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Interaction between Ca^{2+}, K^+, carbamazepine and zonisamide on hippocampal extracellular glutamate monitored with a microdialysis electrode

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1 Multiple components of hippocampal glutamate release were examined by study of Ca^{2+}- and K^+-evoked hippocampal extracellular glutamate release using an *in vivo* microdialysis glutamate biosensor in urethane-anaesthetized rats. In addition, the effects of the antiepileptic drugs, carbamazepine (CBZ) and zonisamide (ZNS) perfused through the probe on glutamate release were assessed.

2 Basal glutamate levels were below detection limits ($\sim$0.1 μM). An increase in extracellular KCl (from 2.7 to 50 and 100 mM) increased extracellular hippocampal glutamate levels to 9.2 ± 1.4 and 20.0 ± 2.6 μM, respectively, calculated from the area under curve (AUC) for 60 min.

3 This KCl-evoked glutamate release consisted of three components: an initial transient rise, a late gentle rise, and late multiple phasic transient rises.

4 An increase in or removal of extracellular $CaCl_2$ levels respectively enhanced and reduced the 50 mM KCl-evoked hippocampal glutamate release (AUC for 60 min) from 9.2 ± 1.4 to 12.4 ± 2.1 and 5.8 ± 0.9 μM.

5 Perfusion with 100 μM CBZ or 1 mM ZNS inhibited both the 50 mM KCl-evoked hippocampal glutamate release (AUC for 60 min) from 9.2 ± 1.4 to 5.5 ± 1.1 and to 5.8 ± 1.3 μM, respectively, as well as the stimulatory effects of Ca^{2+} on KCl-evoked hippocampal glutamate release.

6 These results suggest that both CBZ and ZNS may reduce epileptiform events by inhibiting excitatory glutamatergic transmission.

Keywords: carbamazepine; glutamate; microdialysis electrode; spreading depression; zonisamide

Introduction

Carbamazepine (CBZ), a frequently prescribed major antiepileptic drug, has a wide clinical spectrum of use in simple and complex partial seizures (Loiseau & Duche, 1995), and provides a useful alternative in the treatment of trigeminal neuralgia (Fromm *et al*., 1984), schizophrenia (Neppe, 1982), mood disorder (Okuma *et al*., 1990) and anxiety disorders (Kerk *et al*., 1992). A novel antiepileptic drug, 3-sulfamoylmethyl-1,2-benzisoxazol (zonisamide; ZNS), is effective for the treatment of generalized tonic-clonic seizures, secondarily generalized seizures and simple and complex partial seizures (Rogawski & Porter, 1990; Seino *et al*., 1995). Due to the rarity of serious side effects and the potent antiepileptic effect, ZNS has been considered one of the major antiepileptic drugs in Japan (Seino *et al*., 1995).

Essentially two mechanisms of action of both CBZ and ZNS in the central nervous system have been proposed (Rogawski & Porter, 1990; Macdonald, 1995; Seino *et al*., 1995). One involves the action of CBZ and ZNS on neuronal ion channels. Both CBZ and ZNS reduce sustained, high-frequency, repetitive firing comprised of Na^+ channel activity (Rogawski & Porter, 1990; Macdonald, 1995; Seino *et al*., 1995). The other involves the actions of CBZ and ZNS on synaptic transmission including neurotransmitter receptors and release (Rogawski & Porter, 1990; Macdonald, 1995; Seino *et al*., 1995). Thus, CBZ has binding affinity to adenosine receptors (Marangos *et al*., 1983; Okada *et al*., 1997a) and peripheral benzodiazepine receptors without affecting other receptors (Rogawski & Porter, 1990) whilst ZNS binds to the γ-aminobutyric acid (GABA)-benzodiazepine receptor-ionophore complex (Rogawski & Porter, 1990; Mimaki *et al*., 1991). Further, therapeutic plasma concentrations of both CBZ and ZNS enhance monoamine synthesis and transmission (Okada *et al*., 1992, 1995, 1997b).

Several neurochemical experiments have indicated that therapeutic concentrations of CBZ and ZNS act on excitatory glutamatergic function (Rogawski & Porter, 1990; Macdonald, 1995; Takano *et al*., 1995; Waldmeier *et al*., 1996; Owen *et al*., 1997). For example, CBZ reduced both the glutamate receptor agonist-induced neuronal response (Rogawski & Porter, 1990; Macdonald, 1995) and *in vivo* glutamate release (Waldmeier *et al*., 1996). Whether ZNS influences the glutamatergic system has not yet been clarified fully. ZNS did not suppress the epileptic activity induced by injection of kainate into the amygdala (Takano *et al*., 1995), but reduced ischaemia-induced glutamate release (Owen *et al*., 1997).

Glutamate is the principal excitatory neurotransmitter in the central nervous system, and excessive release of glutamate may produce seizures in epileptic patients (Carlson *et al*., 1992; Ronne-Engstrom *et al*., 1992; During & Spencer, 1993) and in various animal models (Rogawski & Porter, 1990; Ueda & Tsuru, 1994; Macdonald, 1995; Walker *et al*., 1995). Early studies utilizing *in vivo* microdialysis, however, failed to demonstrate a significant elevation of extracellular glutamate levels during seizures induced by the administration of several types of proconvulsant under conditions whereby glutamate uptake was inhibited (Millan *et al*., 1991, 1993). More recently, hippocampal extracellular glutamate levels have been shown to increase following seizure onset, or to be associated with

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seizure onset in epileptic patients with partial seizures (Carlson *et al*., 1992; Ronne-Engstrom *et al*., 1992; During & Spencer, 1993). Furthermore, in epileptic patients, the elevation of hippocampal extracellular glutamate levels was not observed during or after discharges without epileptic seizures (Ronne-Engstrom *et al*., 1992). Amygdaloid kindling has now been shown to be accompanied by a progressive, transient, stimulus-induced elevation of extracellular glutamate levels during the first minute post-stimulus in both hippocampi (Ueda & Tsuru, 1994). These findings suggest that the mechanisms of epileptic seizure and non-epileptic convulsions differ depending upon the occurrence of enhancement of glutamatergic transmission.

In order to characterise the various types of hippocampal glutamate release in more detail, we determined Ca^{2+}- and K^{+}-evoked hippocampal glutamate release by using an *in vivo* microdialysis electrode. In addition, we studied the effects of antiepileptic drugs, CBZ and ZNS, on Ca^{2+}- and K^{+}evoked hippocampal glutamate release.

Methods

Glutamate biosensor preparation

The dialysis electrode (0.25 mm diameter; 2 mm exposed membrane), which was purchased from Sycopel International Ltd. (London, UK), was fixed to a microdialysis probe (0.22 mm diameter; 2 mm exposed membrane, EICOM Co., Kyoto, Japan) and Teflon-coated twin (each spaced 0.1 mm apart) stainless steel recording electrodes (0.08 mm diameter, Unique Medical Co., Tokyo, Japan). Figure 1 shows the detailed structure of the microdialysis glutamate biosensor. The dialysis electrode was filled with a phosphate-modified Ringer's solution (PMRS) containing (in mM): 142.0 NaCl, 2.7 KCl, 1.0 $MgCl_2$, and buffered to pH 7.40 with 0.7 NaH_2PO_4/3.6 Na_2HPO_4. The dialysis electrode was prepared by filling it with PMRS and immersing the membrane in a beaker of 5 mM O-phenylenediamine (Nacalai Tesque INC, Kyoto, Japan) in PMRS bubbled with 100% N_2 for 15 min while constantly stirring the PMRS. The dialysis electrode was connected to an EPS-800 (EICOM Co., Kyoto, Japan), and a voltage clamp was switched on at +650 mV for 15 min to induce electropolymerization, with continuous bubbling and stirring. Upon completion of electropolymerization, the potential was switched off. The dialysis electrode membrane was removed from the O-phenylenediamine solution, and the membrane portion was stored in PMRS. After 20 min, the solution in the dialysis electrode was replaced with fresh PMRS using a perfusion pump, and the current was set at +650 mV and allowed to stabilize. Optional ascorbate (Nacalai Tesque INC, Kyoto, Japan), dopamine (Nacalai Tesque INC, Kyoto, Japan), serotonin (Nacalai Tesque INC, Kyoto, Japan) and noradrenaline (Nacalai Tesque INC, Kyoto, Japan) calibrations were then carried out while the bulk solution was being stirred, and a small amount of either concentrated ascorbate (less than 500 μM), dopamine (less than 10 μM), serotonin (less than 10 μM) or noradrenaline (less than 1 μM) was added. This procedure tested the efficacy of O-phenylenediamine coverage to avoid such compounds from being oxidized electrochemically using the working electrode (Asai *et al*., 1996).

Glutamate oxidase, which was purchased from Yamasa Co. Ltd. (Chiba, Japan), was dissolved in PMRS. The dialysis electrode was set at a potential of +650 mV and filled with PMRS containing the glutamate oxidase (100 $U.ml^{-1}$). An analysis of the calibration was then performed based on a concentrated L-glutamate solution (Walker *et al*., 1995; Asai *et al*., 1996).

Materials and microdialysis system preparation

Male Wistar rats (Clea, Japan), weighing 250–300 g, were housed under conditions of constant temperature (25±2°C)

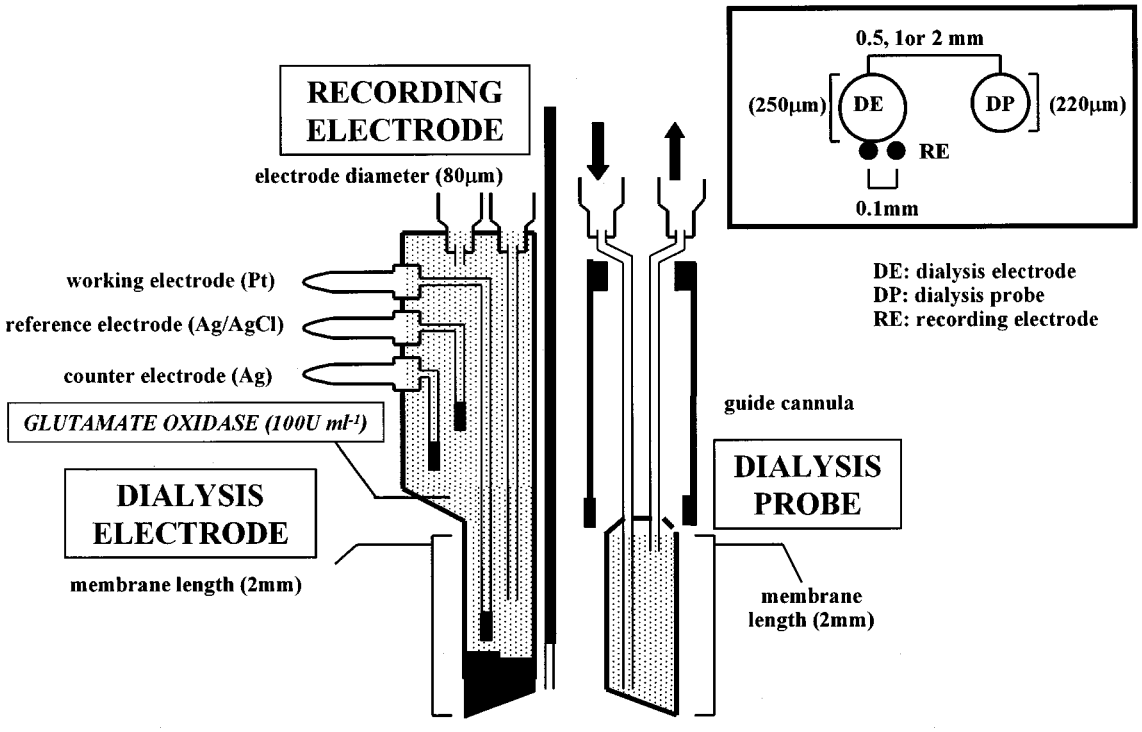

Figure 1 Diagram of the microdialysis glutamate biosensor. It is composed of a dialysis electrode for measurement of extracellular glutamate, a dialysis probe for applying the various agents and a recording electrode for analysis of neuronal firing frequency.

with a 12 h light-dark cycle. The experimental protocols used in this study were approved by the ethical committee of Hirosaki University.

Each rat was anaesthetized with 1.0 $g.kg^{-1}$ urethane (s.c), placed in a stereotaxic frame (Asai *et al*., 1996) and rectal temperature was kept at 37°C using a heating pad (KN-474, Natume Co., Tokyo, Japan). The glutamate biosensor was implanted into the hippocampus (A = −5.8 mm, L = 4.8 mm, V = −4.0 mm relative to the bregma; Okada *et al*., 1997c,d) and the perfusion experiments were started at least 6 h after the hippocampal extracellular glutamate levels had reached a plateau. The perfusion rate was maintained at 1.0 $\mu l.min^{-1}$, using a modified Ringer's solution (MRS) composed of (in mM): 143 NaCl, 2.7 KCl, 1.2 $CaCl_2$, 1.0 $MgCl_2$, 0.2 ascorbate, with 2.0 NaH_2PO_4 and 1.1 Tris to adjust to pH 7.4 (Okada *et al*., 1996a,b). To study the effects of an increase in extracellular Ca^{2+} or K^+ levels ($CaCl_2$- or KCl-evoked) on hippocampal glutamate release, MRS including either 3.4 mM $CaCl_2$ (high Ca^{2+} level MRS: HCMRS), 50 mM KCl (intermediate K^+ level MRS: IKMRS), 100 mM KCl (high K^+ level MRS: HKMRS), both 3.4 mM $CaCl_2$ and 50 mM KCl (high Ca^{2+} and intermediate K^+ level MRS: HCIKMRS), both Ca^{2+}-free and 50 mM KCl (intermediate K^+ level MRS with no added calcium: FCIKMRS), or both Ca^{2+}-free and 40 mM $MgCl_2$ (FCMRS) was infused for 60 min (Okada *et al*., 1996a). The ionic composition was modified and isotonicity was maintained using an equimolar reduction in NaCl. To study the effects of CBZ and ZNS on basal hippocampal extracellular glutamate levels, and $CaCl_2$- or KCl-evoked hippocampal glutamate release, 100 μM CBZ or 1 mM ZNS was dissolved in each perfusing solution (MRS, HKMRS, IKMRS, HCIKMRS, or FCIKMRS). The composition of each MRS is specified in Table 1.

Recording the neuronal firing frequency

The neuronal firing frequency was recorded by a telemeter (Unimec Co., Tokyo, Japan) which was set at a bandpass of 0.1 – 3 kHz, and was fed into the computer as a discharge rate.

Diffusion rate of carbamazepine and zonisamide

To estimate the rates of diffusion of CBZ and ZNS through the membrane, the dialysis probes were perfused at a flow rate of 1.0 $\mu l.min^{-1}$ and placed in the perfusing solution *in vitro*. The temperature was maintained at 37°C during dialysis using a perfusion warmer (Okada *et al*., 1997a,d, 1988). The amount of CBZ and ZNS that diffused through the dialysis tube into the extramembrane solution in 120 min was determined by high-performance liquid chromatography (HPLC) according to the method of Juergens (Juergens, 1987).

Statistics

The differences between the mean hippocampal extracellular glutamate levels under the conditions of perfusion with each type of perfusate, with or without antiepileptic drugs, were analysed using two-way analyses of variance with Tukey's multiple comparison test. The level of statistical significance was set at $P < 0.05$.

Results

The *in vitro* limits of detection at a signal-to-noise ratio of 5 : 1 was in the range of 0.1 μM. The microdialysis electrode could not determine the absolute basal extracellular glutamate levels during perfusion with MRS (pre-stimulation) due to the insufficient sensitivity of the electrode but did allow real-time monitoring of the changes in extracellular glutamate levels induced by various stimuli (e.g. 50 mM or 100 mM KCl).

Diffusion of carbamazepine and zonisamide through the dialysis probe

Diffusion estimates of CBZ and ZNS perfused through the dialysis tube for 120 min were 20.2 ± 1.9% and 18.7 ± 2.6%, respectively. This indicates that the effective concentrations of CBZ and ZNS in the brain tissue were 20.2 ± 1.9 μM and 187 ± 26 μM, respectively and such concentrations are within the therapeutic range (Masuda *et al*., 1979).

Effects of 100 mM KCl on hippocampal extracellular glutamate levels and neuronal firing frequency

The effects of 100 mM KCl-evoked stimulation (HKMRS perfusion) on hippocampal extracellular glutamate levels and neuronal firing frequency are represented in Figure 2. When the distance between the microdialysis electrode and the probe was 0.5 mm, the application of HKMRS (100 mM KCl) for 60 min produced multiple phasic rises in extracellular glutamate levels (20.0 ± 2.6 μM), which was calculated from the area under the curve (AUC), and increased the neuronal firing frequency from 17.2 ± 4.6 Hz to 254.3 Hz (maximum) at 9.43 min. The mean firing frequency during perfusion with HKMRS was 69.9 ± 18.5 Hz. These 100 mM KCl-evoked multiple phasic rises of glutamate release were composed of an initial transient rise, which was dependent upon the increase in neuronal firing frequency, and followed by late phasic rises, which were independent of neuronal firing frequency. When the distance between the microdialysis electrode and the probe was 1 mm, the neuronal firing frequency-dependent, KCl-evoked initial transient rise in extracellular glutamate levels was abolished, whereas the gentle rise and multiple phasic transient rises, which were coincident with a transient

Table 1 Composition of modified Ringer's solutions

	NaCl	*KCl*	*$CaCl_2$*	*$MgCl_2$*	*NaH_2PO_4*	*Na_2HPO_4*	*Tris*
MRS	143.0	2.7	1.2	1.0	2.0	0	1.1
HKMRS	45.7	100.0	1.2	1.0	2.0	0	1.1
IKMRS	95.7	50.0	1.2	1.0	2.0	0	1.1
HCMRS	132.9	2.7	3.4	1.0	2.0	0	1.1
FCMRS	144.4	2.7	0	1.0	2.0	0	1.1
HCIKMRS	85.6	50.0	3.4	1.0	2.0	0	1.1
FCIKMRS	97.1	50.0	0	1.0	2.0	0	1.1
PMRS	142.0	2.7	0	1.0	0.7	3.6	0 (mM)

reduction of the neuronal firing frequency, still occurred. When the distance between the microdialysis electrode and the probe was 2 mm, the initial transient rise was not seen and the gentle rise was reduced in size. However, multiple phasic transient rises were observed.

Effects of Ca^{2+} and K^+ on hippocampal glutamate release

The effects of Ca^{2+} and K^+ on hippocampal extracellular glutamate levels, as determined by the microdialysis electrode with the distance between the electrode and the probe set at 0.5 mm, are shown in Figures 3, 4 and 7a,b,c,d,e. Application of either HKMRS (100 mM KCl and 1.2 mM $CaCl_2$), IKMRS (50 mM KCl and 1.2 mM $CaCl_2$), HCIKMRS (50 mM KCl and 3.4 mM $CaCl_2$) or FCIKMRS (50 mM KCl with no added calcium) for 60 min produced multiple phasic rises of extracellular glutamate levels. These KCl-evoked multiple phasic rises in glutamate release consisted of an initial transient rise and late phasic rises. The increases in extracellular glutamate level induced by perfusion with HKMRS for the initial transient rise (23.0 ± 1.8 μM) and late phasic rises (18.5 ± 3.0 μM), were more than those induced by perfusion with IKMRS (initial transient rise 15.5 ± 1.1 μM and late phasic rise 6.0 ± 1.5 μM; $P<0.01$ (Figures 3 and 7c,d). The 50 mM KCl (IKMRS)-evoked multiple phasic rises in extracellular glutamate levels, calculated from the AUC of the initial transient rise and late phasic rises, were increased and decreased by an increase (HCIKMRS) and a decrease (FCIKMRS) in extracellular Ca^{2+} level ($P<0.05$), respectively (Figures 3 and 7a,b). In addition, switching the perfusion medium from HKMRS to MRS did not reduce the extracellular glutamate level that was previously elevated by HKMRS. In some cases, the extracellular glutamate levels were decreased and increased by perfusion with FCMRS (N = 4/6) and HCMRS (N = 2/6), respectively (Figure 4). However, this was not a significant change in the extracellular glutamate levels under the conditions of perfusion of MRS with FCMRS and HCMRS (Figure 7e).

Effects of antiepileptic drugs on basal, Ca^{2+}- and K^+-evoked hippocampal glutamate release

The effects of 1 mM ZNS (estimated effective concentration: 187 μM) and 100 μM CBZ (estimated effective concentration: 20.2 μM) on basal hippocampal extracellular glutamate levels, when the distance between the microdialysis electrode and the probe was set at 0.5 mm, are shown in Figures 4 and 7f. At least 3 h after perfusion with MRS, when the extracellular glutamate level had reached a plateau, the perfusion medium was switched to MRS containing either 1 mM ZNS or 100 μM CBZ. Neither 1 mM ZNS nor 100 μM CBZ caused an elevation measurable above basal extracellular glutamate levels (Figures 4 and 7f).

The effects of 1 mM ZNS and 100 μM CBZ on $CaCl_2$- and KCl-evoked hippocampal glutamate release, when the distance between the microdialysis electrode and the probe was set at 0.5 mm, are shown in Figures 5 and 6, respectively. At least 3 h after perfusion with MRS containing 1 mM ZNS or 100 μM CBZ, when the extracellular glutamate level had reached a plateau, the perfusion medium was switched to either HKMRS, HCIKMRS, IKMRS or FCIKMRS containing either 1 mM ZNS or 100 μM CBZ. Both ZNS and CBZ inhibited the initial transient rise as well as the late phasic rises in KCl-evoked hippocampal glutamate release (Figures 5, 6 and 7c,d). The stimulatory effect of 3.4 mM $CaCl_2$-evoked stimulation on 50 mM KCl-evoked hippocampal glutamate release was also significantly inhibited by both ZNS and CBZ ($P<0.05$, Vs no antiepileptic drugs) (Figures 5, 6 and 7a,b).

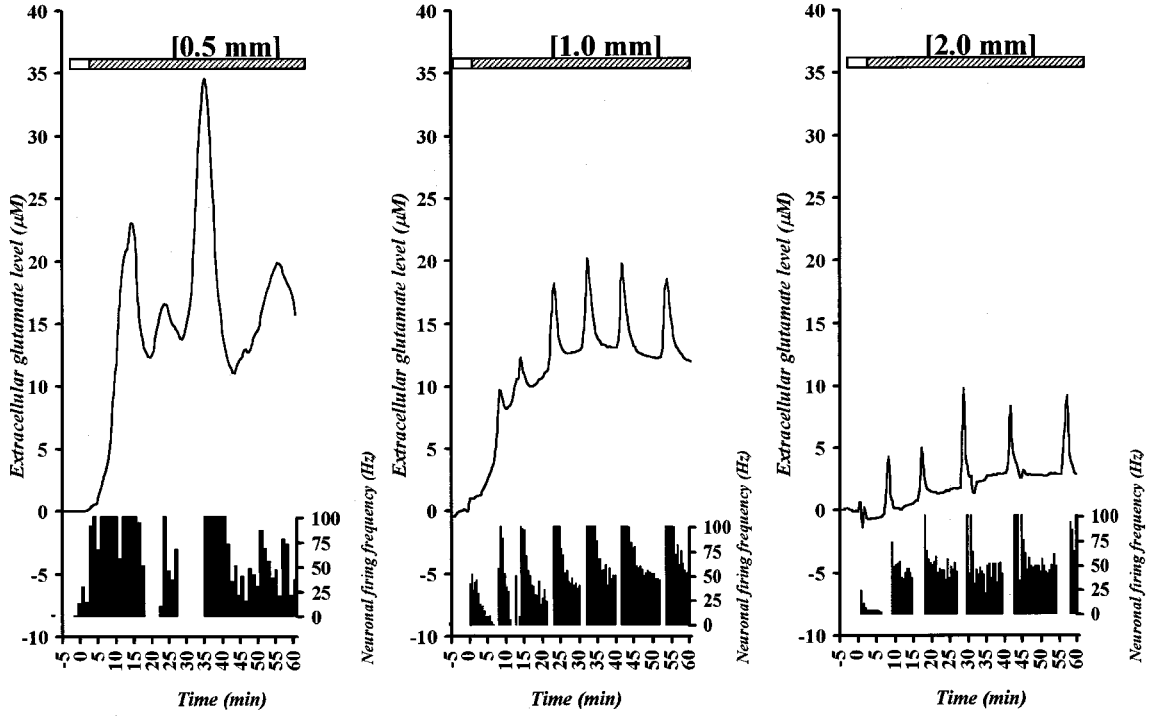

Figure 2 Effects of distance between microdialysis electrode and probe on 100 mM KCl-evoked hippocampal glutamate release and neuronal firing frequency. The left ordinate indicates the hippocampal extracellular glutamate level (μM) and the right ordinate represents an increase in the neuronal firing frequency (Hz) by perfusion with HKMRS. The open and striped portions of the upper bar show the perfusion with MRS and HKMRS, respectively.

Comparison between the effects of CBZ and ZNS on KCl-evoked hippocampal glutamate release

A comparison of the effects of 1 mM ZNS and 100 μM CBZ on $CaCl_2$- and KCl-evoked hippocampal glutamate release is shown in Figure 7. ZNS (IKMRS: 39.3±5.46% reduction; HKMRS: 20.0±6.4% reduction) and CBZ (IKMRS: 19.0±7.0% reduction; HKMRS: 10.7±8.0% reduction) inhibited the initial transient rise of 50 and 100 mM KCl-evoked glutamate release ($F=49.2$, $P<0.05$); the inhibitory effect of ZNS was stronger than that of CBZ ($P<0.05$) (Figure 7c). On the other hand, both ZNS (IKMRS: 32.7±15.8% reduction; HKMRS: 29.3±12.4% reduction) and CBZ (IKMRS: 66.1±17.9% reduction; HKMRS: 60.1±14.9% reduction) inhibited the later phasic rises in KCl-evoked glutamate release ($F=24.7$, $P<0.05$). The inhibitory effect of

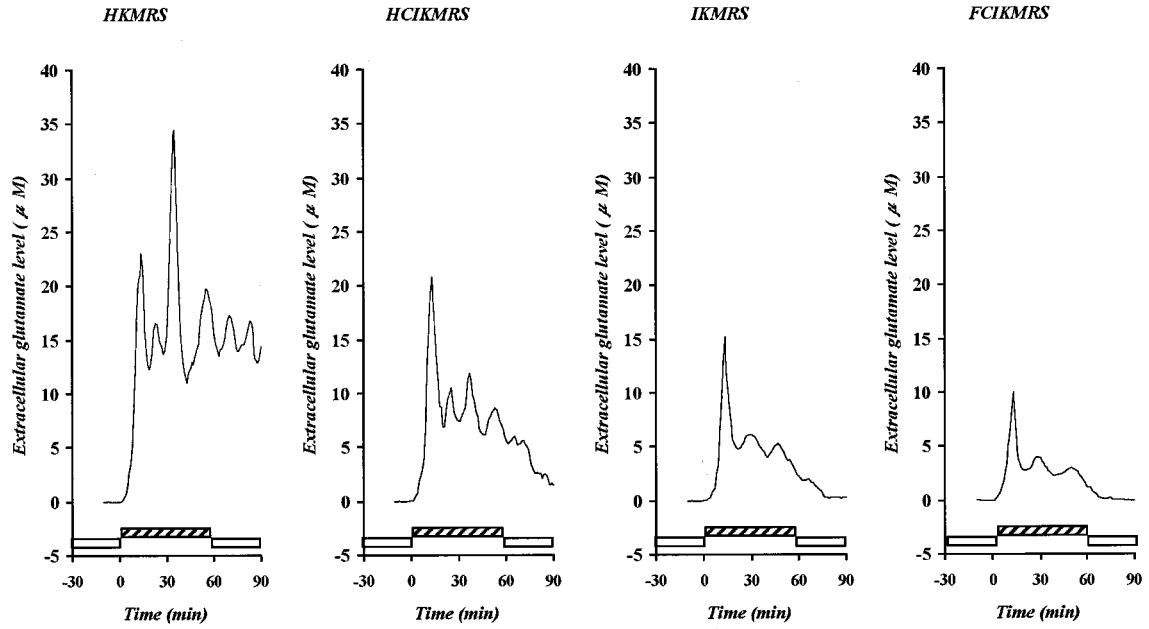

Figure 3 Changes in hippocampal extracellular glutamate level (μM) with time by sustained perfusion of HKMRS (100 mM KCl), IKMRS (50 mM KCl), HCIKMRS (50 mM KCl and 3.4 mM $CaCl_2$) or FCIKMRS (50 mM KCl with no added calcium). The open bars show the perfusion with MRS. The striped bars show the perfusion of HKMRS, HCIKMRS, IKMRS or FCIKMRS.

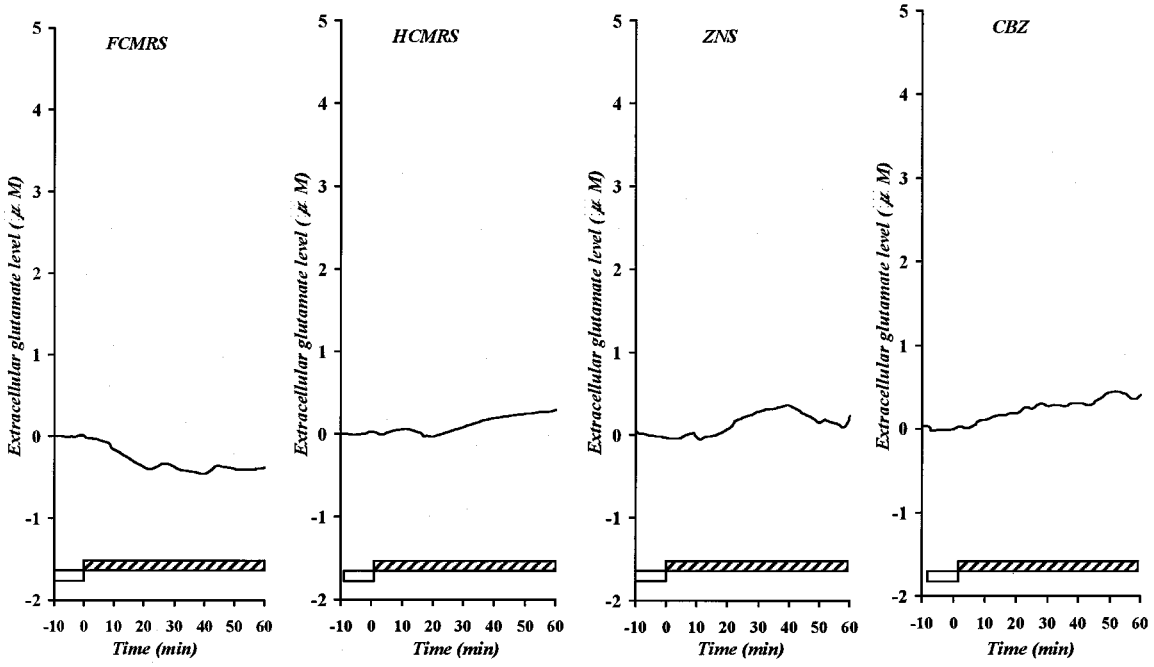

Figure 4 Changes in hippocampal extracellular glutamate level (μM) by sustained perfusion of FCMRS (MRS with no added calcium), HCMRS (3.4 mM $CaCl_2$), 1 mM ZNS or 100 μM CBZ. The open bars show the perfusion with MRS. The striped bars show the perfusion of FCMRS, HCMRS, 1 mM ZNS or 100 μM CBZ.

ZNS on the late phasic glutamate release was weaker than that of CBZ ($P<0.05$) (Figure 7d). The stimulatory effects of Ca^{2+} on KCl-evoked initial transient rise of extracellular glutamate level were significantly inhibited by both of these antiepileptic drugs ($F=8.86$, $P<0.05$); the inhibitory effect of ZNS was stronger than that of CBZ ($P<0.05$) (Figure 7a). The stimulatory effects of Ca^{2+} on KCl-evoked late phasic rises of extracellular glutamate level were also significantly inhibited by both of these antiepileptic drugs ($F=21.9$, $P<0.01$); the inhibitory effect of CBZ was stronger than that of ZNS ($P<0.05$) (Figure 7b).

Discussion

Many previous results as well as the present study have demonstrated that basal extracellular glutamate is K^+-sensitive and Ca^{2+}-insensitive (Westerink *et al*., 1987, 1988; Obrenovitch *et al*., 1993, 1996; Zilkha *et al*., 1995), although one study has reported a reduction of hippocampal extracellular glutamate level in response to removal of Ca^{2+} from the perfusate (Rowley *et al*., 1995). In that report, perfusion with Ca^{2+}-free perfusate reduced the extracellular glutamate level by about 0.1 μM (Rowley *et al*., 1995). The limit of

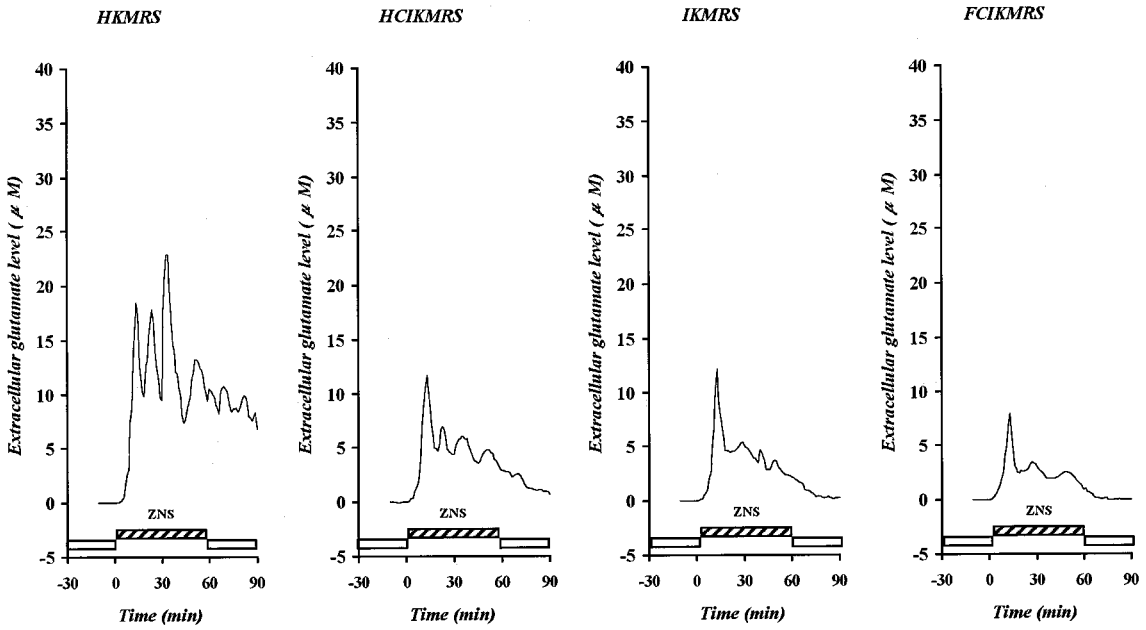

Figure 5 Effects of ZNS on K^+- and Ca^{2+}-evoked hippocampal extracellular glutamate (μM). The open bars show the perfusion with 1 mM ZNS. The striped bars show the perfusion of each HKMRS (100 mM KCl), IKMRS (50 mM KCl), HCIKMRS (50 mM KCl and 3.4 mM $CaCl_2$) or FCIKMRS (50 mM KCl with no added calcium) containing 1 mM ZNS.

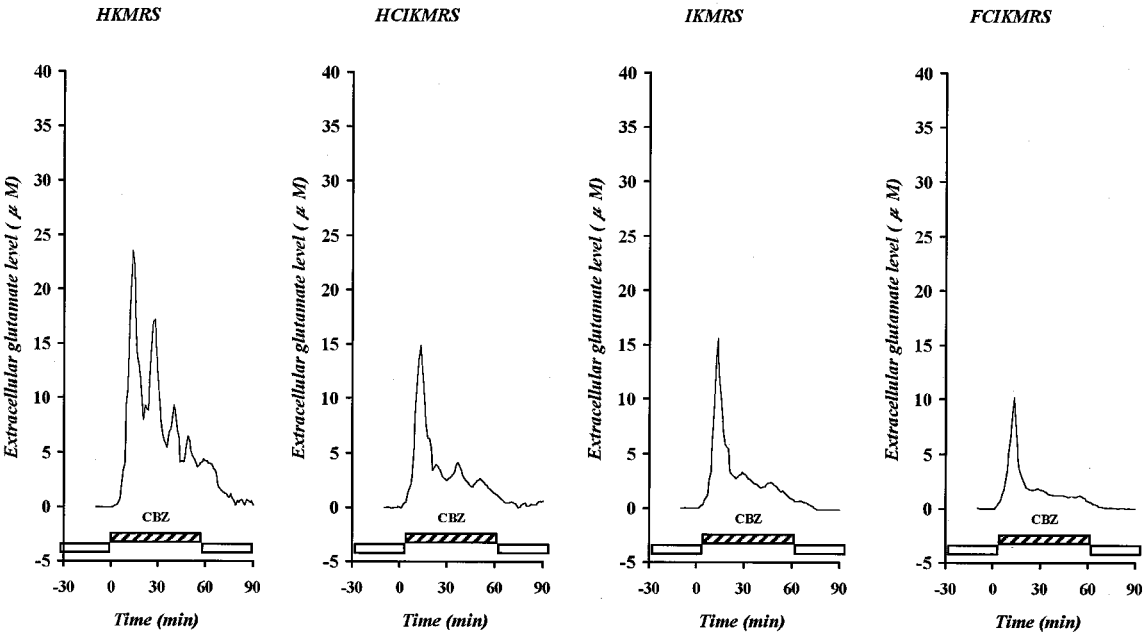

Figure 6 Effects of CBZ on K^+- and Ca^{2+}-evoked hippocampal extracellular glutamate (μM). The open bars show the perfusion with 100 μM CBZ. The striped bars show the perfusion of each HKMRS (100 mM KCl), IKMRS (50 mM KCl), HCIKMRS (50 mM KCl and 3.4 mM $CaCl_2$) or FCIKMRS (50 mM KCl with no added calcium) containing 100 μM CBZ.

detection of this microdialysis electrode adopted in the present study was in the range of 0.1 μM. Therefore, quantification of the reduction of hippocampal extracellular glutamate level in response to removal of Ca^{2+} will require an enhanced sensitivity of this microdialysis electrode.

Obrenovitch and his colleagues (Obrenovitch *et al*., 1993, 1996; Zilkha *et al*., 1995) demonstrated that the K^+-evoked glutamate release had several components: (a) a Ca^{2+}-dependent initial rise which was neuronal activity-independent, (b) after this initial rise, a series of Ca^{2+}-dependent phasic rises associated with neuronal activity including spreading depression, and (c) a small glutamate overflow which persisted in the absence of Ca^{2+}. The present study has confirmed a Ca^{2+}-dependent, K^+-evoked glutamate release. Increasing the space between the microdialysis electrode (recording site) and the probe (stimulation site) abolished the initial transient rise in hippocampal extracellular glutamate levels and reduced the late gentle rise. Thus, it is suggested that the initial transient rise and the late gentle rise are generated by an increase in the extracellular K^+ levels.

On the other hand, the late multiple phasic transient rises in hippocampal extracellular glutamate levels might be induced by spreading depression, since the late multiple phasic transient rises were recorded when the neuronal firing frequency was drastically depressed. Spreading depression is characterized by a transient depression of spontaneous and evoked nerve electrical activity that propagates successively to the adjacent tissue (Nicholson & Kraig, 1981; Koroleva *et al*., 1992). In addition, several investigators have already demonstrated that the release of glutamate and other neuromodulators (e.g. purines) are responsible for the development of spreading depression (Kaku *et al*., 1994; Obrenovitch *et al*., 1996). Surprisingly, in this study, the multiple phasic transient rises were independent of the spacing between the recording

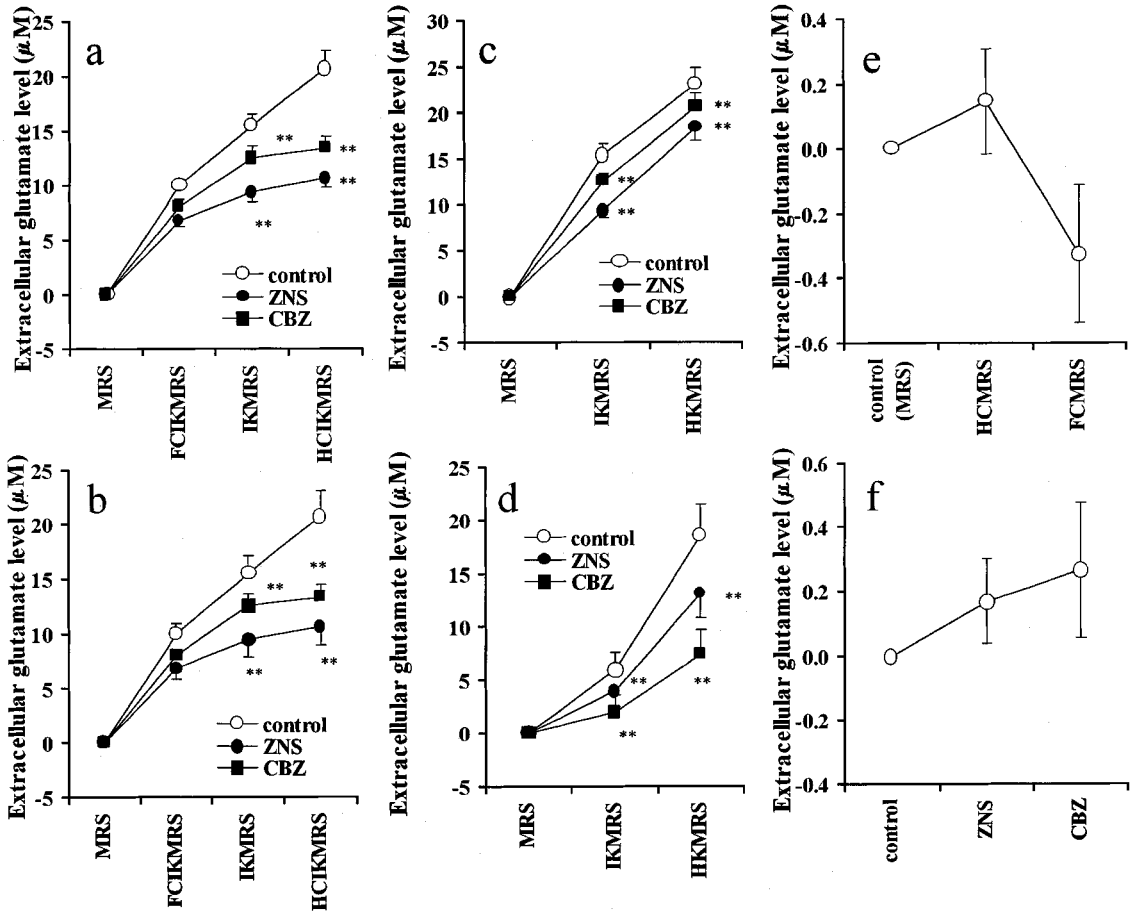

Figure 7 Comparison between the effects of antiepileptic drugs on K^+-evoked hippocampal glutamate release. The hippocampal extracellular glutamate release was measured in perfusates for 60 min during perfusion with HKMRS (100 mM KCl), IKMRS (50 mM KCl), HCIKMRS (50 mM KCl and 3.4 mM $CaCl_2$), FCIKMRS (50 mM KCl with no added calcium), HCMRS (3.4 mM $CaCl_2$) or FCMRS (MRS with no added calcium) containing 1 mM ZNS (●), 100 μM CBZ (■) or no agent (○: control). Figure 7a indicates the effects of ZNS and CBZ on the stimulatory effects of Ca^{2+} on K^+-evoked initial transient rise of extracellular glutamate (mean ± s.e.m., N = 6). Figure 7b indicates the effects of ZNS and CBZ on the stimulatory effects of Ca^{2+} on late phasic rises of K^+-evoked hippocampal glutamate release. Ordinate indicates the AUC levels of late phasic rises of K^+-evoked hippocampal glutamate release (mean ± s.e.m., N = 6). Figure 7c indicates the effects of ZNS and CBZ on K^+-evoked initial transient rise of extracellular glutamate (mean ± s.e.m., N = 6). Figure 7d indicates the effects of ZNS and CBZ on the stimulatory effects of K^+-evoked late phasic rises of hippocampal glutamate release. Ordinate indicates the AUC levels of late phasic rises of K^+-evoked hippocampal glutamate release (mean ± s.e.m., N = 6). Figures 7e and 7f indicate the effects of Ca^{2+} and antiepileptic drugs on basal extracellular glutamate levels, respectively. Ordinate indicates the AUC levels of basal extracellular glutamate levels (mean ± s.e.m., N = 6). These data were analysed statistically by one- or two-way analysis of variance with Tukey's multiple comparison test (**: $P < 0.01$, * $P < 0.05$ vs control).

and stimulation sites (from 1 to 2 mm), suggesting a different scenario from the initial transient and late gentle rises. The conflicting point, in the present study, that the frequency of multiple phasic transient rises was reduced more by 0.5-mm spacing than by 1- to 2-mm spacing between the microdialysis electrode and the probe can be explained by the observation that the flow of dialysate through the microdialysis probe inhibited the propagation of spreading depression (Obrenovitch *et al*., 1995). In addition, perfusion of solutions through the microdialysis probes can change the environment around the probe by diffusion across the membrane of, for example, various ions, adenosine and/or ascorbate (Benvemiste, 1989; Walker *et al*., 1995).

The microdialysis methods combined with either HPLC (Carlson *et al*., 1992; Ronne-Engstrom *et al*., 1992; During & Spencer, 1993; Millan *et al*., 1993), an enzyme cycling technique (Ueda & Tsuru, 1994), or an enzyme flow injection assay (Millan *et al*., 1991) have been used to measure extracellular glutamate levels in studies of the pathogenesis of convulsions. In these previous reports, only those with higher sampling frequency ($\sim$1 min intervals) and higher glutamate detection sensitivities could demonstrate the convulsion-related elevation of glutamate release (Carlson *et al*., 1992; Ronne-Engstrom *et al*., 1992; During & Spencer, 1993; Millan *et al*., 1993; Ueda & Tsuru, 1994). However, even these experiments could not confirm whether seizure-related multiple phasic rises of glutamate release were present since these methods still have an inadequate time resolution. These classical methods have thus far failed to detect a series of frequent transient elevations of the K^+-evoked glutamate release component (including spreading depression). The present study has demonstrated the formation of K^+-evoked glutamate release using real-time monitoring with high time resolution (msec order), utilizing an *in vivo* microdialysis electrode.

CBZ has been reported to inhibit veratridine-induced glutamate release from rat cortex without affecting hippocampal veratridine-induced glutamate release, an action that was suggested to result from modulation of inhibition of Na^+ channel activity by CBZ (Waldmeier *et al*., 1996). There are no similar data concerning the effects of ZNS on glutamate release. However, Owen *et al*. (1997) demonstrated that an ischaemia-induced extracellular glutamate rise was reduced by pretreatment with ZNS and may be attributable to an inhibition of Na^+ channel activity. However, this hypothesis cannot explain the differences between the effects of CBZ and ZNS on K^+-evoked multiple phasic rises of glutamate release without affecting basal extracellular glutamate levels.

In addition, in the present study, both CBZ and ZNS inhibited the stimulatory effects of Ca^{2+} on K^+-evoked hippocampal glutamate release. This result suggests that both CBZ and ZNS inhibit voltage-sensitive Ca^{2+} channels although the particular subtypes that are affected by CBZ and ZNS have yet to be established (Rogawski & Porter, 1990; Macdonald, 1995; Seino *et al*., 1995; Okada *et al*., 1997a). The difference between the effects of CBZ and ZNS on K^+-evoked hippocampal glutamate release might be caused by selective actions of these antiepileptic drugs.

In conclusion, the present study has demonstrated that K^+-evoked hippocampal glutamate release consists of three phases: an initial transient Ca^{2+}-dependent rise which was neuronal activity-dependent; a slow rise and multiple phasic transient rises which were Ca^{2+}-dependent and independent of neuronal activity (spreading depression). The inhibitory effect of ZNS on the initial transient rise was stronger than that of CBZ, whereas the inhibitory effects of CBZ on the series of late multiple phasic transient rises and the slower rise of K^+-evoked hippocampal glutamate release were more effective than those of ZNS. The new method used in the present study, that of real-time monitoring of glutamate release using an *in vivo* dialysis electrode, should prove useful for studying the mechanisms of action of antiepileptic and other CNS-active drugs.

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