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^{3}H-L-Glutamate Binding and ^{3}H-D-Aspartate Release From Hippocampal Tissue During the Development of Pentylenetetrazole Kindling in Rats

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SCHRÖEDER, H., A. BECKER, U. SCHRÖEDER AND V. HOELLT. *^{3}H-L-Glutamate binding and K^+-stimulated ^{3}H-D-aspartate release from hippocampal tissue during the development of pentylenetetrazol-induced kindling in rats.* PHARMACOL BIOCHEM BEHAV **62**(2) 349–352, 1999.—Previous studies have proposed that there is an increase in the density of glutamate binding sites after pentylenetetrazol (PTZ) kindling, whereas the glutamate release is not altered. Little is known about the time course of these changes. Therefore, we studied ^{3}H-L-glutamate binding to hippocampal membranes and K^+-stimulated ^{3}H-D-aspartate release from hippocampal slices of rats given PTZ 3, 7, and 13 times up to a fully kindling state. After three PTZ injections, amino acid release from hippocampal tissue slices was significantly enhanced in comparison to controls, whereas ^{3}H-L-glutamate binding was not altered. After seven injections of PTZ, specific glutamate binding to hippocampal membranes tended to increase, and K^+-stimulated ^{3}H-D-aspartate release from rat hippocampal slices was normalized. The kindled state characterized by generalized clonic–tonic seizures was reached after 13 PTZ injections, and it was accompanied by an enhancement in the density of glutamate binding sites, whereas the chemically evoked amino acid release remained unchanged. It can be concluded that the amino acid release is increased in the early phase of PTZ kindling development, whereas after completion of kindling, the density of excitatory amino acid binding sites is enhanced. © 1999 Elsevier Science Inc.

^{3}H-D-Aspartate release Glutamate binding Hippocampus Kindling

KINDLING is an in vivo model of epilepsy that is produced by repeated application of initially subconvulsant electrical or chemical stimuli culminating in generalized clonic motor seizure activity (5,12).

The enhanced seizure susceptibility induced by kindling is a long-lasting, probably permanent, alteration of the neuronal excitability involving different neurotransmitter systems (2,6,13). Much interest has been concentrated on central excitatory systems. Especially in the glutamatergic system an increased glutamate release, an altered glutamate receptor subtype gene expression, or receptor density measured after electrically induced kindling were found (3,4,9,10,15,20,21).

Little is known about the changes of the glutamatergic neuronal mechanisms after chemical kindling induced by repeated applications of initially subconvulsive doses of pentylenetetrazole (PTZ). Previously we have demonstrated that l-glutamate binding was increased in the hippocampus and cortical regions of PTZ kindled rats, whereas the K^+-stimulated ^{3}H-D-aspartate release from hippocampal slices of kindled rats as a nonmetabolizable marker (23) of Ca^{2+}-dependent and independent glutamate release was unchanged (17–19). Furthermore, it was shown that after PTZ kindling the learning perfomance measured by two-way avoidance reaction in the shuttle box, which is connected with an intact hippocampal formation was disturbed (1). PTZ kindling resulted in cell damage in hippocampal subregions (16) in response to an excessive glutamate release.

However, the contribution of synaptic processes of the glutamatergic neurotransmission in the time course of PTZ

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kindling formation is unknown. Therefore, the goal of the present study was to determine the chemically evoked amino acid release from hippocampal slices and the specific glutamate binding to hippocampal synaptic membranes in the time course of PTZ kindling.

METHOD

Kindling

For all procedures followed, ethical approval was sought prior to the experiments according to the requirements of the National Act on the Use of Experimental animals (Germany).

Male Wistar rats (8 weeks old, 200–240 g) were injected with 35 mg/kg pentylenetetrazole (PTZ, Serva, Germany) IP once every 48 h up to 13 injections (three animal groups with 3, 7, and 13 PTZ injections) and after each injection the resultant seizures were scored according to a modified five-point scale described by Becker et al. (1). Controls received saline injections alone. To equate handling and injection experience, all animals received the same total number of injections. For example, animals receiving three PTZ injections, received 10 saline injections prior to PTZ treatment.

The animals were considered to be kindled after having received 13 PTZ injections and after reaching at least three consecutive stage 4 or 5 seizures.

To study the effect of a acutely evoked PTZ seizures a single convulsive dose of 60 mg/kg PTZ was administered to rats. Controls received saline, although the animals were sacrified at definite points after repeated PTZ injections. The experiments run over a period of 3 months.

Binding Experiment

Twenty-four hours after the 3rd, 7th, and 13th final PTZ or saline injections, animals were decapitated, hippocampi were dissected out, and crude membrane fractions were prepared. ^{3}H-L-Glutamate (specific activity: 1.43 Tbq/mmol, NEN-Dupont) binding was assayed in membrane fractions prepared from hippocampi of saline and PTZ-treated rats, as described by Schröder et al. (18). For the binding assay, the membranes were incubated in 30-mM Tris-HCl buffer (pH 7.4) containing 2.5 mM $CaCl_2$ for 40 min at 37°C. The nonspecific binding was determined in parallel tubes by addition of 100 μM unlabeled L-glutamate.

Amino Acid Release

Freshly prepared 400 μm-thick hippocampal slices (by use of a McIlwain tissue chopper) were incubated with 19.2 nM ^{3}H-D-aspartate (specific activity 962 GBq/mmol, Radiochemical Centre Amersham, UK) for 10 min under aeration with carbogen (O_2/CO_2–95/5%), transferred into superfusion chambers and rinsed by a calcium-free medium. Afterwards, the superfusion was started with Mg^{2+}-free oxygenated Krebs-Henseleit solution at a flow rate of 0.5 ml/min. From 21 to the 25 min after that the medium was changed to a Krebs-Henseleit solution containing 48 mM KCl. Perfusate was collected in 1-min fractions up to 30 min, and assayed for radioactivity using a dioxane-containing scintillation cocktail and a Beckman 6000 LL counter.

Protein content was estimated in aliquots of tissue homogenates using the technique by Lowry et al. (11) with BSA as standard. Samples were expressed as dpm per mg protein and calculated as the sum of fractions over the stimulation phase (K^+ from the 21 up to the 25 min).

For statistical calculations the nonparametric Mann–Whitney *U*-test was used, and significance was considered at the $p < 0.05$ level.

RESULTS

PTZ Kindling

The development of PTZ-induced kindling is shown in Fig. 1. After three PTZ injections a seizure stage 2 was reached. After the 7th PTZ injection rats responded with a mean seizure stage of 3.7 and "fully" kindled rats with seizure stage 4.7.

The acute application of 60 mg/kg PTZ results in generalized clonic–tonic seizures (stage 5).

^{3}H-L-Glutamate Binding to Synaptic Membranes of Hippocampus

Under the incubation conditions used in this study, binding of ^{3}H-L-glutamate to hippocampal synaptic crude membranes from control rats appeared to be specific and saturable (single binding site) with an apparent K_d of 456 ± 82 nM and a B_{max} of 15.8 ± 2.0 pmol per mg protein, and is not contaminated by uptake processes. A low rate of glutamate binding to the transporter cannot be excluded. The Scatchard plot of the glutamate binding to hippocampal membranes of kindled rats with a K_d of 518 ± 69 nM and a B_{max} of 26 ± 4 pmol/mg protein demonstrated an increase of the density of the binding sites in comparison to controls.

Using ligand concentrations of 50 nM, the specific ^{3}H-L-glutamate binding to synaptic membranes displayed a significant enhancement in fully kindled rats in comparison to saline controls (Fig. 2, $U = 3$). After three and seven PTZ injections the specific ^{3}H-L-glutamate binding to hippocampal crude synaptic membranes remained unchanged. However, mention should be made of the fact that the glutamate binding evaluated in controls did vary. However, independently from binding level in controls, in hippocampal tissue, PTZ kindling re-

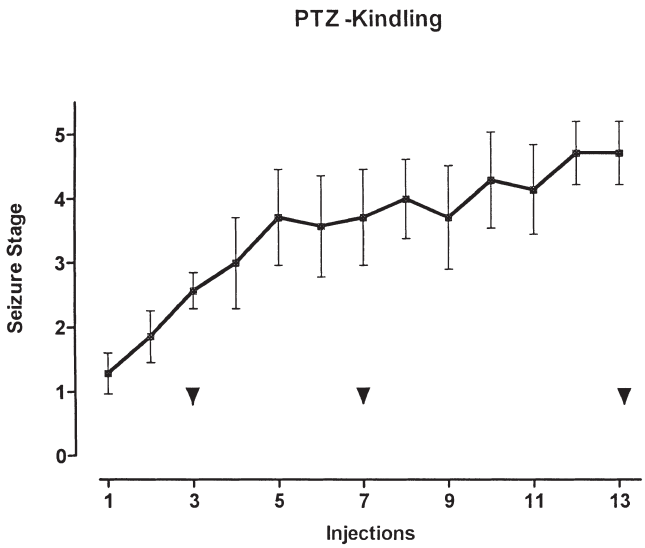

FIG. 1. Development of seizures of rats to PTZ (35 mg/kg, IP) after repeated (3, 7 and 13, indicated by arrows) PTZ injections up to the fully kindled state (means + SEM, $n = 6$).

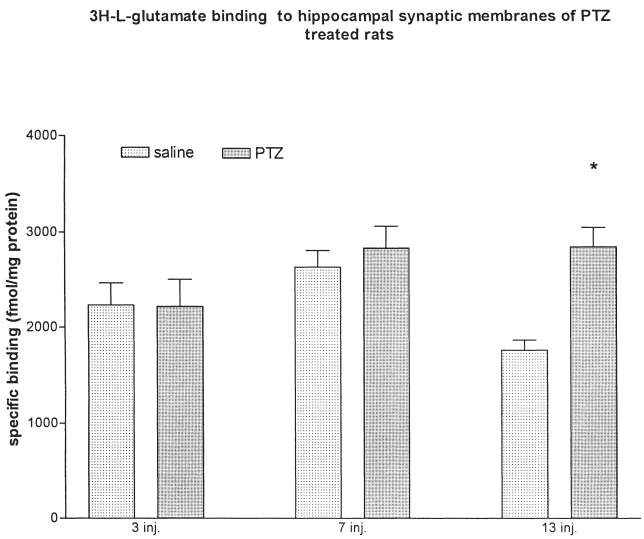

FIG. 2. The specific ^{3}H-L-glutamate binding to hippocampal synaptic membranes of rats treated with PTZ (3, 7 and 13 times) compared to saline controls (in fmol/mg protein, means + SEM, $n = 6$, $^*p < 0.05$).

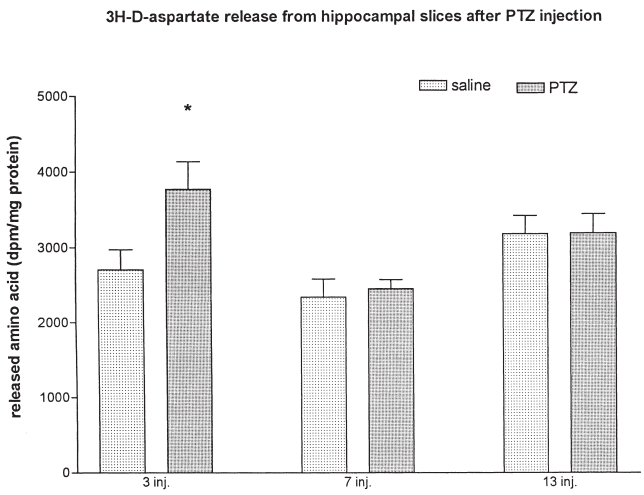

FIG. 3. The K^+-stimulated ^{3}H-D-aspartate release from hippocampal slices of rats given PTZ 3, 7 and 13 times up to fully kindled state in comparison to saline-treated controls (means + SEM, $n = 6$, the sum of fractions over the stimulation phase S1 in dpm/mg protein, $^*p < 0.05$).

sulted in an enhancement of amino acid binding over that of controls (18).

Moreover, acute application of a convulsive doses (stage 5) of PTZ (60 mg/kg IP) did not alter the density of glutamate binding sites in the hippocampus (saline control—2529 ± 141 fmol/mg protein, PTZ-treated rats—2472 ± 136 fmol/mg protein, $n = 6$).

^{3}H-D-Aspartate Release

The K^+-stimulated ^{3}H-D-aspartate release from hippocampal slices (Fig. 3) was unchanged after PTZ kindling (13 injections of PTZ) compared to saline controls ($U = 12$). In contrast, the K^+-stimulated amino acid release from hippocampal slices of rats applicated with three PTZ injections was significantly enhanced in comparison to control animals (Fig. 3, $U = 5$), whereas the K^+-stimulated ^{3}H-D-aspartate release from hippocampal slices of rats measured after seven PTZ injections was found to be not altered ($U = 11$). Acutely given PTZ (60 mg/kg) is unable to alter the chemically evoked amino acid release from hippocampal slices compared with saline-treated controls (not shown).

DISCUSSION

Kindling has become the most studied animal model of epilepsy (5,12). This plastic-adaptive process is postulated to evoke a chain reaction of cellular and molecular events of altered neurotransmission systems, especially by activation of glutamate receptors (2).

The important finding of the present study is the different response of the synaptic processes of the glutamatergic system within the hippocampal formation during the development of PTZ kindling in rats. After the first injections of PTZ the transmitter release as a fast responsible presynaptic process is enhanced (Fig. 3). At this time the glutamate binding sites were found to be unchanged. After the seventh PTZ injection as well as in the case of fully kindled state, the amino acid release becomes normalized, whereas the glutamate binding sites as a pre- and postsynaptic parameter are upregulated in fully kindled rats (Fig. 2) by increased density of the receptor that persisted over a period of months (19).

In the present study, the glutamate binding sites were not differentiated into glutamate receptor subtypes, but in a previous study we found that the enhancement of the glutamate receptor density after PTZ kindling in rats is mainly related to an increase of the metabotropic glutamate receptor in the hippocampus (17). A specific role of the different glutamate receptor subtypes in the development of chemical and electrical kindling cannot be excluded.

In relation to the physiologically evoked changes of the excitability of the hippocampal formation it is interesting that a similar time course of altered glutamatergic events could be detected during induction and maintenance of long-term potentiation (LTP) of the perforant pathway, which was much faster in onset and only short lasting (19).

Acute PTZ application did not alter either the glutamate binding sites or amino acid release in the hippocampus (18). Therefore, it can be assumed that the specific changes of the glutamatergic system may be a correlate of plastic changes of the synaptic system due to the development of PTZ kindling.

In contrast to our results, after completion of electrical kindling in the amygdala or hippocampal formation an increase in glutamate release is described by ex vivo as well as microdialysis studies. This effect is often discussed as a trigger of seizure induction in the electrical kindling model (4,8, 14,22). The increase of glutamate binding sites in response to PTZ kindling of rats (Fig. 3) is comparable with findings described after electrical kindling of rats (3,21). In this context, it is interesting that these data are in a good agreement with the findings of Hosford et al. (7), indicating an increased glutamate binding in hippocampus tissue of epileptic patients.

Summerizing our data it can be concluded that the pre- and postsynaptic processes of the glutamatergic neurotransmis-

sion are involved in the mechanisms underlying chemical and electrical kindling in a quite complex manner. In both models an activation of the excitatory glutamatergic neurons of the brain in the development of a kindling state is needed probably with a varied importance of the studied synaptic parameters.

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REFERENCES

1. Becker, A.; Grecksch, G.; Rüthrich, H. L.; Pohle, W.; Marx B.; Matthies, H.: Kindling and its consequences on learning in rats. Behav. Neural Biol. 57:37–43; 1992.
2. Cain, D. P.: Long-term potentiation and kindling: How similar are the mechanisms. Trends Neurosci. 12:6–10; 1989.
3. Cincotta, M.; Young, N. A.; Beart, P. M.: Unilateral up-regulation of glutamate receptors in limbic regions of amygdaloid-kindled rats. Exp. Brain Res. 85:650–658; 1991.
4. Geula, Ch.; Jarvie, P. A.; Logan, T. C.; Slevin, J. T.: Long-term enhancement of K^+-evoked release of *l*-glutamate in entorhinal kindled rats. Brain Res. 442:368–372; 1988.
5. Goddard, G. K.; McIntyre, D. C.; Lech, C. K.: A permanent change in brain function resulting from daily electrical stimulation. Exp. Neurol. 25:295–330; 1969.
6. Green, J. G.; Greenamyre, J. T.: Bioenergetics and glutamate excitotoxicity. Prog. Neurobiol. 48:613–634; 1996.
7. Hosford, D. A.; Crain, B. J.; Cao, Z.; Bonhaus, D. W.; Friedman, A. H.; Okazaki, M. M.; Nadler, J. V.; McNamara, J. O.: Increased AMPA-sensitive quisqualate receptor binding and reduced NMDA receptor binding in epileptic human hippocampus. J. Neurosci. 11:428–434; 1991.
8. Kaura, S.; Bradford, H. F.; Young, A. M.; Croucher, M. J.; Hughes, P. D.: Effect of amygdaloid kindling on the content and release of amino acids from the amygdaloid complex: In vivo and in vitro studies. J. Neurochem. 65:1240–1249; 1995.
9. Kraus, J. E.; Yeh, G.-C.; Bonhaus, D. W.; Nadler, J. V.; McNamara, J. O.: Kindling induces the long-lasting expression of a novel NMDA receptors in the hippocampal region CA3. J. Neurosci. 14:4196–4205; 1994.
10. Kraus, J. E.; Nadler, J. V.; McNamara, J. O.: Regulation of alternative splicing of NMDAR1 in the kindling model. Mol. Brain Res. 41:97–104; 1996.
11. Lowry, O. H.; Rosebrough, R. N.; Farr, A. L.; Randall, R. J.: Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193:265–275; 1951.
12. Mason, C. R.; Cooper, R. M.: A permanent change in convulsive threshold in normal and brain-damaged rats with repeated small doses of pentylenetetrazol. Epilepsia 13:663–674; 1972.
13. McNamara, J. O.: Analysis of the molecular basis of kindling development. Psychiatr. Clin. Neurosci. 49:175–178; 1995.
14. Meldrum, B.: The role of glutamate in epilepsy and other CNS disorders. Neurology 44:14–23; 1994.
15. Oka, J.-I.; Yamada, M.; Goto, M.; Fukuda, H.: Diazepam restores the increased ^{3}H-glutamate binding to hippocampal synaptic membranes in amygdaloid-kindled rats. Neurosci. Res. 4:185–194; 1987.
16. Pohle, W.; Becker, A.; Grecksch, G.; Juhre, A.; Willenberg, A.: Piracetam prevents pentylenetetrazol kindling-induced neuronal loss and related learning deficits. Seizure 6:467–474; 1997.
17. Schröder, H.; Becker, A.: The role of glutamate receptors in pentylenetetrazole kindling of rats—A neurochemical study. Neuropharmacology 35:A28; 1996.
18. Schröder, H.; Becker, A.; Lössner, B.: Glutamate binding to brain membrane is increased in pentylenetetrazol-kindled rats. J. Neurochem. 60:1007–1011; 1993.
19. Schröder, H.; Schlichthaar, R.; Krug, M.: Changes of glutamatergic synaptic mechanisms after PTZ kindling and LTP. Neuroscience 60:337–342; 1994.
20. Watkins, J. C.: Excitatory amino acids and central synaptic transmission. Trends Parmacol. Sci. 5:373–376; 1984.
21. Wu, K.; Wasterlain, C.; Sachs, L.; Siekewitz, P.: Effect of septal kindling on glutamate binding and calcium/calmodulin-dependent phosphorylation in a postsynaptic density fraction isolated from rat cerebral cortex. Proc. Natl. Acad. Sci. USA 87:5298–5302; 1990.
22. Yamagata, Y.; Obata, K.; Greengard, P.; Czernik, A. J.: Increase in synapsin I phosphorylation implicates a presynaptic component in septal kindling. Neuroscience 64:1–4; 1995.
23. Zablocka, B.; Domanska-Janik, K.: PAF antagonist, BN52021, inhibits ^{3}H-D-aspartate release after ischemia in vitro. Neuroreport 6:85–88; 1994.