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Increased Intercellular Communication in Mouse Astrocytes Exposed to Hyposmotic Shocks

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Abstract

When exposed to 20% and 35%, but not to 50% hypotonic solutions, mouse astrocytes recovered their volume within a few minutes, which coincided with the activation of nonjunctional conductances. Conductance of gap junctions between astrocyte pairs also increased after exposure to a 35% hypotonic shock; however, this effect began at 3 min after the shock, when cells had partially recovered their initial volumes. During the first minute of exposure to 20% and 35% hypotonic stimuli, there was a transient monophasic increase in intracellular calcium levels; exposure to 50% hypotonic solution led to intracellular Ca^{2+} oscillations. The differences in time courses of nonjunctional conductance changes, Ca^{2+} alterations, and intercellular coupling suggest that distinct second messenger pathways are involved in each response. The velocity of mechanically evoked calcium waves propagated among the astrocytes increased at 7.5 min after 35% hypotonic shock. This increase was not seen with 20% or 50% hypotonic stimuli and is not ascribable to the increase in junctional conductance because it was blocked by suramin, a P2 purinergic receptor antagonist. Given that the transduction pathways activated during cell swelling (e.g., generation of phospholipases, phosphokinases, arachidonic acid) exert inhibitory effects on astrocytic gap junctions (Giaume and McCarthy, 1996), it is proposed that the increased junctional conductance during hypotonic shock is due to increased number of channels, perhaps triggered by the initial Ca^{2+} signals (Dolmetsch et al., 1997). As a functional consequence of the increased coupling and enhanced extracellular propagation of Ca^{2+} waves, spread of signaling molecules throughout the glial network is expected to be significantly enhanced during hypotonic stress. The increased intercellular communication between mouse astrocytes in response to hypotonic challenge thus occurs via both gap junction-dependent and -independent mechanisms and presumably provides neuroprotective effects following nervous system injury

Keywords

gap junctions; junctional conductance; connexins; RVD; Ca^{2+} waves

INTRODUCTION

The wide variety of ion channels, transporters, and receptors expressed on astrocytic plasma membranes provides these glial elements with a machinery able to receive and respond to different CNS signals. Thus, astrocytes can be viewed as active participants in the maintenance of brain function because they can protect and interact with surrounding neurons. Furthermore,

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the presence of abundant gap junction channels, which are permeable to ions and molecules up to 1 kDa, between astrocytes allow these cells to coordinate their physiology and to participate in various brain functions as an organized syncytium for exchange of electrical and chemical signals.

Connexin43 (Cx43) is the major constituent of gap junctions in astrocytes (Dermietzel et al., 1989, 1991; Giaume et al., 1991a; Micevych and Abelson, 1991; Scherer et al., 1995). However, Cx43 distribution is not uniform in astrocytes in different brain regions (Naus et al., 1990; Yamamoto et al., 1990; Matsumoto et al., 1991; Micevych and Abelson, 1991; Batter et al., 1992; Belliveau and Naus, 1995) and Cx43 expression is regulated during brain development (Dermietzel et al., 1989) and in response to lesions and traumatic situations (Rohlmann et al., 1993; Hossain et al., 1994a,b; Theriault, 1997). Besides these long-term (1 - 2 hr) controls of gap junctional properties that account for developmental and post-traumatic regulation of Cx43 expression, the strength of astrocytic coupling may also be affected in the short-term (seconds or minutes) by neurotransmitters, nucleotides, and peptides (Dermietzel et al., 1991; Giaume et al., 1991b; Giaume et al., 1992; Enkvist and McCarthy, 1992, 1994; Muller and Kettenmann, 1995). Thus the functional extent of the astrocytic network may be regarded as transiently and dynamically expanding and contracting.

In a number of pathological states affecting the CNS, including ischemia, trauma, and hypoxia, astrocytic swelling is a prominent event (Chiang et al., 1968; Bourke et al., 1980; Long, 1981; Kimelberg, 1992). It was previously reported that astrocytic cell-cell coupling decreased when cells were swollen by hyposmotic stimuli (Kimelberg and Kettenmann, 1990), a response that would be expected to have profound effects on astrocytic function, especially in decreasing the clearance of substances through the syncytium for removal from the CNS at the glial endfeet surrounding the capillaries.

Because of the potential importance of astrocyte coupling in dissipating ionic gradients during anisomotic conditions, we investigated the effects of hyposmotic shocks on astrocyte junctional conductance using the dual voltage clamp technique and measured the time course of cell volume changes and the properties (velocity, amplitude, and efficacy) of intercellular calcium waves in confluent cultures of astrocytes exposed to anisomotic conditions. Cell volume measurements performed using the dye dilution method with calcein-AM loaded mouse astrocytes revealed that volume restoration occurred rapidly after swelling the cells with 20% and 35% but not after 50% hyposmotic shocks. The dual voltage-clamp technique applied to pairs of astrocytes exposed to 35% hyposmotic shocks revealed, contrary to the previous report (Kimelberg and Kettenmann, 1990), that junctional conductance was substantially increased. Such increase in junctional conductance began 3 min after the onset of nonjunctional currents; the nonjunctional currents, which are most probably responsible for cell volume recovery, were activated within a few seconds after the shock and inactivated with a half-time similar to that measured for volume restoration. The velocity of mechanically evoked calcium waves was significantly increased 5 min after bathing the cells with 35% hyposmotic solution; however, such increase was found not to be related to changes in junctional conductance because the increase in velocity was prevented by treatment with suramin, a purinergic P2 antagonist (King et al., 1996; Bolego et al., 1997). These results thus indicate that under such stressful conditions the astrocytic syncytium remains a functional network in which the diffusion of substances is enhanced by gap junction-dependent mechanisms, and the coordination of cell activity by Ca^{2+} signaling is also strengthened through gap junction-independent mechanisms.

MATERIALS AND METHODS

Cell Culture

Purified primary astrocyte cell cultures were obtained from whole brain tissue that was dissected from neonatal mice (GJA1M1 strain, obtained from Jackson Laboratories, Bar Harbor, ME; although the strain is heterozygous for the Cx43 knockout allele, this study used wild type progeny). After trypsinization (0.1% trypsin at 37°C), the dissociated cells were plated either on coverslips or on confocal dishes (glass-bottomed microwells, model #15, Maktek) containing Dulbecco's essential culture medium supplemented with 45% (vol/vol) Ham's F12 medium, 10% (vol/vol) fetal calf serum, penicillin (50 µg/ml), streptomycin (50 µg/ml), glutamine (2 mM), buffered to pH 7.3 with 21 mM sodium bicarbonate. Cells were maintained in 5% CO₂, 95% air atmosphere at 37°C, at 100% relative humidity. About 90% of the cells were shown to be immunopositive for glial acidic fibrillary protein (GFAP). Confocal microscopy experiments were performed using confluent primary cultures (1 to 2-week-old cultures), and electro-physiological experiments were performed on freshly dissociated cells. In one set of confocal microscopy experiments, nonconfluent cell cultures were employed to test whether changes in the time course of cell volume regulation might be related to cell density (see Results).

Electrophysiology

Changes in junctional and nonjunctional conductances during hyposmotic shocks were followed in astrocytes using the dual whole cell voltage clamp technique. Freshly dissociated pairs of astrocytes were voltage clamped at holding potentials of 0 mV and 200 ms duration command steps (ΔV) of 10 mV (from -100 mV to +100 mV) were presented to one cell. Currents were recorded in the pulsed cell (I_1) and in the cell clamped at 0 mV (I_2). Junctional current was measured as $-I_2$ and nonjunctional current as $I_1 - I_2$; junctional and nonjunctional conductances were calculated as $-I_2/\Delta V$ and $(I_1 - I_2)/\Delta V$, respectively (Spray et al., 1981). Patch pipettes were filled with 140 mM CsCl; 10 mM EGTA; 5 mM MgATP (pH 7.25). The cells were bathed in a solution containing 140 mM NaCl; 2 mM KCl; 2 mM CaCl₂; 1 mM BaCl₂; 2 mM CsCl; 1 mM MgCl₂; 5 mM HEPES (pH 7.2). Hyposmotic shock was applied by superfusing the cells with a 35% diluted bath solution. Whole-cell and junctional currents were followed before and during the osmotic shock.

Confocal Microscopy

Volumetric measurements—Cell volume changes under hyposmotic conditions were measured using the optical method of indicator dilution. Confluent cultures of astrocytes (1 – 2 weeks old) were loaded with 0.5 – 1.0 µM calcein-AM for 30 min and fluorescence examined using confocal microscopy (Nikon RCM 8000). In one set of experiments, nonconfluent cultures of astrocytes were also used. Prior to the osmotic shock, a few series of 15–20 1 µm thick z-section confocal images were obtained at a single wavelength (excitation: 488 nm; emission: 540 nm), using the Nikon 40x water immersion objective (N.A. 1.15, working distance 0.2 mm) at 1 min intervals on cells bathed in 200 µl normal solution (same solution as described for the electrophysiological studies) in order to control for photobleaching. Cells were then subjected to hyposmotic shocks (20%, 35%, and 50%) by adding 1 ml of the diluted solution and additional series of z-sections through the cells were acquired at 1 – 2 min intervals for a duration of 10 min following the shock. The images were saved on an optical magnetic disk recorder (OMDR) as an average of 8 frames of single wavelength images and then played back for analysis using Polygon-Star software (Nikon). The gray level (number of pixels per area) within the region of interest (circular spots containing ~400 pixels) of the largest optical z-section of the cells were averaged and then used for analysis of calcein fluorescence intensity. Half-times ($T_{1/2}$) for cell volume recovery were calculated by fitting a Boltzmann equation of the type: $F = \{(F_1 - F_2)/(1 + \exp(t - T_{1/2})/\Delta t)\} - F_2$; where F_1 and F_2 are fluorescence intensity

values at the beginning and at completion of RVD, respectively, and Δt is the time interval between F_1 and F_2 .

Calcium measurements—Changes in intracellular calcium induced by the osmotic shocks were analyzed in confluent cell cultures. Astrocytes, plated as described above on glass-bottomed microwells, were loaded with Indo-1-AM (10 μ M; Molecular Probes, Eugene, OR) at 37 °C for 45 min, after which they were rinsed and used for confocal microscopy. Intracellular Ca^{2+} changes were measured in loaded astrocytes bathed in the same solution used for electrophysiological and volumetric measurements. The ratio of Indo-1 fluorescence intensity emitted at two wavelengths (390–440 nm and 440 nm) was imaged using an UV laser with excitation at 351 nm. Ratio images were acquired at 0.5 – 1 Hz after background and shading corrections using the Nikon real time confocal microscope (RCM 8000) with UV large pinhole and Nikon 40x UV corrected water immersion objective (N.A. 1.15, working distance 0.2 mm). Indo-1 fluorescence ratio images were continuously acquired before and after exposure to the three hyposmotic solutions. The ratiometric images were saved on the OMDR as the average of 16 or 32 frames of ratio images and then played back for measurements of changes in calcium level using Polygon-Star software (Nikon). The gray levels (number of pixels per area) within the region of interest (circular spots with radii of 6.41 μ m, containing ~ 200 pixels) were averaged and then used for analysis.

Velocity, amplitude, and efficacy of calcium wave spread—Calcium waves between Indo-1-AM loaded astrocytes were evoked by mechanical stimulation of one cell in the confocal field using a glass micropipette, as previously described (Scemes et al., 1998). Conduction velocities were calculated by dividing the distances (μ m) between the stimulated and nonstimulated cells by the time interval (sec) between the half-maximal calcium increases within the stimulated and responding cells. Amplitudes of calcium waves were considered as the maximal increments in intracellular calcium observed in responding cells, calculated for each cell as the value of Indo-1 fluorescence ratio rise at the peak of the response divided by the basal fluorescence ratio value acquired before the induction of the calcium waves. The efficacy of calcium spread between glial cells was considered to be the proportion of cells responding with an intracellular calcium increase in relation to the total number of cells within the field of view.

Calcium wave velocity, amplitude, and efficacy were measured before and at 2.5 min intervals for 10 min following the osmotic shocks. The contribution of ATP-mediated calcium wave spread between cultured astrocytes exposed to hyposmotic shocks was evaluated in astrocyte cultures exposed to 100 μ M suramin (a purinergic P2 antagonist: see King et al., 1996; Bolego et al., 1997).

RESULTS

Cell Volume and Intracellular Ca^{2+} Changes Induced by Hyposmotic Stimuli

When swollen by hyposmotic stimuli, astrocytes (and most other vertebrate cells) are capable of returning to their normal volume by a process termed regulatory volume decrease (RVD; see Hoffmann and Simonsen, 1989). For astrocytes, RVD is achieved by the efflux of intracellular osmolytes (K^+ , Cl^- , amino acids) through conductive pathways accompanied by osmotically obliged water (Kimelberg, 1990b; Pasantes-Morales et al., 1994a,b).

Using the optical method of dye dilution, mouse astrocyte volume changes under hyposmotic conditions were followed using real time confocal microscopy. As shown in Figure 1, the magnitude of cell swelling (indicated by the decrease in calcein fluorescence intensity) was proportional to the amplitude of the osmotic shock. These results thus indicate that this dye dilution method is suitable for estimating cell volume changes under anisosmotic conditions.

After the initial phase of cell swelling, mouse astrocytes exposed to 20% and 35% hyposmotic solutions decreased their volumes, as seen by the recoveries in calcein fluorescence intensities to values close to the original ones (Fig. 1A-C). Such volume recovery, however, was not observed in astrocytes exposed to 50% hyposmotic solution, at least during the 10 min observation period following the shock (Fig. 1D).

Under conditions where RVD was present, the halftimes ($T_{1/2}$) of volume recovery were calculated to be 2.52 min for cells exposed to 20% hyposmotic solution and 3.53 min for cells exposed to 35% hyposmotic shock (Fig. 1B, C). Cell density did not affect the $T_{1/2}$ of RVD, as evidenced by recovery of cell volume in nonconfluent cell cultures of astrocytes exposed to 20% hyposmotic solution; half-time ($T_{1/2} = 2.58$ min; Fig. 1A) was similar to that obtained for confluent cultures ($T_{1/2} = 2.52$ min; Fig. 1B).

For many cell types (see McCarthy and O'Neil, 1992), including rat astrocytes (O'Connor and Kimelberg, 1993) but not human UC-11MG astrocytoma cells (Medrano and Gruenstein, 1993) or lymphocytes (Grinstein and Smith, 1990), regulatory volume decrease is a Ca^{2+} -dependent process.

Differently from results obtained previously with rat astrocytes (O'Connor and Kimelberg, 1993; Schliess et al., 1996; Fischer et al., 1997), however, hyposmotic stimuli did not induce a biphasic calcium response in mouse astrocytes; the rapid increase in cytosolic calcium level in mouse astrocytes was not followed by a sustained plateau (Fig. 2). Both 20% and 35% hyposmotic solutions induced single calcium spikes that lasted for ~ 10 sec (Fig. 2A,B). Interestingly, 50% hyposmotic solution induced intracellular Ca^{2+} oscillations (Fig. 2C) with a frequency of 0.06 ± 0.01 Hz; these oscillations were synchronized in the great majority of the cells present in the confocal field and did not last more than 60 sec, probably because calcium spike amplitude decayed over time (Fig. 2C).

The amplitude of these calcium transients as well as the number of cells displaying increases in intracellular calcium levels were dependent on the magnitude of the osmotic shocks (Fig. 2D), as has been described for rat astrocytes (Fischer et al., 1997). Indo-1 fluorescence ratio increased 1.07 ± 0.01 -fold (peak/basal; $N = 4$ cells) in 25% of the cells when exposed to 20% hyposmotic solution, increased 1.13 ± 0.03 -fold (peak/basal; $N = 24$ cells) in 66% of the cells when exposed to 35% hyposmotic solution, and increased 1.35 ± 0.02 -fold (peak/basal; $N = 47$ cells) in all the cells when exposed to 50% hyposmotic solution.

Temporal Correlation Between Cell Volume Changes and Membrane Conductance

Because data presented above showed that cultured mouse astrocytes were able to reduce their volumes when exposed to 20% and 35% hyposmotic solution, but not after exposure to a 50% hyposmotic shock, analysis of membrane conductance changes (nonjunctional and junctional) during RVD were analyzed in astrocytes exposed only to 35% hyposmotic solutions, a condition similar to that employed previously to analyze conductance changes in rat astrocytes (Kimelberg and Kettenmann, 1990).

Hypotonic-induced swelling clearly caused an increase in whole cell currents in astrocytes (Fig. 3A), as was previously described for rat astrocytes (Kimelberg et al., 1990a). This outwardly rectifying current started to develop a few seconds after the shock, increasing in magnitude during the first 2 min and then decaying 4 min after the shock (Fig. 3C). Given that CsCl was present inside the pipette, such conductance (reversal potential close to zero mV) probably is a volume-sensitive chloride conductance as reported for several cell types (Jackson et al., 1996; Strange et al., 1996). Furthermore, the time course of activation and decay of this conductance paralleled the time course of cell volume changes, with volume recovery completed at ~ 5 min after the shock. The half-time ($T_{1/2} = 3.35$ min) of the decay in

nonjunctional currents was very similar to that obtained for RVD ($T_{1/2} = 3.53$ min; Fig. 1C) that followed exposure to 35% hyposmotic shock.

Differently, however, from results obtained on rat astrocytes (Kimelberg and Kettenmann, 1990), junctional conductance was found to increase substantially when astrocytes were exposed to 35% hyposmotic solution (Fig. 3B,C). Such junctional conductance increase was observed 3 min after the stimulus and was maintained for the entire period of the osmotic shock. Such increase was totally reversed by replacing the external hyposmotic solution by the control, isosmotic one (data not shown).

Intercellular Calcium Waves

Because changes in coupling observed after the exposure of astrocytes to hyposmotic shock may be expected to have significant effects on the diffusion of substances through the astrocytic syncytium, and because calcium waves have been reported to be a mechanism by which coordination is attained in the glial network, the velocity, amplitude, and efficacy of intercellular calcium waves, triggered by mechanical stimulation were measured in confluent cultures of astrocytes exposed to anisomotic solutions.

Velocity of calcium waves—Under isosmotic control conditions, mechanical stimulation of one cell induced an increase in intracellular calcium; a few seconds after the stimulus, almost all the cells in the confocal field displayed increases in cytosolic Ca^{2+} . The velocity with which this phenomenon propagated from the stimulated cell to the neighbors was variable among cultures, ranging from 15.35 ± 1.08 $\mu\text{m}/\text{sec}$ to 21.99 ± 1.21 $\mu\text{m}/\text{sec}$ (Fig. 4). When bathed in 20%, 35%, and 50% hyposmotic solutions, the velocity of these calcium waves was significantly altered ($P < 0.0002$; Anova Analysis of Variance) only when the cells were bathed in the 35% hyposmotic solution. Under this condition, the velocity of calcium wave increased from 15.35 ± 1.63 $\mu\text{m}/\text{sec}$ to 23.14 ± 1.7 $\mu\text{m}/\text{sec}$ and to 27.69 ± 1.39 $\mu\text{m}/\text{sec}$ at 7.5 min and at 10 min after the shock, respectively (Fig. 4B).

When cells were returned to the initially isosmotic condition 10 min after exposure to the 35% hyposmotic solution, the velocity of calcium wave propagation returned to basal levels during the first 5 min, increasing again to 34.37 ± 6.63 $\mu\text{m}/\text{sec}$ after 7.5 min (Fig. 5A).

These increases in the velocity of calcium wave were totally prevented by continuously exposing the cells to 100 μM suramin (a P2 purinergic receptor antagonist) (Fig. 5B), suggesting that the increased velocity of Ca^{2+} waves is mediated by extracellular diffusion of ATP.

Amplitude of calcium waves—The amplitudes of mechanically induced calcium waves, measured as Indo-1 fluorescence ratio rise at the peak of the response divided by the basal fluorescence ratio value acquired before the induction of the Ca^{2+} wave, were modified by the three hyposmotic solutions employed (Fig. 6). Calcium wave amplitude was decreased at 2.5 min and at 10 min after the 20% hyposmotic shock (Fig. 6A), whereas exposure of astrocytes to the 35% hyposmotic solution caused an attenuation of the Ca^{2+} wave amplitude only at 2.5 min after the shock (Fig. 6B). However, the amplitudes of calcium waves propagating among the astrocytes were decreased during the entire 10 min exposure to 50% hyposmotic solution (Fig. 6C).

Efficacy of Ca^{2+} wave propagation—The efficacy of the propagation of mechanically induced Ca^{2+} waves, measured as the proportion of cells responding with an intracellular calcium rise in relation to the total number of cells within the confocal field, was not affected by any of the three hyposmotic solutions used. About 98-100% of the cells in the field of view

that were participating in the propagation of the waves before the osmotic shock persisted signaling during the entire exposure to 20%, 35%, and 50% hyposmotic shocks.

These results describing the properties of calcium waves under anisomotic conditions indicate that hypoosmotic stress does not impair Ca^{2+} signaling within the astrocytic network. Even with an attenuated amplitude, the calcium waves traveled among the cells without decreased conduction velocity or efficacy of transmission. Extracellular diffusion of signaling molecules seems to play an important role on the modulation of this intercellular communication.

DISCUSSION

In many pathophysiological conditions, astrocyte swelling is a prominent event (Chiang et al., 1968; Bourke et al., 1980; Long, 1981; Kimelberg, 1992). After swelling by hyposmotic media, astrocytes restore their volume by losing ions, amino acids, and osmotically obliged water (Kimelberg et al., 1990b; Pasantes-Morales et al., 1994a,b); this process of cell volume restoration is termed regulatory volume decrease (RVD).

Similarly to rat cortical astrocytes, mouse astrocytes are shown to undergo RVD after swelling in response to moderate (20–35%) hyposmotic shocks. Nonjunctional conductances shown to be activated within a few seconds after the 35% hyposmotic stress and decaying with a half-time similar to that of RVD are the likely pathways for osmolyte efflux involved in cell volume restoration. These conductances, which reverse at membrane potentials close to zero mV (CsCl in the pipette and in the bath), most probably represent Cl^- channels activated during cell volume changes that have been previously described to play a role in RVD in a variety of cell types (Jackson et al. 1996; Strange et al., 1996).

Although the exact biochemical events that underly RVD still remain to be determined, initial studies (O'Connor and Kimelberg, 1993) pointed to a possible direct control of K^+ and/or Cl^- channels by Ca^{2+} , thereby modulating the efflux of KCl and, hence, RVD. It has been shown that rat astrocytes respond to hyposmotic stimuli with a biphasic increase in intracellular Ca^{2+} , consisting of a transient increase followed by a sustained plateau (O'Connor and Kimelberg, 1993; Schliess et al., 1996; Fischer et al., 1997). The initial transient component of the Ca^{2+} response has been shown to be mainly due to Ca^{2+} release from intracellular stores (O'Connor and Kimelberg, 1993) that are sensitive to bradykinin and ATP but not to ryanodine (Fischer et al., 1997). The second component or plateau phase of the Ca^{2+} response, which is predominately dependent on extracellular Ca^{2+} concentration (Fischer et al., 1997) and which mirrors the time course of RVD (O'Connor and Kimelberg, 1993), has been proposed to promote osmolyte efflux during astrocyte RVD (O'Connor and Kimelberg, 1993).

Differently from responses obtained on rat astrocytes, mouse astrocytes exposed to 20%, 35%, and 50% hyposmotic solutions display only the initial transient component of the Ca^{2+} response, without the sustained plateau phase. Because the transient (10–20 sec) increments in intracellular calcium levels did not parallel the time course of RVD observed when the two lower hyposmotic solutions were employed and because the long-lasting (60 sec) cytosolic Ca^{2+} oscillations occurred under conditions where volume restoration was not achieved (50% hyposmotic condition), it is unlikely that mouse astrocyte RVD is a strictly Ca^{2+} -dependent process.

Although further experiments are needed to verify the extent to which the initial Ca^{2+} rise contributes to mouse astrocyte RVD (see McCarthy and O'Neil, 1992), the results discussed above suggest that other regulatory processes might control the ionic channels involved in RVD in this system. One such pathway involves phosphatidylinositol (PI) hydrolysis mediated by phospholipase (PL) C. In rat astrocytes, hyposmotic stress increases PI hydrolysis (Bender et al., 1992); agonists that stimulate PI turnover accelerate hyposmotic RVD (Bender et al.,

1993), and inhibition of PI hydrolysis by blockade of PLC activity prevents rat astrocyte RVD (Bender et al., 1993).

In mouse pancreatic acinar cells, stimulation of PI hydrolysis resulted in cellular uncoupling (Somogyi and Kolb, 1989) and accelerated RVD (Ngezahayo and Kolb, 1990), leading to the hypothesis that in rat astrocytes the acceleration of hyposmotically induced RVD triggered by agonists that stimulate PI hydrolysis was related to a decrease in intercellular communication (Bender et al., 1993).

In this context, it would be predicted that astrocytes within the syncytium would display a slower RVD than the isolated, noncoupled cells. Contrary to this expectation, however, no difference between the half-times of regulatory volume decrease was obtained for astrocytes within or isolated from the network, as measured by exposing high and very low densities of cell cultures to 20% hyposmotic shocks. This result thus indicates that the strength of coupling does not affect astrocyte regulatory volume decrease, at least under conditions where cells are uniformly exposed to the hyposmotic stimulus.

More interesting, but contrary to previous work performed on confluent cultures of rat astrocytes (Kimelberg and Kettenmann, 1990), is the observation described here that junctional conductance is increased in pairs of mouse astrocytes exposed to 35% hyposmotic stress. Given that under this hyposmotic condition neither the onsets nor the time courses of changes in junctional conductance coincided with changes in nonjunctional conductance, different intracellular steps than those leading to nonjunctional channel activation are presumably involved in controlling/modulating junctional channels.

Conductance of gap junctions formed by connexin43 (Cx43), the main gap junction protein expressed in astrocytes (Dermietzel et al., 1989, 1991; Giaume et al., 1991a; Micevych and Abelson, 1991; Scherer et al., 1997), is affected by phospholipases (PL), phosphokinases (PK), and arachidonic acid (Giaume and McCarthy, 1996), all of which are involved in the transduction pathway related to PI hydrolysis, which was reported to be activated during cell swelling (Bender et al., 1992, 1993). Because these stimuli have been shown to exert inhibitory effects on astrocyte coupling, the increased junctional conductance obtained with 35% hyposmotic shock is likely due to increased number of gap junction channels between the cells. Such a result could arise from redistribution of connexin protein from one intra-cellular compartment to another or from increased connexin synthesis. It is even plausible that transcription factors induced by Ca^{2+} signals (Schliess et al., 1996; Dolmetsch et al., 1997) might be responsible for the increase in junctional conductance during exposure of mouse astrocytes to hyposmotic stimuli.

It was recently reported that the amplitude and duration of intracellular calcium signals control differential activation of transcription factors in B lymphocytes; the transient increase in intracellular Ca^{2+} selectively activates NF- κB and c-Jun N-terminal kinase within 4 min, whereas the low and sustained Ca^{2+} plateau activates NFAT within 1 min (Dolmetsch et al. 1997). For astrocytes, calcium-dependent activation of MAP kinases Erk-1 and Erk-2 was shown to occur in primary cultures 10 – 20 min after exposure to hyposmotic stimuli; the activation of these extracellular signal-regulated kinases (Erk) was shown to be independent of the action of PLC or PKC but dependent on the presence of extracellular calcium, i.e., on the sustained Ca^{2+} plateau (Schliess et al., 1996). Whether the transient Ca^{2+} increment observed in mouse astrocytes exposed to hyposmotic shocks is the signal that leads to higher Cx43 expression and consequently to the junctional conductance increase recorded 3 min after the 35% hyposmotic shock must be further investigated.

It is clear from the data obtained here that hyposmotic stress does not decrease junctional communication between astrocytes, an effect that would be expected to impact negatively on

CNS function. On the contrary, the increased junctional conductance recorded in mouse astrocytes exposed to hyposmotic shocks would facilitate the dissipation and homeostasis of K^+ ions (Kuffler and Nicholls, 1966) and the diffusion of metabolites (Tabernero et al., 1996; Giaume et al., 1997) through the syncytium.

Besides these functional implications of improved junctional communication between astrocytes exposed to hyposmotic stimuli, cooperative cell activity coordinated by intercellular calcium waves (see Sanderson, 1996) may also be enhanced. Although the velocity of calcium waves spreading through the astrocytic network was not affected by the increased junctional conductance, it was enhanced by gap junction-independent mechanisms sensitive to suramin (a purinergic P2 antagonist). The fact that the increased junctional conductance was not reflected by an increased calcium wave velocity is not surprising. Cell cultures of Cx43 knockout (KO) astrocytes propagate calcium waves with velocity, amplitude, and efficacy that is not very different from wild-type astrocytes, indicating that the safety factor for Ca^{2+} wave propagation is so high that the residual 5% coupling contributed by other connexins in KO astrocytes can support this process (Scemes et al., 1998; but see Naus et al., 1997).

This study thus reveals that, in contrast to a previous report, astrocyte intercellular communication responds to hyposmotic stimuli by signal transduction events enhancing dissipation of ionic gradients and long-range signaling through Ca^{2+} waves. Both gap junction-dependent and gap junction-independent mechanisms are shown to contribute, resulting in an effective expansion of the glial communication compartment. As a consequence of increased metabolite clearance and interglial signaling, this enhancement of intercellular communication between astrocytes is likely to provide a neuroprotective effect following traumatic or ischemic injury to the CNS.

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Fig. 1.

Time course of cell volume changes measured from non-confluent (A) and confluent (B) cultures of astrocytes exposed to a 20% hyposmotic shock and confluent cultures exposed to 35% (C) and 50% (D) hyposmotic shocks. Cell volume changes were evaluated using the optical method of indicator dilution in which fluorophore intensity was measured in confocal images (Nikon RCM 8000 confocal microscope). When exposed to hyposmotic shocks, the relative fluorescence intensity of calcein-AM loaded astrocytes rapidly decreased to levels proportional to the amplitudes of the osmotic shocks. After that period, corresponding to cell swelling, relative fluorescence intensity returned toward control values, indicating completion of RVD. No volume recovery was observed when cells were exposed to 50% hyposmotic

solution (D). The half-times ($T_{1/2}$) of RVD, obtained by curve fitting as described in Materials and Methods, are displayed on the graphs.

Fig. 2.

Intracellular calcium changes in astrocytes induced by hyposmotic solutions. **A, B, C** illustrate fluorescence ratios corresponding to calcium levels in two representative cells in a field exposed to different hyposmotic solutions. Exposure to 20% and 35% hyposmotic solutions induced transient increases in Indo-1 fluorescence, indicating intracellular calcium rises, that lasted for about 10 sec (**A** and **B**); the most diluted solution (50%) induced oscillatory Ca^{2+} increments lasting for about 60 sec (**C**). The amplitudes of these calcium responses and the number of cells (top of the bars) displaying such calcium transients were lower in astrocytes bathed in 20% than in cells bathed in 35% and 50% hyposmotic solutions (**D**); see text for details.

Fig. 3.

Magnitudes of (A) whole-cell (nonjunctional) and (B) junctional currents that developed following exposure of pairs of astrocytes to 35% hyposmotic solution. The time courses of the relative changes in conductances (junctional and nonjunctional) are shown in part C. Using the dual whole-cell voltage clamp technique, membrane potentials of both cells were held at 0 mV. Rectangular voltage steps (10 mV) of 200 ms duration were applied to one cell of the pair using Pclamp-6 software to clamp membrane potential to values between -100 mV and +100 mV. The instantaneous current - vs - voltage plots are shown as (I vs V [the weak voltage sensitivity of astrocyte junctional conductance (Dermietzel et al., 1991; Giaume et al., 1991) is not apparent in this plot due to the brief voltage pulses]. The current changes (ΔI) were measured as the difference between pre- and posthyposmotic current responses to applied voltages. Nonjunctional currents were calculated by subtracting the junctional current (flowing through the nonpulsed cell) from that flowing through the pulsed-cell.

Fig. 4.

Calcium wave propagation between astrocytes exposed to (A) 20%, (B) 35%, and (C) 50% hypotonic solutions. Astrocytes were loaded with Indo-1-AM and fluorescence ratio measured using the Nikon RCM 8000 confocal microscope. Images were acquired at 1 Hz. The velocity of calcium waves was obtained by dividing the distance between the stimulated and the non-stimulated cells by the time interval between the half maximal calcium rises of the stimulated and responding cells (see Scemes et al., 1998). The velocity of calcium wave propagation increased only after 5 min of exposure of astrocytes to 35% hypotonic solution (B) and was not statistically affected by treating the cells with either 20% (A) or 50% (C) hypotonic solutions.

Fig. 5. Velocity of calcium waves measured in confluent cultures of astrocytes exposed to 35% hypotonic shock in the absence (**A**) and in the presence (**B**) of 100 μM suramin. (**A**) The increases in the velocity of calcium waves observed at 7.5 and 10 min after exposure to 35% hypotonic solution and at 7.5 min and 10 min after returning the cells to the originally isotonic solution were abolished by 100 μM suramin (**B**). Astrocytes were loaded with Indo-1-AM and fluorescence ratios measured using the Nikon RCM 8000 confocal microscope. Images were acquired at 1 Hz.

Fig. 6.

Amplitude of calcium wave spread among confluent cultures of astrocytes exposed to (A) 20%, (B) 35%, and (C) 50% hypotonic solutions. Top portions show Indo-1 fluorescence ratio values obtained from astrocytes before (basal; light gray bars) and at the peak (dark gray bars) of the response to mechanically induced Ca^{2+} waves. Bottom parts show the amplitude of calcium waves under anisotonic conditions that were obtained dividing Indo-1 fluorescence ratio values at the peak of the response by the basal levels.