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Uridine release during aminopyridine-induced epilepsy

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Uridine, like adenosine, is released under sustained depolarization and it can inhibit hippocampal neuronal activity, suggesting that uridine may be released during seizures and can be involved in epileptic mechanisms.

In an in vivo microdialysis study, we measured the extracellular changes of nucleoside and amino acid levels and recorded cortical EEG during 3-aminopyridine-induced epilepsy. Applying silver impregnation and immunohistochemistry, we examined the degree of hippocampal cell loss.

We found that extracellular concentration of uridine, adenosine, inosine, and glutamate increased significantly, while glutamine level decreased during seizures. The release of uridine correlated with seizure activity. Systemic and local uridine application was ineffective. The number of parvalbumin- and calretinin-containing interneurons of dorsal hippocampi decreased. We conclude that uridine is released during epileptic activity, and suggest that as a neuromodulator, uridine may contribute to epilepsy-related neuronal activity changes, but uridine analogues having slower turnover would be needed for further investigation of physiological role of uridine.

Keywords: Uridine; Adenosine; Inosine; EEG; Hippocampus; Microdialysis; Epilepsy; Rat

Introduction

Epileptic activity is based on broken excitatory–inhibitory balance in the brain tissue (Mody et al., 1992). It is a complex mechanism that leads to epileptic firing, and involves all transmitters and modulators of a certain brain area. Because change in the fast and major transmitter systems has serious side effects, increased attention was given to modulators and endogenous factors influencing epileptic activity that could prevent seizures. Along that line of investigations, it turned out that it is not only the gamma-aminobutyric acid (GABA) that compensates the excitatory activity of glutamate in epilepsy but a tonic adenosinergic inhibition is also a major component of sustained inhibition during seizures (Chin, 1989). Adenosine was shown earlier to reduce glutamate-induced excitotoxicity via A_1 receptors by inhibiting glutamate release (Dolphin and Archer, 1983) and/or hyperpolarizing the postsynaptic neurons (de Mendonca and Ribeiro, 1993; Dolphin et al., 1986). This suggests a cytoprotective role in epilepsy (de Mendonca et al., 2000; Fredholm, 1997), protecting neurons against glutamate-induced excitotoxicity (Rudolphi et al., 1992). However, adenosine can also enhance the glutamatergic excitotoxicity via A_{2A} receptors (Jacobson, 1998; Li et al., 2001; Ribeiro, 1999; Von Lubitz et al., 1999), thus adenosine could induce apoptotic cell death in chronic applications (Abbracchio and Cattabeni, 1999; Abbracchio et al., 1995). Because adenosine can be both cytoprotective and cytotoxic—depending on the receptor activated—thus it can be pro- or anticonvulsive in epilepsy (Klitgaard et al., 1993). That is why adenosine derivatives are not good antiepileptic drug candidates.

Investigating nucleosidergic systems, it turned out that adenosine is only one component of the nucleoside microenvironment of neurons that changes in depolarization. Recently, several pieces of evidence indicate that the concentration of other purine nucleosides, like guanosine (Lara et al., 2001; Schmidt et al., 2000) and adenosine metabolites (inosine, hypoxanthine, xanthine) increase in the extracellular space during epilepsy and ischemia (Berman et al., 2000; Lewin and Bleck, 1981; Phillis et al., 1994; Zhang and Niu, 1994). Thus, the question arises whether nucleosides other than adenosine can also be involved in the modulation of neuronal activity in epilepsy because nucleoside receptors and transporters are not highly specific and it is known that several nucleoside-binding proteins exist. If it is so, the non-adenosine nucleosides

Abbreviations: ABC, avidin–biotin peroxidase complex; ACSF, artificial cerebrospinal fluid; 3-AP, 3-aminopyridine; 4-AP, 4-aminopyridine; CA1, hippocampal Cornu Ammonis 1; CA2, hippocampal Cornu Ammonis 2; CA3, hippocampal Cornu Ammonis 3; CB, calbindin; CR, calretinin; DAB, 3-3′-diaminobenzidine tetrahydrochloride; EEG, electroencephalogram; GABA, gamma-aminobutyric acid; HPLC, high performance liquid chromatography; IgG, immunoglobulin G; PB, phosphate buffer; PV, parvalbumin; SD, standard deviation; s.o., stratum oriens; s.p., stratum pyramidale; s.r., stratum radiatum; TB, Tris-buffer; TBS, Tris-buffer saline; UV, ultraviolet.

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might be novel targets of anti-epileptic drug research if they fail to induce apoptosis as adenosine does.

In the present study, we focused on uridine because in a very early study, chronic, large-dose uridine administration had anti-convulsive effect in a frog epilepsy model (Roberts, 1973; Roberts et al., 1974) and later it was confirmed by studies on humans having epileptic seizures as well (Page et al., 1997). Since our aim was to search for neuronal mechanisms of uridinergic modulation of neuronal activity, formerly we demonstrated that uridine and adenosine are coreleased during kainate-, ouabain-, and high potassium-induced sustained depolarization in a TTX-insensitive manner in the hippocampus and thalamus of rats (Dobolyi et al., 2000). Uridine inhibited the firing rate of hippocampal neurons (Dobolyi et al., 1999) and evoked Ca^{2+} signals in rat synaptosomes (Kardos et al., 1999). Nucleosides are released by sustained reverse transport when cells reach the reversal potential of the nucleoside transporter (Baldwin et al., 1999). Concerning the putative targets of uridine, it is known that uridine interacts with the $GABA_A$ receptor benzodiazepine-binding site (Guarneri et al., 1983, 1985) as well as with neuropeptides (Agnati et al., 1986) and with the dopaminergic system (Myers et al., 1995). The release and neuronal signaling effects of uridine are supported by behavioral observations showing that uridine could influence sleeping behavior of rats (Inoue et al., 1984a,b, 1990, 1995). This suggested a putative uridine receptor responsible for the sleep-promoting action of uridine (Kimura et al., 2001). Thus, we claim that uridine is a real neuromodulator candidate. However, no data are yet available about the release of uridine during epileptic seizures that could directly support the involvement of endogenously released uridine in the generation of epileptic activity as formerly described in frog and human studies (Page et al., 1997; Roberts, 1973; Roberts et al., 1974). Therefore, in the present investigation, uridine release was measured in the hippocampus during pharmacologically induced epileptic seizures and the correlations of EEG activity, uridine, amino acid, and nucleoside release and seizure-induced anatomical changes in the hippocampus were established using the 3-aminopyridine (3-AP) epilepsy model. We also did attempt to modify seizure activity by local and systemic application of uridine.

Materials and methods

Animals

Twenty-one adult male Sprague–Dawley rats (250–300 g) were used. Animals were kept in standard conditions having a 12 h light–dark cycle. Rats were supplied with food and water ad libitum. Experiments were carried out on the basis of local ethical rules in accordance with the Hungarian Act of Animal Care and Experimentation (1998, XXVIII, section 243/1998), which is in conformity with the regulation of animal experiments in the European Community. All efforts were made to minimize pain and suffering and to reduce the number of animals used.

Materials

3-AP and the applied chromatographic standards (purine and pyrimidine bases, ribonucleosides, deoxyribonucleosides, and amino acids) were purchased from Sigma Co. (St. Louis, MO, USA). HPLC grade organic solvents were produced by Merck Co. (Darmstadt, Germany). The artificial cerebrospinal fluid (ACSF) containing 144 mM NaCl, 3 mM KCl, 1 mM $MgCl_2$, and 2 mM $CaCl_2$ was a nonpyrogenic perfusion fluid product of Heim Pál Hospital (Hungary).

Surgery procedures

Animals were anesthetized in 1% Halothane–air mixture and secured in a stereotactic frame (David Kopf, US). Holes were drilled into the skull to insert stainless-steel screw electrodes above the frontal and the parietal cortices unilaterally. A reference electrode was placed above the cerebellum. Microdialysis probes were implanted into both sides of the ventral hippocampi (at A: −5.2, L: 4.5, V: −6.8 coordinates from bregma) with the nose bar positioned at −3.3 mm on the basis of the rat brain atlas of Paxinos and Watson (1982). Probes were inserted slowly, for 30 min, to reduce tissue damage. One hour after the insertion of the microdialysis probes, sample collection and EEG recording was started. The body temperature was measured with a rectal thermometer and adjusted with a heating lamp. During the experiment, the concentration of Halothane was kept constant between 0.3% and 0.5%. In uridine injection experiments, a venous canula was inserted into the left femoral vein before starting the dialysis study. The canula was made from a polyethylene tube (29-Gauge) and attached to a 1-ml syringe through an infusion tap allowing the injection and washing-in of uridine through the femoral vein. Uridine was dissolved in physiological salt solution; uridine concentration was 10 mM and was applied using a 1 mg/kg dose in a single injection.

EEG recording and data processing

EEG was monitored during the whole experiment using a Grass EEG 8B model EEG machine at 0.1 Hz to 1 kHz bandwidth. Data collection was performed with a CED 1401 data processing system using SPIKE2 v2.1 and Signal v1.85 software for CED. The sampling rate was 500 Hz. Thirty-second-long samples were stored and power spectrum analysis was performed for measuring the intensity of epileptic activity. The power spectra of 20–30 Hz were integrated for characterizing epileptic activity.

Microdialysis experiments

Microdialysis probes were made as described previously (Juhász et al., 1989). Briefly, hollow fibers (Travenol, cut-off at 5000 Da, o.d. 0.2 mm, length of the active surface: 3 mm) were adjusted into 23-Gauge, stainless-steel tubings. Fused silica capillaries (o.d. 50 μm) guided by stainless-steel tubes of 27-Gauge were used as the inlet and outlet of the probe. The parts of the dialysis probes were assembled using epoxy glue. Artificial cerebrospinal fluid (ACSF) was used for perfusion. The pH of the ACSF was adjusted to 7.0–7.2 immediately before the beginning of perfusion. The flow rate was 1 μl/min, and from our earlier data, a 20% concentration recovery was estimated (Juhász et al., 1989).

A 1-h recovery period was necessary after surgery to establish a reliable baseline for nucleosides and amino acids (Dobolyi et al., 2000). Samples were collected after a recovery period of 60 min for the three control samples, then at every 30 min to enhance the resolution of amino acid measurement. Ten microliters of the 30-μl sample was used for amino acid analysis and three times 20 μl were pooled for nucleoside analysis. After collecting three control samples, the perfusion fluid in the right probe was changed

to ACSF containing 3-AP (500 mM, pH 7.0–7.2) for 4.5 h. During 3-AP perfusion, samples were collected from the contralateral hippocampus (left probe) every 30 min. Samples collected from the right probe were not analyzed because they contained 3-AP in high concentration. In three experiments, 3-AP was washed out with ACSF for 4.5 h from the right side of the hippocampus. After finishing the experiments, probes and screw electrodes were removed, the wound was closed, and rats were let to recover from anesthesia. After the acute microdialysis experiments, rats were kept alive for histological studies for a 1-day, 3-day, and 1-month period. Sham-operated rats were used for control applying the same method as described above, but perfusing pure ACSF only during the whole microdialysis experiment.

To measure the uridine concentration changes in the brain following the i.p. injection of uridine, we implanted microdialysis probes into the hippocampus of three rats as described above. After 1 h of control period, 100 mg/kg uridine was applied i.p. Five hours later, 500 mg/kg uridine was injected i.p. Uridine and uracil levels were measured to establish the turnover rate of uridine and the relative elevation of uridine was also measured. I.v. application of such a high concentration of uridine was impossible because of its osmotic effects in the blood.

HPLC analysis of nucleosides and amino acids

Nucleoside analysis required merging of three samples to get 60 μl perfusate necessary for the full-scale HPLC assay developed by our laboratory (Dobolyi et al., 1998). The basal level of nucleosides was obtained from the mean of the three control samples, giving a mean basal concentration value for each animal. The basal concentration of nucleosides measured in the dialysate depends on the extracellular concentration recovery inside the probe, which varies slightly from probe to probe. Therefore, changes of nucleoside content of samples collected during and after 3-AP administration were calculated as percentages of the control value. Separation and detection of nucleosides was performed on a HP 1100 series HPLC system with diode-array-detector using Hewlett-Packard Hypersil ODS reversed-phase column (200 × 2.1 mm) filled with C18, 5-μm spherical packing material. The column was cooled and the column temperature was 10°C. Eluent A was 0.02 M formiate buffer containing 0.55% acetonitrile, pH 4.35. Eluent B was 0.02 M formiate buffer containing 40% acetonitrile, pH 4.35. The gradient profile was as follows: 0% B eluent at 0–10 min, 10% at 22 min, and 100% at 30 min. The flow rate was 350 μl/min. Compounds were detected in UV at 254 nm against a reference light beam of 350 nm. Chromatographic peaks were identified from the retention times of external standards.

Ten microliters out of the 30 μl samples was used for amino acid analysis. The basal levels of amino acids were determined as the mean value of three control samples. The changes in amino acid content of samples collected during and after 3-AP administration were calculated as percentages of the control. To measure amino acid concentrations, precolumn derivatization with orthophthal-dialdehyde (OPA) was applied. Derivatization was performed in the presence of mercaptoethanol at pH 10.5 and the OPA derivatized amino acids were detected by 305–395-nm excitation and 430–470-nm emission filters of a fluorescent detector on a Pharmacia AminoSys chromatography system. Detection limits for amino acids were 0.5–5 pmol in 10 μl. Separation of amino acids was performed by HP Hypersil ODS reversed phase columns (200 × 2.1 mm, C18, 5 μm). Eluent A was 0.1 M phosphate buffer containing 4 v/v% tetrahydrofuran, pH 6.0; eluent B was 70% acetonitrile mixed with 0.1 M phosphate buffer adjusted to pH 6.0 with NaOH. The gradient profile was: 0% B at 0 min, 11% B at 2 min, 22% B at 17 min, 50% B at 21 min, 100% B at 25 min, 0% B at 35 min. Chromatograms were evaluated by PE Nelson 2000 software. External standards of 10 μM amino acids were injected after every 12 samples.

Control values were calculated as the mean of the results of three control samples. Data were expressed as the percentage of the control values measured with the same microdialysis probe and compared with Student's t test for correlated samples. Changes were regarded significant if $p < 0.05$. Data are presented as mean ± SD values. Statistica for Windows 5.0 (StatSoft Inc.) software was used for statistical analysis.

Histological procedures

Perfusion and tissue sectioning

The rats had 1-, 3-, and 30-day survival periods, respectively. Sham-operated and normal control animals served as controls with the same survival periods as the epileptic rats. Animals were deeply anesthetized by Equithesin, and perfused through the heart first with saline (1–2 min) followed by a fixative (30 min) containing 0.05% glutaraldehyde, 4% paraformaldehyde, and 0.2% picric acid in 0.1 M phosphate buffer (pH 7.4). Brains were removed from the skull, and postfixed in the same fixative for 1–3 h. Blocks of the hippocampus and overlying neocortex were dissected, and sectioned by Vibratome at 60 μm. Following intense washing in phosphate buffer, alternate sections were processed for silver impregnation and immunocytochemistry.

Silver impregnation

The applied procedure was developed by Gallyas et al. (1980). Sections were kept in the pretreating solution (4% NaOH and 5% NH_4OH) for 2 × 5 min, then in the impregnating solution (0–0.8% NaOH, 2.5% NH_4OH, 0.5% $AgNO_3$) for 10 min, then sections were washed in washing solution (0.5% Na_2CO_3 and 0.01% NH_4NO_3 in 30% ethanol) for 3 × 5 min and for 1 min in developing solution (0.4–0.6% formaldehyde and 0.01% citric acid in 10% ethanol, pH 5.0–5.5), followed by 3 × 10 min wash in 0.5% acetic acid.

Immunohistochemistry

The sections were incubated in 2% bovine serum albumin dissolved in TBS (45 min), followed by incubation in rabbit anticalretinin primary antiserum (Resibois and Rogers, 1992; Rogers and Resibois, 1992), or in rabbit anticalbindin D28K antiserum (CB, Code No. R 202) (Baimbridge and Miller, 1982; Baimbridge et al., 1982), or rabbit antiparvalbumin antiserum (Code No. R 301) (Baimbridge and Miller, 1982; Baimbridge et al., 1982). Sections were treated with the primary antisera (at a dilution of 1:5000 for calretinin (CR), 1:2000 for calbindin (CB), and 1:1000 for parvalbumin (PV) for 2 days at 4°C. For the visualization of immunopositive elements, biotinylated antirabbit IgG (1:300, Vector) was applied as secondary serum followed by avidin-biotinylated horseradish-peroxidase complex (ABC, 1:300, Vector). The immunoperoxidase reaction was developed by 3,3′-diaminobenzidine tetrahydrochloride (DAB, Sigma) dissolved in Tris-buffer (TB, pH 7.6). After the final washes in PB, the sections were dehydrated and embedded in XAM neutral medium.

Quantitative analysis: cell counting

Four to five representative sections with complete penetration of the staining were chosen for the cell number analysis from two controls and from six 3-AP-treated animals (survival time: 1 month). Each PV- and CR-positive cell, together with the outlines of the hippocampus, was drawn by camera lucida, the areas of the subregions [dentate gyrus (GD), CA3 + CA3c, CA1 + CA2] were measured by the Soft Imaging System GmbH analySIS 2.1 software. In each control and 3-AP-treated hippocampi, the cells were counted in the dentate gyrus, CA3, and CA1 (together with CA2). The cell number was determined for unit area (mm^2). The number of cells and the subregion areas from different sections of the same rat have been pooled. The average cell number per unit area of subregion was calculated and one-way ANOVA was used for statistical analysis with the Origin 6.1 software.

Results

The changes of EEG activity during 3-AP-induced epileptic seizures

In 3-AP-induced epileptic state, the EEG activity first showed high-amplitude slow waves, which occurred synchronously in the hippocampus and in the cortex under Halothane anesthesia (Fig. 1A). In 30 min following perfusion of 500 mM 3-AP into the right hippocampus via microdialysis probe, few spike-and-wave discharges were observed in the EEG activity recorded from the cortical surface (Fig. 1B). In 1 h after 3-AP perfusion, epileptiform spike-and-waves became more and more frequent and a marked increase in the power of the 20–30 Hz frequency band occurred (Fig. 1E). In this stage of epilepsy, 30–60-s-long ictal and some minutes of interictal periods alternated. In the postictal periods, a few spike-and-wave patterns were observed (Fig. 1B). In the recovery period, when 3-AP was washed out with ACSF, the number and duration of ictal periods decreased in the first 30 min. In the second 30-min period of washout, no or very short ictal periods occurred (Fig. 1C). One or two hours following the recovery from anesthesia, wet-dog shakes, masseter jerks appeared occasionally in all rats, suggesting some residual epileptic activity.

Changes in nucleoside and amino acid levels in the extracellular space during and after 3-AP application via the microdialysis probe

The baseline concentrations of nucleosides calculated from samples collected 60 min after the implantation of probes were as follows: uracil: 1.11 ± 0.67 μM; uric acid: 2.23 ± 1.36 μM; hypoxanthine: 4.79 ± 2.59 μM; xanthine: 0.40 ± 0.27 μM; uridine: 0.71 ± 0.27 μM; 2′-deoxycitidine: 0.85 ± 0.43 μM;

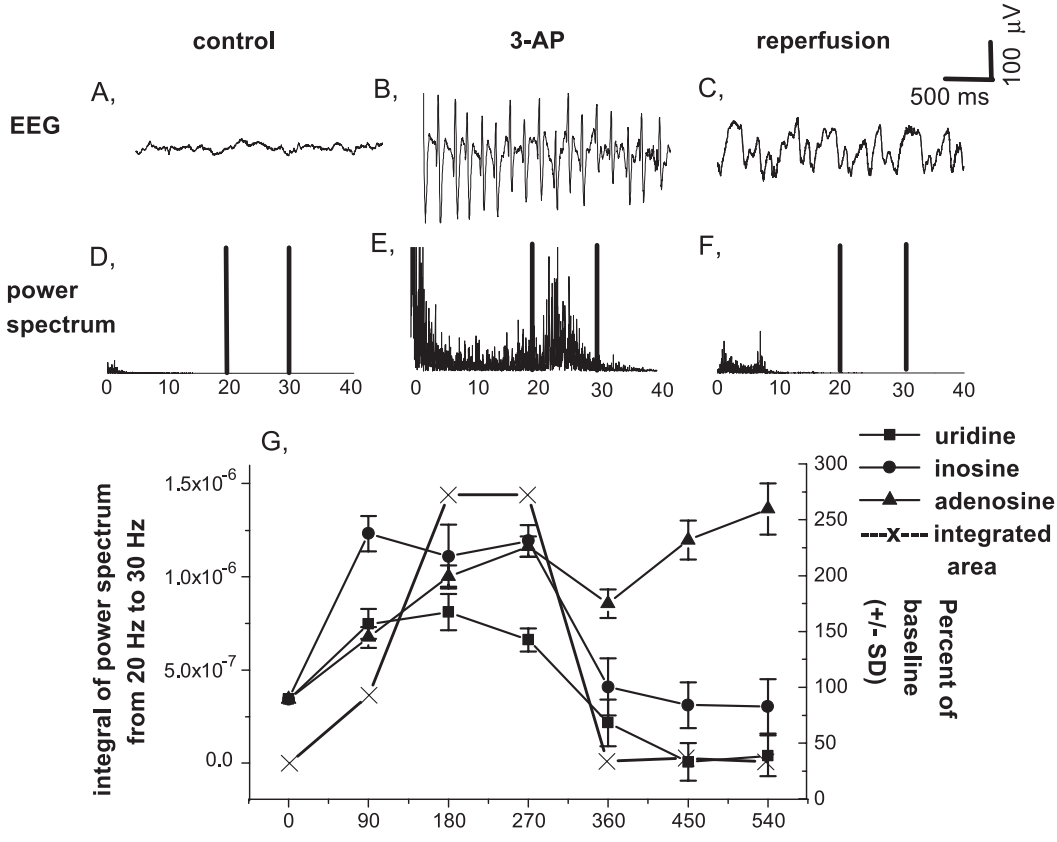

Fig. 1. (A) Normal EEG activity; (B) seizure activity during 3-aminopyridine administration; (C) EEG during washout; (D) the power spectrum of the normal EEG activity in Halothane anesthesia; (E) the power spectrum of EEG in ictal periods; (F) the power spectrum of EEG during washout; (G) correlation of the changes of uridine, inosine, and adenosine levels with the changes of the 20–30 Hz power spectra. (uridine: the changes in the concentration of extracellular uridine ± SD; inosine: the changes in the concentration of extracellular inosine ± SD; adenosine: the changes of the concentration in the extracellular adenosine ± SD; integrated area: the area of the integral of power spectra from 20 to 30 Hz of the EEG).

2′-deoxyuridine: 0.90 ± 0.25 μM; inosine: 0.42 ± 0.32 μM; guanosine 0.26 ± 0.02 μM; thymidine: 0.17 ± 0.50 μM; adenosine: 0.95 ± 0.21 μM; deoxyadenosine: 1.00 ± 0.32 μM.

Extracellular levels of uridine, adenosine, and inosine changed during and after application of 3-AP (Fig. 2). Adenosine, uridine, and inosine levels increased significantly ($p < 0.05$). A moderate increase of hypoxanthine and a small decrease in xanthine was also observed, but the changes have never reached the level of significance. Concentrations of uracil, uric acid, deoxycytidine, deoxyuridine, guanosine, thymidine, and deoxyadenosine were unchanged during 3-AP application (data are not shown).

The baseline concentrations of amino acids calculated from samples collected 60 min after implantation were as follows: aspartate: 0.88 ± 0.17 μM; glutamate: 4.99 ± 1.91 μM; asparagine: 0.98 ± 0.24 μM; serine: 15.4 ± 1.46 μM; glutamine: 33.72 ± 2.64 μM; glycine: 9.56 ± 1.67 μM; threonine: 5.13 ± 0.66 μM; taurine: 1.96 ± 0.41 μM.

During 3-AP application, the extracellular level of glutamate increased, while the glutamine concentration decreased significantly ($p < 0.05$) (Fig. 2). Increase in threonine and taurine was small and not significant ($p > 0.05$). The level of aspartate, asparagine, serine, and glycine remained at the control value.

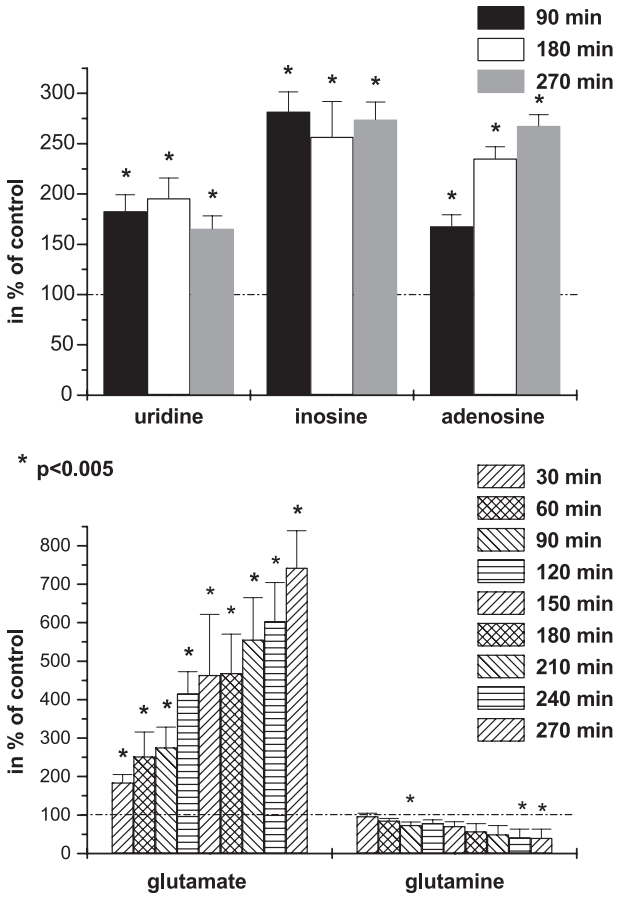

Fig. 2. Extracellular concentration changes of uridine, adenosine, inosine, glutamate, and glutamine as percentages of the control during 3-AP application. The horizontal line is drawn at 100%, which represents the control value. Stars represent significant changes ($p < 0.05$) in uridine, adenosine, inosine, glutamate, and glutamine levels during 3-AP perfusion.

GABA concentration was below the detection limit in several samples; therefore, data are not shown.

Correlation between EEG activity and nucleoside level during 3-AP application via the microdialysis probe

Correlating uridine release with epileptiform activity, we observed that uridine level increase during 3-AP application preceded the frequent appearance of cortical epileptiform discharges. The concentration of uridine and adenosine in the extracellular space increased parallel with the frequency of spike-and-wave discharges as it was observed from power spectra in the 20–30 Hz frequency band (Fig. 1G). During washout of 3-AP, the concentration of uridine in the dialysis probe decreased gradually, and epileptic discharges in the EEG disappeared parallel with the decrease of uridine and inosine, while the increase of adenosine concentration was sustained during the washout of 3-AP.

3-AP-induced morphological changes in the hippocampus

The site of the probes and morphological changes of the principal cells and interneuron types were examined on both sides. The mechanical damage of the probes was restricted to the temporal cortex and to the ventral hippocampus, as it was visible only in a limited number of caudal sections. After 1-month survival, only a thin glial scar was found at the track of the probes. Animals with misplaced probes (6 out of 21) were excluded from the study. Sham-operated animals were also examined, and they did not differ from the controls.

The pattern of principal cell loss caused by unilateral intrahippocampal perfusion with 3-AP was tested with silver impregnation, visualizing the degenerating neurons, and with CB immunostaining, which stains the vulnerable CA1 pyramidal cells and dentate granule cells. The changes of nonprincipal cells were examined by immunostaining for PV and CR, since these cell types proved to show different sensitivity to epileptic injury. No principal cell loss was detectable in the hippocampal hemispheres by silver impregnation at any survival times after 3-AP-induced epileptiform activity, and no change was observed in the CB-immunostaining either (Figs. 3A,B). However, the number of CR-positive interneurons was reduced significantly in the CA1 and CA3 regions of the dorsal hippocampi in both hemispheres (Figs. 3C and 4), but those in the hilus were preserved. Cell counts confirmed the decrease of the number of PV- and CR-positive cells (Figs. 4 and 5). PV-containing fibers were mostly spared, but part of the PV-positive cell bodies and dendrites disappeared in patches in the dorsal CA1 region of 3-AP-treated animals (Fig. 3E), whereas they were spared in the CA3 region and in the dentate gyrus in each survival group. The changes were similar in both hippocampal hemispheres. The differences were present only in the dorsal hippocampi; ventral hippocampi were similar to the sham-operated control.

The time course analyses showed that the changes of interneurons could be observed even in the 1-day survival group. The 3- and 30-day survival groups showed no further degeneration or recovery compared to the 1-day survival group. The morphological changes were present in the dorsal hippocampi of both sides, even on the contralateral side, which did not receive 3-AP directly. Therefore, the 3-AP-induced epileptic seizures caused a moderate injury in the most sensitive interneuronal cell types only in the dorsal hippocampi, while the principal cells remained undamaged.

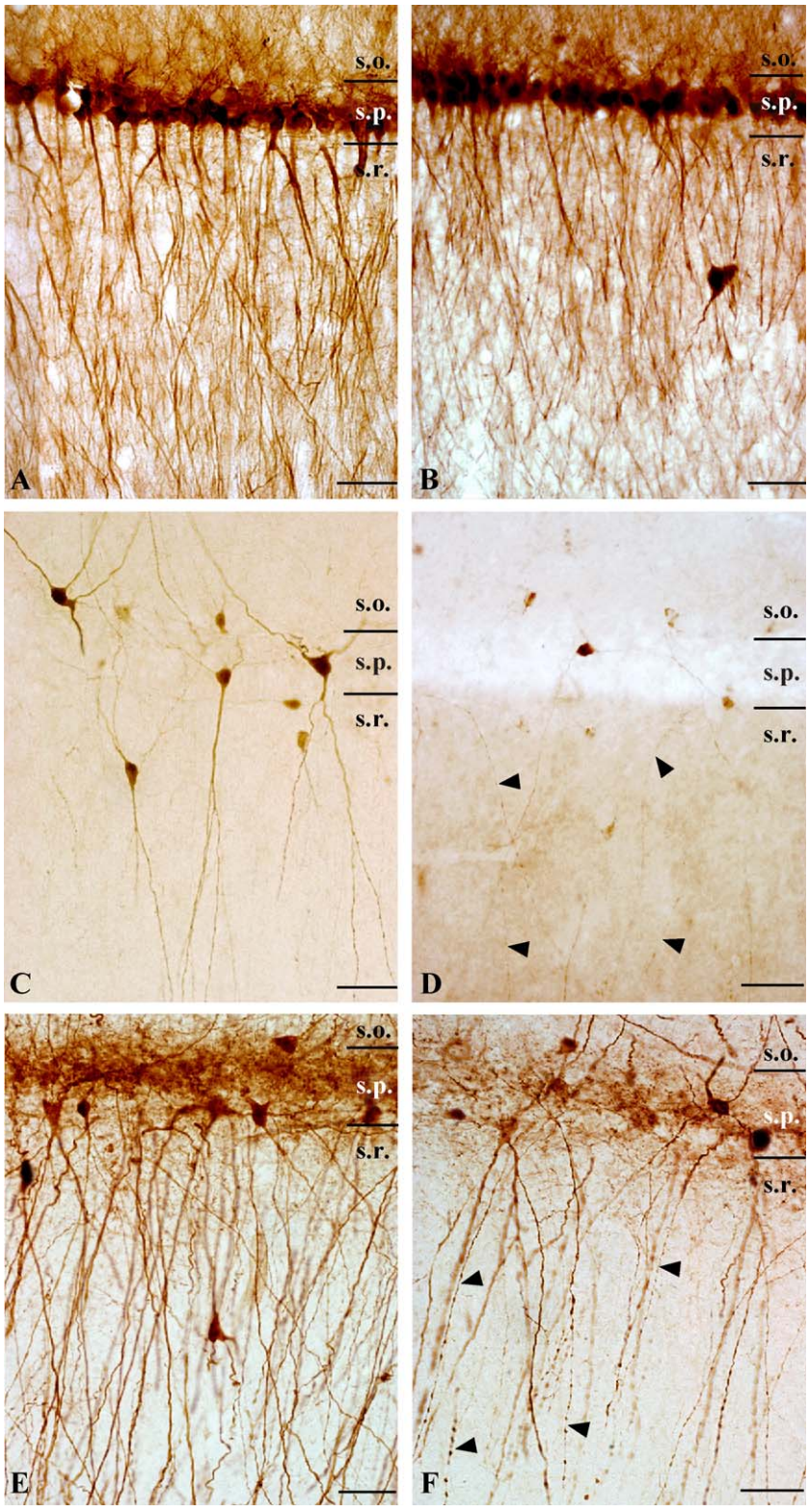

Fig. 3. High-magnification light micrographs of the CA1 subfield from sham-operated (A, C, E) and 3-AP-treated animals (B, D, F). No alterations were found in the number and distribution of CB-immunostained principal and nonprincipal cells compared to the control (A, B) after 30 days following the seizure. CR-positive cells in the CA1 region are reduced in number in the dorsal hippocampus following 3-AP treatment (D), and their dendrites become varicose and fragmented (arrowheads). The number of PV-immunopositive cell bodies decreased in patches in 3-AP-treated animals, whereas their dendrites and axonal arbors partially retained their PV-immunoreactivity (F). The dendrites are varicose and some of them fragmented (arrowheads). Layers of the CA1 region are indicated by solid lines; s.o.: str. oriens; s.p: str. pyramidale; s.r.: str. radiatum. Scale bars: A–F: 50 μm.

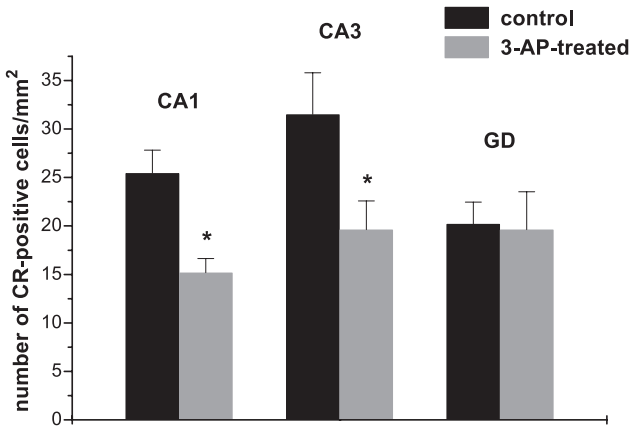

Fig. 4. Quantitative analysis of CR-positive cells in the contralateral dorsal hippocampus 30 days following seizures. Bars represent the mean and SD values of cell numbers in each subregion. Stars represent significant changes ($p < 0.05$). The number of CR-positive cells decreased significantly in the CA1 and CA3 subfields, but did not change in the area of dentate gyrus (GD: dentate gyrus).

The effect of uridine administration in the 3-AP model of epilepsy

Firstly, uridine was injected intravenously when the 3-AP application started. In uridine plus 3-AP treated rats, the maximum elevation of the extracellular level of uridine was 195.64 ± 10.23%. When uridine was not applied together with 3-AP, its concentration increased to 175.46 ± 19.53% (Fig. 6) in ictal periods. Also, the i.v. injection of 1 mg/kg uridine did not increase the level of uridine in the brain of rats more than the ictal activity itself. There was no significant difference between epileptic activity with and without uridine injection on the basis of the EEG.

Because the 1 mg/kg i.v. injection failed, we tried to apply a high dose of uridine using 100 and 500 mg/kg i.p. I.v. application of such a high dose of uridine is not possible because of its osmotic effects in the blood. Even 500 mg/kg uridine was not able to reduce the 3-AP seizures. We also measured the elevation of uridine in the brain after an i.p. injection of 100 and 500 mg/kg uridine. The dose

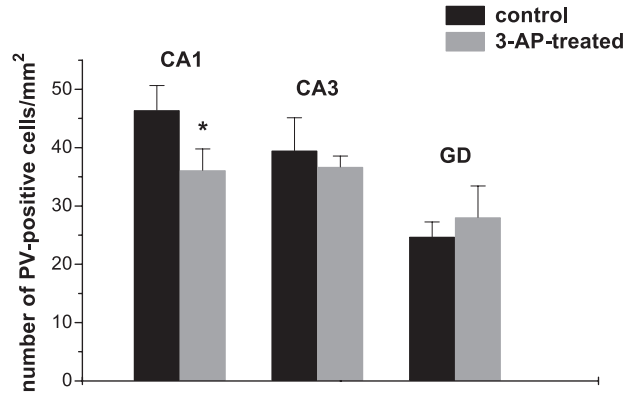

Fig. 5. Quantitative analysis of PV-positive cells in the contralateral dorsal hippocampus after 30 days following seizures. Bars represent the mean and SD values of cell numbers in each subregion. Star represents significant changes ($p < 0.05$). The number of PV-positive cells decreased in the CA1 subfield, but did not change in the CA3 subfield and in the area of dentate gyrus (GD: dentate gyrus).

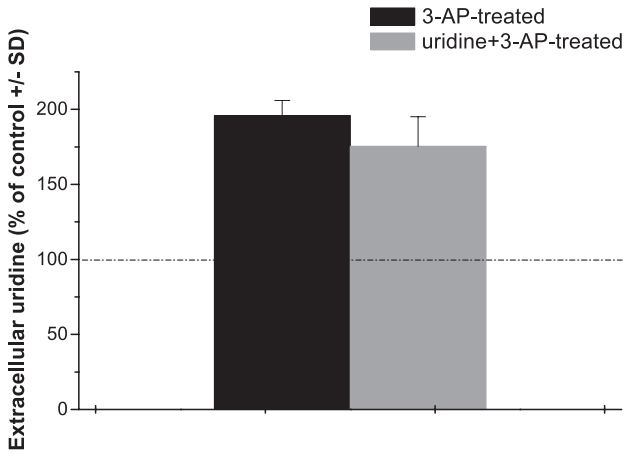

Fig. 6. Maximum elevation of extracellular uridine concentrations in percentage of the control during 3-AP-induced epileptic activity and during systemic uridine administration in 3-AP treatment.

of 100 mg/kg uridine caused an increase of 130 ± 10.8% in the uridine, and 545 ± 24.6% in the uracil level in the hippocampus. Applying 500 mg/kg uridine, the extracellular uridine level increased to 468 ± 23.5% and the uracil level increased to 773 ± 42.2%. The higher increase in uracil than in uridine indicated the extremely rapid turnover of uridine, which was even more sped up by injecting the rats with a high dose of uridine.

Discussion

In the present study, we demonstrated that uridine, adenosine, and inosine were released from hippocampal cells during epileptic activity evoked by perfusion of 3-AP, which also induces increased glutamate release and morphological changes. We failed to confirm the already-described antiepileptic effect of uridine with a single systemic injection of uridine.

We applied the 3-AP model of epilepsy in the present study because of its ictal–interictal character. Application of 4-AP or 3-AP is known to result in epileptic activity in anesthetized rats (Medina-Ceja et al., 2000; Pena and Tapia, 1999; Szente and Boda, 1994). Both 3-AP and 4-AP are potent K^+ channel blockers binding with the same affinity to the ion channels (Nino and Munoz-Caro, 2001). In recent studies, application of 4-AP was the usual drug of choice for inducing epilepsy (Medina-Ceja et al., 2000; Pena and Tapia, 1999), but we claim that 3-AP as a powerful convulsant appears to be more useful in dialysis studies, since it has better diffusion in brain tissue (Szente and Baranyi, 1987; Szente and Pongrácz, 1979).

Pyrimidines, related to purines, can be synthesized de novo within mammals. The ring structure is assembled through a multistep pathway from simple precursors to make the base orotic acid, which is then converted by a multifunctional enzyme to nucleotides. During cell turnover, nucleotides are broken down, although the greater proportion of pyrimidines is not excreted but salvaged (Connolly and Duley, 1999). Nucleosides are released by depolarization via reverse transport (Baldwin et al., 1999). They have no specific transporters, i.e. the same transporter transports different nucleosides, including uridine (Baldwin et al., 1999; Cass et al., 1999). Therefore, when the membrane potential reaches the reversal potential of nucleoside transporters, the nucleoside micro-

environment of cells will change (Dobolyi et al., 2000). In case of the 3-AP epilepsy model, however, ictal and interictal EEG patterns could be observed, so cells do not stay continuously at the transporter reversal potential, but only for a short period of time. Our present finding that uridine release correlates with the ictal periods confirms that uridine release under ictal depolarization significantly exceeds the uridine uptake capacity of the cells of the central nervous system.

With the aim of understanding the mechanisms whether uridine can really influence epileptic activity, we have already disclosed the effects of uridine on neuronal excitability. When we applied uridine by microdialysis probes, it inhibited the spontaneous unit activity in the hippocampus (Dobolyi et al., 1999). This suggests that uridine could be involved in inhibitory mechanisms. In the same experiments, however, uridine proved to be excitatory in other structures as it was observed in the case of thalamic neurons (Dobolyi et al., 1999). Uridine generated fast Ca^{2+} signals in synaptosomes (Kardos et al., 1999), suggesting that it may stimulate transmitter release. Thus, at the present level of understanding the effects of uridine on neuronal excitability, it is hard to predict whether uridine, coreleased with adenosine in epilepsy, is a general inhibitory neuromodulator, or its inhibitory effects revealed in the hippocampus are based on the uridinergic excitation of GABAergic interneurons. In the present study, our attempt to modulate epileptic seizures by systemic injection of uridine did not help in understanding the mechanism of uridine action in the nervous system, because even a single high dose of uridine, injected i.v. failed to elevate the extracellular level of uridine in the brain, so the maximum concentration of extracellular uridine during 3-AP-induced seizures was not elevated by exogenously applied uridine in our study. There are many reasons why the injection of uridine was inefficient. Uridine may not be able to get through the blood–brain barrier; or it has a high metabolic rate; or it diffuses slowly in the brain tissue. It has to be noted that local injection of uridine in dialysis experiments resulted in a very limited spread of uridine in the brain tissue, as it has only diffused 300–400 μm from the source (Dobolyi et al., 1999). Thus, we believe that for getting more detailed information about the physiological roles of uridine in the nervous system, the target protein(s) of uridine should be found and a nonmetabolizing uridine agonist and/or antagonist has to be developed.

It is important to note that the extracellular concentration of adenosine and inosine (the metabolite of adenosine) increased parallel with the frequency of epileptiform discharges. The level of adenosine remained high after washing out 3-AP, while the level of uridine returned to the baseline level during washout. In contrast to uridine, adenosine has a sustained elevation extending far longer than the seizure itself, which allows the development of late and prolonged effects of adenosine. The neuromodulatory role of adenosine depends on a balanced activation of inhibitory A_1 receptors and mostly facilitatory A_{2A} receptors (Cunha, 2001; Lopes et al., 2002). The elevation of adenosine level in the extracellular space can be both cytoprotective and cytotoxic (Abbracchio and Cattabeni, 1999). It may explain the severe side effects of adenosine application (Abbracchio et al., 1995; Von Lubitz et al., 1999).

High levels of extracellular glutamate are known to accompany epileptic activity in the hippocampal formation. Enhanced glutamate release has been described in different epilepsy models (Beal, 1992; Lancelot and Beal, 1998; Medina-Ceja et al., 2000; Meldrum, 1992; Pena and Tapia, 1999), and elevated glutamate level is likely to be responsible for inducing excitotoxic cell death (Pollard et al., 1994).

The recorded epileptiform activity and the elevated level of extracellular glutamate together with the morphological changes of hippocampal neurons suggest that 3-AP-induced seizure activity results in excitotoxic events. The unilateral 3-AP injection excited a large number of CA3 pyramidal cells, granule cells, and hilar mossy cells in the ventral hippocampus. The bilateral projection of hilar mossy cells to the dentate gyrus and the projection of ventral CA3 pyramidal cells to both the CA3 and the CA1 subfield of the dorsal hippocampus explain that morphological alterations were also found in the contralateral hippocampus. Interestingly, morphological changes were observed only in the dorsal hippocampi, while ventral regions remained preserved. The epileptic activity probably caused excitotoxic cell death (Choi, 1987, 1992; Meldrum and Garthwaite, 1990) of the most sensitive CR-positive cells in the CA1 and CA3 regions, whereas the number and morphology of CA1 and CA3 pyramidal cells was unchanged. CR-positive cells form a subpopulation of inhibitory interneurons and they terminate on different other inhibitory interneurons. As a consequence, this slight decrease in the number of CR-containing neurons may cause a decrease in the synchronization of interneurons in the CA1 and CA3 regions, since CR-containing cells are known to selectively innervate—and possibly synchronize—other inhibitory neurons in rats (Gulyás et al., 1996). Thus, we claim that a less-synchronized and in turn less-efficient inhibition enhances excitotoxic cell death.

The survival of hilar CR-positive cells is unexpected, since they were highly vulnerable in other models (Freund and Maglóczky, 1993; Maglóczky and Freund, 1993, 1995). This suggests that 3-AP injection via microdialysis probes might have been largely confined to CA3, which has only sparse projection back to the dentate gyrus (Li et al., 1994). We have also demonstrated a decrease of PV content in the somata and dendrites of CA1 interneurons, while their axons mostly retained their immunoreactivity. Disappearance of PV from cell bodies with preservation of PV-positive axon collaterals has been described earlier in epilepsy and ischemia (Bazzett et al., 1994; Johansen et al., 1990; Maglóczky and Freund, 1995; Tortosa and Ferrer, 1993; Wittner et al., 2001). Whether this may cause any functional impairment of PV-containing interneurons is unknown. Thus, 3-AP-induced epileptic seizures caused a mild damage, only moderately injuring the most sensitive interneuronal cell types in the dorsal hippocampi, and the large mass of principal cells remained intact. Therefore, the concentration changes of the measured extracellular amino acids and nucleosides were caused by epileptic activity rather than by the death and disintegration of cells. The morphological changes in the applied 3-AP epilepsy model suggest that uridine release increases in the case of epileptic activity, which does not result in extensive loss of vulnerable cells.

In former studies, antiepileptic effect of exogenously applied uridine was suggested (Page et al., 1997; Roberts, 1973; Roberts et al., 1974). In our study, we failed to reproduce the data obtained by chronic application of uridine by a single dose of uridine. Even 500 mg/kg uridine did not affect seizure activity. The most possible reason of failure in reproducing antiepileptic effect of uridine in our studies was the rapid metabolism of uridine, converting uridine excess to uracil. Our present data and our previous findings about the effects of uridine on neuronal firing rate clearly demonstrated that uridine could be released endogenously in correlation with

epileptic activity, and the endogenous release of uridine might be sufficient for the uridine to have neuromodulatory effects if it is sustained for a long-enough time. Despite the obvious difficulties of demonstrating the antiepileptic effect of uridine in pharmacological epilepsy models, our data presented here and those we published earlier, together with the findings of Roberts in the 1970s and Page in the 1990s (Page et al., 1997; Roberts, 1973; Roberts et al., 1974), suggest that there is a strong background already available about the possible neuromodulatory role of uridine and its involvement in mechanisms of epileptic activity. In conclusion, further research is needed to find the uridine-binding site or receptor and to develop agonists or antagonists for better understanding of the role of uridine in neurodegeneration induced by epileptic seizures.

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