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Gap junction hemichannels in astrocytes of the CNS

J. C. Sáez[1,2], **J. E. Contreras**[1,2], **F. F. Bukauskas**[2], **M. A. Retamal**[1], and **M. V. L. Bennett**[2]
[1]Departamento de Ciendas Fisiológicas, Pontifida Universidad Católica de Chile, Santiago, Chile
[2]Department of Neurosdence, Albert Einstein College of Medicine, Bronx, NY, USA

Abstract

Connexins are protein subunits that oligomerize into hexamers called connexons, gap junction hemichannels or just hemichannels. Because some gap junction channels are permeable to negatively and/or positively charged molecules up to ~ 1kDa in size, it was thought that hemichannels should not open to the extracellular space. A growing amount of evidence indicates that opening of hemichannels does occur under both physiological and pathological conditions in astrocytes and other cell types. Electrophysiological studies indicate that hemichannels have a low open probability under physiological conditions but may have a much higher open probability under certain pathological conditions. Some of the physiological behaviours of astrocytes that have been attributed to gap junctions may, in fact, be mediated by hemichannels. Hemichannels constituted of Cx43, the main connexin expressed by astrocytes, are permeable to small physiologically significant molecules, such as ATP, NAD^+ and glutamate, and may mediate paracrine as well as autocrine signalling. Hemichannels tend to be closed by negative membrane potentials, high concentrations of extracellular Ca^{2+} and intracellular H^+ ions, gap junction blockers and protein phosphorylation. Hemichannels tend to be opened by positive membrane potentials and low extracellular Ca^{2+}, and possibly by as yet unidentified cytoplasmic signalling molecules. Exacerbated hemichannel opening occurs in metabolically inhibited cells, including cortical astrocytes, which contributes to the loss of chemical gradients across the plasma membrane and speeds cell death.

Keywords

astroglia; cell signalling; connexins; connexons; ischaemia

Introduction: general aspects of connexins and hemichannels

Connexins are transmembrane proteins that belong to a gene family of 19 and 20 members in mouse and human, respectively (Willecke *et al.* 2002). The nomenclature most widely used to refer to the different members of the connexin family uses Cx followed by the predicted molecular mass of the protein (in kDa, e.g. Cx43) (Beyer 1990). To indicate the species of origin a short prefix may be added (e.g. rCx43 for Cx43 from rat). Most connexin genes have similar organization in which the entire coding sequence is in a single exon; Cx36 is exceptional in that the coding sequence is contained in two exons. Alternative splicing of upstream elements may result in different promoters expressed in specific tissues (Willecke *et al.* 2002). Many vertebrate cells express more than one connexin type. A few connexins are expressed in only one tissue; others are expressed more widely. Newly synthesized connexins form hexamers, in particular intracellular compartments, which may

Correspondence: Juan C. Sáez, Departamento de Ciencias, Fisiológicas, Pontificia Universidad, Católica de Chile, Santiago, Chile.

differ among connexins (Martin *et al.* 2001, Sarma *et al.* 2002). In formation of gap junction channels, a hexamer or hemichannel (or connexon) is contributed by each cell. In this presentation, we consider the properties of hemichannels that are not apposed to a hemichannel in another cell, and we will refer to them simply as hemichannels.

The location at which connexins oligomerize into hemichannels is connexin type-dependent. Cx43 assembles in the trans Golgi apparatus, and Cx32 assembles in the ER (Musil & Goodenough 1993, Rahman *et al.* 1993, Díez *et al.* 1999, Martin *et al.* 2001, Sarma *et al.* 2002). Assembled hemichannels are transported to the plasma membrane through at least two different pathways, one resistant and the other sensitive to brefeldin A (George *et al.* 1999, Martin *et al.* 2001), a Golgi apparatus disruptor. Those transported through the brefeldin A sensitive pathway (e.g. Cx43 and presumably Cx32 and Cx50) travel to the plasma membrane via vesicles of 100–150 nm and those transported through the brefeldin A resistant pathway (e.g.Cx26) might directly integrate into the plasma membrane through a mechanism not yet understood (Zampighi *et al.* 1999, Ahmad & Evans 2002, Gaietta *et al.* 2002, Sarma *et al.* 2002). In an *in vitro* cell-free transcription/ translation system, Cx26 is integrated directly into plasma membranes post-translationally (Ahmad &; Evans 2002). Some cells expressing at least two connexins form heteromeric hemichannels (containing more than one type of connexin) (Jiang &; Goodenough 1996, Locke *et al.* 2000, Berthoud *et al.* 2001, Martínez *et al.* 2002), as well as homomeric hemichannels. Homomeric hemichannels of different connexin types in one cell may form junctions in distinct membrane domains (Spray *et al.* 1991, Guerrier *et al.* 1995). Junctions with a different type of hemichannel in each cell are termed heterotypic; not all possible connexin combinations will form heterotypic junctions.

The presence of hemichannels on the cell surface has been documented with several experimental approaches. Freeze-fracture replicas of the plasma membrane of *Xenopus* oocytes expressing Cx50 exhibit a new population of intramembrane particles (~9 nm in diameter) associated with a whole-cell current (Zampighi *et al.* 1999). Using an anti-Cx26 antibody that reacts with a region of the C-terminus of Cx26 and immunofluorescence, Cx26 has been localized at the membrane of the dendritic tips of horizontal cells of a teleost fish where gap junctions are absent, suggesting that Cx26 might form hemichannels (Janssen-Bienhold *et al.* 2001, Kamermans *et al.* 2001). Moreover, in human polymorphonuclear cells treated with proinflammatory agents, but not in resting cells, Cx43 hemichannels have been detected by immunofluorescence using an antibody directed to a region of extracellular loop 1 of Cx43 (Brañes *et al.* 2002). The relative levels of Cx43 hemichannels located in the surface of osteocytic MLO-Y4 and NRK–cells have been measured by Western blot analysis of biotinylated cell surface proteins (Musil & Goodenough 1993, Cooper & Lampe 2002, Plotkin *et al.* 2002). Functional studies using electrophysiological recording in solitary horizontal cells of catfish retina and cells of the urinary bladder epithelium of *Necturus maculosus* have identified membrane currents activated by lowering extracellular Ca^{2+} concentration and applying positive membrane potentials. Those currents showed pharmacological and permeability properties corresponding to hemichannels, but the connexin type expressed is unknown (DeVries & Schwartz 1992, Malchow *et al.* 1994, Vanoye *et al.* 1999). Definitive demonstrations of hemichannel-mediated currents have been obtained in coupling incompetent cell lines transfected with known connexins. Additional approaches to demonstrate the existence of functional hemichannels include comparison of wild type cells and cells from animals with a specific connexin gene knocked out or knocked down by antisense oligonucleotide treatment (Li *et al.* 1996, Contreras *et al.* 2002b). Gap junction blockers have also been used to inhibit hemichannel mediated currents and to decrease permeability to various gap junction permeable tracers (see below).

Exchange of ions and small molecules between the cytoplasm and extracellular space depends, in part, on mechanisms that control the number of hemichannels found at the cell surface and their open time. Under physiological conditions, hemichannel opening accounts for the release to the extracellular space of small molecules, some of which are likely to play a role in cell-cell signalling. It is likely that hemichannels serve as one pathway for uptake of small nutrients present in the extracellular milieu and they may have a role in several other physiological functions. In addition, placing cultured cells in conditions that mimic aspects of ischaemia, such as metabolic inhibition, induces opening of hemichannels, which accelerates the progression to cell death. The identification of diseases in which excessive hemichannel opening occurs and the discovery of pharmacological agents that selectively block these channels could provide new tools to reduce cell death from these diseases as well as from ischaemia.

Opening and closing hemichannels

Because many gap junction channels exhibit a large non-specific permeability, hemichannels in the cell surface were expected to remain closed until appropriate pairing with a hemichannel in a contacting cell (e.g., Bennett *et al.* 1991). The earliest indication of hemichannel opening came from the finding that Cx46 expressed in *Xenopus* oocytes resulted in cell swelling and death (Paul *et al.* 1991). Shortly afterwards, the appearance of membrane currents with pharmacological properties similar to that of gap junction channels in retinal horizontal cells exposed to low extracellular Ca^{2+} concentrations were attributed to hemichannels (DeVries & Schwartz 1992). In subsequent years, macroscopic and microscopic membrane currents mediated by hemichannels formed by known connexin types have been recorded in heterologous expression systems, including *Xenopus* oocytes and several cell lines such as HeLa and RIN cells. Membrane currents mediated by hemichannels have also been recorded from primary cells, including horizontal neurons in fish retina, rat cardiac myocytes and rat cortical astrocytes (John *et al.* 1999, Kondo *et al.* 2000, Kamermans *et al.* 2001, Zhang & McMahon 2001, Contreras *et al.* 2002b, Stout et al. 2002). Agents favouring opening and closing of hemichannels are indicated in Fig. 1.

While under resting conditions opening of Cx46 expressed in *Xenopus* oocytes might be explained by the lack of endogenous regulatory factors present in the lens (see below), hemichannels formed by other connexins are opened by application of positive membrane potentials and/or low extracellular [Ca^{2+}] solutions. Cx46 hemichannels exhibit two voltage gating mechanisms (Trexler *et al.* 1996), a fast form (<1 ms transition time) gating between the fully open state and a substate and a slow form (>5 ms transition time) gating between the open state and the fully closed state, possible transiting through several intermediate states. Thus, hemichannel gating has properties similar to gating of cell-cell channels as described earlier (Bukauskas &; Peracchia 1997, Bukauskas *et al.* 2001). Low extracellular [Ca^{2+}] promotes opening of hemichannels in the exogenous expression systems mentioned above and in several mammalian cells, including Novikoff cells (Li *et al.* 1996), cardiac myocytes (John *et al.* 1999, Kondo *et al.* 2000), astrocytes (Hofer &; Dermietzel 1998, Stout *et al.* 2002, Contreras *et al.* 2002b), and human osteoblast-like cells (Romanello &; D'Andrea 2001). Cx43 hemichannel opening can be induced in cultured astrocytes by mechanical stimulation (Stout *et al.* 2002) and in osteocytic MLO-Y4 cells by treatment with alendronate, a bisphophonate used in the treatment of bone diseases (Plotkin *et al.* 2001). How alendronate induces opening of hemichannels remains unknown. Opening of hemichannels can be enhanced with quinine or quinidine in addition to the application of positive membrane potentials or low extracellular [Ca^{2+}] (Malchow *et al.* 1994, White *et al.* 1999, Stout *et al.* 2002, Ripps *et al.* 2002) (Fig. 1), suggesting the existence of the hemichannel activator. Quinine does not affect the functional state of Cx43 and Cx46 gap junction channels expressed in *Xenopus* oocytes (White *et al.* 1999), although it blocks

Cx36 channels with high potency (Srinivas *et al.* 2001); it is possible that it interacts with connexin domains exposed in hemichannels but not in cell–cell channels. The quinine effect on hemichannels cannot be explained solely by the quinineinduced intracellular alkalinization, because the action is still observed using 80 mM HEPES into the recording patch pipette (Dixon *et al.* 1996). The membrane current mediated by Cx38 hemichannels endogenously expressed by *Xenopus* oocytes is enhanced by quinine in a concentration-dependent fashion. The Hill coefficient of 1.9 suggests that the binding of at least two molecules of quinine is required to produce the effect (Ripps *et al.* 2002).

Membrane currents mediated by hemichannels are greatly reduced at negative membrane potentials or when the extracellular[Ca^{2+}] is >1 mM (DeVries & Schwartz 1992, Ebihara & Steiner 1993, Pfahnl & Dahl 1999, Zampighi *et al.* 1999, Jedamzik *et al.* 2000, Ripps *et al.* 2002). Cx46 hemichannels are also blocked by extracellular application of Ni^{2+}, Co^{2+} or Mg^{2+} (Ebihara & Steiner 1993, Ebihara *et al.* 2003) (Fig. 1). Closure of Cx43 hemichannels has been observed after extracellular application of La^{3+} (Kim *et al.* 1999, Kondo *et al.* 2000, Contreras *et al.* 2002a,b) or Gd^{3+} (Kondo *et al.* 2000, Stout *et al.* 2002) (Fig. 1). Several gap junction channel blockers, including octanol, heptanol, carbenoxolone, oleamide, halothane, 18-α-glycyrrhetinic acid and 18-β-glycyrrhetinic acid block hemichannels (Li *et al.* 1996, 2001, John *et al.* 1999, Zampighi *et al.* 1999, Quist *et al.* 2000, Bruzzone *et al.* 2001a, Franco *et al.* 2001, Kamermans *et al.* 2001, Contreras *et al.* 2002a, Eskandari *et al.* 2002, Ripps *et al.* 2002, Stout *et al.* 2002). Nevertheless, pharmacological sensitivities of hemichannels formed by different connexin types differ. While Cx50 hemichannels expressed in *Xenopus* oocytes are blocked by niflumic acid, diphenyl-2-carb-oxylate and octanol, Cx46 hemichannels are resistant to these agents (Eskandari *et al.* 2002). The inhibitory effect of octanol on Cx50 hemichannels is not altered by the extracellular [Ca^{2+}] and it is only observed when octanol is applied on the extracellular side of the hemichannels (Eskandari *et al.* 2002). These differences in pharmacological sensitivities suggest that in the future it will be possible to control the number of functional hemichannels in a connexin specific manner. These findings encourage the development of pharmacological tools that could be useful to demonstrate the physiological role of hemichannels formed by different connexins and in the treatment of diseases in which hemichannel gating mechanisms are implicated. For example, several Cx32 mutations causing X-linked Charcot-Marie-Tooth neuropathy increase Cx32 hemichannel opening (Castro *et al.* 1999, Abrams *et al.* 2002).

In teleost horizontal cells bathed in Ca^{2+}-free Ringer's solution and exposed to positive potentials, Cx35 hemichannels can be closed by retinoic acid (Zhang & McMahon 2001). The degree of inhibition of horizontal cell hemichannels by co-application of Ca^{2+} and retinoic acid is less than the sum of inhibition by Ca^{2+} or retinoic acid alone, suggesting that their actions are not independent (Zhang 8c McMahon 2001). Low intracellular pH also induces closure of hemichannels formed by various connexins (Trexler *et al.* 1999, Zampighi *et al.* 1999, Beahm & Hall 2002, Ripps *et al.* 2002). A short exposure of Cx46 hemichannels to low pH induces a rapid and reversible closure termed pH gating and a longer exposure induces a poorly reversible or irreversible closure termed pH inactivation (Trexler *et al.* 1999).

In mammalian cell lines, opening of Cx43 hemichannels induced by low extracellular [Ca^{2+}] is blocked by activation of a protein kinase C-dependent pathway (Li *et al.* 1996, Liu *et al.* 1997). Similarly, the membrane current mediated by Cx46 hemichannels expressed in *Xenopus* oocytes is greatly reduced by activation of protein kinase C (Ngezahayo *et al.* 1998, Jedamzik *et al.* 2000). Therefore, protein phosphorylation may induce gating that keeps hemichannels closed. Moreover, Cx43 is substrate for mitogen activated protein (MAP) kinases (Warn-Cramer *et al.* 1998), also termed extracellular signal regulated kinases

(ERKs). MAP kinases can be activated by tyrosine kinase receptors, such as the epidermal growth factor receptor, or through transduction pathways that activate protein kinase C via a cross-talk mechanism (Rivedal & Opsahl 2001, Ruch *et al.* 2001), leading to closure of Cx43 gap junction channels (Berthoud *et al.* 1993, Oh *et al.* 1993). A similar mechanism might operate on Cx43 hemichannels. In support of this notion, reconstituted Cx43 hemichannels remain preferentially closed after phosphorylation with purified MAP kinase, and phosphatase treated Cx43 forms functional hemichannels (Kim *et al.* 1999) (Fig. 1). It is surprising that MAP kinase keeps hemichannels closed without affecting gap junctional communication. Subcellular compartmentalization of MAP kinase may permit access to hemichannels but not to gap junction channels. Alternatively, protein phosphatases may have less access to phosphorylated hemichannels than to phosphorylated gap junction channels, thus increasing the level of hemichannel phosphorylation induced by basal MAP kinase activity.

Perch Cx35, but not skate Cx35, presents a consensus site for protein kinase A phoshorylation. Accordingly, perch but not skate Cx35 hemichannels are closed by activation of protein kinase A with 8Br-cAMP, a membrane permeant derivative of cAMP (Mitropoulou & Bruzzone 2003). Lens connexins (Cx44, Cx46 and Cx56) are phosphoproteins and are detected as multiple bands in immunoblots of lens homogenates (Paul *et al.* 1991, Berthoud *et al.* 1994, Gupta *et al.* 1994), but in oocytes they show the same electrophoretic mobilities as *in vitro* translated proteins (Paul *et al.* 1991, Gupta *et al.* 1994, Ebihara *et al.* 1995). In sheep lens fibres treated with a casein kinase I inhibitor, an increase in dye coupling has been observed, suggesting that phosphorylation of the lens fibre connexins by casein kinase leads to closure of gap junction channels (Cheng & Louis 2001). Cx46 can be phosphorylated by the endogenous casein kinase I activity of the lens (Cheng & Louis 1999). A similar mechanism might keep lens fibre hemichannels closed to maintain the high input resistance of these cells. Deficient regulation of phosphorylation of these connexins in *Xenopus* oocytes might explain the hemichannel opening at physiological voltages. In addition, the relatively negative potential at which these hemichannels open (−20 mV) (Ebihara & Steiner 1993) is closer to the resting membrane potential of oocytes (−30 to −60 mV) (Purnick *et al.* 2000) than to that of rat lens fibres (−60 to −75 mV) (Takeshita *et al.* 1993, Cheng *et al.* 2000).

Enhanced or reduced opening has also been observed in hemichannels formed by mutated and chimeric connexins. Two mutations linked to congenital cataract, Asn63Ser and frame shift 380, reduce opening of Cx46 hemichannels and formation of cell-cell channels (Pal *et al.* 2000). In Cx50, which like Cx46 forms hemichannels in oocytes, the mutation Cx50Hisl61Asn blocks hemichannels activity, but not gap junction formation (Beahm & Hall 2002). Several C-terminal mutations of Cx32, including some of those identified in X-linked Charcot–Marie–Tooth disease, prevent the formation of functional hemichannels (Castro *et al.* 1999). A chimeric connexin consisting of Cx32 with the first extracellular loop replaced by the corresponding Cx43 sequence forms hemichannels in *Xenopus* oocytes and has been useful in studying gating mechanisms (Pfahnl *et al.* 1997, Oh *et al.* 2000).

Conductance and permeability of hemichannels

Unitary activity of hemichannels has been recorded for Cx30, Cx32, Cx43, Cx45, Cx46 and Cx50 (Trexler *et al.* 1996, Ebihara *et al.* 1999, Valiunas & Weingart 2000, Abrams *et al.* 2002, Contreras *et al.* 2002b, Eskandari *et al.* 2002, Valiunas 2002). In Cx30, Cx43, Cx45 and Cx46, the unitary conductance is about twice that of the corresponding cell–cell channels, as would be predicted from simple series arrangement of two hemichannels in forming a single cell–cell channel. The discrepancies may be accounted for by differences in experimental conditions and solutions. All these hemichannels open at positive membrane

potentials and low extracellular [Ca^{2+}], but lens connexins of different species (i.e. rCx46, bCx44 and chCx56) also open at physiological voltages (Paul *et al.* 1991, Ebihara & Steiner 1993, Gupta *et al.* 1994, Trexler *et al.* 1996). Lens fibres have a high input resistance (Ebihara *et al.* 1995), indicating the absence of open hemichannels under physiological conditions. As discussed above, this apparent contradiction may result from differences in post-transcriptional modification of these connexins in oocytes and in lens fibres. The biophysical properties of the macroscopic current generated in *Xenopus* oocytes that coexpress Cx45.6 and Cx50 differ from those induced by the expression of either Cx45.6 or Cx50 alone, indicating the formation of heteromeric hemichannels with distinct properties (Ebihara *et al.* 1999).

The permeability of hemichannels has been evaluated using a number of fluorescent tracers. Cx43 hemichannels are permeable to Lucifer yellow, ethidium bromide, carboxyfluorescein, 7-hydroxycoumarin-3-carboxylic acid and fura-2 (Li *et al.* 1996, Liu *et al.* 1997, Kondo *et al.* 2000, Contreras *et al.* 2002a, Plotkin *et al.* 2002, Stout *et al.* 2002) (Fig. 2). Interestingly, Cx43 hemichannels appear to be impermeable to propidium iodide, unlike Cx43 cell-cell channels (Elfgang *et al.* 1995). Cx46 hemichannels show a strong preference for cations and display marked inward rectification in symmetric solutions (Trexler *et al.* 1996). However, oocytes expressing Cx46 hemichannels take up negatively charged tracers, such as carboxyfluorescein (MW 376) (Hu & Dahl 1999) Cx46 hemichannels are only slightly permeable to Lucifer yellow, cadaverin, and biotin-X (MW 873), suggesting a molecular weight cutoff of about 1 kDa (Hu & Dahl 1999). Cx32 hemichannels are anion selective, and substitution of the first extracellular loop (E1) of Cx32 into Cx46 makes the chimeric Cx46 channels anion preferring, indicating that fixed charges in E1, positive in Cx32 and negative in Cx46, influence charge selectivity of the hemichannels (Trexler *et al.* 2000). Cx45 hemichannels are permeable to Lucifer yellow and propidium iodide and comparison of Lucifer yellow diffusion through Cx45 hemichannels and gap junction channels reveals that the latter are less permeable (Valiunas 2002).

Cx43 hemichannels are permeable to small molecules, including NAD^+, ATP and IP_3 (Bruzzone *et al.* 2001a,b, Romanello & D'Andrea 2001, Arcuino *et al.* 2002, Stout *et al.* 2002). Similarly, cells expressing Cx32 release ATP, suggesting that it can permeate Cx32 hemichannels (Arcuino *et al.* 2002). Part of the evidence for permeation through hemichannels is that fluorescently labelled dextrans of somewhat higher molecular weight are not taken up by cells expressing Cx43 (Arcuino *et al.* 2002, Contreras *et al.* 2002a, Stout *et al.* 2002). Similarly, stachiose fluorescein (MW 1146) is not taken up by oocytes expressing Cx46 (Hu &; Dahl 1999).

In addition to gating, the number of hemichannels in the surface membrane will affect exchange of substances between the intracellular and extracellular milieu. As the half-life of several connexins (not including those in the lens) is known to be a few hours (Fallon & Goodenough 1981, Traub *et al.* 1987, 1989, Musil *et al.* 1990, Beardslee *et al.* 1998, Gaietta *et al.* 2002), it is likely that the number of hemichannels in the plasma membrane is affected by conditions that alter the turnover of these proteins. Inhibition of casein kinase 1 increases the number of surface hemichannels, suggesting a role of this kinase in assembly of hemichannels into cell-cell channels (Cooper &; Lampe 2002). Thus, total movement through hemichannels could be regulated over periods of seconds to hours by gating and by synthesis, insertion and retrieval of hemichannels from the plasma membrane.

Hemichannels in astrocytes under physiological conditions

Different cell types of the adult CNS, including neurons, astrocytes, oligodendrocytes and microglia, express different sets of connexins (Shiosaka *et al.* 1989, Dermietzel *et al.* 1997,

2000, Nadarajah *et al.* 1997, Nagy *et al.* 1997, 1999, Rash & Yasumura 1999, Sohl *et al.* 2000, Teubner *et al.* 2000, Venance *et al.* 2000, Eugenín *et al.* 2001, Oguro *et al.* 2001, Rash *et al.* 2001a,b, Altevogt *et al.* 2002, Long *et al.* 2002, Meier *et al.* 2002, Parenti *et al.* 2002), and for neurons and astrocytes at least the expression pattern changes during development (Dermietzel *et al.* 1991, Leung *et al.* 2002).

In vivo, astrocytes express Cx43 and low levels of Cx26, Cx30, and possibly Cx40, and Cx45 (Nagy *et al.* 1997, 1999, Dermietzel *et al.* 2000). In cultured rat astrocytes, unitary conductances of gap junction channels are similar to those observed in Cx43 transfectants (Dermietzel *et al.* 1991, Giaume *et al.* 1991, Moreno *et al.* 1994, Kwak *et al.* 1995, Bukauskas et al. 2001). Moreover, astrocytes cultured from Cx43-deficient mice exhibit very little gap junctional communication (Naus *et al.* 1997, Scemes *et al.* 1998, Dermietzel *et al.* 2000).

As most gap junction channel blockers also block hemichannels (see above), suppression of Ca^{2+} waves by gap junction blockers does not differentiate between two distinct possible mechanisms of propagation: communication through gap junctions and mediation by ATP released from hemichannels diffusing in extracellular space (Cotrina *et al.* 1998). Other data confirm the extracellular nature of the signalling, including propagation across cell free spaces, dependence on flow of extracellular fluid, sensitivity to purinergic antagonists and visualization of released ATP in the extracellular space (Hassinger *et al.* 1996, Cotrina *et al.* 1998, Guthrie *et al.* 1999, Arcuino *et al.* 2002). Moreover, low extracellular Ca^{2+} concentrations or application of quinine, two conditions known to open hemichannels, elicit local and propagating Ca^{2+} signals (Stout *et al.* 2002). Mechanical stimulation triggers ATP release apparently through Cx43 hemichannels and thus initiates propagation of Ca^{2+} waves in astrocytes and other electrically inexcitable cells (Arcuino *et al.* 2002, Stout *et al.* 2002). Astrocytes bathed in low $[Ca^{2+}]$ medium or mechanically stimulated show membrane currents and uptake of small fluorescent dyes inhibited by flufenamic acid or Gd^{3+} (Stout *et al.* 2002, Arcuino *et al.* 2002). It remains uncertain whether ATP release evokes ATP release in neighbouring cells (and whether propagation is passive or active and regenerative). Astrocytes in low extracellular $[Ca^{2+}]$ show an increased intracellular $[Ca^{2+}]$ response to ATP, whereas astrocytes in high extracellular $[Ca^{2+}]$ show a decreased intracellular $[Ca^{2+}]$ response to ATP. These results suggest that astrocytes possess a mechanism for coupling between extracellular $[Ca^{2+}]$ and the release of Ca^{2+} from intracellular stores, which may be important in pathological conditions associated with low extracellular Ca^{2+} such as seizures or ischaemia (Zanotti &; Charles 1997). Opening of Cx43 hemichannels in HOBIT osteoblastic cells is likely to participate in Ca^{2+} waves evoked by mechanical stimulation, and IP_3 uptake evokes Ca^{2+} transients, suggesting a possible contribution of IP_3 to wave propagation (Romanello &; D'Andrea 2001).

How do hemichannels open? To answer this question, Braet *et al.* (2003) used caged IP_3 or Ca^{2+} and found that photoliberating IP_3 in a single cell within a confluent culture of endothelial cells triggers an intercellular Ca^{2+} wave that is prevented by 18-α-glycyrrhetinic acid, the connexin mimetic peptide gap 26 (a 13-residue peptide with the same sequence as a region of Cx43 E1, suramin (a purinergic receptor blocker), apyrase (an ATPase) or purinergic receptor desensitization. The ATP release induced by uncaging IP_3 was inhibited by buffering the intracellular $[Ca^{2+}]$ with BAPTA. The effect induced by gap 26 with its extracellular application, was reversible in 10–20 min, which is too rapid to be an action on junction formation, and did not block intercellular dye coupling; these properties suggest that gap 26 acts by blocking hemichannels (Braet *et al.* 2003). The block of IP_3 induced hemi-channel opening by BAPTA suggests that IP3 acts by an indirect mechanism, such as dephosphorylation of the connexin or generation of another intracellular signal.

The role of hemichannels in ATP release is not entirely clear. In HOBIT cells overexpressing Cx43, the amount of ATP released under basal and mechanically stimulated conditions is similar to the non-transfected cells, ruling out a major involvement of connexin hemichannels in ATP release in these cells (Romanello *et al.* 2001). Moreover, Ca^{2+} waves between cells transfected with Cx43 were not blocked by 18-α-glycyrrhetinic acid or octanol (Cotrina *et al.* 1998). In addition, oleamide blocks gap junctions and propagation of Ca^{2+} waves (Guan *et al.* 1997), but does not block the permeability of Cx43 hemichannels to NAD^+ (Bruzzone *et al.* 2001b). The discrepancies in published data may result from the existence of multiple pathways of ATP release and from differences in mechanism in different cell types and under different physiological conditions. The ionotropic ATP receptor P2X is permeable to small fluorescent dyes, either positively or negatively charged, including Lucifer yellow, ethidium bromide and YOPRO-1 (Ralevic & Brunstock 1998, Virginio *et al.* 1999, Ferrari *et al.* 2000, Bisaggio *et al.* 2001, Hubscher *et al.* 2001), and could be permeable to ATP. In addition, there is evidence of vesicular release of ATP through a pathway resistant to flufenamic acid and anandamide, two gap junction blockers (Coco *et al.* 2003).

NAD^+ is another signalling molecule proposed to permeate hemichannels. Fibroblasts deficient in Cx43 expression do not release NAD^+, but those transfected with Cx43 release NAD^+ by passive diffusion (Bruzzone *et al.* 2001a). These data provided an explanation of the old paradox of intracellular NAD^+ being the substrate for an ectoenzyme, CD38, that catalyses its conversion to cADPR. CD38 also mediates uptake of cADPR, which is a potent activator of rhyanodine receptors. Autocrine/paracrine Ca^{2+} signalling mediated by cADPR generated from NAD^+ released through Cx43 hemichannels has been proposed to enhance cell proliferation and shorten the S phase of 3T3 fibroblasts (Franco *et al.* 2001). The same autocrine/paracrine mechanism may cause glutamate release from astrocytes leading to delayed Ca^{2+} transients in neurons in coculture (Verderio *et al.* 2001). In addition, Cx43 hemichannels mediate glutamate release from astrocytes under low calcium conditions that might be reached during strong stimulation or ischaemia (Ye *et al.* 2003). From data of this kind, hemichannels can be considered a new component in the tripartite interaction of pre- and post-synaptic elements and astrocytes proposed elsewhere (Araque *et al.* 1999). Quist *et al.* (2000), by using atomic force microscopy and dye uptake assay, examined extracellular Ca^{2+}-dependent modulation of cell volume. They found that Cx43 hemichannels participate in regulation of cell volume in response to small changes in extracellular [Ca^{2+}] (from 1.8 ≤ 1.6 mM) under isosmotic conditions. A similar mechanism might operate in astrocytes in pathological as well as physiological conditions.

Role of hemichannels in pathological conditions

Under normal conditions, astrocytes support nervous system function by supplying neurons with lactic acid as metabolic fuel and glutamine for generation of glutamate, and by maintaining the extracellular milieu particularly with respect to neurotransmitters and K^+ (e.g. Chen &; Nicholson 2000). In numerous pathological conditions, including stroke and head trauma, tissue perfusion is deficient and ischaemia is a common factor. Ischaemia involves hypoxia, deficiency of nutrients and accumulation of metabolic products that can be toxic. After short periods of ischaemia followed by reperfusion, neurons are the main cell type affected. Focal ischaemia can induce necrosis of glial cells as well as neurons in the core of the lesion, and delayed neuronal death can occur days later in the ‘penumbra’, the region adjacent to the core.

In response to lack of oxygen, astrocytes switch to anaerobic metabolism (Peuchen *et al.* 1996), a property that contributes to their surviving periods of ischaemia that are lethal to neurons. The enhanced generation of lactic acid by anaerobic astrocytes in conjunction with

the deficient tissue perfusion raises the concentration of this organic acid reducing neuronal viability (Goldman *et al.* 1989, Nedergaard *et al.* 1991). Thus, injuries that astrocytes tolerate but that alter their metabolism could indirectly affect neuronal susceptibility to the same injury. As the intensity and/or the duration of the ischemic episode increases the functions of astrocytes supportive of neurons (and themselves) are likely to become less and less efficient. The precise steps leading to astrocyte death are not yet established, but death is preceded by progressive membrane depolarization, cellular acidosis, decreased glucose utilization and ATP depletion. Studies in primary astrocyte cultures treated with metabolic inhibitors or oxygen/glucose deprivation have been useful in identifying the time course and mechanism of various astrocyte responses induced by ischaemia. Simultaneous inhibition of the glycolysis and mitochondrial respiration causes a drastic ATP depletion paralleled by a progressive reduction of electrochemical gradients across the plasma membrane (Harold &; Walz 1992). As membrane depolarization takes several minutes, it is plausible that it results, at least in part, from Cx43 hemichannel opening (Fig 2 and Fig 3; Contreras *et al.* 2002a). In support of this possibility, increases in macroscopic membrane current and unitary events ascribable to hemichannels induced by metabolic inhibition in HEK293 cells are reduced to near control levels by halothane, a hemichannel blocker (John *et al.* 1999). Moreover, in metabolically inhibited rabbit ventricular myocytes halothane or heptanol prevents the TTX and nifedipine resistant increase in intracellular $[Na^+]$ and $[Ca^{2+}]$ (Li *et al.* 2001). Both in HEK293 cells and rabbit ventricular myocytes the membrane conductance increased after metabolic inhibition, and the increase has a reversal potential close to zero, suggesting that the increase is mediated by a nonselective membrane channel (John *et al.* 1999, Kondo *et al.* 2000). Accordingly, metabolically inhibited astrocytes take up positive (ethidium, +1 as the Br^- salt) and negative (Lucifer yellow, −2) fluorescent dyes from the extracellular medium (Fig. 2; Contreras *et al.* 2002a). The dye uptake is blocked by La^{3+} or 18-α-glycyrrhetinic acid, both hemichannel blockers, and precedes plasma membrane break down as evidenced by lactate dehydrogenase release to the extracellular medium or cell uptake of dextran-LY (10 kDa) too large to pass through hemichannels (Contreras *et al.* 2002a). More convincing, dye uptake induced by metabolic inhibition is not seen in mouse astrocytes lacking Cx43 for atleast 3 h (Contreras *et al.* 2002a).

The mechanism that induces Cx43 hemichannel opening during metabolic inhibition remains elusive. The possibility that Cx43 dephosphorylation is necessary for opening of hemichannels in astrocytes is supported by the following findings: (i) reconstituted Cx43 hemichannels previously treated with phosphatase, but not those phosphorylated by MAP kinase, are active, i.e. they open and close (Kim *et al.* 1999); (ii) confluent cultures of metabolically inhibited astrocytes contain mostly dephosphorylated Cx43 (Li &; Nagy 2000, Martínez & Sáez 2000, Contreras *et al.* 2002a). To establish that dephosphorylation is necessary for opening of Cx43 hemichannels during metabolic inhibition will require direct evaluation of the state of phosphorylation of the Cx43 forming hemichannels. Gating of hemichannels may be mediated by generation of free radicals, which is increased in metabolically inhibited astrocytes (Taylor *et al.* 1996), and free radical scanvengers, such as trolox and melatonin, reduce opening of hemichannels by metabolic inhibition (Contreras *et al.* 2002a). Direct evaluation of the redox state of Cx43 should further extend our knowledge of this putative gating mechanism. A further indication of specific signalling pathways is that inhibitors of lipoxygenase, but not of cyclooxygenase, prevent opening of hemichannels (Contreras *et al.* 2002a).

The extent of injury following focal ischaemia (transient or permanent) is not established immediately after arterial occlusion; infarct volume expands over time. Episodes of spreading depression have been linked to this secondary increase in size of the infarct. Tissue bordering the infarction fails to repolarize following spreading depression and is incorporated into the lesion implying that infarcts may expand after each episode of

spreading depression (Rawanduzy *et al.* 1997). Passage of potentially harmful cytosolic molecules between cells in the infarct and surrounding cells might cause amplification of injury in focal ischaemia. Octanol, a gap junction blocker, markedly reduces the size of necrotic foci in a rat model of stroke (Rawanduzy *et al.* 1997). However, most gap junction blockers are unspecific; halothane and octanol also reduce potency of chemical synapses (Puil & El-Beheiry 1990, Puil *et al.* 1994), and gap junction blockers inhibit spreading depression (e.g. Martins-Ferreira & Ribeiro 1995, Nedergaard *et al.* 1995, Rawanduzy *et al.* 1997).

Specific approaches with gene knockout or antisense have revealed important neuroprotective actions of connexins during tissue injury. In heterozygous Cx43 KO mice, which exhibit reduced Cx43 expression, infarct size after stroke is greater than in wild type mice (Siushansian *et al.* 2001). In mixed astrocyte neuron cultures, inhibition of astrocyte coupling with gap junction blockers increases neuronal vulnerability to oxidative stress (Blanc *et al.* 1998) or glutamic acid toxicity (Ozog *et al.* 2002). In addition, FGF-2 upregulates Cx43 in rat embryonic day 14 midbrain cultures and promotes survival of dopaminergic neurons; oleamideinduced cell uncoupling abolishes this survival promoting effect (SiuYi Leung *et al.* 2001). In contrast, neuronal cell death in hippocampal slice cultures 48 h after oxygen/ glucose deprivation is less in slices from Cx43 KO mice than in slices from wild type mice (Frantseva *et al.* 2002a). Similarly, treatment with antisense oligodeoxy-nucleotides for Cx26 and Cx32 or for Cx43 reduces cell death caused by oxygen/glucose deprivation, and carbe-noxolone is neuroprotective (Frantseva *et al.* 2002a). Similar results were obtained in a model of traumatic brain injury (Frantseva *et al.* 2002b). In contrast, Cx32 KO mice show enhanced sensitivity to cerebral ischaemia (Oguro *et al.* 2001), suggesting gap junction mediated neuroprotection. In astrocytes and glioma cell lines subjected to metabolic inhibition, oxidative stress, or Ca^{2+} overload, propagation of damage from dying to resistant cells is mediated by gap junctions (Lin *et al.* 1998). Several other cell lines subjected to various insults show bystander killing mediated by Cx43 gap junctions (Azzam *et al.* 2001, Huang *et al.* 2001, Burrows *et al.* 2002, Sanson *et al.* 2002).

In summary, opposite roles for gap junctional communication in cell death have been demonstrated in different paradigms. Gap junctions can mediate metabolic cooperation and be neuroprotective. Alternatively, gap junctions can contribute to the propagation of injury. Relative numbers can affect the outcome; many resistant or healthy cells can rescue a few badly injured cells; a large number of dying cells can overcome the capabilities of a cell that would otherwise survive. In studies to date using gap junction blockers and KO animals, a contribution of hemichannels cannot be excluded. In a medium rich in nutrients and protective agents, such as vitamins that can scavenge free radicals, enhanced hemichannel opening could facilitate uptake of those extracellular compounds favouring cell survival. Conversely, prolonged hemichannel opening would lead to rise in intracellular Ca^{2+} and Na^{+}, loss of K^{+} and intracellular metabolites, and finally cell death.

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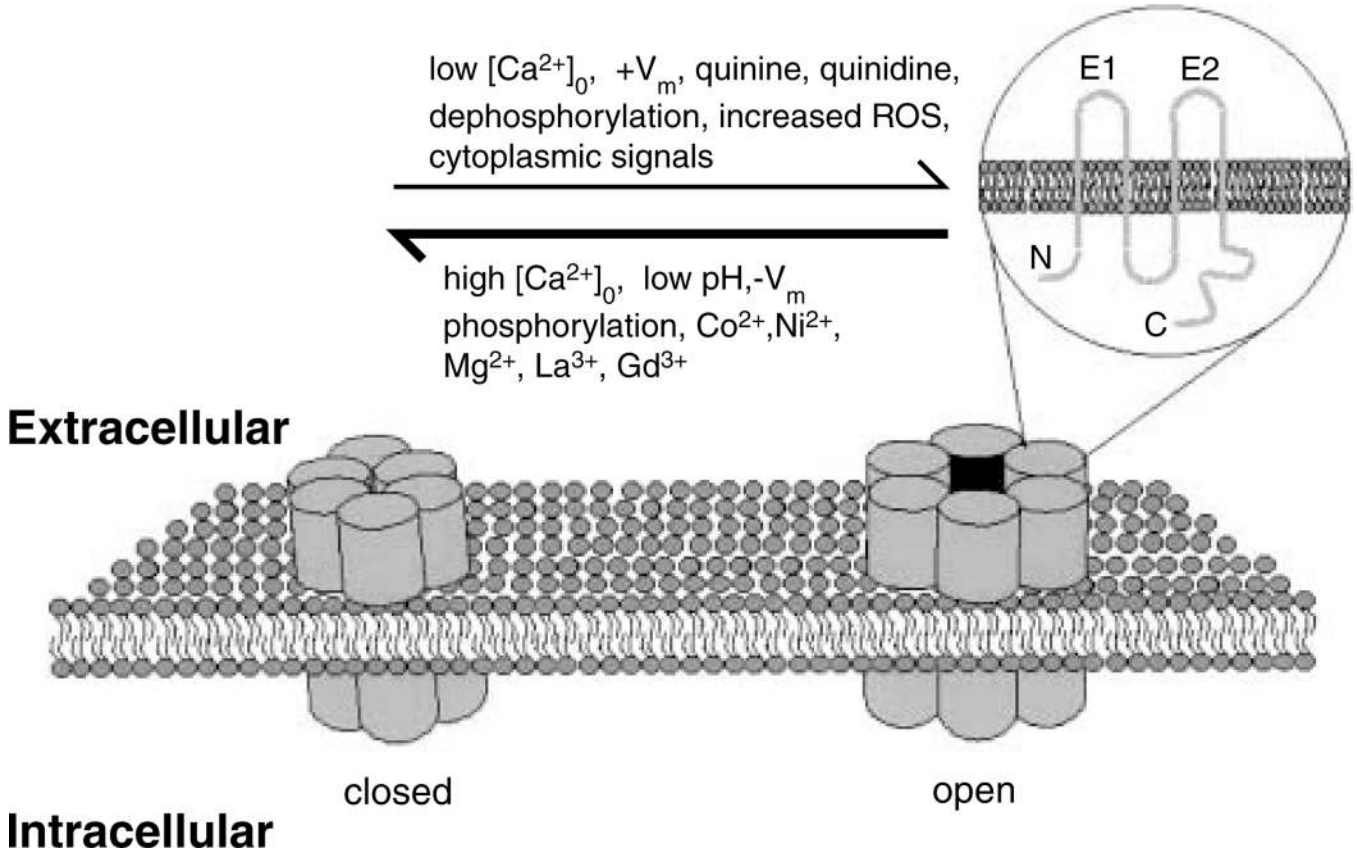

Figure 1.

Conditions that open or close hemichannels. Connexins are tetraspan proteins with both amino and carboxy terminals on the cytoplasmic side of the membrane (inset, upper right). Each hemichannel contains six connexin molecules and under physiological conditions are preferentially closed (left). Open probability of hemichannels is increased in low $[Ca^{2+}]_O$, at positive membrane potentials ($+V_m$), by such pharmacological agents as quinine and quinidine, and after metabolic inhibition leading to dephosphorylation and increase in reactive oxygen species (ROS) and generation of as yet unidentified cytoplasmic signals. Open probability is decreased by high $[Ca^{2+}]_O$, negative membrane potentials ($-V_m$), low intracellular pH, phosphorylation of cytoplasmic domains, polyvalent cations, including Co^{2+}, Ni^{2+}, Mg^{2+}, La^{2+} and Gd^{3+}, and low intracellular pH and other gap junction blockers.

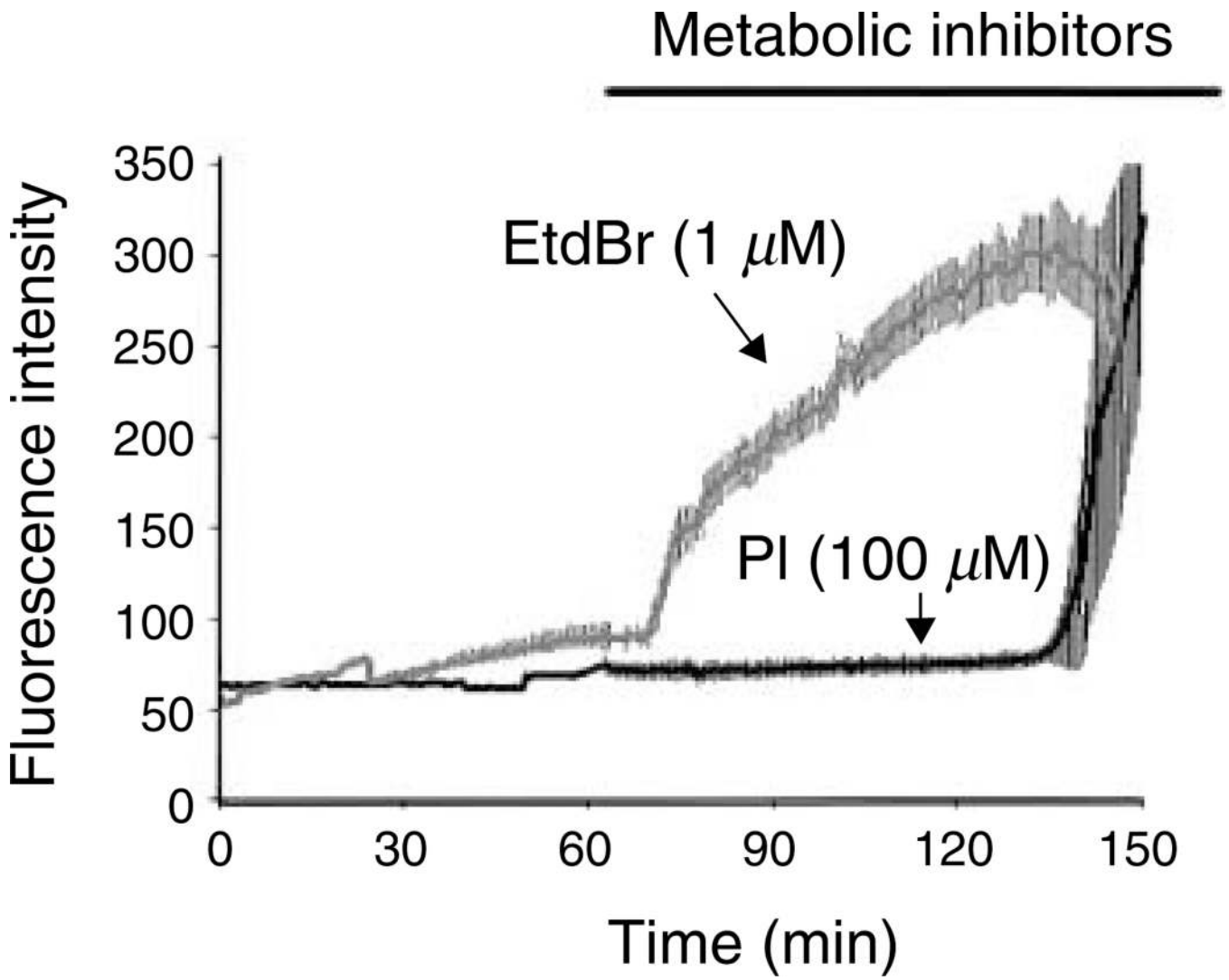

Figure 2.

Time course of ethidium bromide (EtdBr) and propidium iodide (PI) uptake in metabolically inhibited astrocytes. Confluent cultures of rat cortical astrocytes were treated with iodoacetic acid and antimycin A and the time course of EtdBr and IP uptake was measured (in separate experiments). EtdBr uptake began a few minutes after application of metabolic inhibitors (at 60 min). PI uptake was minimal until ~ 130 min of metabolic inhibition at which time uptake increased abruptly. Other experiments indicate that this delayed uptake is because of the loss of membrane integrity.

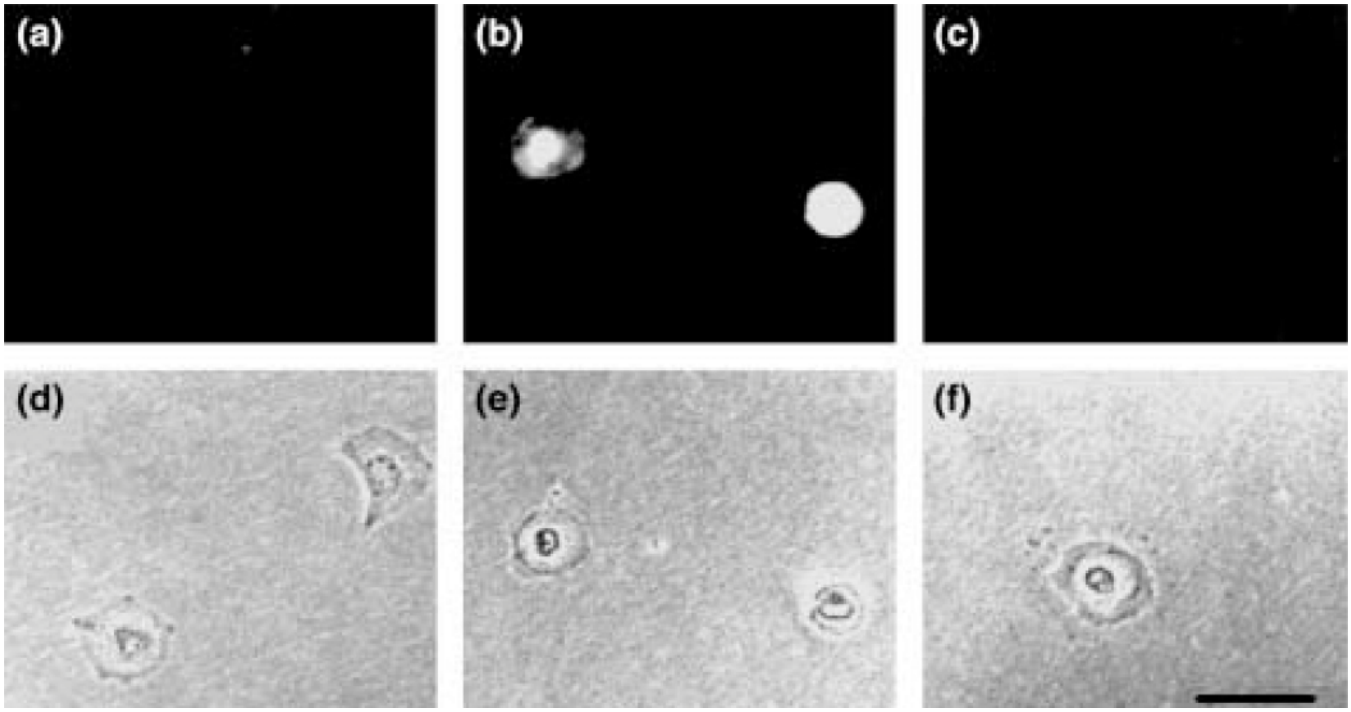

Figure 3.

Ethidium bromide uptake by astrocytes treated with metabolic inhibitors is blocked by 18-glycyrrhetinic acid. Four hours after plating at a very low density (200 cells/60 mm diameter culture dish) cortical rat astrocytes were exposed to iodoacetic acid plus antimycin A for 75 min or kept in control medium. Ethidium bromide (50 μ_M) was then applied for 2 min to cells in the control medium (a), to metabolically inhibited cells (b), and to metabolically inhibited cells treated with 75 μ_M 18β-glycyrrhetinic acid for 5 min before the dye application (c). Cells treated only with metabolic inhibitors were stained (b), and cells under control conditions or treated with metabolic inhibitors plus the gap junction blocker remained unstained; (d), (e) and (f) are phase contrast views of the fluorescent views showed in (a), (b) and (c), respectively. Bar 20 μm.