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Effects of diazepam on extracellular brain neurotransmitters in pilocarpine-induced seizures in rats

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Abstract

The present study was undertaken to gain insights into the mechanism of action of diazepam in focally-evoked pilocarpine-induced seizures by concomitantly assessing the changes produced in the extracellular levels of glutamate, GABA (γ-aminobutyric acid) and dopamine. In vivo microdialysis, coupled to continuous monitoring of electrocorticographic (ECoG) recordings, was performed in freely moving rats. Intrahippocampal perfusion with 10 mM pilocarpine (40 min, 2 μl/min) produced limbic seizures. A single dose of intraperitoneal diazepam (5 mg/kg) was administered 2 h after pilocarpine perfusion was started. Dialysates were sampled both from hippocampus and cerebellum and analysed by microbore liquid chromatography. Diazepam produced instant inhibition of behavioural and ECoG seizure activity. Pilocarpine-induced increases in the extracellular levels of glutamate and dopamine in hippocampus were promptly reduced by diazepam. No concurrent alterations in pilocarpine-induced increases in the extracellular levels of GABA in either hippocampus or cerebellum were seen. Pilocarpine enhanced cerebellar glutamate levels only transiently and levels returned to baseline before diazepam administration. No further changes in cerebellar glutamate levels were observed with diazepam. Our findings suggest that the anti-convulsant action of diazepam against pilocarpine-induced seizures is associated with a prompt attenuation of extracellular hippocampal glutamate overflow without concurrent alteration of pilocarpine-induced increases in endogenous GABA levels. Diazepam also significantly decreased pilocarpine-induced increases in extracellular dopamine levels within the hippocampus. No immediate alterations of the basal levels of the neurotransmitters monitored were observed with diazepam.

Keywords: Microdialysis; Electrocorticography; Glutamate; GABA (γ-aminobutyric acid); Dopamine; Pilocarpine; Diazepam; Hippocampus; Cerebellum

1. Introduction

Systemic diazepam is a well-established therapeutic approach to produce a prompt anti-convulsant effect when administered during an episode of seizures. Diazepam and other benzodiazepine mostly exert their pharmacological actions by allosterically modulating the $GABA_A$–benzodiazepine receptor complex to produce a facilitatory effect on the GABA (γ-aminobutyric acid)-mediated inhibitory neurotransmission in the CNS (central nervous system) (Macdonald and Olsen, 1994; Costa and Guidotti, 1996; Hoogerkamp et al., 1996). At least part of the mechanism of action of benzodiazepines has been suggested to be mediated by an increase in the adenosine concentration (Phillis and O'Regan, 1988). Adenosine has a putative role in presynaptic inhibition of evoked release of glutamate and other neurotransmitters in the hippocampus. Although studies involving receptor binding and immunocytochemical techniques have greatly illuminated the receptor mechanism of action of benzodiazepines, the modulatory roles of the pertinent neurotransmitters in terms of their extracellular concentrations remain largely obscure.

There is evidence that veratridine or K^+-evoked increases in extracellular glutamate concentrations are decreased by diazepam (Baba et al., 1983). The specific protective effect of diazepam against isoniazid-induced seizures was observed without any concomitant change in extracellular GABA levels (Bernasconi et al., 1985). Acute

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administration of an excessively high dose of diazepam to inbred mutant E1 mice has been reported to produce anti-convulsant effects with a concomitant increase in dopamine levels in both the cortex and hippocampus (Hiramatsu et al., 1988). In view of the extensive use of benzodiazepines for stress and anxiogenic disorders, most investigations were conducted to evaluate the effects of diazepam in stress models. The anxiogenic β-carboline (FG 7142)-induced glutamate efflux in the prefrontal cortex was abolished by pre-treatment with diazepam (5 mg/kg) (Karreman and Moghaddam, 1996). Systemic diazepam has been reported to decrease both the basal and stress-evoked increases in extracellular dopamine concentrations in the medial prefrontal cortex (Finlay et al., 1995). Basal extracellular dopamine has been observed to be significantly decreased by relatively low doses of diazepam in the nucleus accumbens (Invernizzi et al., 1991) and prefrontal cortex (Dazzi et al., 1995) while higher doses are required to produce similar effects in the striatum (Invernizzi et al., 1991). Repeated administration of low doses of diazepam has been shown to increase striatal dopamine levels (Stancheva and Petkov, 1988) while stress activated limbic and cortical dopamine release is reported to be unaffected by diazepam (Imperato et al., 1990). Despite all these interesting reports, data are surprisingly lacking on the effects of diazepam on the modulation of hippocampal and cerebellar extracellular overflow of neurotransmitters that are relevant to seizures.

Most frequently, microdialysis studies have reported no evident change in extracellular glutamate concentrations even during prolonged convulsions induced by systemic chemoconvulsants (Meldrum, 1994). A significant alteration of glutamate and GABA concentrations during chemically-evoked acute seizures has only been reported in a few studies (Wade et al., 1987; Lallement et al., 1991). Recently, we have demonstrated marked augmentation of hippocampal glutamate, GABA and dopamine (Smolders et al., 1997a,b) and cerebellar glutamate and GABA (Smolders et al., 1997a,c) overflow following focally-evoked pilocarpine-induced seizures in freely moving rats. The cerebellum, besides being rich in glutamatergic and GABAergic input and output neurons (Headley and Grillner, 1990), is also important from the point of view of the diaschisis phenomenon (Meyer et al., 1993) (haemodynamic and other changes remote to the ictal focus during an interictal phase). A previous study of a pilocarpine model with anaesthetised animals also reported marked increases in extracellular hippocampal glutamate levels (Millan et al., 1993).

A perfect animal model, exactly reproducing the conditions observed in clinical epilepsy, is yet to be developed. Pilocarpine-induced seizures, however, can reproduce certain morphological, neuropathological, and electophysiological features of human complex partial epilepsy (Isokawa and Mello, 1988; Cavalheiro et al., 1991), as evident from studies conducted on resected tissues upon therapeutic removal from brains of human patients with epilepsy. In addition to systemic administration, convulsant activity can also be effectively reproduced by intrahippocampal perfusion with pilocarpine (Millan et al., 1993; Smolders et al., 1997a,b,c). In this way, we could possibly not only limit the magnitude of some of the peripheral adverse effects of the convulsant drug but also save the animal from entering into the stage of status epilepticus.

The present study was undertaken to gain insights into the role of systemic diazepam in modulating pilocarpine-induced concomitant alterations in the extracellular concentrations of glutamate, GABA and dopamine in rat hippocampus and cerebellum during and following an episode of seizure. Microdialysis experiments coupled to continuous monitoring of electrocorticographic (ECoG) recordings were conducted in freely moving rats.

2. Materials and methods

The protocols for the animal experiments described in this study were performed in accordance with national rules on animal experiments and institutional guidelines as prescribed by the Ethics Committee of the Faculty of Medicine of the Free University of Brussels.

2.1. Surgery and electrophysiological procedure

Male albino Wistar rats, weighing between 270 and 300 g, were kept under standard laboratory conditions (room temperature 22 ± 1°C, 12 h light and 12 h dark cycle, with free access to food and water). They were anaesthetised with an intraperitoneal injection of a mixture of diazepam (5 mg/kg) and ketamine HCl (50 mg/kg) and fixed in a stereotaxic frame. Intracranial guides with cannulae were implanted according to the coordinates of Paxinos and Watson (1986). The coordinates relative to bregma were for the hippocampus: lateral +4.6, anteroposterior −5.6, and vertical +4.6; and for the cerebellum: +3.0, −13, and +3.0, respectively. An array of six cortical electrodes was positioned on the dura mater adjacent to the midsagittal suture, both ipsi- and contralaterally, and fixed with dental cement, as described previously (Smolders et al., 1997a,b). Each rat received a single injection of ketoprofen (4 mg/kg, i.p) following the surgery to provide post-operative analgesia. Immediately after surgery, guide cannulae were replaced with CMA 10 microdialysis probes, membrane length 3 mm (CMA microdialysis, Stockholm, Sweden) and perfusion started with Ringer's solution containing 147 mM NaCl, 4 mM KCl and 1.1 mM $CaCl_2$, at a constant flow rate of 2 μl/min by using a CMA 100 microdialysis pump (CMA microdialysis, Stockholm, Sweden). Dialysate sampling was started after waiting for a minimal period of 24 h following the completion of surgery, so that the animals could sufficiently recover from it. Twenty samples were collected every 20 min from both the hippocampus and cerebellum. Each collection vial was

prefilled with 10 μl antioxidant solution containing 0.02 mM HCl, 0.2% sodium metabisulphite and 0.02% Na_2EDTA. During the collection of the first eight basal samples, the perfusion fluid was composed of Ringer's solution only. For the subsequent two collections (9 and 10), the perfusion fluid to the hippocampus was switched to Ringer's solution containing 10 mM pilocarpine. From collection 11 onwards, the probe was again perfused with Ringer's solution. Until the end of collection 13, the experimental protocol remained exactly the same for both the control and diazepam-treated groups. However, at the start of collection 14, each rat in the diazepam-treated group received a single intraperitoneal injection of diazepam (5 mg/kg) dissolved in saline and propylene glycol (50:50) mixture. The vehicle used was previously tested in a series of experiments and was found to have no significant effects on the levels of any of the neurotransmitters (unpublished data). The effects on the basal levels of the neurotransmitters were evaluated in another group of six animals in which a single dose of diazepam (5 mg/kg) was administered at the beginning of collection nine and a total number of 16 samples each from hippocampus and cerebellum were collected.

2.2. Chromatographic assays

For analysis of dopamine, reversed-phase ion-pair microbore liquid chromatography was used as described previously (Smolders et al., 1996). Chromatographic conditions and precolumn derivatization procedures for the amino acids have been described in detail elsewhere (Smolders et al., 1995). For glutamate, reversed-phase microbore liquid chromatography with gradient elution and fluorescent detection was used. The precolumn derivatization was performed with *o*-phthalaldehyde/β-mercaptoethanol. GABA analysis consisted of reversed-phase microbore liquid chromatography with isocratic elution and electrochemical detection. Precolumn derivatization was performed with *o*-phthalaldehyde/*tert*-butylthiol. Excess thiol was scavenged with iodoacetamide.

2.3. Reagents

Pilocarpine, glutamate, GABA and dopamine were purchased from Sigma Aldrich (St. Louis, MO, USA). Diazepam was obtained from Hoffman la Roche (Basel, Switzerland). β-mercaptoethanol and *tert*-butylthiol were supplied by Janssen Chimica (Beerse, Belgium). Sodium metabisulphite, Na_2EDTA, sodium acetate, decanesulfonic acid and solvents of gradient quality for chromatography

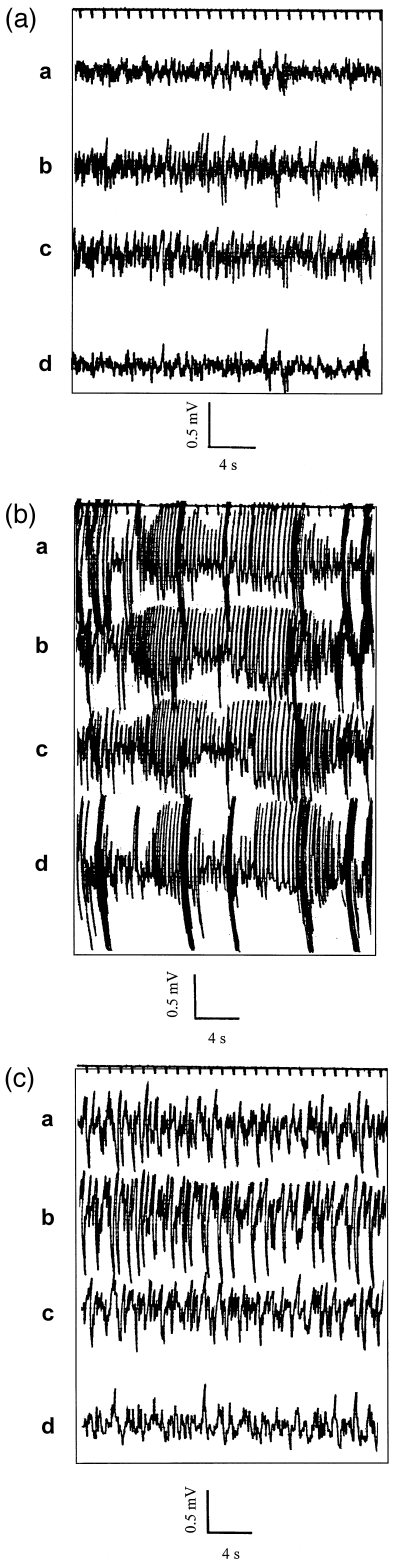

Fig. 1. (a) Ipsilateral ECoG recording showing patterns of a normal electrocorticogram obtained from a rat hippocampus under basal conditions. The ECoG was obtained from six monopolar electrodes [four monitoring (a: central, b and c: parietal and d: occipital), one prefrontal reference, and one ground] implanted in a parasagittal groove on each side of the brain. ECoG was polygraphically amplified and recorded with a time constant of 0.15 s, a high cut-off filter at 70 Hz, and a sensitivity of 500 μV/cm. (b) Ipsilateral ECoG recording showing patterns of a convulsive episode after intrahippocampal pilocarpine administration in a rat. (c) ECoG recordings obtained in the same animal a few minutes after intraperitoneal administration of 5 mg/kg diazepam.

were obtained from Merck (Darmstadt, Germany). All aqueous solutions were prepared in deionised water obtained with a Seralpur Pro 90 CN (Belgolabo, Overijse, Belgium) and filtered through a 0.2-μm membrane filter.

2.4. Statistical analysis

Extracellular dopamine levels in the dialysates were expressed in nanomolar concentrations and amino acids levels in micromolar concentrations without correction for recovery across the dialysis membrane. For determination of the statistical significance of differences in the dopamine and amino acids levels following pharmacological manipulations as compared with mean basal levels, a One-way analysis of variance (ANOVA) for repeated measures and Fisher's protected least significant difference (Fisher's PLSD) post hoc test ($\alpha = 0.05$) were used. Mann–Whitney test was used for comparison between control and treated groups. Statistical analyses were carried out on the percentage of change.

3. Results

3.1. Basal levels of hippocampal and cerebellar glutamate and GABA and hippocampal dopamine

The basal hippocampal dialysate concentrations (mean ± S.E.M) ($n = 18$) were 0.410 ± 0.095 μM for glutamate, 0.069 ± 0.013 μM for GABA and 0.310 ± 0.060 nM for dopamine. The mean basal cerebellar dialysate concentrations were 0.610 ± 0.150 μM and 0.062 ± 0.021 μM for glutamate and GABA.

3.1.1. Control group

3.1.1.1. Behavioural and ECoG changes. During basal collections, the rats mostly remained calm and quiet. ECoG recordings obtained during this time showed a normal pattern of electrical activity (Fig. 1a). A host of behavioural alterations commenced approximately half an hour after intrahippocampal perfusion of 10 mM pilocarpine. The rats assumed a 'semi-erect' posture and exhibited profound gustatory automatisms including head bobbing, teeth chattering, scratching, vibrissae twitchings, salivation and frequent wet-dog shakes. Approximately 1 h after perfusion with pilocarpine, these features progressively developed into intermittent motor limbic seizures characterised by a typical 'praying' posture, facial muscle clonus, forelimb clonus, occasional rearing, and falling and

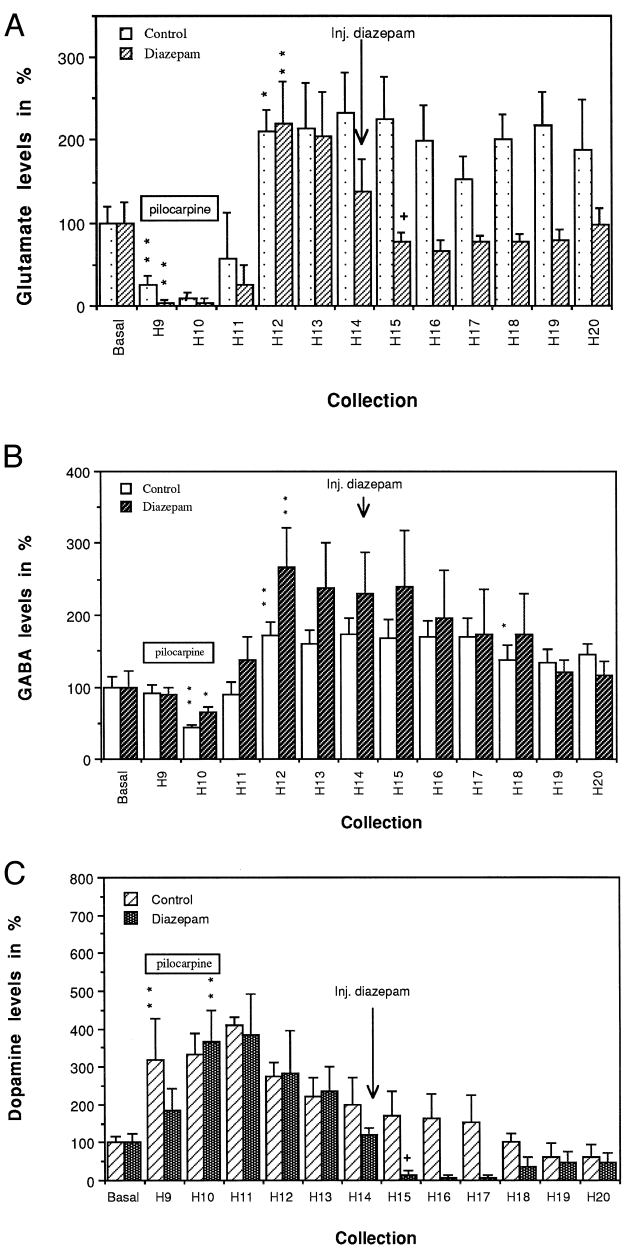

Fig. 2. (A, B, C) Extracellular levels of glutamate (A), GABA (B), and dopamine (C) (in percentage of the baseline level) (mean ± S.E.M.) in the hippocampus (H) under baseline conditions following perfusion with Ringer's solution. Collections H9 and H10 denote collections during intrahippocampal perfusion of 10 mM pilocarpine. Collections H11–H20 denote post-pilocarpine collections during perfusion of Ringer's solution. In the diazepam-treated group, a single intraperitoneal injection of diazepam (5 mg/kg) was given at the beginning of collection 14 in each experiment. For the basal collection, the level is expressed as a percentage (mean ± S.E.M.) of the pooled 160 min (collections 1–8) stable dialysate collections. Each of the remaining bars represents a 20-min collection period. The asterisks denote only the first values significantly different from the corresponding baseline values. Changes in the level of significance from the preceding values have been reindicated with corresponding asterisks. The values significantly different from the baselines are as follows: Group 2A *control*: (collections 9–10, 12–16, 18, 19); Group 2A *diazepam*: (collections 9–10, 12–13); Group 2B *control*: (collections 10, 12–18, 20); Group 2B *diazepam*: (collections 10, 12–16); Group 2C *control*: (collections 9–14); Group 2C *diazepam*: (collections 9–13); ($^{**}P < 0.01$, $^{*}P < 0.05$) (Statistics: Anova and Fisher's PLSD post hoc test). The plus sign denotes the first values that are significantly different from the corresponding control values and are given as follows: Group 2A *diazepam*: (collections 15–19); ($+ P < 0.05$) (Statistics: Mann–Whitney); Group 2C *diazepam*: (collections 15–17); ($+ P < 0.01$) (Statistics: Mann–Whitney).

convulsions. Within the next 60 to 90 min, seizure manifestations gradually became more intense and reached a peak. The intervening period between the appearance of two successive motor seizure manifestations was characterised by increased movement around the cage, grooming, sniffing and profuse salivation. Clear patterns of tonic–clonic seizures were recorded during ECoG monitoring (Fig. 1b).

3.1.1.2. Changes in the extracellular concentrations of glutamate, GABA and dopamine (Fig. 2a, b, c). Intrahippocampal administration of pilocarpine for 40 min resulted in an initial marked decrease in basal hippocampal dialysate concentrations of glutamate to 8% ($P = 0.0001$) and GABA to 44% ($P = 0.0001$). The levels of both amino acids soon increased to attain significant and sustained elevations. More than a two-fold (232%) increase in extracellular glutamate overflow was registered ($P = 0.028$). Simultaneous significant elevations of the extracellular GABA levels to 173% ($P = 0.001$) were also observed. The increase in GABA levels remained evenly sustained until the end of the experiments. Pilocarpine administration also resulted in a highly significant increase in extracellular dopamine levels to 409% ($P = 0.0001$) (Fig. 2a, b, c).

3.1.1.3. Changes in extracellular concentrations of cerebellar glutamate and GABA (Figs. 3 and 4). Cerebellar glutamate levels were transiently (for two collection periods) but significantly increased to 258% ($P = 0.03$), about 40 min after intrahippocampal pilocarpine administration was started. Cerebellar GABA levels were also significantly elevated to 268% ($P = 0.01$) and were associated with a more sustained increase (Figs. 3 and 4).

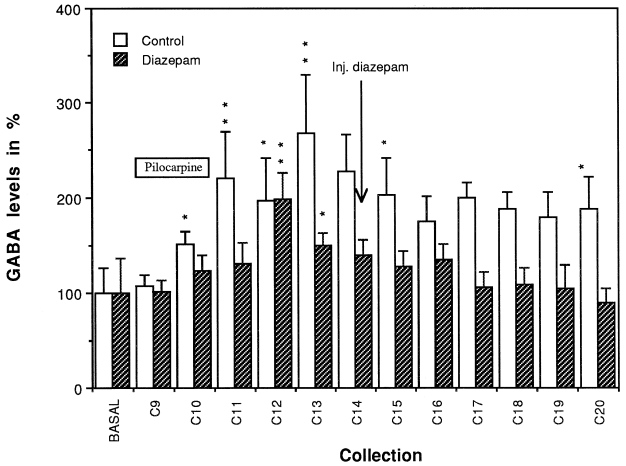

Fig. 4. Extracellular levels of GABA (in percentage of the baseline level)(mean ± S.E.M.) in the cerebellar (C) microdialysates [control group ($n = 6$) and diazepam group ($n = 6$)]. Basal denotes collection under baseline conditions following perfusion with Ringer's solution. Collection C9 and C10 denote collections during intrahippocampal perfusion of 10 mM pilocarpine. Collection C11–C20 denote post-pilocarpine collections during perfusion of Ringer's solution. In the diazepam-treated group, a single intraperitoneal injection of diazepam (5 mg/kg) was given at the beginning of collection 14. For the basal collection, the level is expressed as a percentage (mean ± S.E.M.) of the pooled 160 min (collections 1–8) stable dialysate collections. Each of the remaining bars represents a 20-min collection period. The asterisks denote the first values significantly different from the corresponding baseline values. Only changes in the level of significance from the preceding values are reindicated with corresponding asterisks. The values significantly different from baseline values are as follows: *control*: (collections 10–15, 17, 18 and 20); *diazepam*: (collections 12–16); ($^{**}P < 0.01$, $^{*}P < 0.05$) (Statistics: Anova and Fisher's PLSD post hoc test).

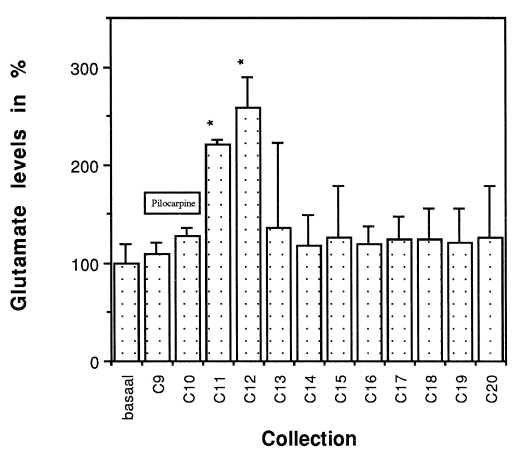

Fig. 3. Extracellular levels of glutamate (in percentage of the baseline level) (mean ± S.E.M.) ($n = 6$) in the cerebellar (C) microdialysates (control group). Basal denotes collection under baseline conditions following perfusion with Ringer's solution. C9–C10 denote collections during intrahippocampal perfusion of pilocarpine. C11–C20 denote post-pilocarpine collections during perfusion of Ringer's solution. For the basal collection, the level is expressed as a percentage (mean ± S.E.M.) of the pooled 160 min (collections 1–8) stable dialysate collections. Each of the remaining bars represents a 20-min collection period. Asterisks denote the values that are significantly different from the baseline value. ($^{**}P < 0.01$, $^{*}P < 0.05$) (Statistics: Anova and Fisher's PLSD post hoc test).

3.1.2. Diazepam-treated group

3.1.2.1. Effects of diazepam in the basal levels. No significant changes in the basal extracellular levels of glutamate, GABA and dopamine in the hippocampus and glutamate and GABA in the cerebellum were observed immediately following the administration of a single dose of intraperitoneal diazepam (5 mg/kg) (data not shown).

3.1.2.2. Behavioural and ECoG changes. Intraperitoneal injection of 5 mg/kg diazepam at the beginning of collection 14 completely abolished all the behavioural alterations induced by pilocarpine. The rats were quiet and appeared to be under the hypnotic effects of diazepam. ECoG recording following diazepam administration showed a pattern of paradoxical sleep waves devoid of a phasic component ($P\theta$) comparable to findings reported earlier (Monmaur, 1981) (Fig. 1c).

3.1.2.3. Changes in the extracellular hippocampal concentrations of glutamate, GABA and dopamine (Fig. 2a, b, c). Following diazepam injection, the extracellular concentrations of glutamate in the hippocampus from collection 15 to 18 were significantly reduced to 67% ($P < 0.05$) as compared to the corresponding elevated levels in the control group. Extracellular levels of dopamine during collection 15, 16, and 17 were significantly reduced to 31% ($P < 0.01$) in comparison to the corresponding levels in the control group. There was no concurrent effect on the extracellular GABA overflow following diazepam administration.

3.1.2.4. Changes in the extracellular cerebellar concentrations of glutamate and GABA (Fig. 4). No significant change in extracellular glutamate overflow was observed with diazepam (data not shown). Although there was no immediate effect of diazepam on cerebellar GABA concentration, elevated GABA levels were not sustained.

4. Discussion

In the present study, we have demonstrated that systemic administration of diazepam can completely abolish focally-evoked pilocarpine-induced seizures. This was evident from concurrent behavioural and electrophysiological changes consistent with the convulsant and anti-convulsant state. This was also reflected by the elicited alterations in the extracellular levels of the neurotransmitters.

During pilocarpine perfusion, the initial response was a significant decrease of glutamate overflow. This is consistent with a previous report in which acetylcholine induced similar effects on glutamate release in hippocampal slice preparations (Marchi et al., 1989). This decrease in glutamate efflux has been described to be due to presynaptic inhibition mediated by muscarinic receptors (Marchi et al., 1989; Marchi and Raiteri, 1996). However, this initial, transient decrease was soon followed by a prolonged marked elevation of extracellular hippocampal glutamate levels. The increase in glutamate overflow began approximately 1 h after the pilocarpine perfusion was started, and was sustained approximately until the end of the experiment. This augmentation of extracellular glutamate concentrations corresponded well with the appearance of focally-evoked seizures, as manifested by changes in behaviour and movement states. These changes soon became overt convulsive seizures, as was evidenced by the ECoG pattern corresponding to tonic–clonic type seizures. These findings, including the initial decrease in glutamate overflow, are similar to our previous observations (Smolders et al., 1997a,b). Elevations of extracellular concentrations of glutamate were also reported with seizures induced by the cholinesterase inhibitor soman (Wade et al., 1987; Lallement et al., 1991) and in epileptic patients (Carlson et al., 1992; During and Spencer, 1993). Systemic administration of diazepam resulted in a prompt and pronounced fall of extracellular hippocampal glutamate levels. This is consistent with the finding that diazepam markedly decreased high K^+- or veratridine-evoked glutamate release in hippocampal slices (Baba et al., 1983). Benzodiazepine-induced inhibition of the presynaptic release of glutamate has also been reported in other in vitro studies but the relation between release inhibition and anti-convulsant action was not clear (Meldrum, 1991). Taking into consideration the diazepam-induced quick decrease in extracellular concentrations of glutamate in our seizure model and the synchronous correlation of this decrease with behavioural modifications and ECoG changes, the modulation of glutamate efflux indeed appears to correspond well with the anti-convulsant action of diazepam.

The involvement of cholinergic mechanisms in pilocarpine-induced epilepsy has been well-documented (Turski et al., 1989). Cholinergic afferents terminate in all pyramidal subfields and in the dentate gyrus (Millan et al., 1993). Excitatory glutamatergic neurons are known to synapse directly on dendrites of principal or pacemaker neurons and also send communicating branches to GABAergic interneurons. By means of their terminals, these GABAergic interneurons can modulate the excitatory input (Roberts, 1986). The GABAergic terminals, which form a dense plexus around the somatic area of pyramidal neurons (Psarropoulou and Daillaire, 1998), form axoaxonic synapses with terminals of excitatory neurons to produce presynaptic inhibition (Roberts, 1986). Indeed, GABAergic terminals have been shown to attenuate muscarinic receptor-initiated postsynaptic excitatory input by proximal inhibition in the hippocampus, so that no amount of excitation causes firing (Psarropoulou and Daillaire, 1998). In a recent study, we have provided in vivo evidence that pilocarpine-induced seizures are initiated by muscarinic receptors and are further mediated by NMDA receptors (Smolders et al., 1997b). There is evidence that benzodiazepines and $GABA_A$ receptor blockers produce opposite modulation of hippocampal acetylcholine release. While flumazenil, a $GABA_A$ receptor antagonist, could significantly enhance hippocampal acetylcholine efflux, diazepam was shown to cause a marked decrease of the neurotransmitter levels (Imperato et al., 1994). Furthermore, potentiation of GABAergic transmission by systemic administration of benzodiazepines resulted in a reduction of the turnover rate of acetylcholine in the hippocampus, which was again blocked by the $GABA_A$ receptor antagonist bicuculline (Imperato et al., 1994). Taken together, these findings strongly suggest that activation of $GABA_A$ receptors is the principal mechanism involved in the action of diazepam against pilocarpine-induced seizures, and the diazepam-induced decreased hippocampal glutamate overflow shown in our results is a likely consequence of the GABAergic presynaptic inhibition leading to attenuation of excitatory input. However, in vitro studies have also shown that diazepam can bind to voltage-dependent sodium

channels to prolong their inactivation period (McLean and Macdonald, 1987) and attenuate voltage-dependent ionic currents in a dose-dependent manner (Ishizawa et al., 1997).

The pilocarpine-induced elevation in cerebellar glutamate levels was only transient and levels returned to baseline before the 14th collection. No further significant alterations were observed in cerebellar glutamate overflow after diazepam administration. Even though the glutamate increase was ephemeral, it corresponded well with the initial period of seizure generation. This may indicate the rapid spread of seizure activity from its primary locus and possibly signifies that the process is secondarily generalized. The rapid decline of cerebellar glutamate levels may indicate that the excitatory currents are not sufficient to sustain the process. We did not see any significant change with diazepam in the basal levels of glutamate in either the hippocampus or cerebellum. This may suggest that diazepam, at least at the used dose, modulates only depolarization-induced glutamate release in these brain regions. However, in a very recent microdialysis study, intraperitoneal triazolam (20 and 100 μg/kg) has been shown to reduce basal glutamate levels in the dorsal hippocampus but not in the cerebellum (Shimuzu et al., 1998). The reduced glutamate levels have been shown to closely correlate with the spatial memory deficits produced by the drug. Although diazepam and triazolam have a similar pharmacodynamic profile, triazolam has been shown to be most efficacious in allosteric modulation of GABA-gated currents, as revealed in a study where these benzodiazepine ligands were tested among others for binding to several recombinant $GABA_A$ receptors (Costa et al., 1994). The discrepancy between our observed effects with diazepam and that of triazolam on the basal hippocampal glutamate levels could possibly be linked to the ability of triazolam to maximize GABA actions at structurally different $GABA_A$ receptors. Moreover, since the study has shown the decrease in hippocampal glutamate release to correlate with the extent of memory deficit, diazepam, which has a lower liability for causing cognition deficit (Costa et al., 1994), would therefore be expected to cause smaller alterations of basal glutamate levels. The effect of triazolam on the basal cerebellar glutamate levels is in agreement with our observed effect with diazepam.

As for glutamate, we also observed an initial marked decrease in extracellular levels of hippocampal GABA. Acetylcholine induced inhibition of GABA release via presynaptic muscarinic non-M_1 receptors was demonstrated in the striatum (Marchi et al., 1990). A sustained increase of extracellular hippocampal GABA concentrations, reminiscent of the pattern of hippocampal glutamate levels, was also observed following pilocarpine perfusion. Pilocarpine induced an initial decrease followed by a pronounced increase of the extracellular levels of glutamate and GABA in the hippocampus, and only a monophasic increase of these transmitters in the cerebellum, as has been discussed in detail elsewhere (Smolders et al., 1997a,b,c). In both the hippocampus and the cerebellum, we observed no immediate changes either of the basal levels or of the pilocarpine-induced augmentation of extracellular GABA levels following diazepam administration. An earlier report has also shown that the protective effect of diazepam against isoniazid-induced seizures is not accompanied by modification of the rate of GABA depletion elicited by isoniazid (Bernasconi et al., 1985). Recent in vitro data suggested that diazepam, in addition to increasing the open probability of chloride channels, also acts by enhancing the conductance of $GABA_A$ channels activated by low ambient concentrations of extracellular GABA (Eghbali et al., 1998). The major effect of benzodiazepines has also been proposed to be mediated by a tonic background of extrasynaptic $GABA_A$ channel activity instead of synaptic transmission (Birnir et al., 1994; Eghbali et al., 1998).

Our results also show marked elevations of extracellular dopamine levels both during intrahippocampal perfusion with pilocarpine and during manifestations of seizure activity. Augmentation of hippocampal dopamine levels during pilocarpine administration could possibly be the result of an increased efflux mediated by presynaptic muscarinic stimulation. A similar increase in dopamine release has been demonstrated in the striatum (Raiteri et al., 1982). We suggest that the increased dopamine overflow, which extended beyond the period of pilocarpine perfusion, is likely to be mediated in response to the generation of seizure activity. There is evidence from in vitro studies that dopamine inhibits the excitability of hippocampal cells by hyperpolarising the resting membrane potential (Benardo and Prince, 1982; Suppes et al., 1985; Smialowski, 1987). In contrast, conflicting results also suggest that the initiation and spread of seizures in the hippocampus is assisted by endogenous dopamine released onto D_1 receptors (Whitton et al., 1992). Albeit oversimplified, dopamine D_1 and D_2 receptors have been implicated in the mediation of excitatory and inhibitory effects, respectively (Kaneko et al., 1993). We did not see any immediate significant change in the basal levels of extracellular dopamine after intraperitoneal diazepam. Although a host of studies have reported a reduction in basal dopamine levels by systemic diazepam in the nucleus accumbens (Invernizzi et al., 1991), prefrontal cortex (Dazzi et al., 1995) and striatum (Invernizzi et al., 1991), conclusive evidence is lacking for diazepam-induced modulation of basal dopamine levels in the hippocampus. We have shown significant attenuation of pilocarpine-induced increases in dopamine levels in the hippocampus following diazepam administration. Although some anti-epileptic drugs have been shown to induce enhanced dopamine release in hippocampus (Whitton et al., 1992; Kaneko et al., 1993; Smolders et al., 1997b) and therefore point to the involvement of dopamine in the suppression of seizures (Starr, 1996), benzodiazepines (Detouchi and Costa, 1973) are notable exceptions. In the

present study, the shift of pilocarpine-induced excitatory behavioural manifestations to extreme quietude and inactivity and the complete suppression of seizures following diazepam injection suggest that reduction of hippocampal dopamine levels is favourable to the anti-convulsant action of diazepam.

The hippocampal dopaminergic fibres originate in the mesencephalic-dopaminergic groups (ventral tegmental area–substantia nigra) (Scatton et al., 1980; Verney et al., 1985). The major source of dopaminergic fibres to the hippocampal formation is the fimbrial pathway. Fimbrial dopaminergic fibres innervate most of the CA1 and CA3 fields and account for 70% of the total hippocampal dopamine levels (Verney et al., 1985). These dopaminergic fibres, therefore, appear to be the ultimate target for the GABAergic inhibitory input in the hippocampus. Diazepam has been suggested to act by terminal regulation of dopamine release by an action on local GABA neurons (Zetterström and Fillenz, 1990; Finlay et al., 1995). Our previous observations also revealed the anti-convulsant effects of systemic administration of vigabatrin (a GABA transaminase inhibitor) were associated with a significant attenuation of pilocarpine-induced increases in dopamine levels (Smolders et al., 1997a). However, local perfusion with vigabatrin did not produce the same effects (Smolders et al., 1997a). Since systemic administration of diazepam can significantly attenuate dopamine levels in other brain regions including the basal ganglia, modulating influences from these areas that further potentiate hippocampal GABAergic inhibition cannot be ruled out. Although the existence of GABAergic afferent pathway(s) providing the inhibitory input onto the hippocampal dopaminergic neurons from the subcortical areas or other brain regions is not very clear, the possibility of a functional relationship between the basal ganglia and the hippocampus has been suggested via a presumptive strio–pallido–septal pathway (La Grutta et al., 1986). This suggestion was made on the basis of findings revealing tonic inhibitory effects of the caudate nucleus on the hippocampus. GABAergic influences on the dopaminergic nigrohippocampal pathway (Scatton et al., 1980; La Grutta and Sabatino, 1990) could also be implicated in the process.

A completely opposite effect was also reported when much higher doses of diazepam (32 mg/kg, i.p) were used, when anti-convulsant effects were accompanied by increases in cortical and hippocampal dopamine levels in inbred mutant E_1 mice (Hiramatsu et al., 1988). Dose and species differences may account for the discrepancy.

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