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Role of connexin-based gap junction channels and hemichannels in ischemia-induced cell death in nervous tissue

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Abstract

Gap junction channels and hemichannels formed of connexin subunits are found in most cell types in vertebrates. Gap junctions connect cells via channels not open to the extracellular space and permit the passage of ions and molecules of ~1 kDa. Single connexin hemichannels, which are connexin hexamers, are present in the surface membrane before docking with a hemichannel in an apposed membrane. Because of their high conductance and permeability in cell–cell channels, it had been thought that connexin hemichannels remained closed until docking to form a cell–cell channel. Now it is clear that at least some hemichannels can open to allow passage of molecules between the cytoplasm and extracellular space. Here we review evidence that gap junction channels may allow intercellular diffusion of necrotic or apoptotic signals, but may also allow diffusion of ions and substances from healthy to injured cells, thereby contributing to cell survival. Moreover, opening of gap junction hemichannels may exacerbate cell injury or mediate paracrine or autocrine signaling. In addition to the cell specific features of an ischemic insult, propagation of cell damage and death within affected tissues may be affected by expression and regulation of gap junction channels and hemichannels formed by connexins.

Keywords

Intercellular communication; Connexon; Intercellular channel; Glia; Neuron; Necrosis; Apoptosis

1. Introduction

1.1. Brain ischemia: a brief overview

Ischemia is a pathophysiological condition present in different degrees in numerous diseases and resulting from neurological insults including embolic stroke, cardiac arrest and brain trauma. A localized embolic infarct is characterized by progression over time, with the ultimate inclusion of more tissue than that killed by the initial ischemic event [61]. Tissue bordering an ischemic lesion (the penumbra surrounding the core) is recruited slowly and progressively into the infarct, as verified by diffusion-weighted magnetic resonance imaging (MRI) of stroke during its maturation [58]. The ischemic core shows rapid necrosis of neurons and glia, including astroglia and oligodendrocytes [138,139]. The penumbra shows delayed cell death (1 or 2 days later) with morphological and metabolic changes characteristic of apoptosis. The continuing expansion of the infarct is not caused directly by the reduction in local blood flow, but by secondary processes, presumably contributed to by

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stress signals or toxic metabolites coming from the infarct. During brain ischemia, loss of ionic homeostasis in neurons and glial cells precedes cell death. Even a brief period of ischemia induces depletion of ATP levels paralleled by a progressive reduction of electrochemical gradients across the plasma membrane [129] followed by metabolic changes characteristic of necrosis and/or apoptosis [148,10]. After ischemia or ischemia/reperfusion neurons are the main cell type that dies, in part because their metabolism is preferentially aerobic. Prolonged ischemia is required to kill astrocytes [137]; their relative insensitivity may be due to their ability to switch from aerobic to anaerobic metabolism [106].

Because of the great susceptibility of neurons to ischemia-induced death (and their importance to brain function), major efforts have been focused on finding death mechanisms, including pathways mediating inappropriate ion fluxes across the neurolemma during and after ischemia. Increased activity of ionotropic neurotransmitter receptors, such as NMDA and GluR2-lacking AMPA receptors, which are permeable to Ca^{2+}, as well as voltage-sensitive ion channels, enhance the early and delayed neuronal death induced by ischemia [80]. Blockers of these channels have been used in pharmacological strategies to reduce ischemia-induced cell death. However, many other processes contribute to cell death and damage propagation. Many glial cell functions are impaired by ischemia not severe enough to kill them, and these dysfunctions may be critical to neuronal viability. Consequently, some studies have focused on astrocyte dysfunctions in death caused by ischemia (reviewed by Chen and Swanson [24]). Astrocytes are the most abundant non-neuronal cells in mammalian brain and in humans constitute ~50% of the total brain volume. Astrocytic functions are essential for normal neuronal activity, and they provide metabolic and structural support to neurons; they control the extracellular concentrations of glutamate, K^+, and H^+ and regulate the volume of extracellular space [43]. Many of these functions are contributed to by “spatial buffering”, which is augmented by coupling of astrocytes through gap junctions. In its initial formulation, K^+ flows into and depolarizes the astrocyte in a high extracellular K^+ region, and this depolarization spreads electrotonically to a low extracellular K^+ region causing K^+ to flow out of the cell. Coupling would also permit cooperation between cells in glutamate uptake and conversion to glutamine. Thus, hyperexcitability and neurotoxicity due to extracellular K^+ and glutamate are reduced [24,55]. In addition, astrocytes modulate neurite outgrowth and provide guidance, which are processes essential for synaptic recovery and remodeling in zones affected by ischemic episodes [24]. That astrocytic gap junctions modulate neuronal survival during and after brain ischemia is supported by numerous studies including some described here. Gap junctions are also found between activated microglia, between many types of neurons, between astrocytes and oligodendrocytes, and in a few somewhat controversial instances between astrocytes and neurons (for review see in this issue (Sfhl et al. [132] and Nagy et al. [157]). To our knowledge, the effect of ischemia on these junctions remains unknown.

1.2. General characteristics of connexins

Connexins constitute a family of transmembrane proteins with at least 19 and 20 members in mouse and human, respectively [151]. Newly synthesized connexins are assembled, depending on the connexin type; in the endoplasmic reticulum or Golgi apparatus to form hexamers known as hemichannels or connexons [83,128]. Hemichannels formed by a single connexin (Cx) type are called homomeric, while hemichannels comprised of different connexins are termed heteromeric. Not all connexin combinations can form heteromeric hemichannels, and not all hemichannels can dock with each other to form functional cell–cell channels (see Harris [56]). After assembly, hemichannels are transported to the surface membrane [48,83]. Growth of gap junctions occurs by insertion of hemichannels, which then dock with hemichannels in the apposed cell at the periphery of existing gap junction plaques [47,75]. How the first channel forms is not clear, and many channels must cluster

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together before the first channel opens [19]. The half-life of several rodent connexins has been found to be between 2 and 5 h (for review see Sáez et al. [125,126]). At the plasma membrane, groups of intact channels forming part of a gap junction are internalized from its central region into one or the other cell [47] and may appear in the cytoplasm as annular junctions. These junctions are accompanied by a bit of cytoplasm from the apposed cell and their internalization requires breaking and resealing of membranes. The internalized junctions are then degraded by both proteosomes and lysosomes [73,67]. The rapid turnover of gap junctions indicates that intercellular coupling could be effectively modulated by changes in the rate of synthesis and/or degradation of connexins. A second class of regulatory mechanisms involves "gating" by which gap junction channels within a junction are made to open or close. Gating stimuli include voltage, H^+, Ca^{2+}, certain lipophilic agents, and protein phosphorylation (for reviews, see Bennett and Verselis [11]; Martínez and Sáez [85]; Harris [56]).

In most systems studied, gap junction channels allow the coordination of intrinsic or elicited metabolic and/or electrical responses of cells in a heterogeneous population [125,126]. These actions are determined in part by the channel conductance and permeability. Some gap junction channels are more permeable to anions; others show preference for cations or exhibit little charge selectivity [38,23]. Hepatocytes expressing Cx26 and Cx32 have gap junctions that are much more permeable to the second messenger IP_3 than cells expressing only Cx26 [98]. Similarly, Cx43 and Cx32 gap junction channels show inverse selectivity to adenosine and ATP, a feature that could influence the metabolic capacity of specific tissues [50]. Moreover, some gap junction channels are electrically quite linear, but others are steeply voltage-dependent [11]. Heterotypic channels formed by voltage-independent and voltage-dependent hemichannels or hemichannels with opposite gating polarity can rectify and have a greater conductance in one direction than in the other [7,146,57,21]. The physiological role of gap junctions will be determined by the signals and metabolites that can pass through them and their total permeability as modulated by their regulatory properties (e.g., voltage dependence).

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1.3. Pattern of connexin expression in the adult mammalian brain

The expression of different connexins varies according to brain region, cell type, and developmental stage. In addition to a brief description here, the reader will find exhaustive reviews on connexin expression in mammalian brain in two articles of this issue (Nagy et al. [157] and Sfhl et al. [132]).

Cx36 is the only connexin that has been reproducibly identified at the ultrastructural level in plaques between coupled neurons of the adult rat brain [115,116], although other connexins are reported at several specific sites and at differing developmental stages (see Bennett and Zukin [12]). Cx36 has been found in the nuclear complex and the neuropil of the inferior olive in normal mice, but not in Cx36 deficient mice [86]. Coupling of glia and neurons by gap junctions in adult mammals it is still controversial [3].

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Astrocytes express Cx43 and lower levels of Cx26, Cx30, Cx40, and Cx45 [90,91,34,116] (see Nagy et al. [157]; Sfhl et al. [132], in this volume). In brain astrocytes, gap junction plaques show colocalization of Cx26, Cx30 and Cx43 [116,93]. However, studies of cultured astrocytes reveal single channel conductance and other biophysical properties consistent with those found in Cx43 transfectants [33,49,89,72,20]. Cx26 is not detected at cell–cell contacts between cultured astrocytes [84], and cultured astrocytes from Cx43 deficient mice do not form functional gap junctions [97]. Thus, Cx43 is thought to be the main functional connexin in cultured astrocytes. Oligodendrocytes express Cx29, Cx32 and Cx47 [116,131,2] (see Nagy et al. [157]; Sfhl et al. [132], in this issue).

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In the brain of adult rats, a few microglia express low levels of Cx43 [41]. In addition, Cx43 and Cx36 have been detected in cultured rat microglia under control conditions [41,102], and the levels of Cx43 increase in microglia recruited to brain stab wounds [41]. Cultured microglia treated with interferon-γ plus TNF-α or bacterial lipopolisaccharide show upregulation of Cx43 expression and form functional gap junctions [41]. In general, gap junction channels expressed by microglia may help to coordinate inflammatory responses [124].

2. Proposed roles of gap junction channels in brain damage in in vitro and in vivo models of stroke and brain trauma

The molecular mechanisms associated with propagation of damage from the ischemic core to adjacent zones are not well understood. Several cellular mechanisms activated during brain experimentally induced ischemia are common to those observed in pathological conditions, such as hemorrhagic stroke, embolic stroke, hypoglycemia, brain trauma and cardiac arrest. Here, we review the proposed roles of gap junction channels in cell death. When coupled cells are subjected to stress or injury, deleterious molecules spread from more injured to less injured cells helping the former and harming the latter; health promoting molecules such as necessary metabolites spread from less injured to more injured cells, harming the former and helping the latter (Fig. 1). Depending on the balance, there can be two different outcomes, bystander killing and rescue of distressed neighbors.

The first evidence obtained in vivo that gap junctions mediated spread of damage in the CNS after focal ischemia was that octanol, a not very selective gap junction blocker, reduces the size of the infarct [117]. This observation is mildly contradicted by subsequent observations that Cx43 heterozygous null mice or mice with astrocytic Cx43 removed by Cre expression are more susceptible to infarct expansion after focal ischemia [130,95,96]. The CA1 hippocampal subfield is more vulnerable to global ischemia and has a higher density of Cx43 gap junctions than the resistant CA3 areas, and damage is reduced by gap junction block with carbenoxolone [114]. These data suggest that damage can propagate through the junctions. In rat brain slices, downregulation of specific connexins with antisense oligonucleotides reduces neuronal cell death 48 h after an episode of hypoxia and glucose deprivation, a model for global ischemia [45]. In a model of brain trauma using organotypic slices from wild type mice and downregulation of Cx32 and Cx26 with antisense oligonucleotides or block by octanol and carbenoxolone or brain slices from Cx43 deficient mice, neuronal cell death was markedly reduced [46]. Moreover, Cx32 deficient mice showed an enhanced sensitivity to global ischemia in the CA1 region of the hippocampus [99]. Results are mixed in in vitro studies as well where gap junction blockers decrease spread of damage [28,29,79,5,62,45,127,22] or increase it [14,101]. In the latter studies using co-cultures of astrocytes and neurons, block of astrocytic gap junctions increased neuronal vulnerability to oxidative stress or glutamic acid [14,101].

A criticism of many of these studies is that known gap junction blockers are quite unspecific, and some of them, such as halothane and carbenoxolone, also reduce transmission at chemical synapses [111,122]. The relatively more specific blocker of Cx36 junctions, mefloquine, may prove useful in disrupting interneuronal communication via gap junctions [31]. Development of more selective drugs as well as transgenic approaches should permit action on specific connexins expressed in particular cell types. Additionally, antisense oligonucleotides, extracellular loop peptides (and potentially small interfering RNAs) may permit relatively precise interference with coupling.

Changes in connexin expression during global ischemia have been reported but their functional significance remains hypothetical. During global ischemia the expression of Cx32

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and Cx26, but not Cx43, increases selectively in the vulnerable CA1 subfield of the hippocampus before the onset of neuronal death [99]. The penumbra of ischemic infarcts in $Cx43^{+/-}$ mice showed an increased level of Cx30 compared with $Cx43^{+/+}$ mice [95]. Treatment of cultured astrocytes with IL-1h for several hours reduces Cx43 levels and cell–cell communication [65,66]. A source of IL-1 could be reactive astrocytes as well as activated microglia. In agreement, gap junctional communication between astrocytes is reduced by activated micloglia in cultures [121,42]. These changes in connexin expression may represent reactive gliosis or be part of adaptive processes that reduce damage or provide tolerance to subsequent ischemic episodes (preconditioning).

Astrocytic gap junction channels show little charge selectivity and they remain open under ischemic conditions in brain slices or cultures [28,78,92,25]. The effects of ischemia on other types of gap junction normally expressed in the brain are unknown.

Gap junction expression affects the expression of other genes [76,63] which can affect cells sensitivity to injury. Gap junction blockers or connexin antisense prevent intercellular communication within a short-time frame and may allow quick recovery. In contrast, connexin knockouts permanently delete a gene, but permit compensatory changes in gene expression. Inducible knockouts allow for greater time and tissue specificity. Contradictory results obtained with these disparate approaches should be interpreted with caution. The development of pharmacological tools to inhibit specifically gap junctions of multiple or single types should lead to clarification of their physiological roles and could provide therapeutic agents for improving the prognosis of patients following ischemic episodes or in other conditions where gap junctional communication may be deleterious.

How does ischemia affect junction channels? It has been reported that in both brain slices and primary astrocyte cultures gap junctional communication is reduced, but not abolished, during ischemia [28,78,92,25]. The reduction in cellular communication could be caused by several gating stimuli including cellular acidosis, rise in free intracellular Ca^{2+} concentration, ($[Ca^{2+}]_i$), and changes in phosphorylation of connexins.

In anaesthetized rats, optical measurements of brain intracellular pH using the vital dye neutral red or ^{31}P-NMR spectroscopy revealed pronounced acidosis ($pH<6.5$) at about 5 min after cardiac arrest [74]. Intracellular acidosis and tissue lactate accumulation were closely correlated. Superfusion of different cell types with millimolar concentrations of weak acids or CO_2 markedly reduces cell–cell communication at $pH<6.5$ [134,135]. Intracellular pH can go as low as 6.1–6.2, if hyperglycemia precedes ischemia [74], suggesting that nutritional status may be critical to the degree of closure of gap junction channels by ischemia. In hippocampal slices, neuronal loss at pH 6.4 is greater in the presence of lactate, which was attributed to a reduction in glutamate uptake [153]. Reduced coupling of astrocytes, here due to acidification, could contribute to reduced glutamate uptake. In contrast to the in vivo situation, the intracellular volume of dispersed cell cultures is negligible compared to the extracellular volume, and organic acids generated during an insult can be diluted many times in the extracellular milieu. The brain slice provides an intermediate between the two. Complete oxygen deprivation applied to rat hippocampal slices causes a reduction in pH of only about 0.2 units [74]. Moreover, adaptation to culture conditions might affect both the cell metabolism and gap junction sensitivity to pH. As mentioned before, the metabolism of astrocytes in culture is more anaerobic and may show a similar adaptation in brain slices. In addition, coupling between astrocytes of brain slices is rather insensitive to intracellular acidification [28]. The penumbra in focal ischemia shows progressive metabolic deterioration culminating in infarction. In vitro preparations are likely to simulate conditions present in the penumbra where intracellular acidification is less pronounced and inhibition of gap junction channels is incomplete.

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Elevation of $[Ca^{2+}]_i$ is another possible cause of reduction in coupling mediated by gap junctions [105]. Fluorometric studies with Fura-2 in whole brain of rats subjected to global ischemia showed that $[Ca^{2+}]_i$ increased 2 min after the beginning of the ischemic insult and continued to increase gradually [94]. This increase in $[Ca^{2+}]_i$ might reduce gap junctional communication between astrocytes.

Dephosphorylation of astrocytic Cx43 is induced by metabolic inhibition and by oxygen deprivation in vivo, in brain slices and in cultured astrocytes [78,28,85,25]. Western blotting with antibodies to phosphorylated and non-phosphorylated forms shows the loss of lower mobility phosphorylated forms and increase in the higher mobility non-phosphorylated form (Fig. 2). Western blotting with an antibody that is specific to the non-phosphorylated form shows the same increase in the non-phosphorylated form. Also, immunolabeling with the antibody to the non-phosphorylated form shows diffuse labeling in the Golgi and ER region in control cultures and no apparent gap junctions (Fig. 3, left panel, control). After metabolic inhibition, there are numerous puncta at cell–cell interfaces and more particulate labeling intracellularly consistent with dephosphorylation of connexin in junctional plaques and possibly increased internalization or aggregation of connexin that has not reached the surface (Fig. 3, middle and right panels). Dephosphorylation of Cx43 in cultured astrocytes is associated with reduced gap junctional communication [78], and metabolic inhibition induces a rapid and marked reduction in intracellular ATP, which would hinder phosphorylation of Cx43 [25]. Moreover, elevation of $[Ca^{2+}]_i$ might activate the Ca^{2+}/calmodulin-dependent protein phosphatase, calcineurin, enhancing protein dephosphorylation. Accordingly, in metabolically inhibited astrocytes cyclosporin A, a calcineurin blocker [53], reduces both the dephosphorylation of Cx43 (Fig. 2) and the reduction in gap junctional communication [78]. Cx43 is widely distributed in brain and other organs and may be similarly affected by ischemia at these sites.

Generation of reactive oxygen species (ROS) is greatly enhanced during partial ischemia and during reperfusion after an ischemic period. In cultured astrocytes, the induction of nitric oxide synthase (by exposure to lipopolysaccharide) correlates with a reduction in gap junctional communication [15]. The latter effect is prevented by incubation with the NOS inhibitor NMMA and with the superoxide anion (O_2^-) scavenger superoxide dismutase. The reaction between NO and O_2^- to form the peroxynitrite anion ($ONOO^-$) may mediate the reduction in coupling. Nitric oxide reduces coupling between neurons [88,120]. However, gap junctions formed by different connexins can be affected oppositely by NO [68] and thus, NO actions in ischemia will require direct evaluation in different cell types and regions of the brain.

It was reported recently that interleukin-1 (IL-1), a pro-inflammatory agent that contributes to stroke-induced brain injury, rapidly (~15 min) reduces coupling between cultured astrocytes by activation of p38/stress-activated protein kinase 2 (p38/SAPK2); the effect occurs without changes in levels of serine phosphorylation of Cx43 [156]. Treatment of (human fetal) astrocyte cultures with IL-1 for several hours reduces Cx43 levels and cell–cell communication [65,66]. A different study failed to observe IL-1 induced decrease in coupling [87]; however, ATP, another molecule massively released following brain insults, reduces coupling by activation of P2Y1 receptors, and IL-1 increases expression of these receptors and the degree of uncoupling [87]. Reoxygenation after hypoxia is followed by a rapid (~15 min) and drastic reduction in astrocytic coupling, which is mediated by arachidonic acid byproducts and internalization of Cx43 gap junction channels [85]. This uncoupling also occurs without changes in the level of Cx43 or its phosphorylation evaluated by Western blot analysis. The reoxygenation-induced uncoupling is prevented by indomethacin, a cyclooxygenase blocker, or melatonin, an antioxidant, and uncoupling is also produced by application of arachidonic acid [84]. The arachidonic acid-induced

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uncoupling is prevented by depletion of extracellular Ca^{2+} or high melatonin concentrations, suggesting the involvement of Ca^{2+} influx and ROS [84,85]. Whether common or independent mechanisms explain the uncoupling of astrocytes induced by IL-1 and arachidonic acid is unknown. But, reperfusion after a period of global ischemia or the partial ischemia in the penumbra of focal ischemia, conditions that elevate arachidonic acid and IL-1 levels, might reduce astrocyte coupling and impair astrocyte network function. Alternatively, reduced gap junctional communication could be protective of astrocytes by reducing propagation of death signals between them. A source of IL-1 could be reactive astrocytes as well as activated microglia. Gap junctional communication between astrocytes is reduced by activated microglia in cultures [121,42].

What signals propagate through gap junctions favoring life or death? Candidate molecules must be gap junction permeable, which as noted depends on size, charge, and connexin. In cells less affected by an ischemic insult the concentration of small molecules required for cell life (e.g., glucose, ATP, ascorbic acid and reduced glutathione) will be higher than in cells that have reached “the point of no return”, and the chemical gradient should favor their transfer from less to more affected cells (Fig. 1). Propagation of cell death could reflect the progressive loss of molecules and ionic conditions required for life and/or the transfer of toxic molecules from dying cells to their less injured neighbors. Irreversibly injured cells most likely contain higher concentration of catabolites (e.g., oxidized glutathione and organic acids) and Ca^{2+} that could flow by simple diffusion to neighboring cells via gap junctions [123] and thereby stress them. The permeability of various types of gap junction channels to toxic agents such as ROS remains unknown.

3. General features of hemichannels in their cellular functions

Not so long ago opening of unapposed connexin hemichannels at the cell surface was thought to be unlikely because of the relatively nonselective conductance and high permeability of gap junction channels; open hemichannels would drown the cell in Na^{+} and Ca^{2+} and cause loss of vital metabolites. Initially opening of hemichannels was observed in Xenopus oocytes expressing, possibly overexpressing, specific connexins, and the oocytes rapidly died unless high Ca^{2+} was applied to keep the hemichannels closed [104]. Subsequently, other lens connexins, including bovine connexin 44 and chicken connexin 56 were found to form conductive hemichannels in Xenopus oocytes [54,36]. Other connexins (i.e., Cx32, Cx35, Cx43 and Cx52.6) expressed in Xenopus oocytes generate hemichannels currents enhanced by zero extracellular calcium and/or membrane depolarization [108,150,51,155]. Recent reports demonstrate that hemichannels in mammalian cells can be functional under physiological and pathological conditions (for review see Bennett et al. [13]; Sáez et al. [125,126]; Goodenough and Paul [52]; Ebihara [37]). However, there remains some skepticism as to the validity of this work [133,103].

Several groups have studied the biophysical properties of hemichannels, partly to give insight into properties of the cell–cell channels. The use of excised patches containing one or a few hemichannels permits characterization of responses to rapid application of various pharmacological agents and improved resolution of voltage gating. Site directed mutation of connexins then expressed in oocytes facilitates structure function studies. For reasons as yet unclear hemichannel activity is difficult to evoke in mammalian cells. Single cell–cell channel activity can be recorded with double whole cell clamp, but to see unmodified single channels, cells must be put into apposition while recording from them. Single channels can be seen in well coupled cells by use of blocking agents to reduce the number of active channels to one or a few; this approach is problematic for some properties such as kinetics, but most known blockers act by reducing open probability and do not affect single channel conductance (see Verselis et al. [147]).

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Studies of hemichannels have revealed important structural properties of hemichannels and gap junction channels. For example, hemichannels in outside out patches are closed by low pH solutions, but do not remain closed [141]. The timing of the reopening indicates that the H binding site leading to closure is on the cytoplasmic side of the pH gate that occludes the channels. More recently, it was demonstrated that extracellular Ca^{2+} binds to a ring of 12 aspartate residues of the external vestibule of Cx32 hemichannels [51]. Asp-169 of one subunit and Asp-178 of an adjacent subunit appear to form a Ca^{2+} binding site [51]. In the absence of extracellular Ca^{2+}, elevated external Mg^{2+} blocks Cx46 hemichannels as does Ca^{2+}, but Mg^{2+} is less potent [36]; it remains to be determined whether its binding site is the same as that for Ca^{2+}. The gating mediated by Ca^{2+} binding to Cx32 might be similar for hemichannels formed by other connexin types, and it is likely to protect cells from the potentially adverse effects of leaky hemichannels. Moreover, PKC mediated phosphorylation of S368 in Cx43 hemichannels closes them [6], which can explain how in Novikoff cells PKC activation blocks connexin 43 hemichannel opening in low extracellular [Ca^{2+}] [77,81]. Other phosphorylation pathways are likely to be involved in keeping Cx43 hemichannels closed. For example, Cx43 is a substrate for mitogen activated protein kinase (MAPK) [149]; reconstituted Cx43 hemichannels remain closed after phosphorylation by purified MAPK, and phosphatase treated Cx43 forms functional hemichannels ([70], see also Pfahnl and Dahl [107]).

A number of recent studies indicate that in vertebrate cells in primary culture and cell lines, macroscopic non-selective currents and uptake and/or release of otherwise membrane impermeant molecules are mediated by functional hemichannels (Fig. 1). Communication incompetent cells transfected with Cx43 can show dye uptake and gap junction blockers (e.g., octanol, heptanol, carbenoxolone, oleamide, halothane, 18-α-glycyrrhetinic acid and 18-β-glycyrrhetinic acid), trivalent cations, and connexin anti-sense oligonucleotides can prevent dye uptake (e.g., Refs. [77,70,25-27,136]). Expression of connexins increases release of ATP from cell lines in response to mechanical stimulation [28,29]. Moreover, low extracellular [Ca^{2+}] enhances hemichannel opening in solitary retinal horizontal cells [35], a subpopulation of cardiac myocytes [66,71], astrocytes [59,136], human osteoblast-like initial transfectant cells [118,119] and other cell lines [77]. Opening of Cx43 hemichannels has also been induced by mechanical stimulation in astrocytes [136]. In combination with membrane depolarization or low extracellular [Ca^{2+}], pharmacological agents, such as biphosphonates [109], quinidine and quinine [82,136] enhance hemichannel opening in several systems. These data suggest the existence of a hemichannel “agonist” which is not yet identified nor has it been determined whether it acts from the cytoplasmic or extracellular side. Some hemichannel blockers act from only one side [39].

Connexin hemichannels are blocked by hyperpolarization of the plasma membrane [140,143] and low extracellular pH [141,9,13,126], as well as gap junction blockers. The specificity of the blockers is not very great and most of them also block other ionic channels (e.g., Ref. [39]), making difficult the pharmacological demonstration of the hemichannel involvement in different biological processes.

Several caveats should be considered in assigning macroscopic currents or fluxes to hemichannels: (1) connexin transfection or deletion might induce expression of unrelated genes, such as channels and transporters, where they could mediate the putative hemichannel functions; (2) connexins have binding partners and might required for transport and insertion in the surface membrane of the molecules that mediate the presumptive hemichannel actions; (3) hemichannel blockers are not specific; (4) other channels not related to hemichannels, such as ionotropic ATP receptors (P2X receptors), the voltage-dependent anion channel VDAC, and mechanosensitive channels, are permeable to small metabolites and fluorescent tracers and could mediate functions ascribed to hemichannels.

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Single hemichannel recording in mammalian cells provides a much stronger demonstration than the macroscopic studies, because they show channel properties that are largely predictable from those of the cell–cell channels. To date, single hemichannel currents have been characterized for Cx30, Cx43, Cx45, Cx46 and Cx50 (for review, see Bennett et al. [13]). These studies show that the main hemichannel conductance measured at positive potentials is consistent with simple series arrangement of two hemichannels to form a cell–cell channel, i.e., the putative hemichannel conductance is twice that of the cell–cell channel of the same type. Recently, we reported biophysical properties of single Cx43 and Cx43-EGFP (enhanced green fluorescent protein) hemichannels (whole cell recording) and compared them to the properties reported for their corresponding cell–cell [26,27,13]. No channel activity is recorded from untransfected, parental HeLa cells. In transfected cells Cx43 hemichannels have voltage gating transitions like those of the cell–cell channels. There are fast (<1 ms) transitions between the fully open state and a substate and slow (>5 ms) transitions between the fully closed state and the substate or fully open state. The slow transitions are likely to represent transitions through multiple short-lived substates. Cx43 with EGFP attached to its C-terminus (Cx43-EGFP) forms hemichannels that have only slow voltage gating and no substate, like the corresponding gap junction channels. In addition, HeLa cells transfected with Cx43 with EGFP attached at its N-terminus (EGFP-Cx43) show normal connexin distribution and gap junction plaque formation, but no voltage gated hemichannels or functional gap junction channels. The single channel activity had the expected pharmacological sensitivity in that it was blocked by octanol and external La^{3+} and increased by low extracellular $[Ca^{2+}]$ [26,27]. These electrophysiological data indicate unequivocally that Cx43 hemichannels present on the cell surface can open [26,27]. However, the fraction of surface hemichannels that open at all appears to be very low.

There is little detailed information on hemichannel permeability and selectivity, and it has been assumed that they are similar in this respect to their corresponding gap junction channels. From dye uptake measurements, Cx43 hemichannels are permeable to Lucifer yellow, ethidium bromide, carboxyfluorescein, and 7-hydroxicoumarin-3-carboxylic acid [77,81,25,71,136,109]. Cx45 and Cx46 hemichannels are permeable to Lucifer yellow and propidium iodide [112,142]. Fluorescently labeled dextrans of molecular weight of ~1500 kDa are not taken up by cells expressing hemichannels [25,136].

Does hemichannel opening have physiological relevance? Studies showing physiological roles of hemichannels have appeared in the last 4 years. Cx43 hemichannels are involved in cell volume regulation in response to quite small changes in extracellular $[Ca^{2+}]$ in an isosmotic solution [113]. In fibroblasts, Cx43 hemichannels mediate release of NAD^+ to the extracellular medium [17,18]. This finding provided an answer to the old paradox of having an intracellular substrate, NAD^+, for an ectoenzyme, CD38, that catalyzes NAD^+ conversion to cADPR. CD38 also mediates the uptake of cADPR, a potent physiological ligand for the ryanodine receptor found in the endoplasmic reticulum. CD38 is found in the endoplasmic reticulum as well. Low cytoplasmic Ca^{2+} levels may open Cx43 hemichannels in the surface and ER membranes to allow cytoplasmic NAD^+ to reach the active sites of CD38 in the extracellular space and in the ER lumen. The cADPR that forms at these sites, CD38 transports back to the cytoplasm, except that on the extracellular side some diffuses to neighboring cells. Autocrine/paracrine Ca^{2+} signaling mediated by NAD^+ flux through Cx43 hemichannels may enhance cell proliferation and shorten the S phase of the cell cycle of 3T3 fibroblasts [44]. Astrocytes may also use this mode of paracrine signaling [144]. In addition, Cx43 hemichannels in astrocytes and other cells may mediate the ATP release triggered by mechanical stimulation, thereby permitting the extracellular propagation of calcium waves [29,30,136,4]. Recently, it was shown that photo release intracellularly of caged IP_3 induces opening of Cx43 hemichannels and release of ATP to the extracellular

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milieu [16]. This mechanism could underlie non-decremental propagation of Ca^{2+} waves as extracellular ATP leads to intracellular generation of IP_3 in neighboring cells.

The presence of Cx26 on the tips of fish retinal horizontal cell dendrites at the synapses with photoreceptors was inferred from immunofluorescence and immuno-electromicroscopy [69,64]. Currents through open hemichannels at this site were thought to change the potential across Ca^{2+} channels in the photoreceptors in response to activity in the surrounding regions and mediate center surround interactions [69]. In this study, the presence of open hemichannels was deduced from the action of the hemichannel (and gap junction) blocker carbenoxolone. Similar observations were made in horizontal cells of turtle and mouse retina [110,152], although mouse horizontal cells express Cx57 and lack both Cx26 and Cx36 [32,60].

4. Putative role of hemichannels in ischemia-induced damage

As noted above, several pathological conditions, including ischemia, reduce ATP levels and increase membrane permeability leading to influx of Ca^{2+} and to loss of ionic homeostasis and small metabolites important for cell viability. In most brain cell types, including neurons, there appear to be multiple mediators of increased membrane conductance. Activation of ion channels, such as non-specific cation and chloride channels, is known to be elicited by conditions resulting from ischemia (e.g., oxidative stress) [8,100]. Because connexin hemichannels were thought likely to have permeability properties similar to those of intercellular channels, it was also thought that their opening would induce metabolic stress and cell death. Indeed, in the first study of open hemichannels, which was in Xenopus oocytes expressing Cx46, non-selective inward current, swelling, and cell death were observed [104]. Increasing the extracellular Ca^{+2} concentration prevents both the development of currents mediated by opening of hemichannels and subsequent cell death [104,36]. As observed later, myocardiocytes and astrocytes [65,66,25], as well as renal proximal tubule cells [145], subjected to ATP depletion show increased permeability to fluorescent molecules of 300 to 400 Da (Lucifer yellow and ethidium bromide). Dye uptake is prevented by gap junction blockers and precedes uptake or loss of macromolecules (dextrans and lactate dehydrogenase), indicating that uptake also precedes plasma membrane breakdown. Block of hemichannels delays the membrane breakdown ultimately caused by metabolic inhibition, suggesting that open hemichannels contribute to cell stress. HEK-293 cells transfected with Cx43 show greater macroscopic currents and Lucifer yellow uptake than parental (non-transfected) cells, when they are subjected to metabolic inhibition [66]. Metabolic inhibition induces dye uptake in primary cultures of astrocytes from wild-type mice but not from homozygous Cx43 knockout mice or mice with astrocyte-specific inactivation of the Cx43 gene [25]. Taken together, these data strongly suggest the involvement of Cx43-hemichannels in dye uptake elicited by metabolic inhibition.

Cx32 hemichannels expressed in oocytes exhibit atypical gating at negative voltages and low Ca^{2+} [51]. This phenomenon does not occur at positive potentials. The non-canonical gating observed at negative potentials requires further investigation.

As expected, HeLa cells expressing Cx43 are more susceptible to killing by metabolic inhibition than are parental cells (Fig. 4). This difference is ascribable to Cx43 hemichannel opening. Two mechanisms have been proposed to open hemichannels during metabolic inhibition, dephosphorylation of Cx43 and action of ROS directly on the connexins or on accessory molecules. Cx43 dephosphorylation does correlate with ethidium bromide uptake induced by metabolic inhibition [25]. However, cyclosporin A, which blocks calcineurin and prevents dephosphorylation of Cx43 by metabolic inhibition (Fig. 2), does not reduce ethidium bromide uptake to a level below that induced by metabolic inhibition alone (Fig.

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5). Conversely, trolox, a free radical scavenger, does not prevent dephosphorylation induced by metabolic inhibition (Fig. 2) but greatly reduces dye uptake (Fig. 5). The previous results were obtained in cells cultured in medium without fetal bovine serum (FBS), because MI in the presence of FBS largely prevents dephosphorilation and delays dye uptake even if it is added after 45 min of metabolic inhibition (Fig. 6). The serum effect might be explained by activation of intracellular pathways by serum factors. Since hemichannels appear to have physiological functions (see above), their functional state may be modulated by signals, such as hormones, neurotransmitters and growth factors.

Astrocyte hemichannels mediate glutamate efflux under low calcium conditions [154]. Thus, hemichannel opening induced by metabolic inhibition may contribute to the accumulation of extracellular glutamate and other metabolites observed during and after ischemia (Fig. 1).

It has been suggested that moderate and/or transient activity of hemichannels is compatible with a physiological function and cell life, but that massive and/or prolonged opening accelerates cell death [25-27]. The data shown here reinforce the idea that opening of hemichannels formed by Cx43 can play a role in ischemic damage. Opening of hemichannels may be increased by some human connexin mutations, which may promote cell death by this novel gain of function. Cx30 mutations associated with hidrotic ectodermal dysplasia may cause cell death by increased opening of hemichannels [40] (Luis C. Barrio, personal communication). Also, Cx32 hemichannel opening is enhanced by a mutation that causes with X-linked Charcot–Marie–Tooth disease [1]. Cx50 with a His176Gln mutation is lethal when expressed in oocytes because of increased opening of hemichannels [9]. Thus, opening of connexin hemichannels appears to be one mechanism underlying connexin related pathologies.

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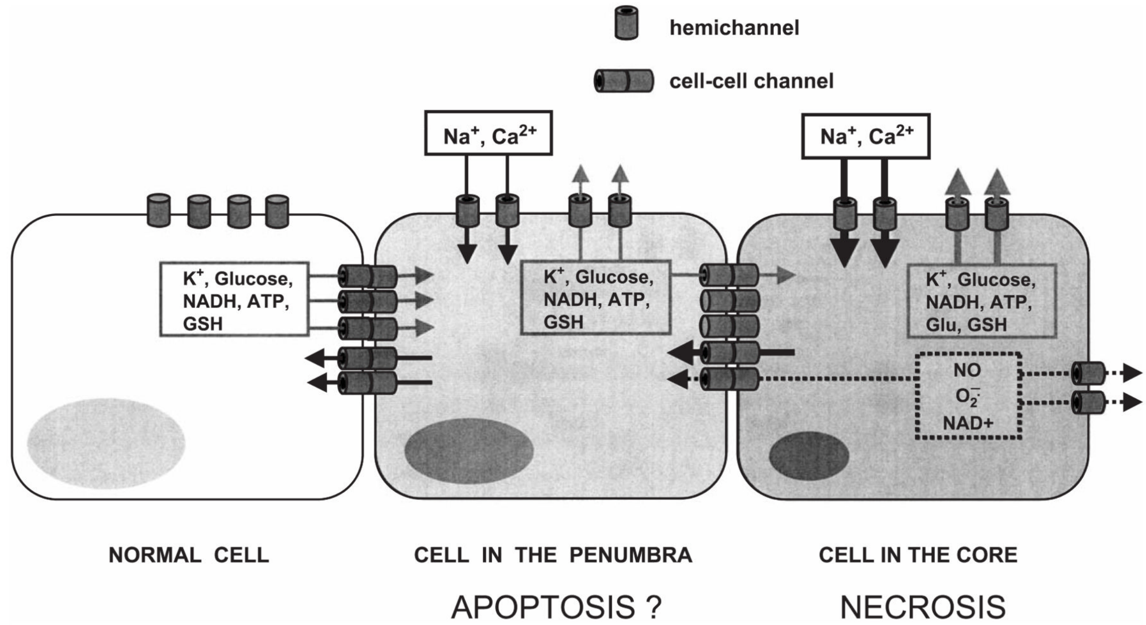

Fig. 1.

Scheme of the possible actions of connexin channels and hemichannels during and after ischemia. Hemichannels or gap junction channels with a dark center are open and are permeated by ions and small molecules. Those without a dark center are closed. Molecules required in normal metabolism (e.g., K^+, glucose, NADH and reduced glutathione: GSH) can be released to the extracellular milieu through open hemichannels or can be transferred through gap junction channels from healthier cells (normal) to cells in the penumbra and from there to cells of the core (gray arrows). In the opposite direction, ions and small potentially toxic molecules present in high concentration in injured cells (e.g., nitric oxide: NO, superoxide ion: O_2^-, and NAD^+) could be transferred through gap junction channels from injured cells to healthier cells (dotted line and arrow), contributing to the propagation of conditions that could promote cell death. In addition, open hemichannels contribute to collapse of the transmembrane ionic gradients by allowing the entry (black arrows) of extracellular ions (e.g., Na^+ and Ca^{2+}) and loss of K^+ and small metabolites such as glutamate.

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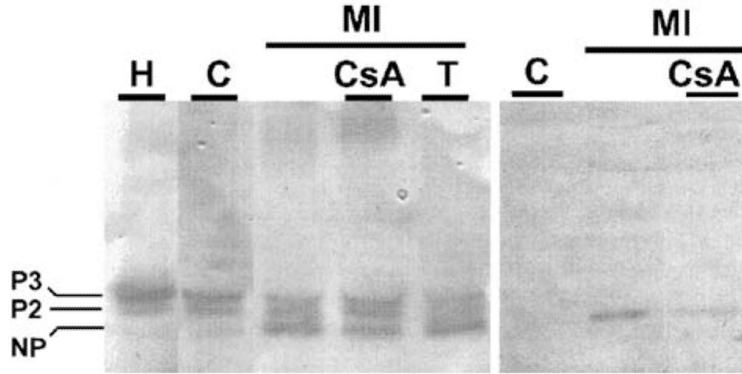

Fig. 2.

Dephosphorylation of Cx43 induced by metabolic inhibition is reduced by inhibition of calcineurin but not by trolox, a free radical scavenger. Left panel: Immunoblot using an antibody that reacts with all phosphorylated and the nonphosphorylated forms of Cx43. P2 and P3 represent phosphorylated forms of Cx43, NP is the more rapidly migrating, nonphosphorylated form. Left panel: 75 min of metabolic inhibition (MI) with iodoacetate (0.3 mM) and antimycin A (5 ng/ml) causes a shift towards less phosphorylation. H: heart tissue to show Cx43 bands. C: control astrocytes show little NP. MI markedly reduces P2 and P3 and increases NP. Inhibition of calcineurin with 2 μM cyclosporin A (CsA) reduces the degree of dephosphorylation by MI. Trolox (T, 100 μM) does not affect dephosphorylation by MI. Right panel: Immunoblot using an antibody that reacts preferentially with the NP form of Cx43. C: control astrocytes show little NP Cx43. The NP form is present in astrocytes after MI for 75 min, but its level is much lower in astrocytes under MI for 75 min in the presence of CsA.

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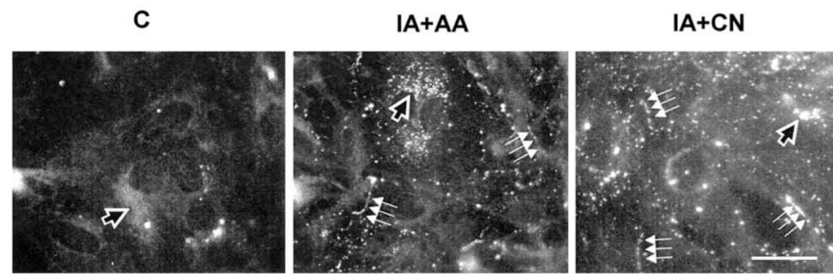

Fig. 3.

Immunoreactivity of nonphosphorylated Cx43 is increased in metabolically inhibited astrocytes. Immunofluorescence of Cx43 in cultured astrocytes was evaluated using an antibody that reacts preferentially with the nonphosphorylated form of the protein (Zymed). Rat cortical astrocytes under control conditions (C) showed diffuse intracellular reactivity likely to correspond to the Golgi apparatus or ER (arrow) and gave little indication of Cx43 at cell appositions. Astrocytes after 75 min metabolic inhibition (MI) with 0.3 mM iodoacetic acid (IA) plus 5 mg/ml antimycin A (AA) or 1 mM potassium cyanide (CN) showed more particulate or vesicular Cx43 reactivity intracellularly (large arrows) and punctate reactivity located at cell–cell interfaces (small arrows). Bar: 25 μm.

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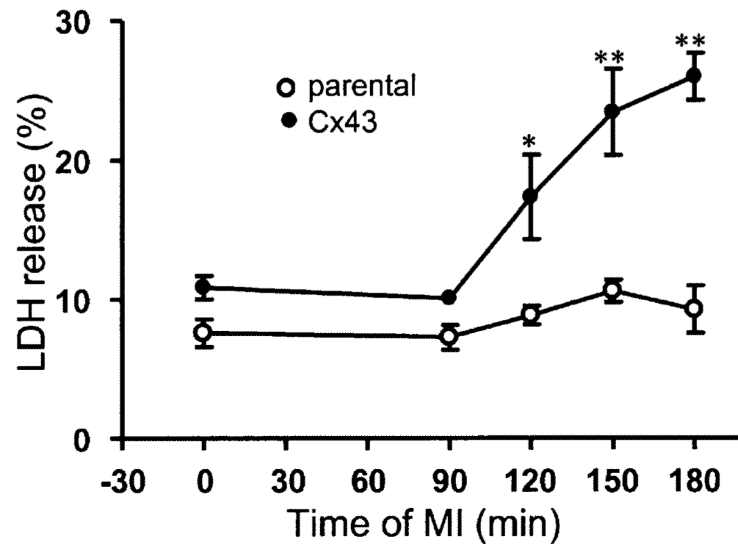

Fig. 4.

Cx43 transfected cells are more susceptible to cell death induced by metabolic inhibitors than are parental cells, a hemichannel action? The LDH activity was measured as described [25], and its release to the culture medium was expressed as the percentage of total LDH activity found in sister cultures [25]. The graph shows the time course of LDH release by parental (open circles) and HeLa Cx43 cells (filled circles) cultured in 96-well plates during metabolic inhibition by antimycin A (10 ng/ml) and iodoacetate (1 mM). LDH release was greater for Cx43 than parental cells. Each plotted point corresponds to the average ±SE (n=6). *$p<0.01$; **$p<0.001$; ANOVA two-way, Bonferroni post-test.

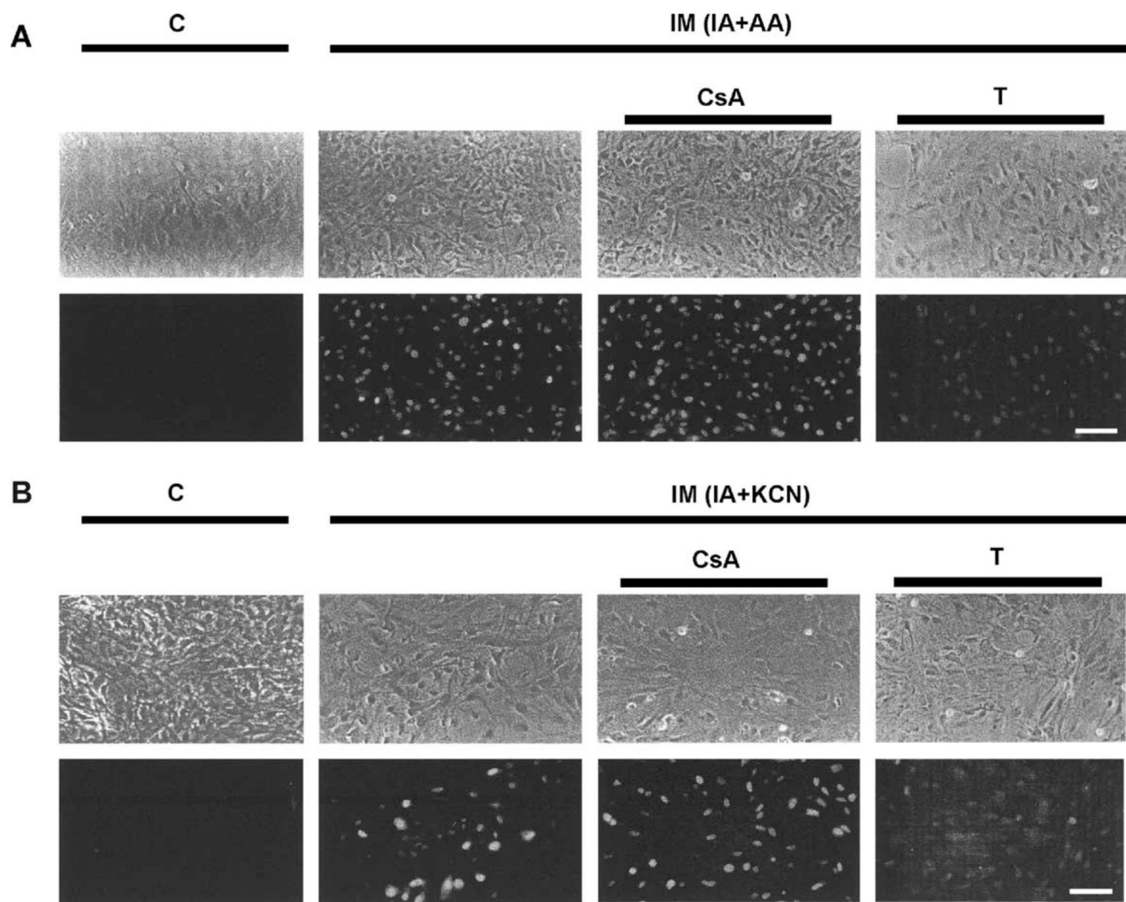

Fig. 5.

The EtBr uptake induced by MI is reduced by a free radical scavenger, trolox (T), but not by an inhibitor of calcineurin, cyclosporin A (CsA). Control (C) astrocytes did not show significant EtBr uptake tested as described previously [25]. Confluent cultures of rat cortical astrocytes were treated with iodoacetic acid (0.3 mM) and antimycin A (5 ng/ml) or 1 mM KCN for 30 min. Then metabolic inhibitors (MI) were washed out and cells were exposed to 2 μM cyclosporin A (CsA) or 100 μM trolox (T) for additional 35 min. Phase pictures in upper row, fluorescence in lower row. Bar: 50 μm.

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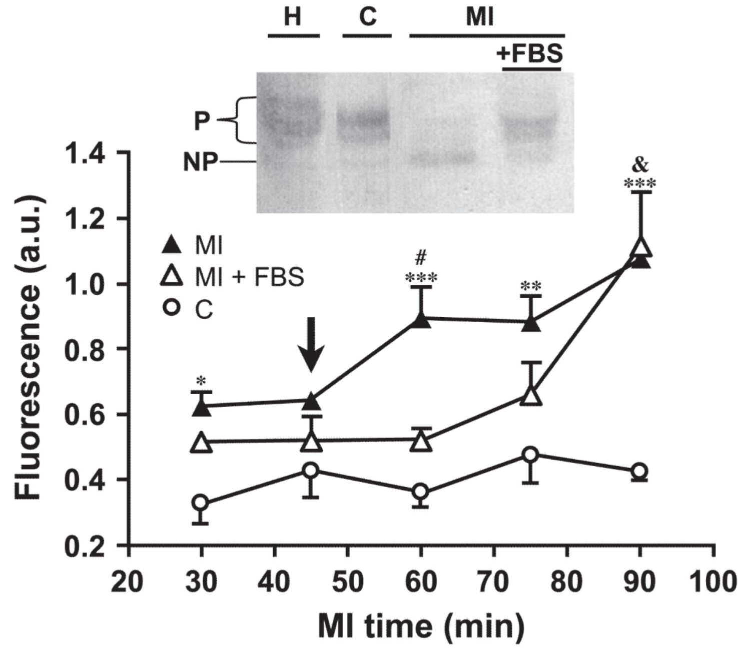

Fig. 6.

Fetal bovine serum delays metabolic inhibition-induced dye uptake and reduces Cx43 dephosphorylation in cortical astrocytes. Rat cortical astrocytes were metabolically inhibited with iodoacetic acid (0.3 mM) and antimycin A (5 ng/ml) for 45 min (up to arrow) and then were incubated in control medium without or with 10% fetal bovine serum (FBS). Metabolic inhibition (MI) induced delayed uptake of ethidium bromide (EtBr, 100 μM) measured fluorometrically as described previously [25]. EtBr uptake was more delayed in the presence of FBS but was independent of FBS at 90 min after the start of the experiment. Open circles, control; closed triangles, MI; open triangles, MI+10% FBS. Each plotted point represents the average value±S.E., n=5. *p<0.05; **p<0.01; ***p<0.001 MI vs. control; & p<0.001 MI +FBS vs. control; #p<0.01 MI vs. MI+FBS. ANOVA two-way, Bonferroni post-test. Inset: Western blot analysis of Cx43 in astrocytes after 45 min MI followed by 30 min in the absence or presence of 10% FBS. MI caused marked dephosphorylation; MI in the presence of FBS caused much less dephosphorylation. H: heart, C: control astrocytes, P: phosphorylated forms of Cx43, NP: nonphosphorylated form of Cx43.