Regulation of Gap Junctional Communication by Astrocytic Mitochondrial K_{ATP} Channels following Neurotoxin Administration in in vitro and in vivo Models

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Key Words

Astrocyte • Neurotoxin • Epilepsy • Gap junction
intercellular communication • Mitochondrial K_{ATP} channel •
Picrotoxin • Spatial buffering

Abstract

It is known that neuronal ATP-sensitive potassium (K_{ATP}) channels and astrocytic gap junctions (GJs) are involved in the mechanism underlying neurodisorders. The K_{ATP} channels exist also in glial cells, and the objective of this study was to determine whether the astrocytic K_{ATP} channels exert their effect on neurotoxin-induced neurodysfunction through regulating the astrocytic GJ function. The results showed that diazoxide, a selective mitochondrial K_{ATP} (mitoK_{ATP}) channel opener, enhanced the GJ coupling, but 5-hydroxydecanoate, a selective mitoK_{ATP} channel blocker that significantly inhibits GJ coupling in vitro did not. Activation of astrocytic mitoK_{ATP} channels alleviated kainic acid-induced dysfunction of GJ intercellular communication. Finally, activation of mitoK_{ATP} channels improved the astrocytic GJ coupling in the hippocampus after seizures due to the colabeling of GJ subunit connexin 43 and connexin 45 with glial marker and was increased substantially by the adminis-

tration of diazoxide. Western blot demonstrated that the mitoK_{ATP} channels regulated the expression of connexin 43 (P2; active form) and connexin 45 in the epileptic hippocampus. These findings demonstrate that activation of astrocytic mitoK_{ATP} channels improves the GJ function in astrocytes, indicating that the effect of the astrocytic mitoK_{ATP} channels on neurotoxin-induced neurodysfunction might be, in part, through the regulation of the GJ-coupled spatial buffering in the hippocampus.

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Introduction

The ATP-sensitive potassium (K_{ATP}) channels, which couple cell metabolism to electrical activity, participate in some important biological processes including neuronal transmission [1, 2]. K_{ATP} channels are located in various parts of the cell, including the surface of the plasmalemmal membrane (sK_{ATP}) and the inner mitochondrial membrane (mitoK_{ATP}) [3–6]. Manipulation of the K_{ATP} channel status in various cellular types and at various intracellular locations has shown a variety of functional roles [3, 7–10]. It has been revealed that neuronal K_{ATP}

channels in the brain are involved in the control of neuronal excitability [11–13] and seizure propagation [1, 14–19]. The roles that glial K_{ATP} channels play are receiving renewed attention due to improved understanding of the molecular events underpinning the physiological functions of glial cells. Recent studies have shown that opening the astrocytic mito K_{ATP} channels with drugs or by physiological stimuli can exert effects on glutamate uptake and astrocytic activation [20–23]. However, the role of astrocytic mito K_{ATP} channels in the brain, in particular their contribution to pathogenesis of neurological disorders such as epileptogenesis, is largely unknown.

The more established function of astrocytes in the brain is in regulating the extracellular milieu. If K^+ regulation fails, pathological events such as epileptiform activity may be initiated [24–26]. One of the key mechanisms proposed to underlie astrocytic K^+ regulation is the spatial redistribution of K^+ termed spatial buffering [27]. Generally, the extensive linkage of astrocytes via the gap junctions (GJs) has been assumed to be essential for spatial buffering [28]. Astrocytic GJ-regulated K^+ buffering is involved in the control of epileptiform activity [24, 29, 30]; however, a large capacity for K^+ clearance was conserved in mice with coupling-deficient astrocytes, indicating that GJ-dependent processes only partially account for K^+ buffering [24]. Astrocytic GJ-regulated K^+ buffering is also believed to remove K^+ by spatial buffering, possibly through inwardly rectifying potassium channels that enable uptake and redistribution of extracellular K^+ [31]. Therefore, we asked whether there is co-operation between the GJs and the astrocytic inwardly rectifying potassium channels, in particular the astrocytic mito K_{ATP} channels, and whether manipulation of the astrocytic mito K_{ATP} channels has an effect on neurotoxin-induced neurodysfunction via regulating spatial buffering.

In the study described here, we examined the effects of mito K_{ATP} channels on gap junctional functions in cultured astrocytes as well as in the hippocampus in a chronic picrotoxin (PTX) kindling epileptic model to determine: (a) whether activation of astrocytic mito K_{ATP} channels regulates gap junctional dye coupling; (b) whether mito K_{ATP} channel openers regulate astrocytic mito K_{ATP} channels against neurotoxin-induced dysfunction of astrocytic GJ intercellular communication (GJIC), and (c) the effect of astrocytic mito K_{ATP} channel-modulated astrocytic GJ coupling on PTX-induced neurodysfunction in vivo.

Materials and Methods

Cell Cultures

Astrocytes and primary neuronal cultures of rat brain were prepared as described previously [32, 33] (for details of methods, see online supplement, www.karger.com/doi/10.1159/000323575). For the experiments of scrape-loading and fluorescence recovery after photobleaching (FRAP), the astrocytic suspension ($\sim 1 \times 10^5$ cells/ml) was cultured on glass coverslips (32 mm in diameter) coated with poly(L-lysine) in a CO_2 incubator.

Intracellular Lucifer Yellow Loading

GJIC was determined by the scrape-loading dye transfer technique [33]. Cultures were incubated for 10 min in Hepes buffer (in mM: 140 NaCl, 5.5 KCl, 1.8 $CaCl_2$, 1 $MgCl_2$, 10 glucose, and 10 Hepes, pH 7.3), and post-washed with Ca^{2+} -free Hepes solution. Loading was achieved by scraping the cultures with a razor blade in the Ca^{2+} -free Hepes buffer containing 0.1% (w/v) lucifer yellow (LY; dilithium salt; Sigma). After incubation ($37^\circ C$) for 8 min, dye transfer between coupled cells was assessed by taking five successive photomicrographs/trial with an inverted microscope equipped with appropriate filters. The number of LY-loaded cell layers reflects the function of GJIC.

Determination of GJIC and Live Imaging

Cultures were incubated ($37^\circ C$) with 15 $\mu g/ml$ 6-carboxyfluorescein diacetate (Sigma) for 20 min and then with D-Hanks' solution, free of 6-carboxyfluorescein diacetate for another 20 min ($37^\circ C$) to allow complete de-esterification. A confocal scanning microscope (Zeiss 510) was used for imaging the 6-carboxyfluorescein diacetate signal. Excitation was provided by the 488-nm line of a krypton/argon laser, and emission was collected at its maximum of 530 nm. After a baseline fluorescence image of the cultures was obtained, the regions of interest (ROIs) were selected to include 4 or 5 cells for bleaching and 5 cells for controlling/dish. ROIs were first scanned with an argon 488 laser at 5–10% power for 5 cycles to determine a measurement of initial fluorescence intensity in the ROI. This value was taken as 100%. Next, ROIs were subjected to photobleaching with an argon 488 laser at full power. The fluorescence intensity of the ROI was then measured at 1, 2, 5 and 8 min after bleaching at 5–10% laser power to measure fluorescence recovery relative to the initial settings in the ROI. All values of fluorescence intensity in the ROIs were obtained by subtracting the background fluorescence from an identical membrane area that did not have detectable fluorescence. The fluorescence recovery percentage at each time point was calculated as:

$$100 [(I_t - I_0)/(I_c - I_0)]$$

where I_t is fluorescence intensity in the ROI at the given time point, I_0 is the intensity of fluorescence in ROI after photobleaching, and I_c is the average value of 5 measurements of fluorescence intensity in the ROI before photobleaching.

Chemical Preparations and Administration

Diazoxide (Dia; 0.1 mM), 5-hydroxydecanoate (5-HD; 1 mM), 18 α -glycyrrhetic acid (AGA; 0.01 mM) and kainic acid (KA; 0.05 mM; all from Sigma) were dissolved in DMSO and then diluted to the required concentrations with the culture medium. For scrape-loading and FRAP cells, we applied the chemicals 1 h before start-

ing the tests. For the Western blot, we treated the cultures with chemicals for 5 days before the protein extraction. For 6-day-old primary neuronal cultures, the neurons were treated with chemicals for 1 h, post-washed and incubated (37°C) with the normal culture medium for another 1 h, and the culture medium (without cells or chemicals) was collected and used to incubate (37°C) the astrocytic cultures for 1 h before starting the FRAP tests.

Animals and Long-Term PTX Kindling Protocols

Male Sprague-Dawley rats (initial body weight 220–250 g) were housed under standard conditions. Animals were cared for and handled in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals (NIH publications).

PTX kindling was done as described [15, 34]. Briefly, rats ($n = 110$) received a daily injection (i.p.) of PTX (2.25 mg/kg; 1.5 mg/ml suspended in saline; Sigma) for 20 days, and control rats ($n = 10$: 6 for Western blot and 4 for immunocytochemical staining) received a daily injection of vehicle for the same period. Rats were observed for 45 min after each administration, and seizures were recorded according to the scale devised by Racine [35]. After stage 4 or 5 seizures on 3 consecutive days, rats were visually video-monitored and monitored by electroencephalogram (EEG) after treatment with PTX. Stage 4 and 5 animals with simultaneous EEG showing epileptiform discharges ($n = 37$) were used in subsequent experiments [36]. The kindled rats were divided into four groups at random ($n = 5$ –6 for Western blot and $n = 3$ –4 for immunocytochemical staining): (1) PTX, (2) PTX rekindling, (3) Dia and (4) 5-HD. These rats were video-monitored (40 h/week) for spontaneous motor seizures for 2 weeks after the 20-day kindling. At 14 days after the last drug treatment of the 20-day protocol, rats in the PTX rekindling, Dia and 5-HD groups were again treated with PTX (2.25 mg/kg), and the sham and PTX groups received the same volume of vehicle alone. Preconditioning with Dia (1.9 mg/kg, 1 mg/ml in saline), a mitoK_{ATP} channel opener [37], and 5-HD (1 mg/kg, 1 mg/ml in saline), a mitoK_{ATP} channel blocker, was done by injection (i.p.) once a day for 3 days, and the last injection was done 1 h before the PTX retreatment. Rats were video-monitored for 45 min after each administration, and seizures were recorded according to the scale devised by Racine [35]. Each animal was EEG monitored in a 4-hour recording period for evaluating the seizure frequency, then killed by decapitation 7 days after the last treatment.

Preparation of Cytosolic Extracts, Crude Synaptic Membrane Fraction and Western Blot Analysis

The whole-cell protein extraction from the cultured astrocytes was done with CellLytic M solution (Sigma) according to the manufacturer's instructions.

We used the method of Danbolt et al. [38] for preparation of cytosolic extracts and crude synaptic membrane fractions from the hippocampus (see online suppl. methods for details). Crude synaptic membrane fractions and cytosolic extracts were frozen and stored at -80°C . Protein determination was done with a modified Bradford assay (Sigma). Samples were normalized to equal concentrations of protein. For Western blot analysis, total protein (50 μg) was separated by electrophoresis in a 12% (w/v) polyacrylamide gel. The blots were incubated overnight at 4°C first with primary antibody connexin 43 (Cx43, 1:2,000; Chemicon), Cx45 (1:2,000; Chemicon), Cx36 (1:1,000; Chemicon) and Cx30

(1:1,000; Chemicon), then with horseradish peroxidase-conjugated IgG (Sigma), and last with an ECL kit. The optical density of the films was quantified by the free software (Image J 1.4) and calculated as a value of integrated densities. Data were converted to relative optical density as percentages with β -actin (1:5,000, Sigma) designated as 100%.

Immunocytochemical Staining and Semiquantification

Every eighth section between bregma -3.30 mm and -5.80 mm was selected for detecting the expression of Cx43 and Cx45 in the hippocampus ($n = 3$ –4; online suppl. fig. S1A; see online suppl. methods for brain section preparation).

Sections were immunostained by blocking, then incubated overnight (4°C) with primary antibodies followed by secondary antibodies conjugated to fluorescent labels. The combination of primary and secondary antibodies is shown in online supplement table 1. All fluorescent conjugated secondary antibodies were purchased from Jackson ImmunoResearch (West Grove, Pa., USA). Primary antibodies were: Cx43 and Cx45 (1:200; all from Abcam, Inc.); glial fibrillary acidic protein (GFAP; 1:500; Harlan), a marker for mature astrocytes; NG2 (1:300; Chemicon), a marker for oligodendrocyte precursor cells; Map2 (1:500; Chemicon), a marker for mature neuron; NeuN (1:500; Chemicon), a marker for mature neuron, and doublecortin (DCX, 1:200; Santa Cruz), a marker for immature neuron.

Immunoactivity was observed and recorded under a confocal microscope (Zeiss 510) by using a multichannel configuration with the same scan setting used for each section. Five views in CA1, CA2, CA3 and dentate gyrus (DG) per section were quantified by using ImageJ 1.41 with the Measure RGB plugin. We used the Co-localise RGB plugin for Cx43/GFAP and Cx45/GFAP, or Cx43/Map2 and Cx45/Map2 colabeling (online suppl. fig. S1B). The density of colabeling from each view was normalized to the total density of Cx43 and Cx45.

Data Analysis

All data are shown as mean $\pm$ standard error of the mean (SEM). The experimental data were analyzed statistically by one-way analysis of variance and post-hoc multiple comparisons with Fisher's least significant difference test. $p < 0.05$ was chosen as the level of statistical significance.

Results

Activation of Astrocytic mitoK_{ATP} Channels Facilitated GJIC

LY diffused into a large population of astrocytes extending over several rows in control astrocytes, but in cultures with AGA, a GJ blocker, LY was confined to the loaded astrocytes along most of the scrape lines (fig. 1). Interestingly, Dia, a selective mitoK_{ATP} channel opener, enhanced the GJIC significantly, and we found the layers of LY-loaded cells were more than twice as many as in the controls and four to five times as many as in the AGA-treated cultures ($p < 0.05$ in each case). However, 5-HD, a mitoK_{ATP} channel blocker, substantially inhibited the

Fig. 1. Astrocytic $\text{mitoK}_{\text{ATP}}$ channels regulate astrocytic GJIC. **a** GJIC was measured by scrape-loading on day 2 (DIV9) and day 5 (DIV12) after the subcultures. Each group included 3–4 dishes and took five successive photomicrographs per trial. Values are mean $\pm$ SEM; * $p < 0.05$. **b** Representative picture of LY-loaded astrocytes on day 2 treated with Dia, a $\text{mitoK}_{\text{ATP}}$ channel opener (**b**₁) and with 5-HD (**b**₂), a $\text{mitoK}_{\text{ATP}}$ channel blocker (bar = 20 μm).

GJIC. When treated with AGA or 5-HD, the robust effects exerted by Dia were not totally blocked, indicating that activation of astrocytic $\text{mitoK}_{\text{ATP}}$ channels facilitated GJIC.

Activation of Astrocytic $\text{mitoK}_{\text{ATP}}$ Channels Attenuated GJ Blocker-Induced Inhibition of Recovery of Fluorescence

Control astrocytes exhibited a progressive recovery of fluorescence after bleaching, but with AGA the recovery of fluorescence was severely blocked (fig. 2a, b). Although activation of astrocytic $\text{mitoK}_{\text{ATP}}$ channels with Dia did not show an elevated recovery of fluorescence compared to the controls at each time point, the levels of comparative fluorescence intensity recovery (CFIRP) were improved substantially by Dia at 5 min in Dia + AGA-treated

ed cultures compared to those in AGA-treated astrocytes ($p < 0.001$). In contrast, blocking of astrocytic $\text{mitoK}_{\text{ATP}}$ channels with 5-HD showed that CFIRP was inhibited severely at each time point, and it was unable to achieve the control level at 8 min (CFIRP 67.7%). Unexpectedly, 5HD + AGA gave control levels of recovery at 8 min.

Opening of Astrocytic $\text{mitoK}_{\text{ATP}}$ Channels Alleviated KA-Induced Inhibition of GJIC

Treatment with KA decreased the CFIRP significantly at 1, 2 and 5 min after bleaching compared to the controls, but Dia alleviated this KA-induced inhibition of GJIC. In contrast, 5-HD and AGA severely blocked the recovery of GJIC, but in the Dia + AGA + KA cultures, the CFIRP was enhanced substantially at each time point compared to that with AGA + KA (fig. 2c, d).

Activation of Astrocytic $\text{mitoK}_{\text{ATP}}$ Channels Reversed KA-Induced Downregulation of Cx43 and Cx30 Protein Expressions in vitro

Western blot analysis showed that KA decreased the expressions of Cx43 (P2) (active form). Dia could reverse the KA-induced downregulation, but the level of P2 form was downregulated substantially in the 5-HD + KA cultures. In addition to changes of the levels of the P2 form, there were changes in the overall levels of Cx43. The differences of total Cx43 expression among groups were similar to the patterns of the P2 form except for Dia + KA versus KA ($p > 0.05$). For Cx30, we saw the same effects regulated by the astrocytic $\text{mitoK}_{\text{ATP}}$ channels, Dia reversed the KA-induced downregulation, while 5-HD further downregulated its expression (fig. 3).

Manipulation of $\text{mitoK}_{\text{ATP}}$ Channels Regulated the Cx Protein Expression in the Hippocampus of PTX-Kindled Rats

The Western blot results showed that in the PTX group during the seizure-free interval, the expression of Cx43 (P2), Cx45 and Cx30 was downregulated significantly compared to the controls. In the PTX-rekindling rats, only Cx43 was substantially upregulated compared to the PTX group. Pretreatment with Dia enhanced the expression of Cx43, Cx45 and Cx30 compared to the PTX group without rekindling, and pretreatment with 5-HD decreased their expression compared to those in the Dia rekindling group. Manipulation of the $\text{mitoK}_{\text{ATP}}$ channels had no significant effect on Cx36 expression after PTX rekindling. In addition, we found a downregulation in the levels of the P0 and P1 form of Cx43 after PTX rekindling with or without pretreatment with Dia/5-HD (fig. 4).

Fig. 2. Astrocytic mitoK_{ATP} channels regulate the astrocytic GJIC. **a** Representative pictures of FRAP. Scale bar, 20 μ m. **b** The effects of astrocytic mitoK_{ATP} channels on GJIC by FRAP. **c** Astrocytic mitoK_{ATP} channels alleviated KA-induced inhibition of astrocytic GJIC. **d** Comparison of GJIC between with and without KA treatment. Values are mean; * $p < 0.05$.

The results of immunofluorescent staining support our Western blot analysis results showing that the expression of Cx43 was downregulated significantly in the PTX group in the whole hippocampus compared to the controls. After PTX rekindling, Cx43 expression was upregulated but still below the level of the controls. Pretreatment with Dia enhanced Cx43 expression significantly only in the CA3 region. In contrast, pretreatment with 5-HD decreased Cx43 expression in the entire hippocampus (fig. 5a and online suppl. fig. S3A–C).

The expression of Cx45 was downregulated significantly in the hippocampus in the PTX group compared to the controls but not the CA3 region, where the level was higher than that in the controls. After PTX rekin-

dling, Cx45 expression was upregulated slightly in the DG and CA1 regions, and increased significantly in the CA2 and CA3 regions. Pretreatment with Dia enhanced its expression significantly in the whole hippocampus, but pretreatment with 5-HD decreased its expression, except in the CA3 region after seizures (fig. 5b and online suppl. fig. S3A–C).

Activation of mitoK_{ATP} Channels Increased Cx Expression in Glial Cells in the Hippocampus of PTX-Kindled Rats

In the PTX group, the levels of Cx43/GFAP or Cx45/GFAP colabeling were not significantly different from those of controls. After PTX rekindling, the level of cola-

Fig. 3. Astrocytic mitoK_{ATP} channels regulated the Cx43 and Cx30 protein expressions after KA treatment in vitro. **a** Representative pictures of Western blots from culture protein extracts. **b** Relative optical density (ROD) of semiquantitative densitometry data from culture protein extracts probed with antibodies against Cx43 and Cx30. Values are mean $\pm$ SEM; * $p < 0.05$.

Fig. 4. mitoK_{ATP} channels regulated the Cx protein expressions in the hippocampus of PTX-kindled rats. **a** Representative pictures of Western blots from hippocampal protein extracts. **b** ROD of semiquantitative densitometry data from hippocampal protein extracts probed with antibodies against Cx43, Cx45, Cx36 and Cx30. Values are mean $\pm$ SEM; * $p < 0.05$; $n = 5-6$.

Fig. 5. mitoK_{ATP} channels regulated the Cx43 (a) and Cx45 (b) protein expressions in the hippocampus of PTX-kindled rats by immunocytochemical staining. **c** mitoK_{ATP} channels regulated the colabeling of Cx43 or Cx45 with GFAP/Map2 in the hippocampus of PTX-kindled rats. Values are mean $\pm$ SEM; * $p < 0.05$; $n = 3-4$.

belonging did not change markedly. Activation of mitoK_{ATP} channels with Dia did increase the levels of colabeling across the entire hippocampus, except Cx45/GFAP in the CA2 region, compared to that in the PTX rekindling rats. In contrast, blocking of mitoK_{ATP} channels with 5-HD did not decrease the levels of colabeling significantly (fig. 5c, 6, 7). Interestingly, we found that Cx43 was highly expressed cytoplasmically in the hippocampus (online suppl. fig. S4–S6). By analyzing the phenotypes of these cells after seizures, we found that Dia robustly increased the total numbers of Cx43/NG2 colocalizing cells in the whole hippocampus (online suppl. fig. S6), but decreased the numbers of Cx43/Map2 colocalizing cells in the CA2 region substantially.

By contrast, the level of Cx45/Map2 colabeling was not changed significantly in the PTX group except in the CA3 region, compared to that of the controls. After PTX rekindling, this colabeling was not altered significantly. Dia increased the levels in some areas compared to that of PTX rekindling, but significant differences

were detected only in the DG region. However, 5-HD substantially decreased the levels in the DG and CA3 regions in comparison to Dia (fig. 5c and online suppl. fig. S4).

Discussion

Main Findings

Here, we report new findings regarding how the astrocytic mitoK_{ATP} channels regulate GJIC in the hippocampus, which might contribute to epileptogenesis in the rat. While activation of astrocytic mitoK_{ATP} channels enhanced the GJ coupling, blocking of astrocytic mitoK_{ATP} channels decreased the astrocytic GJ coupling. Opening astrocytic mitoK_{ATP} channels alleviated KA-induced dysfunction of GJIC. Finally, activation of mitoK_{ATP} channels improved the GJ coupling in the hippocampus in chronic epileptic rats, as we found that the mitoK_{ATP} channel opener substantially increased the levels of Cx43/

Fig. 6. a–q Representative pictures of the Cx43/GFAP or Cx45/GFAP colabeling in DG of PTX-kindled rats regulated by mitoK_{ATP} channels. Scale bar = 20 μm.

GFAP or Cx45/GFAP colabeling in the hippocampus after seizures. We suggest that the mechanism whereby astrocytic mitoK_{ATP} channels contribute to the pathogenesis of neurodisorders, in particular epileptogenesis, might be partly through the regulation the GJ-coupled spatial buffering in the hippocampus, in addition to the neuronal K_{ATP} channel-modulated excitability.

Astrocytic K_{ATP} Channels and GJ Coupling

The role of astrocytes in the brain has come under intense scrutiny in recent years. Astrocytes have been recognized to actively modulate neuronal communication [39], in addition to their more established functions in

regulating the extracellular milieu, which are still incompletely understood. K_{ATP} channels within the brain are enriched in neurons as well as in glial cells [40–42], but until now little was known about the roles of astrocytic sK_{ATP}/mitoK_{ATP} channels in the brain. More recently, it has been revealed that activation of astrocytic K_{ATP} channels, particularly mitoK_{ATP} channels, improves the function of glutamate transporters in astrocytes, and this is supposed to be beneficial for astrocytes in exerting their effects on neuronal function through glutamate transporters to maintain stimulation [23], thus indicating that astrocytes might affect neuronal activity through a variety of specific mechanisms.

Fig. 7. Representative pictures of the Cx43/GFAP or Cx45/GFAP colabeling in CA3 of PTX-kindled rats regulated by mitoK_{ATP} channels. Scale bar = 20 μ m.

In the current study, we showed that activation of mitoK_{ATP} channels enhanced the GJ coupling; in contrast, blocking mitoK_{ATP} channels substantially inhibited GJ coupling in astrocytes [43, 44]. These results indicate that astrocytic mitoK_{ATP} channels exert their effects on neuronal function partly through GJIC. However, this was seemingly inconsistent with an earlier report that Dia inhibited GJ permeability and sK_{ATP} channel blockers facilitated GJ permeability in astrocytes [45]. We think sulfonylureas and membrane openers mimic the effects of ATP on the sK_{ATP} channels, which might suggest that intracellular energy status of the astrocytes might regulate intercellular communication through

GJs. Activation of mitoK_{ATP} channels could sustain its own metabolic machinery [46] and the subsequent increase in ATP would inhibit the sK_{ATP} channels, which would result in an increase in GJ permeability [45]. However, if inhibition of mitoK_{ATP} channels leads to compromised energy reserves of the astrocytes [46], the sK_{ATP} channels would be activated, resulting in a decrease in GJ permeability. Another possibility is that the effect of Dia on GJIC is not dose dependent if its concentration was >0.1–0.15 mM as observed in our experiments (not shown) [45]. Finally, we used the more specific technique of FRAP (versus scrape-loading dye transfer) to verify this regulation by astrocytic mitoK_{ATP} channels on GJIC,

and applying 5-HD, a selective mitoK_{ATP} channel blocker, as a control in which the results were found to be contrary to what happened after Dia administration. A study examining islets and β -cells from the K_{ATP} channel in transgenic mice found that a small percentage of K_{ATP} channel activity of the β -cells is sufficient to maintain strong glucose-dependent functions, but inhibition of GJs caused complete loss of K_{ATP} channel activity in the control of membrane potential [47], which supports our conclusion that K_{ATP} channels and GJs might influence each other synergistically.

However, this will not always be the case because we found that 5-HD + AGA gave a faster recovery of fluorescence at 5 min and 8 min than either drug alone. We did not observe the same phenomenon after treatment with KA, where the effect was very similar to that of either drug alone. Unfortunately, we are not certain what really happened under these severe conditions which might involve multiple mechanisms including ion buffering and metabolic machinery, or the activation of some compensatory mechanisms.

Astrocytic K_{ATP} Channels and Astrocytic GJs in Epileptogenesis

The hallmark of K_{ATP} channels is their regulation by intracellular nucleotides. Inhibition by ATP but stimulation by MgADP [48] links neuronal metabolism to excitability, and thus they are thought to play an important role in protecting cells from neuronal death caused by hypoxia, ischemia or metabolic inhibition [49, 50]. It has been reported that neuronal K_{ATP} channels show an ability to adjust cell excitability [11–13]. Pharmacological and genetic studies have provided evidence to further support the roles of neuronal K_{ATP} channels in the control of neuronal excitability and seizure propagation by depolarizing presynaptic neuron and subsequently by inhibiting the release of excitatory neurotransmitter, or by regulating GABA transporters [1, 14–19]. However, little is known about the roles of astrocytic sK_{ATP}/mitoK_{ATP} channels in epileptogenesis.

Because of the improved understanding of the molecular events underlying the physiological functions of astrocytes, it is believed that reactive astrocytes in epileptic tissue both promote and oppose seizure development through a variety of specific mechanisms [51]. Astrocytic GJs play an important role in the generation of epileptiform events [52–55]. Wallraff et al. [24] observed that slices of mice with GJ coupling-deficient astrocytes displayed a reduced threshold for the generation of epileptiform events, indicating that elevated astrocytic GJ

coupling might abolish the generation of epileptiform activities.

We demonstrated that activation of mitoK_{ATP} channels attenuated the behavioral outcomes of seizures in chronic epileptic rats (online suppl. fig. S2). Furthermore, we found that opening of astrocytic mitoK_{ATP} channels alleviated neurotoxin-induced dysfunction of GJIC in cultured astrocytes and increased GJ coupling after seizure in vivo. Together, our results indicated that the anticonvulsant action of mitoK_{ATP} channel openers [15] stemmed, probably in part, from upregulating the astrocytic GJ coupling against the seizure generation as well as adjusting the astrocytic GJ coupling against the seizure propagation, in addition to the opening neuronal K_{ATP} channels to limit cell excitability. Our results indicate that local alterations in K_{ATP} channel function could have variable effects on the actual level of hyperactivity in the epileptic brain.

Composition of Astrocytic GJs, GJ Coupling and GJ Subunit Protein Expression

Generally, Cx43 and Cx30 are expressed in astrocytes, Cx45 and Cx36 are expressed in neurons, and Cx32 is expressed in oligodendrocytes [56]. Actually, the neuronal-glial coupling is not fully understood [57]. LM immunofluorescence analysis of astrocytic Cx43 revealed that a small portion of GJs (<5%) was distributed along the surface of oligodendrocyte cell bodies (identified by labeling for the oligodendrocyte marker enzyme CNPase) [58, 59]. In the current study, we also found that Cx43 was expressed in neuronal and oligodendrocytic cell bodies (online suppl. S4–S6). It was revealed recently that neurons control the expression of the two major astrocytic GJ proteins by upregulating Cx43 in astrocytes cultured with neurons and by inducing Cx30 to form functional GJ. These effects require close interactions with neurons and are reversed by excitotoxic treatments that lead to neuronal death and, when applied in vivo, trigger significant modifications in the expression of both Cxs in reactive astrocytes [60]. Our results support this opinion. We found that Cx43 was upregulated in the entire hippocampus (fig. 5c), and its colabeling with Map2 (online suppl. fig. S4 and S7) and NeuN (online suppl. fig. S5) was enhanced in the CA2 and CA3 regions after a seizure. After treatment with Dia, Cx43 was upregulated only in the CA3 region (fig. 5b), and its colabeling with GFAP (fig. 5c and fig. 6, 7) and NG2 (online suppl. fig. S6) was enhanced significantly in the entire hippocampus; in addition, colabeling with Map2 was downregulated in the CA2 region (online suppl. fig. S7) at 7 days after seizure.

We think this could be due to the mitoK_{ATP} channels either directly modulating astrocytic GJ coupling or through controlling neuronal activity to regulate Cx expression and its function [60].

Pathophysiological Implications and Perspectives

As mentioned above, pharmacological evidence has suggested that K_{ATP} channel openers are effective anti-convulsants [16–19]. However, little is known about the anticonvulsant effect of activation of astrocytic K_{ATP} channels, particularly of astrocytic mitoK_{ATP} channels, and its possible mechanism. Here, to our knowledge, we show for the first time that the anticonvulsant action of K_{ATP} channels is achieved, at least partly, by enhancing the astrocytic mitoK_{ATP} channel-regulated GJ coupling in addition to the neuronal K_{ATP} channel-modulated excitability. Therefore, the present study reveals a novel pharmacological profile of astrocytic K_{ATP} channel openers as regulators of GJ coupling, and provides a new strategy for anti-epilepsy treatment. Upregulation of Cx43, Cx45 and Cx30 in epileptic tissue (fig. 4, 5a, b) might re-

flect adaptive consequences rather than a causation of seizure activity.

In addition, availability of animals with ablation of astrocytic sK_{ATP}/mitoK_{ATP} channels or astrocytic Cxs or precisely selective openers/blockers of astrocytic sK_{ATP}/mitoK_{ATP} channels or GJs might also promote a better understanding of the proposed roles of astrocytic sK_{ATP}/mitoK_{ATP} channel-regulated GJ couplings in glia-related transcellular signaling pathways involved in the epileptogenesis of the brain.

Acknowledgements

This publication was supported by the National Natural Science Foundation of China (30471840 and 30570644), Natural Science Foundation of Zhejiang Province (Y205127, Y2080272 and R2090266), by the Fundamental Research Funds for the Central Universities, and by the Key Laboratory of Medical Neurobiology of Ministry of Health at Zhejiang University. Special thanks to Dr. Chunhu Yang (Bioelectromagnetics Laboratory, Zhejiang University School of Medicine) and Dr. Gu Weizhong for excellent technical assistance.

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