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Review

Calcium and connexin-based intercellular communication, a deadly catch?

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ABSTRACT

Ca^{2+} is known as a universal messenger mediating a wide variety of cellular processes, including cell death. In fact, this ion has been proposed as the 'cell death master', not only at the intracellular but also at the intercellular level. The most direct form of intercellular spread of cell death is mediated by gap junction channels. These channels have been shown to propagate cell death as well as cell survival signals between the cytoplasm of neighbouring cells, reflecting the dual role of Ca^{2+} signals, i.e. cell death versus survival. Its precursor, the unopposed hemichannel (half of a gap junction channel), has recently joined in as a toxic pore connecting the intracellular with the extracellular environment and allowing the passage of a range of substances. The biochemical nature of the so-called intercellular cell death molecule, transferred through gap junctions or released/taken up via hemichannels, remains elusive but several studies pinpoint Ca^{2+} itself or its messenger inositol trisphosphate as the responsible masters in crime. Although direct evidence is still lacking, indirect data including Ca^{2+} involvement in intercellular communication and cell death, and effects of intercellular communication on intracellular Ca^{2+} homeostasis, support this hypothesis. In addition, hemichannels and their molecular building blocks, connexin or pannexin proteins, may exert their effects on Ca^{2+}-dependent cell death at the intracellular level, independently from their channel functions. This review provides a cutting edge overview of the current knowledge and underscores the intimate connection between intercellular communication, Ca^{2+} signalling and cell death.

Abbreviations: ASK1, apoptosis signal-regulating kinase 1; ATP, adenosine triphosphate; Bcl-2, B-cell lymphoma-2; BH3, Bcl-2 homology domain 3; $[Ca^{2+}]_i$, intracellular calcium concentration; $[Ca^{2+}]_e$, extracellular calcium concentration; cADPR, cyclic ADP-ribose; cAMP, cyclic adenosine monophosphate; cGMP, cyclic guanosine monophosphate; Cx, connexin; CytC, Cytochrome C; DAG, diacylglycerol; ER, endoplasmic reticulum; ERK1/2, extracellular signal-regulated kinase 1/2; GJ, gap junction; GSH, glutathione; HC, hemichannel; IP_3, inositol 1,4,5-trisphosphate; IP_3R, inositol 1,4,5-trisphosphate receptor; ITM2B, integral membrane protein 2B; KID, keratitis, ichthyosis and deafness; MAPK, Mitogen-Activated Protein Kinase; NAD+, nicotinamide adenine dinucleotide; NO, nitric oxide; OGD, oxygen and glucose deprivation; P_2X_7R, P_2X_7 receptor; Panx, pannexin; PIP2, phosphatidylinositol-4,5-bisphosphate; PLC, phospholipase C; PKC, Protein kinase C; PTP, permeability transition pore; ROS, reactive oxygen species; SOCE, store-operated calcium entry; TNF-α, Tumor Necrosis Factor-α.

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1. Introduction

Ca^{2+} acts as a versatile signal and a ubiquitous second messenger in all cell types. It is generally accepted that Ca^{2+} is involved in the regulation of a wide range of cellular processes, including gene expression, secretion, fertilization, proliferation, development, contraction and cell death. To manage this, each cell contains an extensive Ca^{2+} signalling toolkit which is used to assemble communicative systems with very different spatial (oscillations versus waves) and temporal (microseconds to hours) dynamics [1]. At any moment in time, the level of intracellular Ca^{2+} is regulated by a balance between the 'on' reactions that introduce Ca^{2+} into the cytoplasm and the 'off' reactions through which these signals are removed by the concerted action of buffers, pumps and exchangers [2]. Although controlled increases in intracellular Ca^{2+} via release from intracellular stores and influx from the extracellular environment, are indispensable for Ca^{2+} signalling, sustained high intracellular Ca^{2+} concentrations ($[Ca^{2+}]_i$) lead to cell death, through necrosis as well as apoptosis [3–6]. In this regard, the pattern of Ca^{2+} changes – steady or oscillatory changes – has been shown to determine the outcome toward either cell death (sustained Ca^{2+} change) or cell survival (Ca^{2+} oscillations) [7].

These intracellular Ca^{2+} changes may however also be propagated from cell-to-cell by so-called Ca^{2+} waves, contributing to the versatility of Ca^{2+} signalling. A considerable amount of data demonstrates that the intercellular spreading mechanism involves gap junctions (GJs), channels connecting the cytoplasm of neighbouring cells and mediating the transfer of inositol trisphopshate (IP_3) and Ca^{2+} itself through the junctional channel [1,8–10]. In addition to the clear evidence for GJ mediated Ca^{2+} waves, later studies demonstrated that another non-junctional, paracrine mechanism also contributes to the propagation of Ca^{2+} waves. Hemichannels (HCs), half of a GJ channel, play an important role here by forming a pore between the intracellular and extracellular environment and allowing the release of adenosine triphosphate (ATP) into the extracellular compartment which then stimulates neighbouring cells by binding to purinergic receptors [1,10–13]. HCs may also form a pathway for Ca^{2+} entry [14]. GJs and HCs thus contribute to Ca^{2+} signal communication and also influence the cellular Ca^{2+} homeostasis. As a consequence, the diverse effects of these channels on Ca^{2+} homeostasis may contribute to the process of cell death. Furthermore, changes in $[Ca^{2+}]_i$ regulate both GJs and HCs, and thus modulate the overall intercellular communication network [15–17]. In addition, HCs are also influenced by extracellular Ca^{2+} [18]. This dynamic aspect of channel modulation by Ca^{2+} may affect the vulnerability of neighbouring cells to toxic agents and be of paramount importance in determining the rate and extent of the spread of cell death. In this review, we will provide a state-of-the-art overview of GJs and HCs and their role in cell death. We will present what is known about the relationship between Ca^{2+} and intercellular communication in the context of cell death. The role of Ca^{2+} and its upstream messenger IP_3, and other plausible candidates as cell death messengers will be discussed. Finally, we will highlight a possible connection between the intracellular presence of connexins (Cxs)/pannexins (Panxs), the building blocks of these channels, and the regulation of Ca^{2+} homeostasis and cell death.

2. A brief curriculum vitae of gap junctions and hemichannels – role in cell death

GJ plaques consist of numerous intercellular GJ channels of large pore diameter (~6.5–15 Å). They provide cytoplasmic continuity between neighbouring cells by conveying small hydrophilic molecules (<1–1.5 kDa), such as metabolites, ions, amino acids, and second messengers [19]. One GJ channel is composed of two HCs (Fig. 1). Opening HCs facilitates the entry of below 1–1.5 kDa substances (e.g. Ca^{2+}, Na^{+}) [14] or the escape of essential metabolites such as nicotinamide adenine dinucleotide (NAD+) [20,21], ATP [22,23], glutamate [24,25], prostaglandins [26], glutathione (GSH) [25] and IP_3 [27].

In vertebrates, HCs are composed of Cx or Panx proteins [19,28,29]. Although it is still a matter of debate whether GJs formed by Panxs do exist under *in vivo* conditions, they have been shown to form functionally competent HCs [30,31]. Mammalian Cxs constitute a multigene family of proteins with at least 20 members. They are named according to their molecular weight, ranging from 25 to 62 kDa with Cx43 being the most abundant and wide-spread Cx in the human body [32]. So far, three Panxs (Panx1, Panx2 and Panx3) have been characterized in human and rodents. Despite the lack of sequence homology between Cxs and Panxs, they share a similar topology and both display cell-specific expression patterns [28]. Each protein contains four transmembrane regions, two extracellular loops, a cytoplasmic loop, and a cytosolic N-terminal and C-terminal tail (Fig. 1). The abundance of similar proteins that form GJs and HCs suggests specific functions of these channels composed of different Cx/Panx species. This is supported by the observation that the biophysical permeation properties of GJs and HCs depend on the nature of the Cx/Panx proteins that form the channel and clear differences in channel permeability have been shown for various ions, reporter dyes and signalling molecules such as cyclic adenosine monophosphate (cAMP), ATP and IP_3 [33–37].

Intercellular communication via Cx and Panx channels is a necessity for cellular processes which require a rapid coordinated action, such as the synchronized contraction of the cardiac muscle, or, on a more extended time scale, processes like cell differentiation and growth, which underlines its importance in maintaining normal tissue homeostasis [38–40]. However, intercellular communication does not only result in constructive outcomes but can also have detrimental consequences since the very same communication pathway has been shown to contribute to spread of cell death [39,41–43]. This emerging area is surrounded by some controversy, as GJs have been shown to favor but also impede the spread of cell death through the diffusion of respectively pro-apoptotic or anti-apoptotic factors. This mechanism was termed 'bystander death' and 'good Samaritan effect' respectively and results in an exciting move from the conservative model of apoptosis in which cell death is controlled within an individual cell, to a model involving intercellular communication serving to protect or harm the health of the neighbours [41]. Thus, apoptosis, previously thought to limit cell damage by allowing one cell to disconnect from its adjacent cells for the 'grater good', is actually a 'contagious process' propagated by messengers through GJs. Its involvement not only boosts the efficiency of cancer therapy (by triggering cell death in neighbour cells) but also contributes to secondary injury in the context of stroke or brain trauma [43–45].

At the same time, it is generally accepted that HCs have a very low open probability under normal conditions so as to avoid an excessive loss of ions, metabolites and nutritional molecules that could lead to cell death [29]. In line with this, there are at least four mechanisms by which HCs could exert effects on cell death or survival: (i) via the release of paracrine cell death messengers, (ii) via the uptake or release of toxic or essential molecules respectively, (iii) as a signal transduction pathway or (iv) via an effect on mitochondria [43].

Several lines of evidence support a function for Cxs/Panxs that is not related to their channel-forming capabilities in cell death [46–49]. The molecular mechanism by which Cx/Panx proteins exerts this effect on the cell death process is unclear; it might involve direct interactions with cell death regulators since Cxs have been shown to co-localize in the cytoplasm with major cell death regulators of the B-cell lymphoma-2 (Bcl-2) family including Bax, Bak and Bcl-Xl or apoptosis signal-regulating kinase 1 (ASK1)

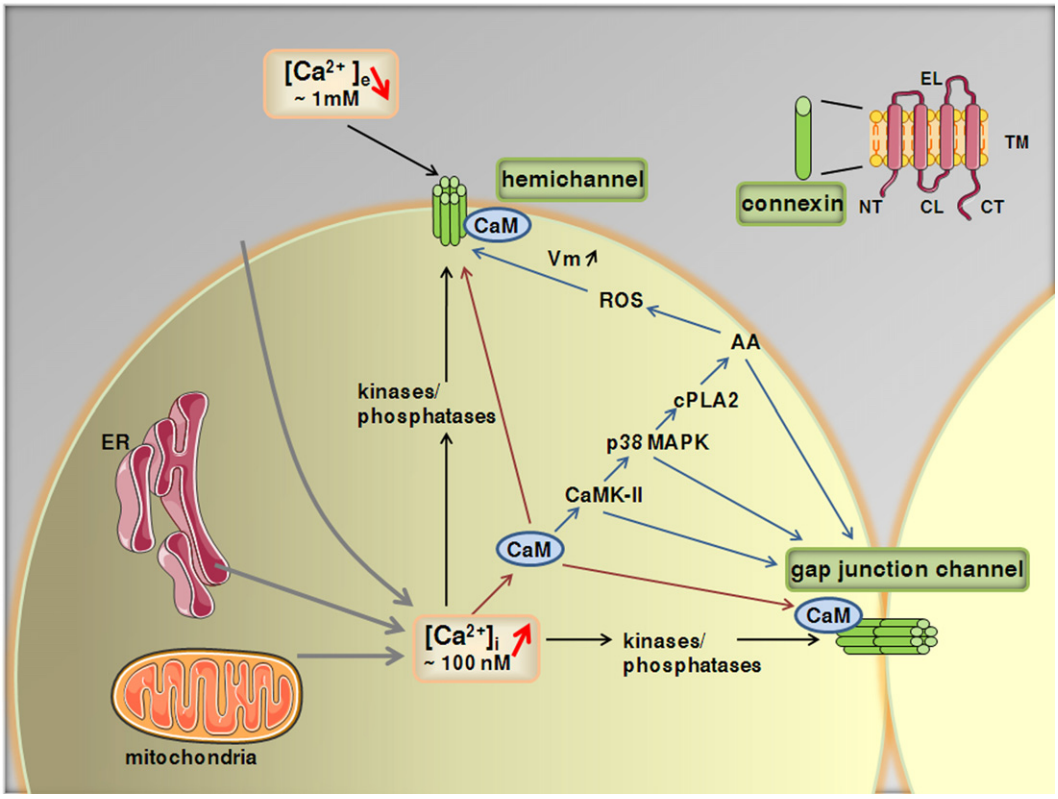

Fig. 1. Effects of $[Ca^{2+}]_i$ on connexin-based channels. GJ channels are composed of two HCs, belonging to the membrane of neighbouring cells. Each HC consists of six Cx proteins that have a topology characterized by four membrane-spanning domains (TM), two extracellular loops (EL), one cytoplasmic loop (CL), one cytoplasmic amino tail (NT) and one cytoplasmic carboxy tail (CT). HCs can also be composed of Panx proteins that have the same topology but no sequence homology. Factors influencing $[Ca^{2+}]_i$ are shown in the left half of the cell and involve Ca^{2+} entry and release from intracellular organelles like the ER and mitochondria. $[Ca^{2+}]_i$ elevation is known to influence GJ permeability and HC activity. A direct effect of Ca^{2+} on Cx channels is unlikely due to the absence of a Ca^{2+} binding site. However, effects via intermediate signalling steps such as activation of kinases or phosphatases (see text), or calmodulin (CaM) have been well documented [15,61]. CaM has been demonstrated to bind to different Cx types [15] and can also activate other signalling paths that affect GJs or HCs. For example, activation of CaM kinase-II (CaMK-II) by CaM, can activate p38 MAPK that has been demonstrated to inhibit GJs [166]. More downstream, subsequent activation of cytosolic phospholipase A2 (cPLA2) may occur resulting in activation of arachidonic acid (AA) metabolism and signalling. AA cascade signalling has been implicated in HC activation via intermediate steps involving ROS and membrane depolarization as possible HC opening messengers [17]. AA is also reported to decrease gap junctional communication, presumably mediated by a direct action on channel proteins [183]. Although most studies indicate a reduction of gap junctional communication in response to an increase of $[Ca^{2+}]_i$, some also reported an increased opening of GJs [115]. In addition, GJ inhibition/stimulation by $[Ca^{2+}]_i$ elevation is known to depend on the magnitude, the duration as well as the source of the $[Ca^{2+}]_i$ increase [15,63–65,77,78]. Not only $[Ca^{2+}]_i$, but also $[Ca^{2+}]_e$ may influence HCs with decreased $[Ca^{2+}]_e$ triggering HC opening [18]. HCs composed of Panxs are not affected by $[Ca^{2+}]_e$ [31].

[50–52]. They are also present, at least parts of the protein like the C-terminal, in the nuclear compartment, and interfering with Cx expression influences a whole array of pro- and anti-apoptotic genes [53,54]. The mechanisms by which Cxs affect gene transcription remain largely unexplored but may involve both direct actions (i.e. cis-acting) or indirect actions (i.e. via transcription factors and mediators of crucial signalling pathways) [55,56]. Cxs or Panxs also reside in intracellular compartments where Ca^{2+} handling is a critical element in the cell death process, namely mitochondria and the endoplasmic reticulum (ER) [57]. Inherent to its overall physiological relevance, it is not surprising that a deregulation of Cx/Panx expression and concomitant intercellular communication burgeons into a plethora of pathological conditions as will become clear throughout this review.

3. Regulation of intercellular communication by Ca^{2+}

In 1877, Engelmann reported that cardiac cells in direct contact with each other during life became independent as they died. This phenomenon, named 'healing-over', was believed to result from the formation of ionic barriers between injured and uninjured cells [58]. More than a century later, it appeared that intracellular Ca^{2+} plays a vital role in protecting intact cells from leakage of metabolites through GJs, by closing and disconnecting them form damaged cells [59,60]. In addition to posttranslational modification (e.g. phosphorylation), transmembrane voltage (i.e. over the plasma membrane) and transjunctional voltage (i.e. over the GJ), the gating and/or permeability of GJ channels is indeed influenced by changes in cytosolic ion (Ca^{2+} and H^+) concentration [15,61,62]. Both acidosis and increases in $[Ca^{2+}]_i$ are since long known to inhibit gap junctional communication, albeit that the actual levels of $[Ca^{2+}]_i$ elevation necessary to suppress GJs is rather variable (from nanomolar to micromolar) and seems to depend on Cx and cell type [63–65]. Ca^{2+} is also reported to affect the functional state of Cxs and Panx HCs. A reduction of the extracellular Ca^{2+} concentration ($[Ca^{2+}]_e$) or an increase in $[Ca^{2+}]_i$ open Cx HCs [16–18], while Panx HC opening is enhanced by an increase in $[Ca^{2+}]_i$, but is insensitive to variations in $[Ca^{2+}]_e$ [11,31]. Other signals or conditions that control the functional state of Cx HCs include membrane depolarization [66], mechanical stimulation [12], changes in phosphorylation status [67], changes in redox status [68], reactive oxygen species (ROS) [69], nitrosylation of the Cx protein [70], ischemia/hypoxia [44,71,72], and also certain Cx mutations [73–76]. Many of these signals are involved in the cascades leading to cell death and are also tightly connected to the Ca^{2+} homeostasis within the cell, as will become clear later on.

It is not entirely understood whether Ca^{2+} acts on GJ channel gating directly or through some intracellular intermediates (Fig. 1).

In some cases, the source of Ca^{2+} appears to play an important role as an increase in intracellular Ca^{2+} resulting from capacitive Ca^{2+} entry appears to be much more effective in inhibiting GJs than that caused by partial Ca^{2+} release from internal stores [77]. Even micromolar bulk increases in $[Ca^{2+}]_i$ induced by the ionophore ionomycin were unable to reduce gap junctional communication, in contrast to thapsigargin-induced store-operated Ca^{2+} entry (SOCE) [78]. This highlights the point that high local submembrane Ca^{2+} concentrations (near the pore of SOCE) are needed to close GJs. It seems however unlikely that Ca^{2+} acts directly on the Cx channels since this would require clusters of negative charges present on the cytosplasmic side of the pore. The only conserved acidic amino acid facing the cytosol is a glutamate between the fourth transmembrane domain and the C-terminal domain, and six such residues per HC are insufficient to account for the Ca^{2+}-dependent gating, sometimes reported to be in the nanomolar range [15]. Many experimental studies suggest that it may involve intermediates such as kinases (e.g. p38 Mitogen-Activated Protein Kinase (MAPK) [79] or protein kinase C (PKC) [80]) or phosphatases (e.g. calcineurin [81]) activated by Ca^{2+}. Most Cxs are phosphoproteins and in different models, apoptosis was directly correlated with changes in the phosphorylation pattern of the Cxs [61,67,82–84]. Ca^{2+} might also mediate its effects on intercellular communication via calmodulin, a molecule which consists of an N- and C-lobe, each containing specialized domains known as EF-hands that bind Ca^{2+} with affinities in the nanomolar range. Ca^{2+} binding to these domains induces conformational changes enabling calmodulin to interact with receptors. Such interaction was demonstrated with Cx38, Cx32, Cx37 and Cx43, Cx44, and Cx50 [15]. Ca^{2+} triggered HC responses induced by the ionophore A23187 and glutamate activated a cascade of multiple intermediate signalling steps that involved calmodulin, calmodulin-dependent kinase II, p38 MAPK, phospholipase A2, arachidonic acid, lipoxygenases, cyclooxygenases, ROS, nitric oxide (NO) and depolarization [17]. De Vuyst et al. further revealed a very narrow range (around 500 nM) for regulation of HC responses by $[Ca^{2+}]_i$ and an activation and disappearance of these responses by $[Ca^{2+}]_i$ respectively below and above 500 nM [17]. This narrow range of modulation of HC responses by $[Ca^{2+}]_i$ might determine their contribution at the different stages of the cell death process, i.e. opening during the initiation (modest Ca^{2+} increases) while closed toward the end (high Ca^{2+} increases).

Opening of Cx HCs is also critically dependent on the $[Ca^{2+}]_e$ [18]. Various genetic mutation studies in the Cx field have brought forward evidence that $[Ca^{2+}]_e$ is a direct mediator of HC gating. Mutations in Cx32, Cx30 and Cx26 can cause hereditary peripheral neuropathy, hidrotic ectodermal dysplasia, and nonsyndromic hereditary hearing impairments and skin disease respectively. Functional analysis indicated that these phenotypes are caused by abnormal HC opening which adversely affects the viability of the involved cells. In most cases the phenotype could be rescued by elevation of the $[Ca^{2+}]_e$ [73–76]. Gomez-Hernandez and colleagues revealed that the deregulation of the HCs is caused by the mutation of a Ca^{2+} binding site, consisting of 12 Asp residues, which mediates most Ca^{2+} effects on the Cx32 HCs [74]. Furthermore, a mutation in Cx26, causing nonsyndromic hereditary hearing impairment, is located right next this Ca^{2+} binding site and strongly suggests a similar mechanism of disturbed HC gating [73]. In native Cx26 HCs, it was observed that the cytoplasmic but not the extracellular Cx domains rotate in response to a reduction of the Ca^{2+} concentration, thereby closing the HC [85].

4. Intercellular Ca^{2+} signalling and cell death

A complex regulation of Ca^{2+} signalling is required for cell survival since many cellular functions are controlled by Ca^{2+}. Additionally, the communication of cytoplasmic Ca^{2+} signals between cells allows for the transmission of local information to a global level, thereby amplifying the signal and synchronizing the function of a large group of cells. The ubiquitous nature of Ca^{2+} waves in diverse cell types in response to a variety of stimuli, recently adjoined by evidence in *ex vivo* and *in vivo* conditions [86–88], underscores the importance of this kind of signalling events for cell and organ function. In various multicellular systems, GJs and HCs, mostly through release of ATP via Cx as well as Panx HCs, have been implicated in the spread of Ca^{2+} signals to neighbouring cells possibly contributing in this way to the coordination of several physiological processes [1,89–91]. Proposed functions for intercellular Ca^{2+} signalling arise from the developmental field e.g. in the developing retina, where they are implicated in the proliferation and differentiation of neural progenitor cells [92], and in the organization and proliferation of the developing neocortex [93]. Ca^{2+} wave activity is also important in the auditory system, more specifically the inner ear. Here, Cx HCs seem to be a necessity for sustaining long-range Ca^{2+} signal propagation by promoting ATP release while GJ channels allow the diffusion of Ca^{2+}-mobilizing second messengers, presumably IP_3, across coupled cells [94,95]. Currently, most evidence for intercellular Ca^{2+} signalling comes from the brain [96]. The communication of Ca^{2+} signals between cells is a typical feature of glial cells, but might involve other cells since neuronal cells also exchange Ca^{2+} signals among each other as well as with surrounding astrocytes [10,97,98]. The importance of astrocytic Ca^{2+} signals in the brain is related to their central position between the information processing neurons and the vascular cells (the brain cell tripartite). They may influence the synaptic signal transmission due to the release of so-called gliotransmitters such as glutamate [20]. Gliotransmitters are not only expected to influence neuronal activity and brain microcirculation, but also feedback on astrocytes and thus modulate their Ca^{2+} excitability. Furthermore, by sending endfeet to endothelial cells, astrocytes may also affect the vascular cells (i.e. smooth muscle and endothelial cells) to modulate blood vessel diameter and transports across the blood–brain barrier [99].

Next to the observation of intercellular Ca^{2+} signalling in brain slices, in intact organs such as perfused rat liver, or *in vivo* in the intact brain, one should realize that the majority of our knowledge and understanding of intercellular Ca^{2+} signalling derives from *in vitro* culture work on adherent cell monolayers [86,87,97,100]. Although *in vitro* studies indicate that cells can transmit intercellular Ca^{2+} waves, the magnitude of the stimuli necessary to trigger this form of Ca^{2+} signalling is usually considerably higher than would be expected to occur under physiological conditions. Reports of spontaneous intercellular Ca^{2+} waves *in vivo* are becoming available [86], but in view of what is known so far, it is likely that this form of Ca^{2+} signal transmission plays a more prominent role under pathological (cell death-related) rather than under physiological conditions.

4.1. The bidirectional aspect of gap junctional communication and Ca^{2+} signalling in cell death

4.1.1. Ca^{2+}-dependent regulation of gap junctional communication in cell death

Since drastic alterations in Ca^{2+} concentration frequently accompany the onset and amplification of cell death in various pathological conditions [3–7,101–104], it may have a profound effect on the pathological outcome via a deregulation of intercellular communication. This is well exemplified in the case of ischemia-related injury whereby Cx channels have been proposed to contribute to the gradual expansion of apoptotic cell death in the ischemic penumbral region [44]. This pathological condition is associated with a reduction in gap junctional communication and an opening of large-conductance channels that have biophysical properties resembling those of Cx and Panx HCs [71,105]. Although

the molecular mechanisms by which ischemia affects intercellular communication is not known, it is accompanied by a decrease in $[Ca^{2+}]_e$ and an increase in $[Ca^{2+}]_i$ and acidosis, which all have a profound impact on the status of GJs and HCs [59,60,106–108]. The healing-over phenomenon brings forward a rather simplistic scenario for $[Ca^{2+}]_i$ effects on GJs, namely the closure of the junctional channels by elevated $[Ca^{2+}]_i$, thereby isolating the dying cell from its neighbours [58–60]. However, there is much evidence that GJs remain open under ischemic conditions in the brain and in apoptotic cell death occurring spontaneously or as a consequence of serum deprivation [71,82,109–111]. Early apoptotic bodies are indeed still able to transfer fluorescent reporter dyes from neighbouring normal cells in serum-deprived cultures, which indicate that they are coupled to healthy cells via GJs [82]. This suggests that uncoupling of GJs, as a protective measurement, is not an essential feature of cell death. One possible explanation why GJs might escape from the closing actions of $[Ca^{2+}]_i$ elevation relates to the buffering capacity of the cytoplasm and the rapid uptake of Ca^{2+} in the stores which may create large intracellular Ca^{2+} gradients within the cell [112,113]. However, in a study providing a quantitative assessment of the relation between junctional conductance and GJ-mediated cell death, intercellular conductance increased during Cytochrome C (CytC)-induced bystander cell death in *Xenopus* oocyte pairs expressing endogenous Cx38 [114]. It is important to note that although most studies indicate a reduction of gap junctional communication in response to an increase of $[Ca^{2+}]_i$, some also reported an increased opening of GJs [115].

4.1.2. Are Ca^{2+} and/or its second messenger IP_3 the intercellular cell death masters in crime?

As is clear from the previous discussion, GJs have important links to the cellular Ca^{2+} homeostasis, as channels being influenced by $[Ca^{2+}]_i$ but also contributing to deregulation of $[Ca^{2+}]_i$. For GJs, the question of how exactly they contribute to cell death reduces to the search for the molecule(s) responsible for bringing over the cell death message to neighbouring healthy cells or the survival message to the dying cells (Fig. 2). However, many factors influencing the cell death process can readily pass through GJs and exert their effects on adjacent cells. A more precise criterion for a candidate molecule communicating cell death via GJs is, next to its ability to pass through GJ channels, that it has the potency to directly trigger cell death or that it can be converted into a killer molecule under certain conditions. The latter possibility is of special importance given the fact that many of the messengers able to permeate GJs, such as Ca^{2+} and IP_3, are physiological messengers in the very first place. Cusato et al. studied the relation between the gap junctional

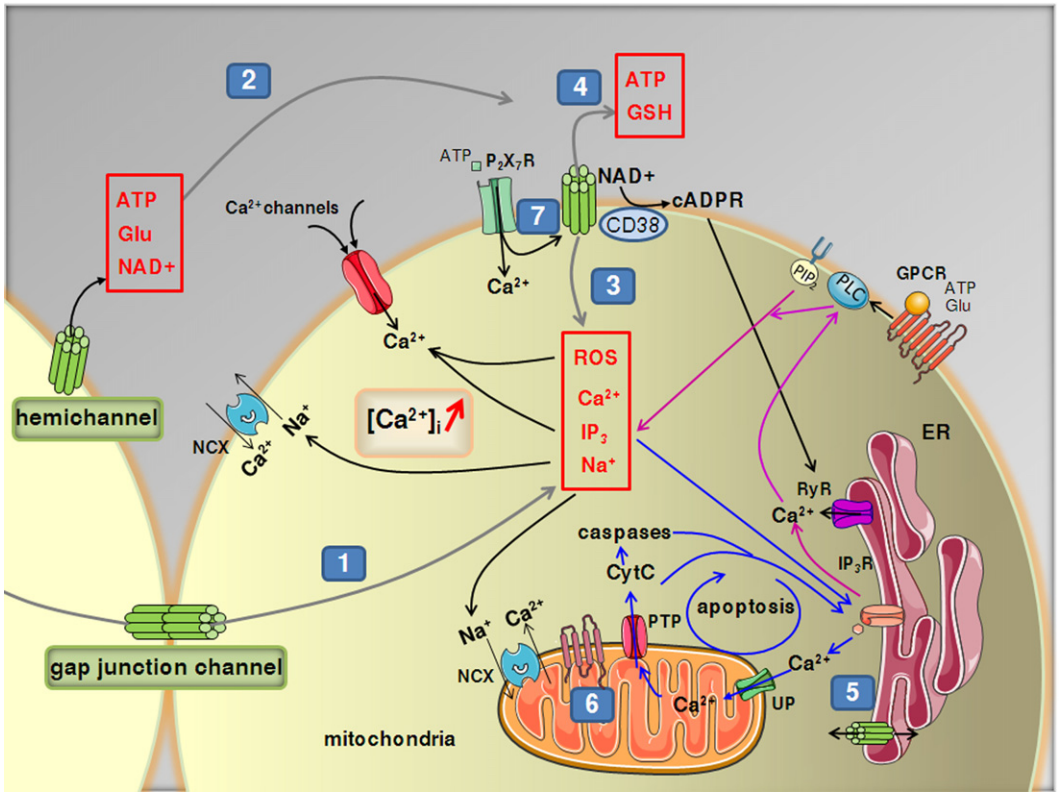

Fig. 2. Mechanisms of Cx channel-dependent (de)regulation of $[Ca^{2+}]_i$. GJs and HCs may affect $[Ca^{2+}]_i$ through a number of mechanisms. GJ channels can accommodate direct exchange of ions (Ca^{2+}) and molecules such as IP_3, ROS and Na^+ leading to $[Ca^{2+}]_i$ changes via IP_3R channels located on intracellular stores (ER) and possibly also the plasma membrane, ROS-activated Ca^{2+} channels and the reverse mode of the Na^+/Ca^{2+} exchanger (NCX) respectively (1) [1,8,141,144]. HCs may alter $[Ca^{2+}]_i$ by various mechanisms: as a paracrine release pathway for molecules that may trigger $[Ca^{2+}]_i$ changes in neighbouring cells by activating G protein-coupled receptors (GPCRs, i.e. metabotropic purinergic or glutamate (Glu) receptors) or Ca^{2+} channels (P_2X_7Rs or glutamate-activated Ca^{2+}-permeable channels), or by conversion of NAD+ to cADPR and subsequent uptake by the CD38 enzyme (2) [13,20,24,151,161]. HC opening may also accommodate the entry of the same molecules transferred through GJs, or the release of essential molecules such as ATP or GSH, resulting in a loss of defense potential (3) [14,25,27,69,157]. Cx proteins, possibly organized as channels, may also play a role by affecting ER Ca^{2+} homeostasis, as demonstrated for Panx1 HCs (5) [9]. Cxs have been reported to be present in the mitochondrial membrane and were suggested to influence mitochondrial functioning directly or through HC formation (6) [57]. HCs composed of Panxs may act as a Ca^{2+} entry route or as a part of the P_2X_7R death complex (7) [152]. Sustained high $[Ca^{2+}]_i$ leads to cell death. Additionally, all of the molecules transferred through Cx channels (depicted in red) have been shown to induce/accompany cell death processes and are tightly linked to $[Ca^{2+}]_i$ as demonstrated in particular for IP_3. A small amount of IP_3 transferred through GJs or taken up by HCs could result in apoptosis since it can activate a vicious circle (depicted in blue) with CytC release and caspase activation as a consequence (see text) [137]. Secondly, IP_3-induced Ca^{2+} release from the ER can provide a positive feedback mechanism for PLC activation and subsequent IP_3 generation (depicted in pink) [133]. This mechanism has put IP_3 in the spotlight as the prototype candidate to communicate the cell death message. The factors or conditions that convert IP_3 from a physiological messenger to a cell death messenger are, however, largely unknown.

conductance of *Xenopus* oocyte pairs after injecting CytC in one oocyte and the survival or death of the uninjected cell. From this study, it became evident that the level of conductance was an important predictor of the outcome. Oocyte pairs coupled by GJs needed to reach a certain threshold of junctional conductance in the order of 6 μS for bystander death to occur [114].

The molecular size of proteins directly involved in the apoptotic pathway, such as CytC, Apaf-1 and the caspases, preclude their passage through the channels. Several studies have suggested that Ca^{2+} and/or IP_3 are plausible candidate mediators of bystander death, based on the work with the intracellular Ca^{2+} chelator BAPTA-AM [114,116,117] and the inositol 1,4,5-trisphosphate receptor (IP_3R) inhibitor Xestospongin C [114]. As already referred to, Ca^{2+} and IP_3 are the major messengers of Ca^{2+} wave propagation. An important observation is that as Ca^{2+} wave fronts travel throughout damaged cells to neighbouring healthy cells, high micromolar $[Ca^{2+}]_i$ levels – high enough to evoke cell death – of intracellular Ca^{2+} can be attained [118,119]. Ca^{2+} waves have indeed been proposed to function as 'death waves' in several experimental models. Neurons are more prone to ischemia-induced damage as compared to astrocytes, and neuronal Ca^{2+} signals can be transmitted to astrocytes, evoking Ca^{2+} waves in astrocytic networks [98]. Hence, necrotic neurons could, in principle, initiate death waves from the ischemic core to trigger apoptosis in astrocytic cells that have relatively normal levels of ATP in the penumbra or border zone. Conversely, the reported protective function of GJs in ischemia might allow astrocytes to survive a potentially necrotic and energy-depleting condition by supplying ATP to astrocytes more closely located to the ischemic core zone. These cells are, however, expected to die later by the energy-consuming steps of apoptosis [120]. Gap junctional communication might also result in a reduction of toxic molecules (i.e. reduction of the amount of the toxic metabolite over a volume of multiple cells) [43,44,52,121–125]. Thus, GJs may exert both a survival and killing function in the same model system. A balance between the spread of survival or apoptotic factors would then, in addition to the severity of the primary insult, determine the outcome, i.e. size of the affected area. In different model systems existing of well-coupled cells, the propagation of death indeed never becomes totally generalized [111,117].

Gap junctional coupling of astrocytes can result in a neuroprotective effect as has been demonstrated in several studies. Gap junctional communication in astrocytes is essential for the control of the composition of the extracellular environment through the capacity of buffering cytotoxic substances, such as free radicals, glutamate and K^+, as well as by providing neurons with nutrients including antioxidants, glucose and ATP [44,126–128]. For example, astrocyte–astrocyte coupling protected neurons against oxidative stress in mixed cultures of neurons and astrocytes. The mechanism remains unclear, but involves a suppression of the accumulation of oxyradicals and stabilization of Ca^{2+} homeostasis in the neurons [122]. Recently, Kozoriz et al. concluded that the C-terminal region of Cx43 is important for this neuroprotective function based on middle cerebral artery occlusion experiments in mice expressing a truncated form of Cx43. A deletion of the C-terminal region resulted in less coupled cells, alterations in GJ channel gating and hemichannel activity, and reduced spread of intercellular Ca^{2+} waves [129]. Reactive astrocytes also exert a protective effect on human melanoma cells treated with various chemotherapeutic drugs by sequestering Ca^{2+} through GJs from the cytoplasm of neighbouring tumor cells. This may provide a mechanistic explanation for the observed resistance of melanoma brain metastases, which are surrounded and infiltrated by activated astrocytes, to chemotherapeutic drugs [130].

Ca^{2+} waves may also play a role in the developing nervous system. UV spot irradiation or mechanical injury of an embryonic anterior pagoda neuron in the leech resulted in the initiation of growth in the axon of a contacting healthy cell. This was associated with Ca^{2+} wave fronts crossing at GJs from the dying cell to the adjacent healthy one. Since the authors did not observe any Ca^{2+} release from internal stores in those neurons at that stage, they attributed the presence of a Ca^{2+} wave to a direct spread of Ca^{2+} through GJs. Consequently, they ruled out the possibility that a molecule such as IP_3 crossed the junctions and released Ca^{2+} from postsynaptic releasable pools [118]. This seems somewhat controversial because the contribution of Ca^{2+} passage via GJs is known to be limited due to its low effective diffusion constant (13–65 $\mu m^2/s$) in the cytoplasm that is related to the presence of Ca^{2+} binding proteins [112,131]. For comparison, the effective diffusion constant for IP_3 is 283 $\mu m^2/s$ [112].

Most currently available evidence points to IP_3 as the prototype candidate to communicate the cell death message. Upon binding to their receptors, classic death ligands such as Tumor Necrosis Factor-α (TNF-α) and Fas ligand can promote IP_3 generation in some cells by initiating phospholipase C (PLC) mediated metabolism of phosphoinositol-4,5-bisphosphate (PIP_2) [119]. Of note, some isoforms of PLC, which function to hydrolyze PIP_2 to IP_3 and diacylglycerol (DAG), are particularly sensitive to Ca^{2+}. An elevation of $[Ca^{2+}]_i$ alone may activate these enzymes or may provide a positive feedback mechanism for PLC activation [132,133]. Moreover, an increase in PLC activation results in a depletion of PIP_2 from the plasma membrane which has been suggested to inhibit Cx43-based cell–cell communication [134]. IP_3 can permeate through GJ channels (MW 486) and trigger the rapid release of Ca^{2+} from the ER and other cellular membranes by its binding to IP_3Rs. Among the intracellular Ca^{2+} release channels, the IP_3R has attracted the most attention in the cell death field because it displays a unique combination of properties including its regulation by Ca^{2+} itself and by members of the Bcl-2 family of pro- and anti-apoptotic proteins [7,135,136]. The ensuing Ca^{2+} changes may trigger CytC release from mitochondria through mitochondrial Ca^{2+} uptake and permeability transition pore (PTP) opening. Released CytC on its turn can interact with IP_3 receptors to potentiate ER Ca^{2+} release and activate caspases which proteolytically cleave the receptor. Both of these mechanisms provide an enhanced ER Ca^{2+} leakage pathway and exacerbate mitochondrial Ca^{2+}-driven CytC release in a vicious circle (Fig. 2 – depicted in blue) [137]. These regulatory characteristics allow IP_3Rs to play a role in the initiation and the amplification of Ca^{2+} dependent apoptotic pathways [119].

In addition, GJs seem to display selective permeability toward IP_3 depending on their Cx composition. Niessen and colleagues demonstrated that microinjection of IP_3 into confluently growing HeLa Cx32 transfectants induced propagation of a Ca^{2+} wave from the injected cell to adjacent cells that was at least three- to four-fold more efficient than in HeLa Cx26 cells and about 2.5-fold more efficient than in HeLa Cx43 transfectants [37]. Differences in selectivity for second messengers may play an important role in the extent to which cell fate-modulating signals are spread since conveying a cell death or survival message through GJ channels has indeed been shown to be a selective and well-coordinated process, involving specific Cxs [138].

4.1.3. What about other cell death messengers?

Other potential cell killer candidates are ROS which are tightly interconnected with Ca^{2+} homeostasis and signalling (Fig. 2). Ca^{2+} overload can promote ROS production in response to various cell death stimuli. The exact mechanism still remains elusive but may rely on PTP opening with release of CytC and GSH- and NADPH-dependent antioxidative enzymes as a consequence [5,139]. ROS may also augment the cellular Ca^{2+} surge by promoting Ca^{2+} entry via L-type channels or Ca^{2+} channel upregulation [122,140]. Additionally, ROS may diffuse via GJs to neighbouring cells as has been proposed in the context of endothelium-dependent contractions

[141]. In turn, oxidative stress, thermal stress and metabolic inhibition also stimulates cell–cell dye coupling and promotes the presence of HCs in the plasma membrane, two effects that were associated with increased recycling of Cx proteins to the membrane [70,142]. These observations suggest that ROS might regulate and exacerbate its own spread via GJs or HCs.

Experimental evidence, again from the ischemia field, brings up Na^+ as plausible cell fate modulator (Fig. 2). After an ischemic episode, cardiac myocytes may respond to reperfusion by hypercontracture, resulting in infarct regions consisting almost exclusively of areas of contraction band necrosis composed of clusters of hypercontracted dead myocytes. The hypercontracture response was reported to be propagated from cell-to-cell via GJs [143]. In an attempt to identify the chemical messenger, hypercontracture was mimicked by microinjection of extracellular media containing 1 mM Ca^{2+} to stimulate sarcolemmal disruption. The propagation was caused by the passage of Na^+ through GJs that, on its turn induced Ca^{2+} entry via the reverse mode of the Na^+/Ca^{2+} exchanger, a major regulator of intracellular Ca^{2+} homeostasis in excitable cells. Additional experiments suggested that the direct passage of Ca^{2+} through GJs, although not sufficient to induce hypercontracture in the neighbouring cell, may have contributed to its development [144].

Other proposed GJ-permeable messengers include cAMP and cyclic guanosine monophosphate (cGMP) as killer molecules, or glucose, ATP or the free radical scavengers ascorbic acid and reduced GSH to favor cell survival. However, direct evidence that these molecules can function as cell fate modulators is still lacking [42,43].

4.2. The bidirectional aspect of hemichannel responses and Ca^{2+} signalling in cell death

The role and functional consequences of HC opening, in response to Ca^{2+} changes or other factors, on cell death is still poorly defined but several possibilities have been put forward (Fig. 2) [43]. Being a pore permeable to ions, HC opening may help in setting off cell death by compromising the cell's ability to maintain ionic homeostasis, for example by excessive entry of Na^+ and/or Ca^{2+}. This hypothesis is supported by a study investigating a mutation in the cytoplasmic loop of Cx26 (G11E) which is responsible for autosomal dominant KID (keratitis, ichthyosis and deafness) syndrome. The presence of mutant Cx26 potentiated the uptake of Ca^{2+} into the cells with necrotic cell death as a consequence. Most notably, an increase in Ca^{2+} uptake was also detected when cells were transfected with the wild-type form, indicating a major function of Cx26 in the regulation of Ca^{2+} flux [145]. The HC pathway also facilitated the uptake of Na^+ and Ca^{2+} in ventricular myocytes during metabolic inhibition [14]. The increase in Ca^{2+} due to HC opening can result in a vicious circle of Ca^{2+}-dependent HC opening and HC-dependent Ca^{2+} rises resulting in cell death. Such vicious cycle has been demonstrated during metabolic inhibition where the rise in Cx32 HC activity was preceded by a small, but rapid increase in $[Ca^{2+}]_i$, which was due to Ca^{2+} entry from the extracellular medium via HCs. This rise in $[Ca^{2+}]_i$, in turn, increased surface expression and stimulated the opening of HCs, leading to increased Ca^{2+} influx [116].

Being a pore permitting diffusion in both directions, HCs could also contribute to $[Ca^{2+}]_i$ rise in an indirect way by the release of various substances which act on receptors on more distant cells. A recent study by Gossman and Zhao revealed that IP_3 can be released by HCs in the cochlea where IP_3-evoked Ca^{2+} waves are important for normal hearing. Moreover, extracellular application of IP_3 triggered an $[Ca^{2+}]_i$ increase. The authors hypothesize that IP_3 might re-enter the neighbouring cells via HC uptake and bind to its receptor, in this way acting as an extracellular mediator to participate in intercellular signalling. Although the lifetime of IP_3 in the cytoplasm is limited, this extracellular pathway may play a crucial role in long distance communication since there are no specific enzymes present to degrade it [27,94]. Most intriguingly, IP_3Rs are not only located within the ER membrane, but have also been reported to be present in the plasma membrane. The role of IP_3Rs in the plasma membrane is still unclear but several lines of evidence suggest they might directly mediate Ca^{2+} entry in response to cytosolic IP_3 [146].

Cx43 HCs also mediate NAD+ transport and functionally interact in the plasma membrane with the ectoenzyme CD38 that converts NAD+ to the Ca^{2+} mobilizer cyclic ADP-ribose (cADPR). CD38 translocates cADPR to the cytoplasm where it binds ryanodine receptors (Fig. 2) [20]. While NAD+ treatment can reduce ischemic brain damage and genotoxic agent-induced death of primary neuronal and astrocyte cultures, it has recently been found to decrease tumor cell survival by a mechanism involving oxidative stress, P_2X_7 receptors (P_2X_7Rs) and an altered Ca^{2+} homeostasis [147].

Release of ATP and other metabolites such as glutamate and prostaglandins through HCs could also contribute to paracrine spreading of the cell death process (Fig. 2). Their extracellular diffusion and action on corresponding receptors on remotely located cells often accompanies a rise in cytoplasmic Ca^{2+} [4,5,104,148,149] that in turn may lead to a new cycle of HC responses [14,16,74]. In a model of cochlear explant cultures, ablation of the hair cell region caused a spatiotemporal spread of extracellular signal-regulated kinase 1/2 (ERK1/2) activation in the supporting cells. This signalling mechanism that acts to promote hair cell death was associated with the generation of an intercellular Ca^{2+} wave in the hair cell region and was dependent on ATP release and the presence of Cx channels [150]. Purinergic P_2X_7Rs have also been shown to increase $[Ca^{2+}]_i$ in response to ATP released via open Panx HCs in oligodendrocytes subjected to oxygen and glucose deprivation (OGD). An increase in $[Ca^{2+}]_i$ via this receptor resulted in death of the oligodendrocytes [151]. In addition, Panx1 HCs appear to be recruited into the P_2X_7R signalling complex to function as a permeabilization pore, capable of passing large molecules in response to activation of the P_2X_7R (Fig. 2) [152,153]. An important observation is that ATP also belongs to a group of 'find-me' signals, released by apoptotic cells to recruit phagocytes. Panx1 HCs have been suggested to mediate the release of these signals during apoptosis. Cleavage of Panx1 by caspases, resulting in a constitutively open channel, seems to be important for this function [154]. Likewise, caspase-mediated truncation of Cxs has also been reported during apoptosis [84,155]. HCs can also influence the cell's energy level by allowing ATP to leak out of the cell [22,117]. A decrease in intracellular ATP has, expectedly, many consequences. First of all ATP plays a decisive role in the balance between cell death via necrosis or apoptosis, and a decrease in its level speeds up the progression of apoptosis toward secondary necrosis *in vitro* [156,157]. Secondly, a change in intracellular ATP level influences the gating of Cx channels as it is an important substrate for protein kinases which determine the phosphorylation status of Cxs [158]. Thirdly, low ATP levels activate glycolysis and reduce the intracellular pH, known to influence HC gating possibly via Ca^{2+} [15,159]. Cx HCs may also be involved in the development of intracellular Ca^{2+} oscillations and thereby determine the outcome toward cell survival [7]. The latter require a regenerative signalling loop, and Ca^{2+}-triggered ATP release via CxHCs followed by autocrine action of ATP on cell-surface receptors and subsequent regeneration of the cytoplasmic Ca^{2+} signal [160].

The Ca^{2+}-dependent release of glutamate from astrocytes has been shown to affect the $[Ca^{2+}]_i$ in adjacent neurons through the activation of glutamate-ionotropic receptors [161]. This is one of the major proposed mechanisms of ischemic brain toxicity and may contribute to neuronal damage in neurodegenerative diseases [161,162]. Ye et al. reported that, astrocytes in culture express-

ing Cx43 release an excessive amount of glutamate and other amino acids when HCs were opened by exposure to low extracellular divalent cation (i.e. low Ca^{2+} and Mg^{2+}) concentrations. Several reports suggest HC opening starting from 1 mM $[Ca^{2+}]_e$ and below [18,25,74] and significant glutamate release through HCs is detected at about 0.1 mM $[Ca^{2+}]_e$, which can occur in the brain during ischemia [24,106,107]. The HC-related astrocytic pathway of glutamate release will add to neuronal (mostly vesicular) sources of glutamate and can be adjoined by the release of glutamate from activated microglia and macrophages through Cx32 HCs [163,164]. A recent study also demonstrated that a combined effect of ATP and glutamate released via astroglial Cx43 HCs mediate neuronal death through activation of Panx1 HCs [165].

Finally, several studies demonstrated that oxidative stress, closely linked to cellular Ca^{2+} homeostasis, could be at the heart of HC opening. Arachidonic acid increased cell permeability mediated by Cx43 HCs and byproducts of its production were suggested to open HCs during metabolic inhibition [17,71]. Retamal et al. presented further evidence that S-nitrosylation by NO of one or more of the three intracellular cysteines of Cx43 might be the underlying mechanism of increased HC opening induced by metabolic inhibition and proinflammatory conditions in astrocytes [70,166]. Of note, NO production can be stimulated by Ca^{2+} [139]. The same mechanism may account for Panx1 channels which are also reported to be sensitive to changes in redox potential [167,168]. Moreover, HCs are not only regulated by these substances but also actively participate in the uptake of ROS, contributing to an expansion of cell death, or the release of glutathione, sensitizing the cells to oxidative stress induced cell injury (Fig. 2) [69,169].

5. Intracellular localization of connexins/pannexins and intracellular Ca^{2+} handling in relation to cell death

Early immunohistochemical studies documented anti-Cx43 immunoreactivity in the plasma membrane in close opposition to the mitochondria. Since mitochondria are known to sequester Ca^{2+}, the authors suggested that the close association between mitochondria and GJs may function to buffer the intracellular Ca^{2+} concentration near the GJs, and thereby regulate their permeability [170]. Later studies revealed a mitochondrial localization of Cx43 in human umbilical vein endothelial cells, myocardial cells, brain cells and liver cells. In rat liver, hepatocyte-specific Cx26 and Cx32, were found to be present in mitochondria and Cx32 has also been reported in heart mitochondria. Cx43 has been observed in the inner as well as outer mitochondrial membranes [171–174]. The functional relevance of mitochondrial Cx43 is, however, still unclear. Cellular stress and ischemic preconditioning have been shown to increase its mitochondrial localization in endothelial cells and cardiomyocytes, respectively. Upon ischemic pre-conditioning, Cx43 translocates from the plasma membrane to the inner mitochondrial membrane in cardiomyocytes via a heat shock protein 90-dependent translocase of the outer membrane-20 complex pathway. Moreover, an increased pro-engraftment survival of insulin-like growth factor 1 preconditioned stem cells was attributed to the translocation of Cx43 into the inner mitochondrial membrane.

Mitochondrial Cx43 was recently found to be a major regulator of cardiomyocyte apoptosis since various GJ inhibitors induced a concomitant release of Ca^{2+} and CytC in isolated mitochondria with apoptosis as a consequence [173]. The GJ inhibitor carbenoxolone also facilitated the Ca^{2+}-induced PTP opening in synaptic rat brain mitochondria by potentiating the dephosphorylation of serine-368 of Cx43 caused by PTP [172]. In addition, Lu and colleagues observed that mitochondria-specific overexpression of Cx43 helped stem cells to avert mitochondrial CytC release, limiting caspase 3 activation and improving cell survival after OGD. These observations suggest that Cx43 could contribute to keep the PTP in a closed state, thereby preventing the detrimental effects of mitochondrial Ca^{2+} overload. At a molecular scale, this might involve integration of Cx43 into the PTP, hydrophobic interactions between Cx43 and CytC or HC-related mechanisms (Fig. 2) [175]. Mitochondrial Cx43 has been shown to contribute to K^+ fluxes in cardiomyocytes presumably by forming HC-like structures [176]. The potential presence of Cx43 in the mitochondrial membrane may relate this protein to the apoptosis-regulating Bcl-2 family of proteins. Bcl-2, which resides in the mitochondrial membrane, plays an essential role in repressing apoptosis by preventing CytC release from mitochondria into the cytosol [177]. Protein structural analysis using computational methods revealed that Cx43 contains a Bcl-2 homology domain 3 (BH3), which has a similar domain organization as the integral membrane protein 2B (ITM2B). ITM2B has a pro-apoptotic role by interacting with Bcl-2. Additional results indicated that proteins with four transmembrane topology are rare but present in the Bcl-2 family. These data suggest Cx43 is a candidate member of the Bcl-2 family [175,178].

Not only mitochondrial Ca^{2+} handling but also ER Ca^{2+} homeostasis appears to be one of the key regulators of cellular life and death. Various apoptotic stimuli evoke the efflux of Ca^{2+} from the ER and subsequent accumulation into the mitochondria. A strategic positioning of the mitochondria close to the sites of IP_3R-mediated Ca^{2+} release from the ER seems to be crucial for this ER-to-mitochondria conduit. This results in the formation of so-called Ca^{2+} 'hot spots' which overcomes the low affinity of the uniporter, a channel responsible for the mitochondrial Ca^{2+} overload, and causes subsequent release of pro-apoptotic molecules, including CytC from mitochondria [5,179]. It is increasingly recognized that a number of the key pro- and anti-apoptotic Bcl-2 family of proteins in the cell are present in the ER membranes and alter the Ca^{2+} permeability of ER membranes e.g. by regulating IP_3R activity [7]. Whether Cxs play a role in ER Ca^{2+} handling is still speculative but since Cx43 is a candidate member of the Bcl-2 family and since Cxs are synthesized and oligomerize in the ER to HCs (there is some controversy as to whether this holds for each member of the Cx family) it is conceivable to assume that Cxs play a role by regulating ionic fluxes over the ER membranes, analogous to what has been suggested in mitochondria (Fig. 2). In line with this hypothesis, Panx1 HCs have been demonstrated to form Ca^{2+}-permeable channels in the ER, contributing to ER Ca^{2+} leak and affecting intraluminal ER Ca^{2+} concentration [9,91]. However, these results should be interpreted with care as they are based on overexpression experiments in cell lines.

6. Conclusion and perspectives

Whether cell death occurs naturally or upon experimental means, cells can alter their fate radically in response to loss of a nearby dying neighbour. Intercellular communication by means of GJs and HCs has been implicated by allowing the passage of messengers that appear during cell death. A key step in understanding this process is to unveil the identity of the signals that communicate the message to surrounding cells.

A substantial body of evidence indicates that intracellular Ca^{2+}-dependent and intercellular communication pathways are coordinately entangled to regulate cell death. The tight connection between Ca^{2+}, intercellular communication and cell death, and the opposed functions (pro-cell death versus pro-survival) that Ca^{2+} as well as Cx-related communication can exert in cell fate, makes Ca^{2+} a master cell death regulator not only at the intracellular but also at the intercellular level [43,119]. An important challenge for future work is to verify whether Ca^{2+} as such or its mobilizing messenger IP_3 are the actual cell death messengers or whether these are epiphenomena involved in the pathways leading

to cell death. A major caveat of currently used plasma membrane permeable compounds applied to investigate the Ca^{2+}- and IP_3-dependency of intercellular cell death spread is the inability of these tools to make distinction between trigger cells, in which cell death is induced, and receptor cells undergoing bystander death [114,116,117]. Another approach addressed to demonstrate the cell death-inducing potency of Ca^{2+} consists of introducing a high concentration of these ions into the cytoplasm (f.e. via microinjection) and subsequently investigating the outcome on bystander death [180]. However, the effect of Ca^{2+} on cell death and also on Cx-related communication has been shown to largely depend on the magnitude, the duration and the source of the Ca^{2+} increase [65], which is not always taken into consideration in such studies. Furthermore, evidence supporting a correlation between deregulation of Ca^{2+} homeostasis and bystander death does not imply its direct participation as messenger of cell death communication. Direct evidence could be obtained by selectively eliminating or allowing the passage of single molecules through GJs and/or HCs which are under investigation as potential cell death messengers. Krutovskikh et al. used oleamide, a neuropeptide which was reported to selectively confine the GJ permeable molecules to Ca^{2+} ions, to study the role of Ca^{2+} in propagating spontaneous cell death through GJs in a rat bladder carcinoma cell line. Application of this substance did not abrogate coordinated cell death by clusters, indicating that Ca^{2+} ions were the most probable cell-killing signals that were spread between these cells [111]. An important concern regarding the use of compounds like oleamide is that its presumed selective GJ actions are based on the observation that it decreased the size of Ca^{2+} waves [181]. However, other second messengers such as IP_3, are much more likely to contribute to Ca^{2+} wave propagation and this can be adjoined by paracrine messengers and communication as well. The use of mutated Cx channels with a selective impermeability for the presumable cell death messenger might substantially contribute to this field. Oh and colleagues favored the hypothesis that reduced or absent transfer of the second messenger cAMP is the primary defect that underlies the S26L mutation in Cx32 which is associated with Charcot-Marie-Tooth disease [182]. Along the same line, Beltramello et al. demonstrated that a Cx26 mutation, associated with deafness, is responsible for a selective defective transfer of IP_3. Of major importance is that the mutation occurs midway in the second transmembrane domain of Cx26 and that this valine residue is conserved in most α- and β-group Cxs, making this an attractive approach to study the role of IP_3 in cell death spread via GJs consisting of different Cxs [94].

It should be realized that the idea of one single molecule being responsible for the communication of and intercellular spread of cell death is a rather unlikely hypothesis. A group of messengers might have to team up and orchestrate their effects to mediate cell death spread via Cx channels, rendering the situation even more complicated. Although the quest for the one and only cell death messenger is still open, one thing is sure: whether it is a direct cell death messenger or not, Ca^{2+} certainly plays a pivotal role in the intercellular spread of cell death. The Ca^{2+} dependence of cell death spread warrants further attention since several pathologies are associated with abnormal Ca^{2+} signalling such as ischemia and can potentially provide a pharmacological target to prevent extensive demise in these conditions. Therefore, further exploration of this research area should be strongly encouraged and is anticipated to produce some major findings in the upcoming years.

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