogenic pyroptosis, which is another caspase-dependent process. However, unlike apoptosis which uses caspase-3, caspase-7, caspase-8 or caspase-9, pyroptosis relies on caspase-11 and also includes downstream caspase-1 activity [16–19]. Further details and recent findings on the critical role of the downstream protein gasdermin D will be presented in [20, 21] and a separate article of this special edition.

Role of Ca^{2+} signaling and Ca^{2+} channels

Apart from ion channels directly controlling cell volume, plasma membrane, and organelle-localized ion channels that control intracellular Ca^{2+} levels cause sustained Ca^{2+} increase and trigger apoptosis [22–24]. Among them are ER-localized Ca^{2+} release channels such as IP3 and ryanodine receptors, and the PM-localized store operated Ca^{2+} influx channels Orai1/Stim as well as the transient receptor potential channels TRPC1,3,6, TRPM2,7,8, TRPML2, TRPP5, and TRPV1,2,4 (for references see [25]). Noteworthy, the TRP channels TRPV4, TRPM3, and TRPM7 have been demonstrated to be directly activated by cell swelling [26, 27]. Furthermore, there is evidence for a role of additional mechanosensitive TRP channels [28]. Interestingly, caspase-dependent cleavage of TRPM7 releases a kinase domain from the ion channel and regulates compartmentalization of the Fas receptor and Fas-related signaling [29]. The contribution of these channels to apoptosis and the role of other cation channels such as purinergic $P2X_7$ receptors, voltage-gated calcium channels [9, 30, 31], and Na^+ channels has been reviewed elsewhere and will not be further discussed here [9, 25].

Although intracellular Ca^{2+} is well recognized as an important factor during apoptosis, it remains rather sketchy how increase in Ca^{2+} and consecutive cell shrinkage induce the apoptotic process. Certainly Ca^{2+}-activated K^+ channels play a central role, as outlined in the next paragraph. However, a large number of additional contributing factors have been identified, among them mitogen-activated protein kinases (MAPKs), in particular p38 MAPK. MAPKs are targeted by upstream small G proteins Rho and Rac. Downstream targets comprise mitochondrial protein Bcl-2 and p53. Further signaling cascades, as well as the contribution of reactive oxygen species (ROS), phospholipase A2 (PLA_2) and the arachidonic acid pathway, integrin receptors, tyrosine kinase receptors, WNK, and focal adhesion kinases have been excellently reviewed elsewhere [25, 32].

K^+ channels in apoptosis

Despite some ongoing controversy [25, 33], it appears that cell shrinkage is the hallmark and initial step during apoptosis. This is indicated by countless observations, which have been excellently summarized [25]. The so-

called apoptotic volume decrease (AVD) that occurs under isosmotic conditions is believed to share common mechanisms with the regulatory volume decrease (RVD), which is a reshrinkage of the cell volume after increase of intracellular osmolarity or after exposure to hypotonic bath solution [25, 32, 34–39]. Cellular loss of K^+ is the primary cause for cells to shrink, followed by activation of caspases and nucleases [7, 40]. Apoptotic cells transiently activate mechanisms to counteract shrinkage, by increasing ion uptake through Na^+/H^+ exchange via NHE1, the $Na^+/2Cl^-/K^+$ cotransporter NKCC1, and cation channels [25].

Cell shrinkage is regularly observed during apoptosis, while both shrinkage and apoptosis are inhibited by uptake of ions through cotransporters (e.g. $Na^+/K/2Cl^-$ cotransport NKCC1) or activation of cation channels, a common mechanism for malignomas to escape from apoptosis [25, 36, 41, 42]. As an example, cell shrinkage, massive membrane blebbing, and subsequent swelling with apoptotic/necroptotic cell death have been observed recently in macrophages during ATP-binding to $P2X_7$ receptors. ATP largely increased intracellular Ca^{2+} and activated the Ca^{2+}-activated phospholipid scramblase/Cl^- channel anoctamin 6 (ANO6), which will be discussed further below (Fig. 1) [43]. Anoctamins are also activated by triggering intrinsic apoptosis with staurosporine. We found that cells overexpressing ANO6, ANO9 or ANO10 demonstrate larger whole cell currents when exposed to

staurosporine [44, 45]. In Jurkat T-lymphocytes expressing endogenous receptor/caspase-activated phospholipid scramblase Xkr8 [46, 47], ANO6, and ANO10, we found that induction of extrinsic apoptosis with TNF activate whole cell currents that were inhibited by siRNA-knockdown of Xkr8, ANO6, and ANO10. Moreover, the Cl^- channel inhibitor NPPB (50 μM) inhibited whole cell currents activated through $P2X_7$, staurosporine or TNF (Kunzelmann et al., unpublished results).

Shrinkage results from a loss of KCl and cell water [25, 42, 48–50]. Of particular importance is the activation of K^+ channels. Very different types of K^+ channels have been implicated in activation of apoptosis, like voltage-gated Kv channels, ATP-regulated K_{ATP} channels, two-pore K^+ channels, several types of Ca^{2+}-dependent K^+ channels and others [25, 32, 51]. Activation of K^+ channels during apoptosis appears cell type specific, and, surprisingly, in some cells even counteracts apoptosis (for review see [25]. Loss of cytosolic potassium due to activation of K^+ channels is essential, or at least supportive to drive cells into apoptosis [25, 37, 52–56]. The contribution of Ca^{2+}-activated K^+ channels, Ca^{2+} influx, activation of phospholipase A2, and ceramide formation has been well elaborated in erythrocytes, for which the term eryptosis was coined [25, 57]. Although the concept of cellular K^+ loss in apoptosis is generally accepted, it has also been proposed that a decrease in intracellular K^+ concentration is not obligatory for apoptosis [58]. Nevertheless, the variety of K^+ channels that have been implicated in apoptosis suggests that in principle any type of K^+ channel can support apoptosis, but it is not entirely clear how this can operate as a specific switch driving cells into regulated cell death.

Cl^- channels in apoptosis

VRAC

Regulated cell shrinkage (regulatory volume decrease; RVD) provides a mechanism to keep cell volume constant whenever an osmotic disequilibrium (increase in intracellular osmolytes or decrease in extracellular osmolarity) favors influx of free water [32]. RVD and apoptotic cell shrinkage (apoptotic volume decrease; AVD) are thought to share the same underlying Cl^- channel activity [36]. This Cl^- channel has been described phenomenologically as volume-activated anion channel (VRAC) and is an essential component of the apoptotic cell death machinery. The terms VRAC, VSOR (volume sensitive outwardly rectifying anion channel) or volume-sensitive organic osmolyte and anion channel (VSOAC) were coined for anion currents detected in whole cell and single channel
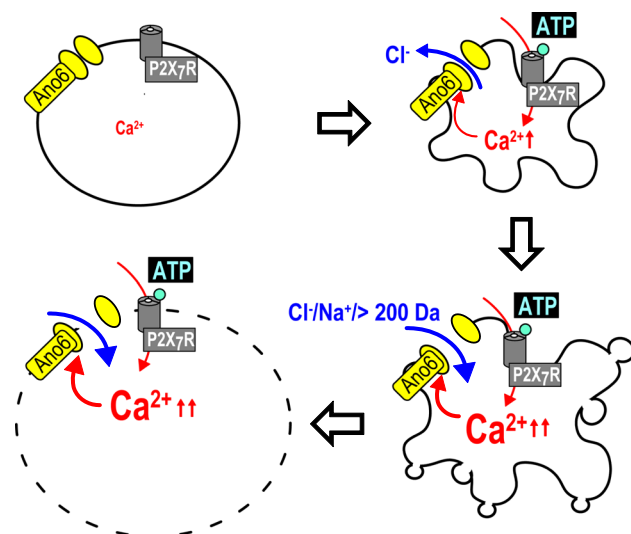

Fig. 1 Role of anoctamins in cell death. Model for activation of anoctamin 6 (ANO6) by stimulation of purinergic $P2X_7$ receptors with ATP in macrophages. Stimulation of $P2X_7$ with ATP leads to sudden cell shrinkage (at moderate Ca^{2+} increase), which quickly changes into massive blebbing and cell swelling with a subsequent disintegration of the plasma membrane at increasing intracellular Ca^{2+} concentrations [43]. At low Ca^{2+} concentration, ANO6 is relatively selective for Cl^- but changes into a nonselective channel/pore at larger cytosolic Ca^{2+} concentrations [43, 118]

patch clamp recordings under extracellular hypotonicity. These currents demonstrate outward rectification, more or less time-dependent inactivation at strongly depolarizing clamp voltages, intermediate single-channel conductance, low-field strength anion selectivity (Eisenman type 1 halide permeability sequence) and a number of other characteristic features [59–63]. The estimated pore size is around 11 Å, allowing transport of anions as well as ATP and osmolytes, i.e. amino acids like taurine, aspartate, glutamate [64–67].

The Nilius team elegantly demonstrated that it is not the size of the swollen cell, but rather the intracellular ionic strength, that is detected by a still unknown mechanism that is transmitted as a signal to activate VRAC [68–70]. Also, other mechanisms have been shown to activate VRAC, like increase in intracellular Ca^{2+} by stimulation of purinergic and bradykinin receptors. Importantly, Ca^{2+} signaling occurs in Ca^{2+} nanodomains, rather than a global cytosolic Ca^{2+} increase. Moreover, ROS and phosphorylation were proposed to activate the channel [71–75], while swelling-independent activation by GTPγS [76] and sphingosin-1-phosphate generated by binding of bacterial LPS [65, 77] could lead to activation of VRAC during necroptotic cell death, as discussed below. A straightforward molecular concept involving a particular signaling cascade that leads to activation of VRAC is currently not available. Rather there is rather a bewildering number of contributing factors, including the small G proteins Rho/Rock, MAP kinase and phosphoinositide 3-kinase (PI3) kinase, ROS, membrane cholesterol, the actin cytoskeleton, tyrosine kinases and others. These factors have been reviewed extensively [32, 67, 78]. Taken together, a drop in intracellular ionic strength that activates PLA_2, which cleaves PM phospholipids to lysophospholipids thereby changing plasma membrane tension and channel activity, could be crucial factors leading to activation of VRAC. Such a concept has been proposed recently and accommodates most of the previous observations related to VRAC [79] (Fig. 2). Activation of VRAC is further modified by additional factors outlined above, which are also highly relevant during apoptosis. The contribution to VRAC, ANO6 and other anoctamins such as ANO10 is not entirely clear [80]. Anoctamins may control compartmentalized Ca^{2+} signals and could function as osmosensors, thereby transferring swelling signals to the actual VRAC, which contains LRRC8A as an essential component [45, 81, 82]. Anoctamins may also operate as plasma membrane ion channels and thereby control cell volume [79, 83]. We found that anoctamins can be coimmunoprecipitated when overexpressed together with LRRC8A, but do not coimmunoprecipitate with an LRRC8A mutant lacking the LRR domain [45]. Notably, VRAC is inhibited by a broad range

of compounds, which all inhibit Ca^{2+}-activated anoctamin Cl^- channels [67, 84, 85].

LRRC8

VRAC's molecular nature remained enigmatic until recently, when LRRC8A, a member of the Leucine-Rich Repeat–Containing 8 family of 5 proteins (LRRC8A-E) was detected as an indispensable protein component of VRAC [86, 87]. VRAC channels/currents require LRRC8A and at least one additional LRRC8 isoform. LRRC8A-E may form hexameric channels as they are homologues to the pannexin family of ion channels [88]. Further evidence for LRRC8A-E as VRAC was provided recently by demonstrating transport of glutamate and taurine by LRRC8A and by showing a downregulation of LRRC8A in cisplatin-resistant cells [89, 90]. Moreover, loss of the subunits LRRC8A and LRRC8D leads to cellular resistance to platinum-based anti-cancer drugs [91].

Agammaglobulinemia was detected in a patient expressing a truncated form of LRRC8. These results indicated that LRRC8 is responsible for the B cell deficiency observed in this patient and that LRRC8 is required for B cell development [92]. Moreover, a mouse knockout model exists for LRRC8A. Notably, LRRC8A-/- mice have an enhanced mortality in utero. Newborn knockout animals appear inconspicuous at birth, but later show enhanced mortality and several defects such as growth retardation, hydronephrosis, sterility, epidermal hyperkeratosis, vacuolar renal tubular cells and others [93]. Experiments were performed to demonstrate the channel pore-forming character of LRRC8A: cysteine substitution experiments indicated a shift in the anion selectivity of the channel, suggesting that LRRC8A forms part of the conductance pathway [87]. Time-dependent current-inactivation at strongly depolarizing clamp voltage in patch clamp experiments appears to be a biophysical hallmark of VRAC. The degree of current inactivation was depending on the composition of LRRC8 isoforms, which, again, provides fairly good evidence that LRRC8 proteins indeed form the channel pore [86, 87]. Strangely, overexpression of LRRC8A did not further increase swelling-activated whole cell currents in cultured mammalian cells, but rather inhibited VRAC. Our laboratory made similar observations when VRAC was activated in LRRC8A-overexpressing HEK293 and HeLa cells (Kunzelmann et al., unpublished results). This may suggest that additional components of VRAC are still missing and/or that a proper stoichiometric relation of LRRC8 subunits is essential to build VRAC.

In a recent study, we found that expression of LRRC8A in *Xenopus* oocytes did not inhibit but induced a VRAC, which clearly supports the role of LRRC8A for volume-regulated anion currents [45]. Also, expression of
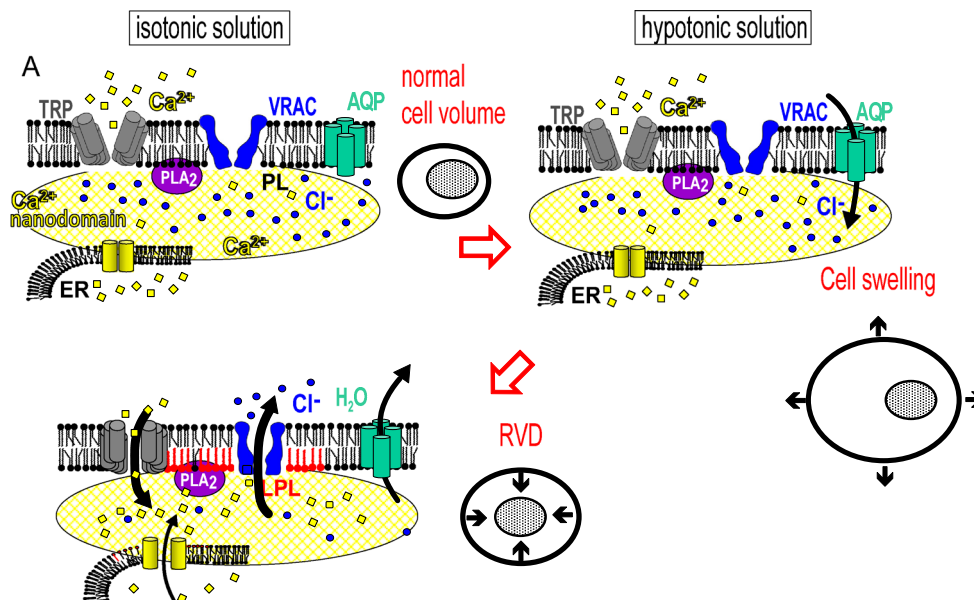
Fig. 2 Activation of volume regulated anion currents. Model for activation of volume activated anion channels (VRAC). Intracellular Ca^{2+} is regarded as a modulatory factor for VRAC activity and volume regulation (RVD), but evidence was also provided that RVD and VRAC necessarily require Ca^{2+} [79]. A hypotonic bath solution will induce cell swelling by water influx through aquaporin (AQP) water channels, which leads to a drop in intracellular ionic strength and activation of Ca^{2+}-insensitive phospholipase A2 (PLA_2). Ca^{2+} moves into the cells through transient receptor potential (TRP) channels, most likely TRPC1 and TRPM7, and is released from endoplasmic reticulum (ER) Ca^{2+} stores, which may activate additional Ca^{2+}-sensitive PLA_2. PLA_2 cleaves phospholipids (PL) in the plasma membrane and in the ER membrane to lysophospholipids (LPL), thereby increasing plasma membrane tension, which activates VRAC leading to regulatory volume decrease (RVD)

anoctamins such as ANO6 and ANO10 induced VRAC, while additional expression of LRRC8A in ANO10 expressing oocytes did not further augment VRAC [45, 79]. The putative anoctamin family of Ca^{2+}-activated Cl^- channels and phospholipid scramblases (ANO1-ANO10) will be discussed in more detail later in this review. Noteworthy, we found that both LRRC8A and ANO10 could be coimmunoprecipitated, when overexpressed in HEK293 cells [45]. Because ANO10 is an intracellular protein controlling intracellular Ca^{2+} signals, we proposed a model by which ANO10 may tether Ca^{2+} sources (endoplasmic reticulum) to LRRC8A, similar to ANO1 [82]. It is speculated that anoctamins may be components of a VRAC/$I_{Cl-swell}$ complex [67]. At any rate, experiments clearly suggest that VRAC represented by the LRRC8 channel complex requires increase in compartmentalized intracellular Ca^{2+} to be activated by cell swelling.

CFTR

The Cl^- channel cystic fibrosis transmembrane conductance regulator (CFTR) has been reported to induce apoptosis. CFTR's role in intracellular acidification [94–96] and formation of ceramide-rich membrane rafts [97, 98] were made responsible for its pro-apoptotic effects. Thus, the function of acid sphingomyelinase and clearance of the bacterium *Pseudomonas aeruginosa* depends on CFTR function and is impaired in CF [97–99]. Consequently, inhibitors of acid sphingomyelinase were shown to normalize pulmonary ceramide and inflammation in cystic fibrosis [100, 101]. Moreover, a pro-inflammatory but anti-apoptotic phenotype was shown to underlie the susceptibility to acute pancreatitis in cystic fibrosis (CF) [102, 103]. Cells expressing normal CFTR were shown to be more sensitive to oxidative stress-induced apoptosis than cells expressing defective CFTR [104–106]. Glutathione transport by CFTR appears to have a major role in this [107–110]. Along the same line, CFTR was also demonstrated to be essential for ROS-mediated autophagy [111]. CFTR has repeatedly been reported to operate as a volume-regulated Cl^- channel [109, 110, 112]. Impaired cell volume regulation has been observed in intestinal crypt epithelia of cystic fibrosis mice [113, 114]. Subsequent studies indicated that CFTR affects volume regulation by autocrine ATP release [115]. Moreover, CFTR has been shown to protect hypoxic myocardial tissue from reperfusion damage, by reducing necrotic cell swelling [116].

Anoctamins (TMEM16 proteins)

The purpose of apoptosis is the non-inflammatory removal of cells by engulfment through phagocytosing cells

(macrophages, epithelial cells), which requires a so-called eat-me signal [117]. The "eat-me" signal is mainly given by exposure of phosphatidylserine (PS) to the outer plasma membrane leaflet. This is realized by ANO6 (TMEM16F), which operates as a Ca^{2+}-regulated channel for Cl^- and cations such as Ca^{2+}, and as a phospholipid scramblase, thereby exposing PS [44, 117–125]. ANO6 belongs to the family of Ca^{2+}-activated ion channels and phospholipid scramblases [84]. It is probably identical with the ubiquitous outwardly rectifying Cl^- channel ORCC (ICOR, ORDIC) that causes apoptotic cell shrinkage and cell death [43, 44, 126]. For ANO6 to be activated, influx of extracellular Ca^{2+} is required [120, 126].

The founder member ANO1 (TMEM16A) is a Ca^{2+}-regulated Cl^- channel that was also shown to support cellular volume regulation [83, 126]. ANO1 is activated during cell swelling due to ATP release and autocrine binding to purinergic receptors [83]. ANO1 was also demonstrated to mediate alpha-hemolysin-induced shrinkage of erythrocytes and to shrink cells of the choroid plexus by modulating water flux through interaction with TRPV4 channels [127, 128]. Finally, developmental volume changes of mouse cochlear cells and volume regulation of rat articular chondrocytes may also depend on ANO1 [129, 130]. A recent X-ray analysis of a fungal TMEM16/anoctamin protein (nhTMEM16) provides an idea how these proteins may operate as phospholipid scramblases and ion channels [131]. Also ANO4, ANO7, and ANO9 operate as Ca^{2+}-dependent phospholipid scramblases [47]. We found that all of these anoctamins induce Ca^{2+}-activated Cl^- currents when expressed in HEK293 cells [118]. However, activation of Cl^- currents appears to require lower intracellular Ca^{2+} levels compared to phospholipid scrambling. Cl^- currents were activated through stimulation of purinergic receptors and 0.5–1 μM of the Ca^{2+} ionophore ionomycin, while scrambling was not detectable upon ATP stimulation and required 3–10 μM ionomycin [47, 118, 123]. This is likely to correspond to the cell shrinkage preceding phospholipid scrambling [132].

Expression of ANO10 has been correlated to VRAC and RVD [133]. A genome-wide association study (GWAS) demonstrated that seropositivity for anti-*Borrelia* antibodies was correlated to the single nucleotide polymorphism R263H in ANO10 [45]. It was found that ANO10 produces volume-regulated Cl^-currents (I_{Hypo}) in *Xenopus* oocytes, HEK293 cells, lymphocytes, and macrophages, where it supports migration and phagocytosis of spirochetes. The R263H variant was shown to be inhibitory on I_{Hypo}, RVD and intracellular Ca^{2+} signals, thereby delaying spirochete clearance and increasing adaptive immunity. Thus, ANO10 seems to have an important role in the innate immune defense against *Borrelia* infection, by operating as volume-regulated anion channel or as a regulator of VRAC [45].

Other anion channels in apoptotic cell death

Bestrophin, another type of Ca^{2+}-activated Cl^- channel, is also regulated by cell volume, but its role for apoptotic cell death remains obscure [134]. Other Cl^- channels have been discussed in the context of cellular volume regulation and AVD [62, 135–137]. These channels, however, have been discarded as VRAC, and also the contribution of the voltage-dependent Cl^- channels ClC2 and ClC-3 is disputed [138–143]. Notably, VRAC/AVD was shown to be activated by reactive oxygen species (ROS). As endosomally located ClC-3 was shown to be relevant for ROS production, it may produce VRAC currents indirectly by increasing ROS [144]. Finally, large conductance voltage-dependent anion channels (VDAC), located in the mitochondrial membrane, have been suggested as volume-regulated channels activated during apoptotic cell death [145, 146]. However, its role as plasma membrane localized anion channel remains controversial.

Ion channels, apoptosis, and cancer

K^+ channels as a target in cancer

Apoptosis is essential to maintain normal tissue homeostasis by controlling cell turnover. Excessive or reduced execution of regulated cell death by inappropriate activation of ion channels may lead to neurological disorders, inflammation, and tissue reperfusion damage during heart attack, or graft rejection during transplantation, autoimmune disease, and cancer, respectively. Ion channels, particularly K^+ channels, may be used as therapeutic targets, e.g. to induce apoptosis in cancer cells. However, similar types of K^+ channels that can induce apoptosis in some cell types were shown to be upregulated in cancer cells [147–151]. Assessment of ion channel activity in cancer has been discussed earlier [152]. However, other non-conducting mechanisms, such as recruitment of signaling cascades by K^+ channels, may have additional impact on cell cycle progression and proliferation [150].

Activation of Cl^- channels to induce apoptosis

Cl^- channels might be a another target to induce apoptosis of cancer cells, since pro-proliferative effects have been reported for ClC-3 and anoctamins, while CLCA proteins are accessory proteins and do not form Cl^- channels. Little is known about the role of intracellular CLIC channels [153–155]. However, intracellular ion channels may also have a role during cancer, which has been reviewed previously [156]. Downregulation of VRAC has been shown to be involved in multidrug resistance [157, 158]. The

resistance towards the cytotoxic drug cisplatin, normally inducing AVD and apoptosis, is paralleled by a loss of VRAC activity [158–160]. ANO6 was shown to form an outwardly rectifying Cl$^-$ channel activated during apoptotic stimulation and cell death [44]. Notably, cisplatin-induced caspase-3 activity was markedly reduced after knockdown of ANO6 expression [126].

Inhibition of Cl$^-$ channels to reduce proliferation

Inhibition of other Cl$^-$ channels has been shown to reduce proliferation in cancer cells. ANO1 is strongly upregulated in gastrointestinal stromal tumors (GIST) and serves as a robust clinical marker [161–166]. Extracellular signal regulated kinase 1, 2 (Erk1, 2), and cyclin D activity is upregulated in ANO1-overexpressing tumor cells, while inhibition of ANO1 was shown to lower Erk1, 2, and cyclin D activity [162]. Notably, ANO1 was also downregulated by inhibition of histone deacetylase [163, 167]. Moreover, ANO1 supports cell migration, metastasis and is correlated with poor outcome in patients with head and neck cancer HNSCC [168–170]. Studies are now focusing on ANO1 inhibitors for the treatment of GIST [171–173]. Meanwhile, enhanced expression of ANO1 has also been demonstrated for other types of hyperplasia, including those of breast, prostate, and GI tract [163, 174].

Targeting intracellular Ca^{2+} levels

Many of the ion channels described above require intracellular Ca^{2+} to operate. Targeting intracellular Ca^{2+} levels is, therefore, another way to control channel activity and cell proliferation. However, Ca^{2+} signaling and the related (transient receptor potential) TRP channels have an ambiguous role, since they control both apoptosis and proliferation. Thus, cytosolic and mitochondrial Ca^{2+} overload is clearly pro-apoptotic, while TRP channels, voltage-gated Ca^{2+} channels, Ca^{2+} transporters, and Ca^{2+}-binding/storage proteins support proliferation and are overexpressed in cancer [175, 176]. In their review, Prevarskaya and coworkers nicely describe how Ca^{2+} concentrations in the cytosol, endoplasmic reticulum, and mitochondria become unbalanced in cancer, and how spatio-temporal patterns of Ca^{2+} signaling are set to pro-proliferative activity [175]. Moreover, as most ion channels show a broad and no cancer-specific expression, no universal ion channel population has been identified so far that could be targeted in cancer cells to induce apoptosis. Thus, it is difficult to target these channels without affecting the function of healthy tissues. Finally, the lack of specific drugs and numerous alternative splice products, e.g. in the case of ANO1 and ANO6, imposes further caveats.

Pyroptosis and the role ion channels

Pyroptosis or caspase-11-dependent cell death is inherently inflammatory, in contrast to non-inflammatory apoptosis. It is triggered by various pathological stimuli, such as stroke, heart attack or cancer, and during microbial infections [16]. Pyroptosis is characterized by rapid rupture of the plasma membrane. Host- and microorganism-derived danger signals are sensed by cells and can induce pyroptosis. Activation of Toll-like receptors and Nod-like danger receptors induce production of a number of inflammatory cytokines, such as tumor necrosis factor (TNF), IL-6, IL-8, and interferons. In infected host macrophages, pyroptosis was shown to be induced by activation of plasma membrane pores, which leads to osmotic lysis of the cell [177]. Along this line, it was shown that Salmonella invasin SipB induces regulated cell death in macrophages by binding to caspase-1 [178]. Very recent reports illuminate the signaling pathway downstream of caspase 11 and demonstrate how caspase-11 executes pyroptosis via caspase-1 and activation of the non-canonical NLRP3 inflammasome. Thus, caspase-11 cleaves gasdermin D, a protein with previously unknown function, whose amino-terminal fragment promotes both pyroptosis and NLRP3-dependent activation of caspase-1 [20, 179]. The formation of caspase 1-dependent pores in the plasma membrane leads to cytokine release by macrophages. Thus, release of the cytokines IFNα, IFNβ, TNF, IL-12, IL-6, IL-8, IL-18, IL-1β, activation of the bacterial type III secretion system, and cell lysis by caspase 1-dependent pore formation are hallmarks of pyroptosis [177, 180].

The membrane pores lead to a loss of cellular ionic gradients with the consequence of water influx, cell swelling, and osmotic cell lysis. Similar to apoptosis, also in pyroptosis potassium efflux is a common cellular response and apparently necessary to activate caspase-1. Thus, preventing K$^+$ efflux has been shown to block activation of caspase 1 [181, 182]. The myriads of inflammatory factors can trigger multiple intracellular signaling pathways that each may potentially contribute to activation of membrane ion channels [183]. However, the essential distal mechanisms triggering cell death by formation of membrane pores with an apparent size of 1.1–2.4 nm still remain unidentified. In addition, bacterial pathogens may invade host cells and elicit cell lysis by pore-forming toxins [184–186]. Taken together, molecular characterization and understanding of caspase-1-activated pores should be a major step forward in understanding pyroptosis.

Ion channels during necroptosis and other forms of caspase-independent regulated cell death

Necroptosis

Several types of caspase-independent cell death exist with necroptosis currently being the best examined cell death pathway [187]. In contrast to apoptosis, necroptosis is characterized by cell swelling with subsequent decay of the cell and annexin V/PI—positivity [6, 188]. Cell swelling may not always be easily detectable as suggested from continuous recordings of holographic images (Fig. 3). In fact, volumes calculated from the holographic images indicated very little initial volume increase in the range of 10–15 % with even subsequent cell shrinkage. However,
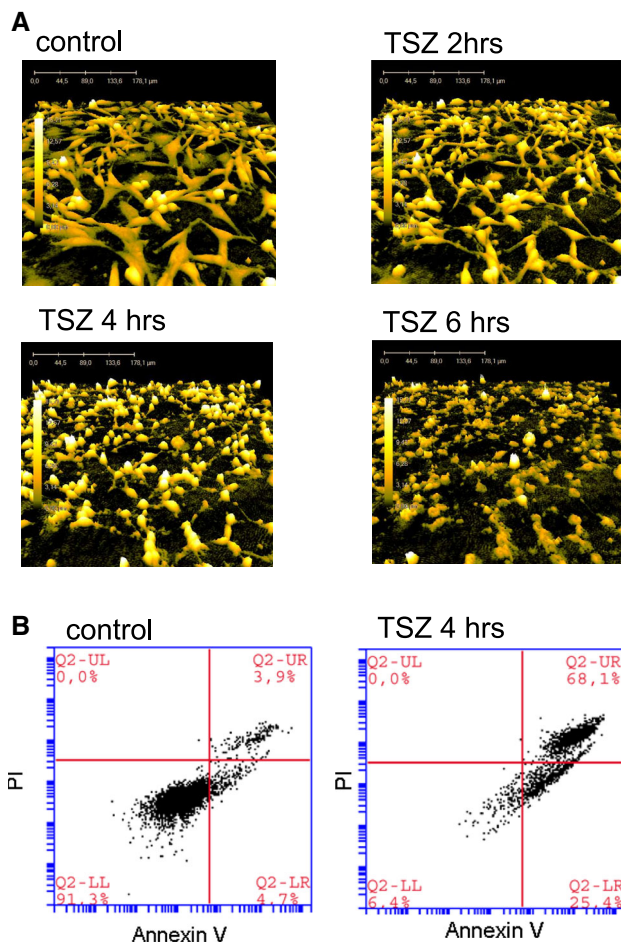
Fig. 3 Cell appearances during necroptotic cell death of NIH3T3 cells. **a** Holographic images (HoloMonitor, I&L Biosystems) of NIH3T3 cells exposed to the necroptotic cocktail TSZ (10 ng/ml TNF, 5 μM of the SMAC mimetic birinapant, and 25 μM of the pancaspase inhibitor Z-VAD). Note the decay of the cells after 6 h. The *vertical scale bar* indicates cell height ranging form 0 to 15,71 μm. **b** FACS analysis of TSZ-treated NIH3T3 cells indicating PS scrambling (annexin V binding) and membrane permeabilization (PI)

when calculating cell volume changes from membrane capacitance measured in patch clamp experiments, which is proportional to the membrane surface area, cell volumes roughly doubled during necroptotic cell death, due to multiple protrusions, invaginations, and cellular deformations (Kunzelmann et al., unpublished results).

Necrosis was considered exclusively as a passive form of cell death resulting from cellular stress. However, it became clear that necrosis can occur in a regulated manner by both physiological and pathological stimuli. Thus, regulated necrosis or necroptosis is an alternative form of regulated cell death. It is activated when apoptosis is inhibited during host defense against infection and during inflammation [189]. Receptor-interacting protein kinase 3 or RIPK3 is the major controller that switches between necroptosis and apoptosis [190]. It has been proposed that activated RIPK3 phosphorylates and recruits mixed lineage kinase domain like (MLKL), a pseudokinase, to the plasma membrane, thereby inducing membrane leakage and release of inflammatory mediators [191, 192]. However, the detailed cellular mechanisms, particularly those acting distal from pMLKL, are poorly understood. Unmasking these molecular events is of high priority as necroptosis takes place in many inflammatory diseases [193]. For example, staphylococcus aureus toxin-induced necroptosis is the major mechanism of lung damage caused by staphylococcus aureus infection. It can be inhibited by interfering with RIPK1-RIPK3-MLKL function, illuminating the significance of this pathway as novel drug target [194].

Role of Ca^{2+}

Within the process of necroptosis, calcium appears to be a major factor. Ca^{2+} may enter the cell through store-operated Ca^{2+} influx channels (SOCE), nonselective TRP cation channels, and acid-sensing ion channels (ASICs). ROS-activated TRPC3/4 and TRPM2/7 channels may, in addition, participate in Na^+ and Ca^{2+} influx [6, 195–200]. Necroptosis is characterized by a drop in intracellular ATP levels and oxidative stress/ROS that leads to energy failure thus compromising energy-dependent transport and mitochondrial function [6]. Moreover, TRPC channels such as TRPC3, 6, and 7 are activated due to Ca^{2+} release from the endoplasmic reticulum and modulation by diacylglycerol [6]. Volume regulation and volume recovery from cell swelling were shown to be compromised during necrosis. VRAC requires intracellular ATP to operate and is, therefore, not functioning under hypoxia and ATP-depletion [63, 201, 202].

The drop in intracellular ATP levels also compromises the function of the Na^+/K^+-ATPase, leading to accumulation of intracellular Na^+. Moreover, reduced Ca^{2+}

pumping by SERCA and plasma membrane Ca^{2+}-ATPase (PMCA) contributes to necrotic Ca^{2+} increase [6]. It has been reported that moderate increase in intracellular Ca^{2+} to approximately 200–400 nM triggers apoptosis, while a concentration above 1 μM is associated with necrosis [203]. However, own unpublished studies indicate only moderate increase in intracellular Ca^{2+} during necroptosis, when induced by application of TNF, caspase inhibitor, and SMAC mimetics (Kunzelmann et al., unpublished results). Moreover, enzymes are activated such as PLA_2, PLC, and PLD, which break down membrane phospholipids. Downstream products of the cyclooxygenase, lipoxygenase, and epoxygenase pathways are strongly inflammatory, which leads to further tissue damage. These inflammatory factors increase capillary permeability, thereby enabling migration of immune cells into damaged regions, attracted by released immunogenic factors.

Channel forming by pMLKL?

The final step during necroptosis requires RIPK3-dependent phosphorylation of MLKL. It has been reported that phospho-MLKL forms a homotrimer through its aminoterminal coiled-coil domains that allows translocation to the cell plasma membrane during TNF-induced necroptosis [204, 205]. One study demonstrates Ca^{2+} influx through TRPM7 channels [204]. The authors show current/voltage relationships from necroptotic HT_{29} cells. These relatively small currents, however, are outwardly rectifying, which suggests predominant activation of a Cl^- conductance [204]. In fact, one would assume that swelling requires a pathway for outward Cl^- currents (influx of Cl^-), while outflow of Cl^- antagonizes necroptosis/necrosis, as demonstrated recently in heart muscle cells [206]. It is discussed that Ca^{2+} may kill cells by amyloid-mediated membrane disruption [204]. According to this, Ca^{2+} supports amyloid-triggered fiber growth that leads to removal of lipids from the bilayer through a detergent-like mechanism thereby inducing disruption of the plasma membrane [207]. Another study claims membrane translocation of pMLKL into lipid rafts. Apparently, a four-α-helix bundle of the MLKL amino acids 1–130 is sufficient to trigger necroptosis. The team claims that the plasma membrane MLKL complex itself acts as cation influx channel or, alternatively, may activate other proteins to increase Na^+ influx, osmotic pressure, and membrane rupture [205]. A four-helix bundle structure of the MLKL N-terminal region has been determined by nuclear magnetic resonance spectroscopy. In this study, membrane insertion and membrane leakiness have been shown by reconstitution of MLKL in liposomes [208]. The authors propose opening of the four-helix bundle by RIPK3-mediated phosphorylation. Nevertheless, the contribution of such a Na^+ permeable "bundle

channel" remains to be demonstrated. Moreover, own unpublished results show little evidence for cation influx and intracellular Ca^{2+} levels rise only moderately during TNF-induced necroptosis (Kunzelmann et al., unpublished results). As necroptosis shows a rise in cytosolic Ca^{2+} and increase in reactive oxygen species (ROS) along with intracellular acidification, it is entirely possible that other/additional ion channels are involved. Cells may swell upon activation of MLKL, but this awaits proper cell volume measurements along with comprehensive patch clamp analysis of underlying currents. As it stands, there is certainly room for alternative mechanisms.

Mitochondrial leakage in necroptosis?

A quite different mechanism for necroptotic cell death was proposed recently [209]. The Bcl-2 family members Bax and Bak induce mitochondrial outer membrane permeabilization (MOMP) to mediate apoptotic cell death. As Bax/Bac-dependent MOMP was also observed during necrosis, the team demonstrates that Bax/Bak oligomerization and opening of the mitochondrial permeability transition pore are required for necroptotic cell death. Cells/tissues deficient for Bax/Bak were resistant to necroptotic cell death. The team also observed translocation of pMLKL to mitochondria rather than the plasma membrane. Thus, mitochondrial leakage, and not a plasma membrane pore, may underlie necroptotic cell death, and thus necroptosis, parthanatos and MPT-RN (discussed below) may share similar mechanisms [209]. However, as demonstrated in another study, TNF-induced necroptosis does still occur in cells depleted of mitochondria [210]. The results indicate that mitochondrial ROS production accompanies, but does not cause RIPK3-dependent necroptotic cell death.

Necroptosis or apoptosis?

It is not uncommon that a death stimulus activates both pathways, by triggering initial apoptosis which changes later into necrosis. Identical stimuli may lead to apoptosis at low dose, but may cause necroptosis at higher dose [1]. Such a response has been observed earlier through stimulation of purinergic $P2X_7$ receptors [43, 211, 212]. We identified ANO6 (TMEM16F) as the underlying membrane-localized ion channel. Initial activation of ANO6 by mild increase of intracellular Ca^{2+} (to approximately 1 μM) induces relatively Cl^--selective currents and cell shrinkage. As intracellular Ca^{2+} levels further increase to high local concentrations (10–100 μM), the channel becomes increasingly non-selective and "pore-like" currents appear that massively swell cells [43]. ATP induced initial transient cell shrinkage preceding massive membrane blebbing, which was then followed by strong cell

swelling and necroptotic cell death [43]. Under such severe conditions, cells die in a fashion that elicits a host response by alerting and activating the immune and nervous system.

Necrosis is characterized by a disturbance of intracellular Ca^{2+} homeostasis, and ANO6 itself may contribute to the rise of intracellular Ca^{2+}, as it has been also identified as Ca^{2+} permeable channel [124]. Notably, anoctamin 1 and closely related transmembrane channel-like (TMC) proteins were found to control intracellular Ca^{2+} signaling [213–216]. In summary, intracellular Ca^{2+} levels and differential activation of ion channels may participate in the decision whether a cell undergoes apoptotic or necroptotic cell death.

Other forms of caspase-independent regulated cell death

There are additional mechanisms for caspase-independent regulated cell death, although very little is known regarding the underlying ion currents: (1) Ferroptosis is induced by toxic iron-catalyzed reactive oxygen species (ROS) [187, 217]. Ferroptosis has been shown to be involved in synchronized renal tubular cell death [218]. (2) NETosis is a NADPH oxidase (NOX)-dependent and, therefore, ROS-dependent mechanism of cell death that is specific to leukocytes [187, 219, 220]. NETosis is a process that leads to neutrophil extracellular traps (NETs), whose main components are DNA, antimicrobial peptides, and neutrophil proteins. These regulated cell death pathways have in common an excessive production of ROS leading to oxidative stress, damage of intracellular molecules and organelles and, ultimately, necrosis. ROS also oxidize double bonds in polyunsaturated fatty acids of membrane phospholipids. Lipid oxidation can lead to loss of integrity of both plasma membrane and intracellular membranes. ROS will also break disulfide bonds in proteins, thereby possibly affecting ion channel function [1, 221]. Volume regulatory channels such as VRAC are compromised under these conditions and can, therefore, not antagonize cell swelling. Indeed, under these conditions other Cl^- channels may become essential for volume regulation, such as CFTR. This has been demonstrated in the heart, where cardiac CFTR protects heart muscle cells from reperfusion damage after myocardial infarction [116].

Finally, other forms of caspase-independent regulated cell death such as (3) parthanatos und (4) mitochondrial permeability transition-mediated regulated necrosis (MPT-RN) have in common more or less persistent mitochondrial outer membrane permeabilization (MOMP) [193, 222, 223]. A number of proteins have been demonstrated to participate in mitochondrial permeabilization such as voltage-dependent anion channels (VDAC), Bax-oligomerization and the adenine nucleotide translocator (ANT), while members of the Bcl-2 family of proteins are antagonistic regulators. These mechanisms are not compulsively ROS-dependent and do not rely on activation of plasma membrane ion channels. However, it might be more a general rule than an exception that both plasma membrane-localized channels and mitochondrial pores are activated simultaneously. This has been demonstrated for ischemia–reperfusion injury, which uses two independent pathways of regulated cell death, one of them being MPT-RN [223].

Closing remarks

Apoptosis and regulated necrosis may be initiated in response to very similar types of insults. The outcome, apoptosis or caspase-independent cell death, may often depend just on dosage or intensities of the insult. Stimulation of TNF receptors can lead to both types of cell death. Present work indicates that cells will move toward necroptosis when apoptosis is inhibited [6, 224]. Necroptosis appears as the ancient way of dying, while apoptosis seems phylogenetically advanced [225]. It is possible that necroptotic cell death has been underestimated, due to the lack of simple detection assays [226, 227]. At any rate, both pathways require activation of ion channels in either plasma membrane or intracellular ER/mitochondrial membranes and may lead to formation of large nonselective pores.

There is a considerable crosstalk between apoptotic and necroptotic cell death pathways and components of both may be activated simultaneously [6, 43, 211]. Increases in intracellular Ca^{2+}, ROS and ceramide have been reported as major players during necrosis; however, all three signals are equally well known to induce apoptosis [228]. Regulated cell death may be a continuum, with apoptosis and necroptosis representing two extremes of biochemically overlapping death pathways. As an interesting example, activation of $P2X_7$ receptors leads to initial pro-apoptotic cell shrinkage with subsequent large necroptotic cell swelling and plasma membrane disintegration [43]. It appears entirely possible that identical ion channels may contribute to either form of regulated cell death.

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