[31P]/[1H] Nuclear Magnetic Resonance Study of Mitigating Effects of GYKI 52466 on Kainate-Induced Metabolic Impairment in Perfused Rat Cerebrocortical Slices

*Pei Tang, *Serguei Liachenko, *John A. Melick, and +Yan Xu

Department of *Anesthesiology and Critical Care Medicine, and †Pharmacology University of Pittsburgh, Pittsburgh, Pennsylvania, U.S.A.

Summary: Purpose: Kainic acid (KA) has long been used in experimental animals to induce status epilepticus (SE). A mechanistic implication of this is the association between excitotoxicity and brain damage during or after SE. We evaluated KA-induced metabolic impairment and the potential mitigating effects of GYKI 52466 [1-(4-aminophenyl)-4-methyl-7,8-methylenedioxy-5H-2,3-benzodiazepine] in superfused rat cerebral cortical slices.

Methods: Interleaved [31P]/[1H] magnetic resonance spectroscopy (MRS) was used to assess energy metabolism, intracellular pH (pHi), N-acetyl-l-aspartate (NAA) level, and lactate (Lac) formation before, during, and after a 56-min exposure to 4 mM KA in freshly oxygenated artificial cerebrospinal fluid (oxygenated ACSF).

Results: In the absence of GYKI 52466 and during the KA exposure, NAA, PCr, and ATP levels were decreased to 91.1 ± 0.8, 62.4 ± 3.9, and 59.1 ± 4.3% of the control, respectively; Lac was increased to 118.2 ± 2.1%, and pH, was reduced from 7.27 ± 0.02 to 7.13 ± 0.02. During 4-h recovery with KA-free ACSF, pHi rapidly and Lac gradually recovered, NAA decreased further to 85.5 ± 0.3%, and PCr and ATP showed little recovery. Removal of Mg2+ from ACSF during KA exposure caused a more profound Lac increase (to 147.1 ± 4.0%) during KA exposure and a further NAA decrease (to 80.4 ± 0.5%) during reperfusion, but did not exacerbate PCr, ATP, and pH, changes. Inclusion of 100 μM GYKI 52466 during KA exposure significantly improved energy metabolism: the PCr and ATP levels were above 76.6 ± 2.1 and 82.0 ± 2.9% of the control, respectively, during KA exposure and recovered to 101.4 ± 2.4 and 95.0 ± 2.4%, respectively, during reperfusion. NAA level remained at 99.8 ± 0.6% during exposure and decreased only slightly at a later stage of reperfusion.

Conclusions: Our finding supports the notion that KA-induced SE causes metabolic disturbance and neuronal injury mainly by overexcitation through non-N-methyl-D-aspartate (NMDA) receptor functions.

Key Words: Brain slice—Status epilepticus—Excitotoxicity—GYKI 52466—Kainic acid—Nuclear magnetic resonance.
We evaluated KA-induced metabolic impairment and the potential neuroprotective effects of GYKI 52466 in superfused rat cerebral cortical slices. Interleaved \(^{31}P/\text{[}^{1}H\text{]}\) nuclear magnetic resonance (NMR) spectroscopy was used to assess energy metabolism, intracellular pH (pH), \(N\)-acetyl-l-aspartate (NAA, a neuron marker) level, and lactate (Lac) level simultaneously. The effects of extracellular \(\text{Mg}^{2+}\), an NMDA-receptor antagonist, were also investigated. We showed that KA-induced overexcitation causes increased accumulation of Lac in the absence of hypoxia, irreversible energy failure, and delayed NAA loss. GYKI 52466, combined with \(\text{Mg}^{2+}\), protected the brain slices from such deteriorating changes.

**MATERIALS AND METHODS**

**Brain slice preparation**

All animals were handled according to a protocol approved by the Institutional Animal Care and Use Committee of the University of Pittsburgh. Wistar-Furth rats (Harlan Sprague-Dawley, OH, U.S.A.) aged 12–13 days were used because of their high susceptibility to the neurotoxic effects of KA and their reliable and quantifiable response to KA in producing SE (15,16). For each NMR study, 10–12 rats were killed by rapid decapitation. Each brain was quickly removed from the cranial cavity and washed in cold, freshly oxygenated (95% \(\text{O}_2/5\% \text{CO}_2\)) artificial cerebrospinal fluid (oxy-ACSF), which normally contains (in mM) \(\text{NaCl} 124, \text{KCl} 5, \text{KH}_2\text{PO}_4 1.2, \text{CaCl}_2 1.2, \text{NaHCO}_3 26, \text{glucose} 10, \text{and MgSO}_4 1.2\) (or 1.2 \(\text{Na}_2\text{SO}_4\) in \(\text{Mg}^{2+}\)-free ACSF). Four cortical slices were obtained from the lateral surfaces of the left and right hemispheres by sliding the brain past a blade fixed to the normal oxy-ACSF with neither KA nor GYKI 52466. After the insult, the perfusate was switched back to a high rate once the ACSF reached the desired level in the chamber. The constant upward flow of the ACSF, in balance with the gravity, kept the brain slices suspended in the center of the probe coils. The chamber temperature was controlled at 37°C by the temperature controller of the NMR spectrometer. The \(\text{PO}_2\), \(\text{PCO}_2\), and pH of the ACSF entering the superfusion chamber were frequently checked with a Corning 178 pH/blood gas analyzer to ensure \(\text{PO}_2 \geq 390\) mm Hg, \(\text{PCO}_2\) of 27–34 mm Hg, and pH of 7.3–7.4.

**KA exposure**

KA and GYKI 52466 were obtained from Research Biochemicals International (Natick, MA, U.S.A.) and were used without further purification. Brain slices were allowed to stabilize in the superfusion chamber for at least 1 h while the magnetic field homogeneity was optimized and 4 sets of \(^{31}P/\text{[}^{1}H\text{]}\) reference spectra were taken. Thereafter, a 56-min KA exposure was started, and the time was arbitrarily designated zero. In addition to the control group that received no KA exposure, there were four insult groups: group 1, 4 mM KA with neither \(\text{Mg}^{2+}\) nor GYKI 52466; group 2, 4 mM KA with 1.2 mM \(\text{Mg}^{2+}\) and without GYKI 52466; group 3, 4 mM KA with \(\text{Mg}^{2+}\) and with 100 \(\mu\text{M}\) GYKI 52466; and group 4, 4 mM KA with both 1.2 mM \(\text{Mg}^{2+}\) and 100 \(\mu\text{M}\) GYKI 52466. After the insult, the perfusate was switched back to the normal oxy-ACSF with neither KA nor GYKI 52466 and superfusion was continued for a 4-h recovery period.

**Interleaved \(^{31}P/\text{[}^{1}H\text{]}\) NMR Spectroscopy**

Interleaved \(^{31}P/\text{[}^{1}H\text{]}\) NMR spectra were acquired continuously throughout the KA exposure and recovery periods using an Otsuka Chemagnetics (Fort Collins, CO, U.S.A.) CMXW-400SLI NMR spectrometer, operating at 162.3 and 401.1 MHz for the \(^{31}P\) and \(^{1}H\) resonance, respectively. A broad-band high-resolution NMR probe (Nalorac, Martinez, CA, U.S.A.) with \(^{1}H\) decoupling channel was used. The nominal 90° pulse widths for \(^{31}P\) and \(^{1}H\) were 12 and 25 \(\mu\text{s}\), respectively. The
pH against the control group. The pH was calculated from the PE-PCr frequency difference, δ, between the phosphoethanolamine (PE) and PCr peaks, using $\Delta pH = 5.63 + \log(\delta - 3.19/6.95 - \delta)$ (21, 22). A second-degree polynomial correction was performed based on the calibration data of Espanol et al. (19), using $\Delta pH = 17.678 - 4.4407x + 0.41929x^2$, where x is the pH calculated from the PE-PCr frequency differences.

Except for pH, all data were expressed as percentage changes relative to the averaged values (100%) before KA exposure. Because even in the control group, which was superfused only with the nominal oxy-ACSF, there was slight deterioration in NAA and Lac levels after 4-5 h of superfusion, the metabolic changes in the KA-insulted groups were further normalized, time point by time point, against the control group.

**Statistical analysis**

All values are mean ± SEM. Statistical analysis was performed as described previously (17, 19). First, a repeated-measures analysis of variance (ANOVA) was used to compare the relative metabolite values at different times points for all groups [i.e., test of null hypothesis, (23)]. If the null hypothesis was rejected, multiple comparisons of three treatment groups (i.e., groups 2-4) against a single untreated group (group 1) were made using Dunnett's test (23) at 11 a priori chosen timepoints, starting at 20 min before onset of KA exposure, with increments by 32 min, and ending at 300 min (i.e., -20, 12, 44, 76, 108, 140, 172, 204, 236, 268, and 300 min). In this test, the means at a given timepoint were ranked first, comparisons were then made by computing the q' statistic (23) between a treated group and group 1 in the order from the largest to the smallest difference in means. If a treated group was not significantly different from group 1, no further comparisons at that timepoint were made for the remaining groups with a smaller mean difference from group 1. A p-value <0.05 was considered statistically significant.

**RESULTS**

Under the control condition, superfused brain slices can be kept healthy for at least 8 h and often for >12 h (17), with no detectable change in pH, PCr, and ATP levels and a slight decrease (<4%) and increase (<5%) in NAA and Lac levels, respectively. In contrast, exposure to 4 mM KA resulted in immediate changes in pH, PCr, ATP, and Lac levels. Representative interleaved [31P]/[1H] NMR spectra before, during, and after 4 mM KA exposure are shown in Fig. 1, demonstrating the high spectral quality obtainable with the superfused live brain slices at the high magnetic field and under the high-resolution condition. The PE peaks in the control 31P spectra in Fig. 1 resulted predominantly from the extracellular inorganic phosphate in the ACSF. Figure 2 compares the changes in PCr, ATP, NAA, and Lac as a function of time among the four insult groups. The shaded time interval indicates the 56-min KA exposure. The changes in pH (data not plotted) were not significantly different among the four groups at almost all time-points, except immediately after KA exposure when group 4 (pH = 7.22 ± 0.01) differed significantly from group 1 (pH = 7.13 ± 0.02). In the presence of 1.2 mM Mg2+ and in the absence of GYKI 52466, 4 mM KA exposure caused NAA, PCr, and ATP levels to decrease to 91.1 ± 0.8, 62.4 ± 3.9, and 59.1 ± 4.3% of the control level, respectively; Lac was increased to 118.2 ± 2.1%, and pH decreased from 7.27 ± 0.02 to 7.13 ± 0.02. During 4-h reperfusion with KA-free oxy-ACSF, pH rapidly and Lac gradually recovered, NAA decreased further to 85.5 ± 0.3% of the control, and PCr and ATP showed little sign of recovery. Removal of Mg2+ from the oxy-ACSF during KA exposure caused a more profound increase in Lac (to 147.1 ± 4.0%) during KA exposure and a further decrease in NAA (to 80.5 ± 0.5%) during reperfusion, but did not exacerbate PCr, ATP, and pH changes. With the inclusion of both 100 μM GYKI 52466 and 1.2 mM Mg2+ during KA exposure, PCr and ATP levels were maintained above 76.6 ± 2.1 and 82.0 ± 2.4% of the control, respectively, during KA exposure and recovered to 101.4 ± 2.4 and 95.0 ± 2.4%, respectively, during the reperfusion. The NAA level remained at 99.8 ± 0.6% during exposure and decreased slightly at a later stage of the reperfusion. The pH was decreased to 7.22 ± 0.01 during exposure but rapidly recovered on KA washout.
FIG. 1. Representative interleaved $^3$P (A) and $^1$H (B) nuclear magnetic resonance (NMR) spectra, obtained (a) before, (b) during, (c) 1.5 h after, and (d) 3 h after 56 min of KA exposure. Peak assignments are phosphoethanolamine (PE), intra- and extracellular inorganic phosphate (Pi), phosphocreatine (PCr), ATP, inositol (Ins), choline (Cho), creatine/PCr (Cr/PCr), glutamate/glutamine (GLx), N-acetyl-aspartate (NAA), kainic acid (KA), and lactate (Lac).

DISCUSSION

KA-induced SE is a well-established model in rodents that reflects some important aspects of human epilepsy (3,9). In studies of strain dependence of KA-induced SE (15,16), Wistar-Furth rats were shown to be highly susceptible to the neurotoxic effects of KA and are reliable and quantifiable (i.e., with low within-group variability) in producing SE. Moreover, such reproducibility is more profound in the immature than in the adult animals (15,24). Although this latter aspect may be attributable to the effects of brain maturity on the viability of cerebrovascular autoregulation in the adult animals, our choice of immature Wistar-Furth rats for the present ex vivo study can nevertheless ensure that the response to KA in the brain slices closely mimics the clinical state of SE.

The KA concentration (4 mM) used in the present study was between the peak blood KA concentration (~1--2 mM) in a systemic administration to induce SE (25), and the concentration for direct intracerebral KA injection was ~5 mM (26). Our pilot study of different KA concentrations (data not shown) confirmed the previous findings with glutamate (17) which showed that slightly higher concentrations of excitotoxin are needed in the superfused brain slices to produce NMR measurable changes comparable to those observed in vivo. As shown in Fig. 2, exposure of the cerebrocortical slices to 4 mM KA caused rapid deterioration of intracellular high-energy phosphorus metabolites. Correlated with this change was a rapid increase in Lac, suggesting that the increase in glycolytic rate after KA exposure was greater than the increase in the rate of oxygen consumption (27), even in the presence of more than adequate oxygenation in ACSF. This finding is in agreement with the in vivo findings of Wasterlain (28) showing that during seizure activity glycolytic metabolism increases 8-fold, oxygen consumption increases 2- to 3-fold; and high-energy utilization increases 2- to 4-fold. Lac levels were partially reversed even during KA exposure and continued to recover after KA washout with normal oxygen ACSF. The partial reversal of Lac during KA exposure was believed to reflect the initial accumulation of Lac in the slices and the subsequent washout by ACSF flow after Lac was transported or leaked (after possible membrane damage) to the extracellular space. Recent in vitro studies (29,30) showed that Lac may also be used as an energy substrate in place of glucose during ATP failure. Our results during recovery appear to support this possibility because in group 4, in which PCr and ATP levels were largely maintained, Lac showed significantly slower recovery than in the other three groups. Unlike Lac, the high-energy phosphates did not recover, implying permanent tissue damage, although loss of nucleotides from cells to the ACSF, which was not recirculated, may be partially responsible for the failure of ATP recovery. The NMR spectroscopic method is neither cell specific nor sensitive to microheterogeneity. Although NAA is mainly located in neurons, existence of a small glial pool in immature brain has been suggested (31,32). Nevertheless, that the NAA level steadily decreases after KA exposure and that GYKI 52466 can significantly prevent such decrease makes us speculate that the NAA changes observed with NMR are mostly associated with neurons (17).

As compared with lack of extracellular Mg$^{2+}$ during KA exposure, marginal attenuation of KA-induced neu-
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FIG. 2. Comparison of relative changes in (a) phosphocreatine (PCr) (b) ATP, (c) N-acetyl aspartate (NAA), and (d) lactate (Lac) levels among the four insult groups: group 1, 4 mM kainic acid (KA) without Mg²⁺ and GYKI 52466 (open squares, n = 3); group 2, 4 mM KA with 1.2 mM Mg²⁺ and without GYKI 52466 (solid squares, n = 4); group 3, 4 mM KA without Mg²⁺ and with 100 μM GYKI 52466 (open circles, n = 3), and group 4, 4 mM KA with 1.2 mM Mg²⁺ and 100 μM GYKI 52466 (solid circles, n = 4). The shaded interval indicates 56-min KA exposure. All data points are mean ± SEM. Where there are no error bars, the SEM is smaller than the size of the symbol. Multiple comparisons of groups 2–4 against the single untreated group (group 1) were made with Dunnett’s test. At a given timepoint, significant differences from group 1 are marked: *p < 0.01; †p < 0.05.

...ronal loss was observed in the presence of 1.2 mM Mg²⁺, an NMDA receptor antagonist, but the energy metabolism was not significantly improved. In cultured neurons (10) KA was shown to produce a concentration-dependent increase in glutamate release, presumably secondary to the activation of AMPA receptors (11). Therefore, partial protection of NAA loss by Mg²⁺, as shown in the present study, may suggest certain involvement of NMDA receptors in the neuronal damage caused by KA-induced SE. However, when the effects of KA on AMPA receptors are blocked by the noncompetitive antagonist GYKI 52466, presynaptic action of KA can actually elicit a dose-dependent decrease in glutamate release and decrease glutamatergic synaptic transmission (11). Indeed, inclusion of 100 μM GYKI 52466 in the absence of Mg²⁺ produced a significantly better protection of the PCr level than that provided by Mg²⁺ alone (Fig. 2).

GYKI 52466, when administered in the presence of Mg²⁺, had a significant mitigating effects against KA-induced energy failure and NAA loss. Because GYKI 52466 is a noncompetitive AMPA receptor antagonist (12,33), the activity of GYKI 52466 clearly involves the action at the AMPA receptors. Recently, combined use of GYKI 52466 with conventional AEDs was shown to potentiate the protective effects of these drugs without producing adverse side effects (13), suggesting that concomitant administration of noncompetitive AMPA receptor antagonists, such as GYKI 52466, with conven-
tional AEDs may offer a novel approach to the treatment of epilepsy (34).

Whether seizure activities, especially KA-induced SE, can cause damage to immature brains remains controversial (35,36). Albala et al. showed (25) that KA caused more severe seizure activities in immature rats (15-day-old pups) than in adult rats, yet histological brain damage, which could be easily identified in adults, was absent in the pups. This led to the common belief that SE produces no structural damage in the young as long as they have not reached adolescence (37). However, in the study of Albala et al. (25), 90% of the immature rats died before being examined histologically. Only the 15-day-old pups that survived showed no histological damage. There was a distinct possibility that the most pups died as a direct or indirect consequence of neuronal damage. As shown in our present study, a concentration of 4 mM KA caused irreversible energy failure and NAA loss in immature brain slices. The deteriorating KA effects can be mitigated by treatment with GYKI 52466. All these findings suggest that the immature brain, if untreated, can be injured by KA-induced overexcitation, and that by blocking the excitotoxicity action at the non-NMDA receptors the ability of the brain slices to withstand a certain degree of KA insult is strengthened.

An ex vivo KA-induced overexcitation model was used to mimic the clinical state of SE. The energy failure and neuronal damage, as measured by the interleaved $^{31}$P/$^1$H NMR spectroscopy, were clearly mediated by the action of AMPA/KA receptors. The noncompetitive non–NMDA-receptor antagonist GYKI 52466 had a significant mitigating effects against KA-induced metabolic impairment.

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REFERENCES


