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ANTIEPILEPTIC ACTION INDUCED BY A COMBINATION OF VIGABATRIN AND TIAGABINE

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Abstract—Vigabatrin, an inhibitor of GABA breakdown by GABA transaminase and of GABA transporter isoform 1 (GAT1), and tiagabine, a highly specific inhibitor of GAT1, have successfully been applied in the treatment of epilepsy. We investigated the effects of individual and combined application of these drugs on GAT1 expressed in *Xenopus* oocytes, and examined the effects on epileptiform discharges in the CA3 area of brain slices of genetically epileptic El and control ddY mice, and on the occurrence of seizures in El mice. Simultaneous application of vigabatrin and tiagabine inhibited epileptiform discharges induced by high-K^+ solution in the brain slices in an antagonistic fashion. The degree of inhibition by tiagabine after pre-treatment with vigabatrin was additive in ddY mice and synergistic in El mice. In Mg^{2+}-free solution, co-treatment by the two drugs produced additive inhibition in slices from both mouse strains, but pre-treatment with vigabatrin produced synergistic inhibition in slices only from ddY mice. In the slices from El mice, a combination of drugs resulted in additive effects in both co- and pre-treatment by the drugs. Although these drugs are also effective *in vivo* at suppressing seizure occurrence in El mice, the combined application does not show synergistic effects, but rather is antagonistic under the experimental conditions in this particular variant of epilepsy. The synergistic inhibition of epileptiform discharges in brain slices may, in part, have originated from the complex interaction with GAT1. In experiments on the GAT1 expressed in oocytes it could be demonstrated that synergistic inhibition occurs only at low concentration (0.1 nM) of vigabatrin. This illustrates that the oocytes may form a powerful test system for drug screening and investigation of complex drug interactions.

These results present a novel interpretation of synergistic inhibition of certain epileptic discharges using vigabatrin and another drug, and that for successful synergistic treatment of epilepsies carefully designed timed dosage regimens are essential. © 2005 Published by Elsevier Ltd on behalf of IBRO.

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Abbreviations: ACSF, artificial cerebrospinal fluid; ANOVA, analysis of variance; GABA, gamma-aminobutyric acid; GAT1, GABA transporter isoform 1; NMDA, *N*-methyl-D-aspartate; ORi, oocyte Ringer's solution.

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The dominating inhibitory neurotransmitter in mammalian brain is gamma-aminobutyric acid (GABA), and the GABAergic system has been shown to play a key role in the occurrence of pathological conditions with mismatched inhibitory synaptic transmission like in epilepsy. For inhibition of epileptic seizures, compounds have been designed that interfere with different steps within the GABAergic system, ultimately leading to enhanced or prolonged activation of postsynaptic GABA receptors. Inhibition of GABA-transaminase by e.g. vigabatrin is supposed to increase the availability of GABA, while inhibition of the GABA-uptake transporter (GAT1) e.g. by tiagabine modulates the amount and dwell time of GABA in the synaptic cleft.

To terminate synaptic transmission, GABA is taken up by transporters, which are driven by an inward-directed Na^+ gradient. In addition, release of neurotransmitter via transporters from synaptic terminals has been reported to be of functional significance (During et al., 1995; Rossi et al., 2000; Wu et al., 2001). Recently, evidence has been presented that vigabatrin is also an effective inhibitor of GABA transport (Leach et al., 1996; Eckstein-Ludwig et al., 1999). The concentration dependence of GABA transport inhibition by vigabatrin is quite complex (Eckstein-Ludwig et al., 1999). Strong inhibition of GABA uptake occurs at nano- to micromolar concentrations, relief of inhibition is observed at micro- to millimolar concentrations, and inhibition again takes place at concentrations above 1 mM. This inhibition has been attributed to a high-affinity block of the GAT1 by vigabatrin in the nano- to micromolar range in addition to vigabatrin being accepted by the transporter as substrate instead of GABA, and competition with GABA as substrate for the transporter in the upper dosage range.

Both vigabatrin and tiagabine are known to exhibit antiepileptic effects on seizures in *in vivo* and *in vitro* epilepsy models, and the combined application has been shown to exhibit synergistic effects in mouse cortex (Leach et al., 1997) and in add-on therapy in humans (Leach and Brodie, 1994). Vigabatrin seems to play a key role in the synergism, since gabapentin in combination with vigabatrin also exhibits synergistic inhibition of field potentials in hippocampal slices of guinea-pig (Lücke et al., 1998). Pre-treatment was more effective than co-application of vigabatrin for suppressing epileptiform discharges (Köhling et al., 2002).

The El mouse, derived from the ddY mouse, was internationally registered in 1964 (Imaizumi and Nakano,

1964) and has been used as an epilepsy model with secondary generalized tonic–clonic seizure (Seyfried and Glaser, 1985; King and Lamotte, 1989). Epileptic seizures of El mice can be induced by sensory stimulation such as tossing. Antiepileptic efficacies of phenobarbital (Honda, 1984) and diazepam (Mizoule et al., 1985) were reported. Electrographic studies suggested that neuronal excitability in the hippocampal formation plays an important role in the epileptogenesis of this model. GABAergic disinhibition of the hippocampal formation has been observed (Ono et al., 1997; Fueta et al., 1998) and the disinhibition of dentate granule cells in the hippocampal slice was restored by application of the GABAergic antiepileptic drug flunitrazepam at clinical concentrations (Ono et al., 1997). Furthermore, we recently provided results indicating reduced GABA release from interneurons in CA1 (Lambert et al., 1996) in El mice leading to decreased GABAergic inhibition. In another study we showed that GABA uptake by the GABA transporter GAT1 expressed in *Xenopus* oocytes was enhanced when the cells were co-injected with hippocampal mRNA from El mice, as compared with mRNA from ddY mice (Fueta et al., 2003). This also suggests a decrease in GABAergic function. Therefore, enhancement of GABAergic inhibition by the uptake inhibitor tiagabine or the transaminase inhibitor vigabatrin may have potential antiepileptic effects.

To elucidate the basic mechanisms of antiepileptic action of combined application of vigabatrin and tiagabine, we started with a simple model system, the *Xenopus* oocyte with the expressed GAT1. We then proceeded to analyze the occurrence rate of epileptiform discharges in the epilepsy model using hippocampal slices from El and ddY mice. Discharges were initiated by high-K^+ or Mg^{2+}-free solution. In the current study we present the potential antiepileptic effects of combined application of the two drugs on epileptiform discharges in the hippocampal slices. Furthermore, we also investigated the occurrence of seizures *in vivo*.

EXPERIMENTAL PROCEDURES

Experiments with oocytes

Females of the clawed toad *Xenopus laevis* were anesthetized with tricaine (MS222; Sandoz, Basel, Switzerland; 1 g/l). Parts of the ovary were removed and treated with collagenase. Full-grown prophase-arrested oocytes were selected for experiments. For expression, cRNA of GAT1 of mouse brain (about 40 ng/oocyte) was injected. The cDNA clone was kindly provided by Dr. J. Fei (Institute of Biochemistry & Cell Biology, Chinese Academy of Sciences, Shanghai, PRC). These cells, together with non-injected control oocytes, were stored at 19 °C in oocyte Ringer's solution (ORi; in mM: 90 NaCl, 2 KCl, 2 $CaCl_2$, 5 morpholinopropane sulfonic acid/Tris adjusted to pH 7.4 with NaOH or Tris) containing antibiotics (in mg/l: 70 gentamicin or 25 streptomycin plus 20 penicillin). Experiments were performed after 3–5 days of incubation at room temperature (22–24 °C).

For determining the maximum transport activity of the neurotransmitter transporter, uptake of [^{3}H]-labeled GABA (Amersham, Braunschweig, Germany) was measured at 90 mM external Na^+ and 100 μM total GABA concentration. The oocytes were incubated for 20 min in a solution (volume 200 μl) of ORi containing the GABA with 15 nM [^{3}H]-labeled GABA (9.25 kBq/200 μl). For determination of non-specific uptake, measurements were performed in parallel with non-injected control oocytes, or with oocytes kept in a Na^+-free incubation solution, where NaCl was replaced by tetramethylammonium chloride. Non-specific uptake is less than 10% of the uninhibited transporter-mediated uptake. To exclude oocytes with high membrane leakage, 1 mM sucrose with 18 μM [^{14}C] sucrose (16 kBq/200 μl; DuPont NEN, Bad Homburg, Germany) was added to the incubation medium (see Schmalzing et al., 1991).

Effects of combined application of vigabatrin and tiagabine have been reported to increase the level of GABA in mouse cortex (Leach et al., 1997). Based on the complex dependency of vigabatrin action on the GAT1 (see introduction and (Eckstein-Ludwig et al., 1999), an explanation for an additive or a synergistic effect had been suggested (Eckstein-Ludwig et al., 2000). We tested this hypothesis experimentally on GAT1 isolated from the other components of the GABAergic system by expressing GAT1 of mouse brain in *Xenopus* oocytes. The GAT1 in its isolated environment is very sensitive to tiagabine, and uptake was completely inhibited by 20 μM tiagabine. Therefore, in the present oocyte experiments we used only 1 μM ($K_{1/2}$ approximately 2 μM; see Eckstein-Ludwig et al., 1999).

Mice

Seizure-susceptible El mice and the control ddY mice, from which the El mice had been derived, were obtained from the breeding colony at the Animal Research Center of the University of Occupational and Environmental Health. They were bred under the conditions of a 12-h light/dark cycle, constant temperature (23±1 °C) and humidity (55±5%), and free-watering and feeding. The experiments were performed under the control of the Ethics Committee of Animal Care and Experimentation in accordance with The Guiding Principle for Animal Care Experimentation, University of Occupational and Environmental Health, Japan that conformed to the National Institutes of Health Guide for the Care and Use of Laboratory Animals, and the Japanese Law for Animal Welfare and Care (No. 221). All efforts were made to minimize the number of animals used and their suffering.

Slice preparation

Transverse slices of 450 μm thickness were obtained from the middle third region of hippocampi with a McIlwain tissue chopper (Campden Instruments Ltd., IN, USA) after decapitation under deep anesthesia with diethylether. The hippocampal slices were transferred to an interface-type recording chamber, where they were allowed to equilibrate for at least 1 h. The chamber temperature was controlled at 32±0.5 °C, and the slices were continuously perfused (1 ml/min) with oxygenated (95% O_2, 5% CO_2) artificial cerebrospinal fluid (ACSF; pH 7.4) of the following composition (in mM): NaCl 124, KCl 2, KH_2PO_4 1.25, $CaCl_2$ 2, $MgSO_4$ 2, $NaHCO_3$ 26, and glucose 10. After 1-h stabilization of slices in the interface chamber, perfusion medium was switched from ACSF to a high-K^+ medium (so-called high-K^+ model) or Mg^{2+}-free (so-called 0-Mg model) medium. For high-K^+ medium, the K^+ concentration was raised from 3.25 to 8.0 mM by adding KCl to ACSF. For Mg^{2+}-free medium, $MgSO_4$ was excluded from ACSF.

In order to examine antiepileptic effects induced by a single application of vigabatrin and tiagabine, or a combination of the drugs, in *in vitro* high-K^+ and 0-Mg slice models, we made six groups for each mouse strain (Fig. 1): (A) control, (B) single application of vigabatrin (B-1) and tiagabine (B-2 for 3 h, B-0 for 0.5 h), (C) simultaneous application of vigabatrin and tiagabine, (D) application of tiagabine at the end of the 2.5 h treatment of vigabatrin. Antiepileptic effects in the multiple groups were compared at the end of the drug perfusion (timing indicated with the thick arrows in Fig. 1). In the present study, 87 slices from 44 El (19±1 week-old) and 85 slices from 43 ddY (20±1 week-old) mice