Long-lasting dephosphorylation of connexin 43 in acute seizures is regulated by NMDA receptors in the rat cerebral cortex

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Abstract. Gap junctions consisting of connexin 43 subunits provide intercellular communication between astrocytes, contributing to their function of maintaining the central nervous system (CNS) microenvironment. Magnetic resonance imaging (MRI) studies demonstrate prolonged astrocyte swelling related to seizures, while in vitro studies show the disruption of intercellular coupling and the cytotoxic swelling of astrocytes in seizure-equivalent environments. We examined the relation between astrocyte swelling and connexin 43 regulation using an in vivo seizure model. Generalised tonic-clonic convulsions were induced in adult rats using intraperitoneally (i.p.)-administered 4-aminopyridine (4-AP). The expression of connexin 43 mRNA and protein was measured in the neocortex. Pre-treatment with N-methyl-D-aspartate (NMDA) receptor antagonist dizocilpine maleate (MK-801) resulted in the amelioration of seizure symptoms and the prevention of connexin 43 dephosphorylation, as well as significantly reduced astrocytic swelling. Dephosphorylation of connexin 43 was shown to reduce astrocytic gap junction (GJ) permeability. Our results therefore suggest that, during acute seizures, a prolonged inhibition of intercellular coupling develops in the astrocyte network. This accounts for the long-lasting astrocyte swelling observed, and potentially impairs neuronal buffering function. The results also imply that this uncoupling is regulated through neuronal and/or glial NMDA-type glutamate receptors.

Introduction

Central nervous system (CNS) seizures are characterised by repeated synchronised neuronal bursts, a subsequent increase in transmitter release, and changes in volume transmission and in the diffusion parameters of the extracellular space. Electric activity and intercellular flow of ions and small molecules are regulated in part by gap junctions (GJs) (>5). These provide neuronal and glial coupling, and are therefore considered candidates for the pathogenesis of epilepsy. Morphological studies have long indicated the presence of GJs between glial cells as well as between neurons (8). GJs are composed of connexin protein subunits that form two hexameric connexon channels on adjacent cell surfaces (9,10). Connexins are encoded by a large multigene family which has more than 20 members in mammals (11). Certain members of this connexin family are cell-specific in their expression; connexin 36 is detected in neurons (12), while connexin 43 is present in astrocytes and in ependyma cells of the CNS (13). The connexon is assembled in the endoplasmic reticulum and the Golgi-apparatus and then inserted into the cell membrane to form an ‘unapposed’ connexon hemichannel (10,14). This subsequently forms an intercellular GJ with a hemichannel of an adjacent cell (10). The conduction properties of the connexin 43 hemichannels are influenced by the phosphorylation state of the connexin protein subunits; dephosphorylation causes the opening of the hemichannel (15) and decreases the formation of GJs (15,16), resulting in their closure. GJs allow the passive flow of molecules below 1 kDa (17); therefore, the regulation of connexons plays an important role in water-ion homeostasis and volume transmission in the brain in both physiological and pathological conditions (18). Astrocyte cultures and brain slices exposed to seizure-equivalent environments, such as high [K+] and, increased glutamate concentration, displayed delayed dephosphorylation of connexin 43 and decreased coupling of astrocytes (19). The reduction of intercellular coupling impairs the efficient trafficking of ions and metabolites and, as such, is likely to have an impact on glial cell volume in seizures. The long-lasting alteration of extracellular volume transmission [i.e., the significant decrease of the apparent diffusion coefficient (ADC) in several forebrain areas] coupled with the swelling of the astrocytes was measured with magnetic resonance imaging (MRI) combined with electron microscopy in rats during acute 4-aminopyridine (4-AP) convulsions (4). In vitro studies elucidated the mechanisms underlying the initial glial swelling in response to high glutamate concentrations.
Table I. Experimental groups and animal numbers.¹

<table>
<thead>
<tr>
<th>Methods</th>
<th>4-AP (5 mg/kg)</th>
<th>MK-801 (1 mg/kg) plus 4-AP (5 mg/kg)</th>
<th>Solvent (0.9% NaCl) control</th>
</tr>
</thead>
<tbody>
<tr>
<td>RT-PCR</td>
<td>9 (3/group)</td>
<td>9</td>
<td>3</td>
</tr>
<tr>
<td>Western blot</td>
<td>15 (5/group)</td>
<td>15</td>
<td>5</td>
</tr>
<tr>
<td>Electron microscopy</td>
<td>9 (3/group)</td>
<td>9</td>
<td>3</td>
</tr>
</tbody>
</table>

¹The evaluation of seizure symptoms was performed in a separate animal group (see Table II).

Table II. Behavioural analysis of the effect of NMDA antagonist on 4-AP seizures.

<table>
<thead>
<tr>
<th>Compounds</th>
<th>GTCS latency (min)</th>
<th>SEM</th>
<th>% animals displaying GTCS</th>
</tr>
</thead>
<tbody>
<tr>
<td>4-AP</td>
<td>30.3</td>
<td>1.4</td>
<td>100</td>
</tr>
<tr>
<td>4-AP plus MK-801</td>
<td>34.2</td>
<td>4.5</td>
<td>27.7*</td>
</tr>
</tbody>
</table>

Tests were conducted in groups of ten animals each. The antagonist was injected intraperitoneally. Ten minutes later, 4-AP was administered, and the latencies of the onset of GTCS were measured from the time of the 4-AP injection (significance, *p*<0.05; ANOVA followed by the post hoc Bonferroni test). GTCS, generalised tonic-clonic seizures; SEM, standard error of the mean.

extracellular glutamate (20) and [K+] (21). Elevated extracellular glutamate levels in seizures are long-lasting - 150 min or more (22) - but the decrease in ADC values lasts much longer - for at least 24 h (4). We therefore investigated the role of the neocortical astrocyte network and the possible role of astrogentic GluRs in pathological volume regulation in 4-AP seizures. Our present experiments targeted the molecular mechanisms of the regulation of connexin 43 in correlation with the measurable swelling of astrocytes in the neocortex of the convulsing rat. We investigated the role of N-methyl-D-aspartate (NMDA) glutamate receptors in connexin 43 regulation and edema formation.

Materials and methods

Animals and treatments. Male Wistar rats weighing 200-250 g were bred in the Central Animal House of the University of Szeged under standard conditions with ad libitum access to food and water. All experiments were conducted in accordance with the European Community Council Directive of November 24, 1986 (86/609/EEC) and the Hungarian Animal Act (1998). Rats were injected intraperitoneally with 4-AP (Sigma, 5 mg/kg 4-AP dissolved in physiological saline, 1.0 mg/ml concentration). The control animals received the same volume of physiological saline i.p. Dizocilpine maleate (MK-801, Sigma) dissolved in physiological saline was administered i.p. 10 min before 4-AP administration at a dose of 1 mg/kg (23). Animal seizure behaviour was evaluated as previously described by measuring the latency of symptoms (24). Animals were observed at 1, 3 and 24 h from the time of 4-AP injection, with 3 repeats in each group (Table I). The pharmacological effect of MK-801 was evaluated separately (10-10 animals in each group; Table II).

Nucleic acid measurements. Animals were anesthetised with diethyl-ether, decapitated, their brains removed, and the neocortex of the right hemisphere frozen in liquid nitrogen. For reverse-transcriptase polymerase chain reaction (RT-PCR), the acid guanidinium-phenol-chloroform method (25) was used to extract total-RNA, 2 μg of which was used for reverse transcription (RT) (26). PCR was carried out with 1 μl of 20 μl RT cDNA product along with components, as previously described (27). The sequence of connexin 43 and glycine-aldhyde phosphate dehydrogenase (GAPDH) primers has been previously described (28,29).

After the linearity of connexin 43 and GAPDH was carefully established, PCR was carried out at 17, 22 and 27 cycles. PCR products were separated on 6% acrylamide gel and stained with ethidium bromide (Sigma). The gels were scanned and the bands evaluated by densitometry using ImageQuant (Typhoon™ 9400 Variable Mode Imager, Molecular Dynamics). The densitometric values of connexin 43 mRNA bands were normalised on GAPDH mRNA detected and amplified from the same samples.

Protein measurements. Neocortex samples were obtained as described above and frozen in liquid nitrogen. Regarding the solubility of the different phosphorylated forms of connexin 43 (9), no detergents were used during protein purification. The second portion of the samples was homogenised in ice cold buffer (pH 7.2) containing 20 mM HEPES, 0.5 mM EDTA and the following protease inhibitors: 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 μM pepstatin A and 2 μM leupeptin (28). To preserve the phosphorylated forms, 10 mM of the phosphatase inhibitor Na2VO4 was added to the lysis buffer in specified samples. Homogenates were centrifuged at 20,000 g for 30 min at 4°C, then the pellet was resuspended in homogenisation buffer. Alkaline phosphatase treatment was carried
out with some modifications (30). With Na3VO4 excluded, 70 μg of pre-cleaned lysate was equilibrated against 100 mM Tris (pH 8.0), 100 mM NaCl, 5 mM MgCl₂, 2 mM PMSF and 0.6% SDS. Half the sample was treated with 2 U/sample alkaline phosphatase at 37°C for 4 h, and the other half was incubated untreated. The reaction was terminated by the addition of 200 mM Na3VO4. Samples were re-dissolved in alkaline phosphatase at 37°C and 0.6% SDS. Half the sample was treated with 2 U/sample μl70 out with some modifications (30). With Na3VO4 excluded, expressed as arbitrary units (means ± STDEV, n=3).

Figure 1. Expression of connexin 43 mRNA following acute 4-aminopyridine (4-AP) seizures. (A) Representative gels exhibiting connexin 43 (Cx43) and the internal control glyceraldehyde phosphate dehydrogenase (GAPDH) at 1 and 3 h following seizure induction. The band indicates 300 bp fragment length. (B) Summarised data. The relative density of the Cx43 bands is expressed as arbitrary units (means ± STDEV, n=3).

Statistical analysis. Data are presented as the means ± STDEV. Statistical analysis of the relative band densities and phosphorylation rates between the indicated groups were performed using the Student's t-test and the Newman-Keuls test. One-way analysis of variance (ANOVA) followed by the Bonferroni post hoc test was used when comparing astrocyte end-feet areas. SPSS 9.0 statistical software was used for analysis and p<0.05 was considered statistically significant.

Results

Behavioural studies. The behavioural effects of the treatments on separate groups of animals were observed (Table I). The i.p. administration of 4-AP caused characteristic behavioural symptoms within 15 min: tremor of the vibrissal and masticatory muscles followed by a generalised tremor of the body musculature (detectable as a continuous fasciculation of the muscles), and then generalised tonic-clonic seizures (GTCS). The main behavioural symptoms disappeared after 90-120 min at most; the animals then displayed mild tremor or brief myoclonic episodes. By the end of the experiment, all the animals had recovered completely. Pre-treatment with MK-801 prevented the development of GTCS in 72.3% of the animals (Table II). The symptoms preceding GTCS (tremor of the vibrissae, tremor of the masticatory muscles, and generalised tremor) were not detected in animals pre-treated with MK-801. In these rats, instead of tremor, the first symptoms were hypotonia of the limbs and unsteady gait. Antagonist administered on its own did not induce any obvious behavioural changes.

Connexin 43 mRNA expression. The levels of GAPDH and connexin 43 mRNA were quantified in neocortex samples collected at 1, 3 and 24 h following 4-AP and the control physiological saline injections (not shown). In the controls as well as in the 4-AP treated animals, the GAPDH messages were not statistically different. Similarly, at no time were density changes observed in the normalised connexin 43 mRNA levels when the 4-AP treated group and the control group were compared (Fig. 1).

Connexin 43 protein phosphorylation. Previous studies have shown that the phosphorylated forms of connexin 43 migrate as separate bands on the gel during electrophoresis (9). These two phosphorylated forms, P1 and P2, migrated at 44 kDa...
and 46 kDa, respectively (Fig. 2A). Non-phosphorylated protein (NP) was detected at 42 kDa (Fig. 2A). To confirm the phosphorylation status of the identified forms, parallel samples were treated with alkaline phosphatase. This resulted in the complete disappearance of the P1 and P2 bands, with a single band left representing the NP form (Fig. 2A). The complete insolubility of the P2 form and the partial insolubility of P1 in Triton X-100 have been reported (9) and were confirmed by our preliminary experiments (data not shown). Following the method of Christ et al. (28), we successfully extracted both the phosphorylated and non-phosphorylated forms of connexin 43 without the use of detergents, achieving optimal protein yield (Fig. 2A). Some of the blots displayed P1 and P2 as a single fused band representing the overall quantity of phosphorylated connexin 43, while in other blots the P1 and P2 forms were visible as separate bands. Because of this slight discrepancy in migration properties, we were not able to analyse the P1 and P2 forms separately. Therefore, during densitometric analysis, P1 and P2 band densities were added together, and their sum was normalised to the density level of the NP band to determine the ratio of phosphorylated/non-phosphorylated connexin 43 molecules. The overall quantity of connexin 43 protein, expressed by the added density of all bands (P1+P2+NP), showed no change in 4-AP treated tissue compared to the control neocortex (data not shown). The phosphorylation status of connexin 43 was unchanged at 1 h (Fig. 2B), but at 3 h (Fig. 2C) following 4-AP treatment there was an ~50% decrease (p=0.0017) in the ratio of phosphorylated versus non-phosphorylated connexin 43 forms in the epileptic animals. Surprisingly, this dephosphorylated status persisted until 24 h post seizure as a significant ~10% reduction.

Figure 2. Expression of connexin 43 protein in the cerebral cortex following brief acute seizures. (A) Representative blots demonstrating the phosphorylation-dephosphorylation of connexin 43 isoforms. The P2 (46 kDa), P1 (44 kDa) and non-phosphorylated (NP, 42 kDa) forms were detected in the control lysate (left), whereas only the NP form was present (right) following alkaline phosphatase treatment (Alk.). (B-D) Original blots demonstrating expression of the phosphorylated and non-phosphorylated forms of connexin 43 at 1 (B), 3 (C) and 24 h (D) following seizure induction. Dephosphorylation is visible at 3 and 24 h in convulsing animals (4-AP) compared to controls. (E) Summarised data of the changes in the phosphorylation of connexin 43 following seizures. Note the decrease in phosphorylation rate at 3 h, which remains significant at 24 h. 4-AP, convulsing animals; control, animals injected with saline; means ± STDEV, n=5, p<0.01 at 3 h; p<0.01 at 24 h.

Figure 3. Connexin 43 dephosphorylation (A and B) and seizure-associated glial swelling (C and D) in the cerebral cortex can be reversed by MK-801 pre-treatment. (A) Representative blots showing the strong presence of phosphorylated connexin 43 after MK-801 pre-treatment 3 h following seizure induction. (B) Summarised data from panel A blots (n=5; p<0.01). (C and D) Representative electron micrographs demonstrating perivascular glial swelling at 3 h post seizure induction (C), and reduced by MK-801 pre-treatment (D). A, astrocyte end-feet; L, capillary lumen; asterisk, red blood cell. (E) Graph summarising percent changes in astrocytic end-feet area at 1, 3 and 24 h post 4-AP injection. Note the prolonged swelling, significant at 1, 3 and 24 h, and the protective effect of MK-801 (average ± STDEV, n=27-33; p<0.001).
in phosphorylation (Fig 2D). To clarify the relation between NMDA receptor activation in 4-AP seizures and the subsequent phosphorylation changes of connexin 43, the NMDA antagonist MK-801 was applied prior to seizure induction. This pre-treatment resulted in a decrease in connexin 43 dephosphorylation at 3 h (Fig. 3A and B), suggesting that the long-lasting seizure effect was the result of an NMDA-dependent mechanism.

Astrocyte end-feet area. In our next experiment, the area of perivascular astrocyte processes following 4-AP seizures with and without MK-801 pre-treatment was measured (Fig. 3C and D). Baseline values of the astrocytic end-feet area in untreated animals were considered to be 100% for the purposes of our analysis. A 464% ± 85 increase in the size of the end-feet compared to baseline was seen at 1 h following seizure induction, followed by a 471% ± 104 and 392% ± 71 increase in size at 3 and 24h, respectively (n=29-34, p<0.001). Swelling was abolished by the application of MK-801 with 310% ± 59, 292% ± 59 and 129% ± 45 end-feet areas in the 1, 3, and 24 h groups, respectively (n=28-32, p<0.001) (Fig. 3E).

Discussion

The present study is the first to demonstrate the post-translational changes of the GJ subunit connexin 43 in vivo in epileptic animals. Our results strongly indicate that, in acute GTCS, connexin 43 is not regulated at the transcriptional level. The expression of connexin mRNA in our neocortical samples was very stable in every animal group. On the other hand, very important phosphorylation changes were observed that were dependent on regulation processes mediated by NMDA glutamate receptors. Several lines of evidence suggest a regulatory role for connexin 43 in adjusting extracellular K+ levels. A likely mechanism is that open GJ contribute low-resistance intercellular coupling between neighbouring cells (33), allowing the passive movement of ions and molecules accumulated during GTCS, such as K+ (32,34) or glutamate (35), via this cellular network. This has been referred to as ‘spatial buffering’ (18).

A decreased phosphorylation rate in the GJ subunit connexin 43 was observed in response to seizures, which corresponds to previous findings in astrocyte cultures. The dephosphorylation of connexin 43 has been observed in response to various pathological stimuli in vitro (19,36,37), for example when reconstituting a seizure-like environment with excess glutamate (1 mM) and high [K+]o (15 mM) in brain slices (19). This dephosphorylation was demonstrated to correspond with a reduction in intercellular coupling (16,38). Similar responses were observed using in vitro models of ischemia and metabolic inhibition (37,39).

Our experiments also indicate that MK-801 attenuates the symptoms of seizures. Pre-treatment prevented the onset of GTCS in more than 70% of the animals. MK-801 is a high-affinity open-channel blocker that blocks the ion channel of the receptor at the NMDA receptor, inhibiting or decreasing the ion fluxes which follow glutamate binding (40). MK-801 administration i.p. and i.c.v. was found to protect against 4-AP seizures in electrophysiological experiments (41). The present experiments provide further data on the important role of glutamate and NMDA receptors in seizure pathology, and prove that the blockade of NMDA receptor is beneficial and decreases glial swelling significantly. These results also increase the possibility of therapeutic applications of NMDA-receptor antagonists for seizure-related brain edema.

The role of the phosphorylation status of connexin 43 in the regulation of GJ permeability has been studied extensively. The balance between the two states is set by the equilibrium between the phosphatase PP-2B/calcineurin (38) and the kinase c-Src (16). Both enzymes are regulated by intracellular Ca2+ levels. As NMDA receptors permit the influx of Ca2+, it is possible that the activation of this receptor is related to connexin 43 dephosphorylation through the activation of phosphatase PP-2B/calcineurin. Cytoplasmic Ca2+ may, in response to mGluR stimulation of acute hippocampal slices, be increased by other mechanisms, such as the release from internal stores reported by Potter and McCarthy (42). It may enter the cell via voltage-sensitive channels (43) and metabotropic glutamate receptors during A-4P seizures as assessed by in vivo two-photon microscopy (44). NMDA receptors are known to be expressed in neurons (45), while astrocytes react to glutamate by metabotropic receptors and express NMDA subunits only under pathological conditions such as ischemia or anoxia (46). In contrast, recent studies have clearly demonstrated the presence of functional NMDA subunits in cortical astrocytes in the normal brain (47,48).

MK-801 preserved connexin 43 phosphorylation in glutamate-treated astrocyte cultures (19). Our results show a similar effect in vivo; the blockage of NMDA glutamate receptors by MK-801 inhibited connexin 43 dephosphorylation in astrocytes and, at the same time, significantly reduced cellular swelling. We therefore believe that MK-801 acted not only on neuronal, but also on astrocytic NMDA receptors in our experiments. Consequently, NMDA receptor activation could potentially regulate gap junction coupling in astrocytes by modulating intracellular Ca2+ levels.

Our electron microscopy measurements demonstrate prolonged perivascular astrocytic swelling, which was simultaneous to the impairment of GJ coupling (connexin 43 dephosphorylation). This implies that the latter contributes to seizure-related glial edema. Astrocyte swelling appeared earlier than connexin dephosphorylation, but after 3 h its decrease followed the decrease of dephosphorylation. On the other hand, MK-801 decreased swelling at every time point, implying that it was largely caused by excessive glutamate release. This long-lasting (150 min from the injection of 4-AP) significant elevation of extracellular glutamate has been proven by our previous experiments in 4-AP seizures (22). Therefore, we believe that glial swelling in GTCS has two phases: Phase 1 depends on elevated extracellular glutamate and causes swelling through the entry of Na+ and Ca2+ into the cell (42), and Phase 2 (post-3 h) is mediated by the opening of the hemichannels through the dephosphorylation process and the uncoupling of the astrocytes (15,39). This second phase might
be responsible for the decrease in ADC values at 24 h (4). GJ proteins may exist as hemichannels, and have been shown to respond to dephosphorylation with increased permeability. As such, they provide a new route for solute trafficking between the extra- and intracellular compartments (39). As the density of hemichannels on the cell surface is not known, we cannot estimate their contribution to astrocyte swelling in seizures. Our results suggest that, in addition to other known factors (e.g., aquaporins) (49) GJ and glutamate-dependent mechanisms contribute significantly to the phases of seizure-related glial cell swelling.

In the present study, we demonstrated the dephosphorylation of the GJ protein connexin 43 following seizures. Our results indicate that the swelling of astrocytes and connexin dephosphorylation are related phenomena. Based on previous in vitro data, we propose that connexin 43 dephosphorylation results in the closure of intercellular GJs, which in turn isolates individual astrocytes. This mechanism may inhibit spatial buffering and contributes to long-lasting astrocyte swelling in seizure. The inhibition of astrocytic swelling and the simultaneous prevention of connexin dephosphorylation by MK-801 indicates an NMDA receptor-dependent regulatory mechanism at work in the rat neocortex. These observations may open up a new therapeutic avenue for re-establishing the spatial buffering of the astrocyte network and for helping in the recovery of brain tissue following epileptic events.

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References