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Short communication

Flumazenil prevents diazepam-elicited anticonvulsant action and concomitant attenuation of glutamate overflow

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Abstract

Systemic administration of diazepam (5 mg/kg, i.p.) produced a prompt anticonvulsant effect in pilocarpine-induced seizures in freely moving rats. The anticonvulsant effect was associated with significant attenuation of pilocarpine-evoked increases in extracellular hippocampal glutamate levels to below the baseline levels. The purpose of the present microdialysis study, therefore, was to investigate if the effect of diazepam on glutamate release was mediated at the level of the benzodiazepine γ-aminobutyric acid$_A$ (GABA$_A$) receptor complex to preclude any non-GABAergic mechanisms. Systemic administration of the specific benzodiazepine-receptor antagonist flumazenil (10 mg/kg, i.p.)-elicited complete reversal of diazepam-evoked anticonvulsant action and concomitant attenuation of extracellular glutamate efflux below the baseline levels. This provides evidence that under the given experimental conditions, diazepam-evoked alterations in glutamate overflow associated with the anticonvulsant action were indeed mediated at the level of benzodiazepine-GABA$_A$ receptor complex, possibly involving the modulation of both pre- and post-synaptic sites of the receptor complex.

Keywords: Glutamate; Diazepam; Flumazenil; (Rat)

1. Introduction

Benzodiazepines actions are evidently mediated by allosteric modulation at the γ-aminobutyric acid$_A$ (GABA$_A$) receptor channel (Olsen, 1981; Haefely, 1994). Ionotropic GABA$_A$ receptors can mediate both pre- and post-synaptic inhibition. GABA$_A$-mediated pre-synaptic inhibition leads to inhibition of transmitter release from the excitatory axons (MacDermott et al., 1999). On the other hand, there is significant evidence of non-GABAergic-mediated effects of benzodiazepines, particularly implicating low affinity bindings with voltage-dependent Na^+ channels. Limitation of sustained repetitive firing in spinal cord neuronal cell culture was produced by bindings of benzodiazepines to voltage-dependent Na^+ channels and not to high affinity central benzodiazepine receptors (McLean and Macdonald, 1987; Backus et al., 1991).

Recently, we have demonstrated the anticonvulsant effect of diazepam (5 mg/kg) and the concomitant modulation of extracellular brain neurotransmitters in pilocarpine-induced seizures in freely moving rats. The anticonvulsant action was evident from changes both in behavioural and electrocorticographical recordings. This diazepam-elicited anticonvulsant effect was also associated with significant attenuation of the extracellular hippocampal glutamate levels below the baseline values (Khan et al., 1999). The findings provided the first in vivo evidence to a couple of previous in vitro reports demonstrating diazepam (Baba et al., 1983) and flurazepam (Vellucci and Webster, 1985) to reduce K^+-evoked glutamate release in rat hippocampal and spinal cord slices, respectively.

The plausible mechanisms by which diazepam can attenuate pilocarpine-induced increases in glutamate release may include either an augmentation of GABAergic inhibition, mediated both at the pre- and post-synaptic GABA$_A$ receptor sites and/or a reduction of voltage-dependent ionic currents. Since it is reasonable to assume an implication of voltage-dependent Na^+ channels in the action of

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benzodiazepines and since depolarization-evoked release of neurotransmitters is a function of voltage-gated Na^+ channels, we deemed it important to evaluate if the attenuation of glutamate was independent of diazepam's action at benzodiazepine-$GABA_A$ receptor level.

The present study was, therefore, designed to investigate if the reduction of glutamate levels concomitant with the anticonvulsant action of diazepam was mediated at the level of benzodiazepine-$GABA_A$ receptor complex by blocking the action of diazepam on the receptor with flumazenil, the specific benzodiazepine receptor antagonist.

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 for Animal Experiments of the Faculty of Medicine of the Free University of Brussels (V.U.B).

2.1. Surgery

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 (25 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: lateral +4.6, anteroposterior −5.6 and vertical +4.6. Post-operative analgesia was provided to each rat by giving a single injection of ketoprofen (4 mg/kg, s.c.). 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). Dialysate sampling was started after waiting for a minimal period of 24 h following the completion of surgery, permitting the animals to sufficiently recover. A total of twenty samples were collected, each at every 20-min interval. During the collection of the first eight basal samples, the perfusion fluid was composed of Ringer's solution only.

2.2. Experimental protocol

The experiments were broadly classified into four groups.

(1) Pilocarpine control (pilo/control) group: Consisted of first eight basal collections. During collection 9–10, the hippocampus was perfused with 10 mM pilocarpine in Ringer's solution. From collection 11 onwards, the perfusion fluid was switched back to Ringer's.

(2) Pilocarpine/diazepam (pilo/diaz) group: Consisted of first eight basal collections followed by intrahippocampal perfusion of pilocarpine during collections 9–10. A single injection of diazepam (5 mg/kg, i.p.) was given at the beginning of collection 14.

(3) Pilocarpine/diazepam/flumazenil group: Same as the diazepam group except that a single flumazenil injection (10 mg/kg, i.p.) was given immediately after diazepam was injected.

(4) Basal/flumazenil group: The rats received a single intraperitoneal injection of flumazenil (10 mg/kg, i.p.) at the beginning of collection 14.

All rats that did not receive diazepam and/or flumazenil were given corresponding vehicle(s) injections at the beginning of collection 14.

2.3. Chromatographic assays

Chromatographic conditions and precolumn derivatization procedures for the glutamate assay have been described in detail elsewhere (Smolders et al., 1995). Reversed phase microbore liquid chromatography with gradient elution and fluorescent detection was used. The precolumn derivatization was performed with o-phthalaldehyde/β-mercaptoethanol.

2.4. Reagents

Diazepam, pilocarpine and glutamic acid were purchased from Sigma (St. Louis, MO, USA). Flumazenil was obtained as a gift from Roche. Diazepam was dissolved in a mixture of saline, propylene glycol and ethanol (3:3:4). Flumazenil was suspended in a minimal amount of Tween-80 and adjusted to the final volume by adding saline. β-mercaptoethanol was obtained from Janssen Chimica (Beerse, Belgium). 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.

2.5. Statistical analysis

Extracellular glutamate levels in the dialysates were expressed in micromolar concentrations without correction for recovery across the dialysis membrane. Basal values in the figures (i.e. 100% baseline values) were the mean of eight stable neurotransmitter levels obtained in conditions before drug administration with standard error on the mean (S.E.M). For determination of the statistical significance of differences in the neurotransmitter levels following phar-

macological manipulations in time within one experimental group, one-way analysis of variance (ANOVA) for repeated measures and Fisher's protected least significant difference (Fisher's PLSD) post hoc tests ($\alpha = 0.05$) were used. Mann–Whitney's test ($\alpha = 0.05$) was used for comparison between two-subgroups on a certain time point.

3. Results

The mean basal concentration of extracellular glutamate was 0.541 ± 0.063 μM ($n = 23$).

3.1. Effects of 10 mM pilocarpine (pilo / control group) ($n = 6$)

3.1.1. Behavioural manifestations

Intrahippocampal administration of 10 mM pilocarpine resulted in the manifestations of limbic seizures. Approximately half an hour after intrahippocampal perfusion of 10 mM pilocarpine behavioural changes started to manifest with the appearance of gustatory automatisms including head bobbing, teeth chattering, vibrissae twitching, salivation and frequent wet dog shakes. In around another half an hour, these excitatory features progressively developed into intermittent motor limbic seizures characterised by facial muscle clonus, forelimb clonus, rearing and convulsions. During the subsequent 60 to 90 min, seizure manifestations gradually increased to reach a peak.

3.1.2. Changes in extracellular glutamate levels

Intrahippocampal perfusion of pilocarpine (40 min) resulted in an initial significant decline in the basal hippocampal extracellular glutamate levels to 30% ($P = 0.0014$) (see Fig. 1). This was immediately followed by a significant and sustained augmentation of glutamate levels to more than twofold (265%) ($P = 0.0001$) of the baseline values.

3.2. Effects of 10 mM pilocarpine and diazepam (5 mg / kg, i.p.) ($n = 6$)

3.2.1. Behavioural manifestations

Until collection 13, the behavioural manifestations were the same as that of the pilo/control group. Intraperitoneal injection of 5 mg/kg, diazepam at the beginning of collection 14 completely abolished all the behavioural alterations induced by pilocarpine in around 5 min. The rats were quiet and appeared to be under the hypnotic effects of diazepam. This effects lasted until the end of an experiment.

3.2.2. Changes in extracellular glutamate levels (Fig. 1)

After the initial significant decline in the basal hippocampal dialysate concentrations of glutamate to 39% ($P = 0.0016$) during intrahippocampal perfusion of pilocarpine, the extracellular levels of glutamate soon markedly increased to more than twofold (209%) ($P = 0.0001$) of the baseline levels. Intraperitoneal injection of diazepam

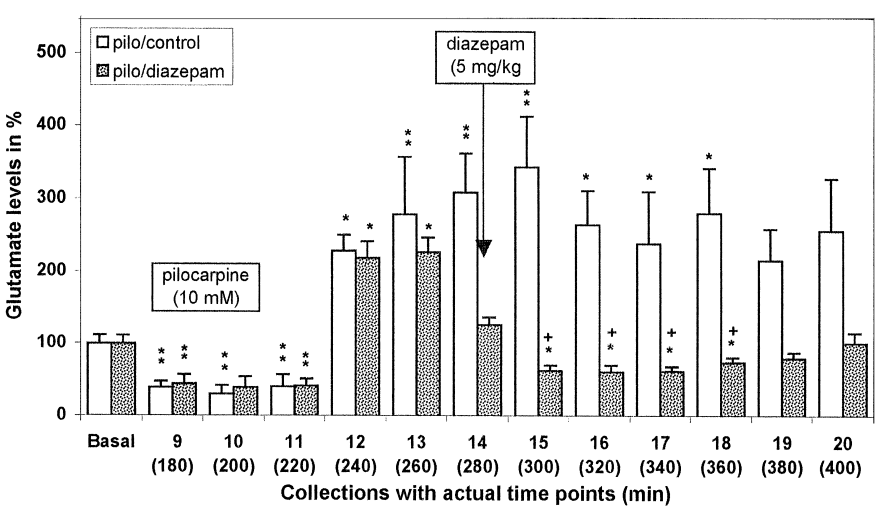

Fig. 1. Effects of intrahippocampal pilocarpine (10 mM) alone (pilo/control group) ($n = 6$) and pilocarpine in combination with systemic diazepam (5 mg/kg, i.p) (pilo/diazepam group) ($n = 6$) on the extracellular levels of glutamate (in percentage of the baseline level) (mean ± S.E.M). For the basal collection (collections under baseline conditions), the level is expressed as a percentage (mean ± S.E.M) of the pooled 160-min (collections 1–8) stable dialysate concentrations during which the probes were perfused with Ringer's solution only. Each of the remaining bars represents a 20-min collection period, indicated by the corresponding collection number and the actual time point (min). The arrow pointing the bar indicates the time point of corresponding drug administration. The horizontal bar captioned "pilocarpine" represents the length of intrahippocampal perfusion of 10 mM pilocarpine. The asterisks denote the values significantly different from the corresponding baseline values ($^{**}P < 0.01$, $^{*}P < 0.05$) [Statistics: Anova and Fisher's PLSD post hoc test]. The plus sign (+) denotes the values that are significantly different from the corresponding values in the pilo/control group ($^{+}P < 0.05$) [Statistics: Mann–Whitney].

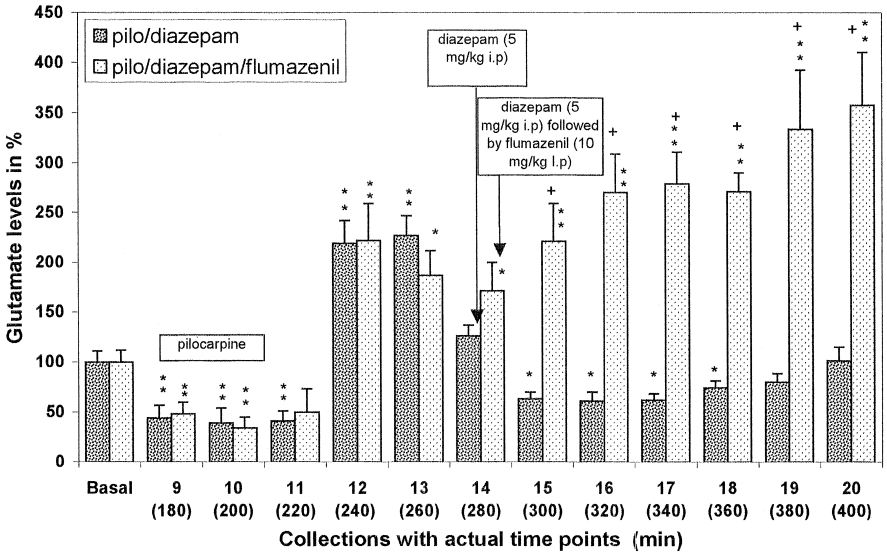

Fig. 2. Effects of pilocarpine in combination with systemic diazepam (5 mg/kg, i.p) (pilo/diazepam group) ($n = 6$) (same as in Fig. 1) and in combination with systemic diazepam and flumazenil (10 mg/kg, i.p) (pilo/flumazenil/diazepam group) ($n = 6$) on the extracellular levels of glutamate (in percentage of the baseline level) (mean ± S.E.M). For the basal collection (collections under baseline conditions), the level is expressed as a percentage (mean ± S.E.M) of the pooled 160-min (collections 1–8) stable dialysate concentrations during which the probes were perfused with Ringer's solution only. Each of the remaining bars represents a 20-min collection period, indicated by the corresponding collection number and the actual time point (min). The arrow pointing the bar indicates the time point of corresponding administration of the drugs. The horizontal bar captioned "pilocarpine" represents the length of intrahippocampal perfusion of 10 mM pilocarpine. The asterisks denote the values significantly different from the corresponding baseline values ($^{**}P < 0.01$, $^{*}P < 0.05$) [Statistics: Anova and Fisher's PLSD post hoc test]. The plus sign (+) denotes the values that are significantly different from the corresponding values of pilo/control group ($^{+}P < 0.05$) [Statistics: Mann–Whitney].

prevented pilocarpine-evoked augmentation in glutamate overflow. More strikingly, the glutamate efflux was significantly decreased to 61% between collections 15–18, as compared to the baseline level ($P = 0.0061$).

3.3. Effects of 10 mM pilocarpine, diazepam (5 mg/kg, i.p.) and flumazenil (10 mg/kg, i.p.) (n = 6)

3.3.1. Behavioural manifestations

The behavioural manifestations were basically the same as that of the pilo/control group. Intraperitoneal injection of diazepam did not give any protection against pilocarpine-induced seizures in the presence of flumazenil, as the animals continued to suffer from intermittent motor limbic seizures.

3.3.2. Changes in extracellular glutamate levels

Intrahippocampal perfusion of pilocarpine resulted in immediate attenuation of extracellular glutamate levels to 33% ($P = 0.0024$). This was followed by pronounced elevation of glutamate levels and unlike in the pilo/diaz group, the elevation remained sustained following the administration of diazepam and flumazenil. The extracellular glutamate levels increased up to 357% ($P = 0.0001$) and the values from collection 15–20 were significantly different from the corresponding values in the pilo/diaz group ($P = 0.01$) (see Fig. 2).

3.4. Effects of flumazenil (10 mg/kg, i.p.) on the basal glutamate levels (n = 5)

A single intraperitoneal injection of flumazenil did not produce any significant changes in the glutamate levels as compared to the baseline values (data not shown). There were also no appreciable changes observed in the normal behaviour of the rats.

4. Discussion

We are reporting here that flumazenil can antagonize diazepam-evoked anticonvulsant effect and the concomitant alterations of hippocampal extracellular glutamate overflow in pilocarpine-induced seizures in freely moving animals.

Intrahippocampal perfusion of pilocarpine clearly produced the induction of limbic seizures in the rats as were evident from pronounced behavioural alterations. The period of a peak in seizure manifestations also corresponded well with the pronounced augmentation of extracellular glutamate levels. The perfusion of pilocarpine, however, was accompanied by an initial decline in the basal extracellular glutamate levels, possibly owing to an activation of the pre-synaptic muscarinic receptors to produce an inhibition of neurotransmitter release (Marchi et al., 1989,

1990). The initial attenuation and subsequent increases of extracellular glutamate levels during pilocarpine-induced seizures are in accordance with our previous studies (Smolders et al., 1997a,b; Khan et al., 1999, 2000).

When diazepam was administered at a peak of seizure activity, the convulsive episodes promptly subsided. This anticonvulsant action of diazepam was also evident from continuous electrocorticographical recording monitored in our previous study (Khan et al., 1999). The extracellular glutamate levels were also significantly reduced as compared to the control values. The attenuation of glutamate levels did not stop at that point, but went further down significantly below the baseline values. The reduction of pilocarpine-induced increased efflux of glutamate appeared as a concomitant feature whenever a protective effect was manifested by any of the several anticonvulsants that we have tested so far in the focal pilocarpine model (Smolders et al., 1997a,b,c; Khan et al., 2000). Such diminution of glutamate promontory appeared to be a reciprocal effect in response to the arrest of seizures. However, unlike the effect of diazepam on glutamate efflux in the present and in our previous study (Khan et al., 1999), under no circumstances did the glutamate efflux decline significantly below the baseline values. This gives a strong indication that diazepam-evoked reduction of glutamate overflow is not solely a corollary to the seizure arrest, but involves additional mechanism(s). Two possible mechanisms may additionally contribute to the glutamate efflux to diminish: the first, via a modulation of pre-synaptic ionotropic $GABA_A$ receptors (MacDermott et al., 1999) and the second, via a possible modulation of voltage-dependent Na^+ currents, which are present in the pre-synaptic terminals. To exclude one of these possibilities, we blocked the action of diazepam on the benzodiazepine receptors with the specific benzodiazepine receptor antagonist flumazenil. The resultant effects did not support any action other than that is mediated solely at the benzodiazepine-$GABA_A$ receptor level as flumazenil completely reversed both the anticonvulsant action and the glutamate attenuation. The specificity of flumazenil in this regard is proven as several authors have demonstrated that the antagonist has no effect on the action of diazepam on voltage-dependent Na^+ channels (McLean and Macdonald, 1987; Backus et al., 1991; Ishizawa et al., 1997). The present data, therefore, provide evidence that diazepam-evoked attenuation of glutamate release is indeed a receptor-mediated effect at the level of benzodiazepine-$GABA_A$ receptor complex. We further hypothesise that diazepam-elicited effects may involve the modulation of both pre- and post-synaptic $GABA_A$ receptors.

Interestingly, unlike their effect on the evoked release of amino acids, benzodiazepines, like diazepam (Khan et al., 1999) and flurazepam (Vellucci and Webster, 1985), do not influence the basal amino acids efflux. In the present study, flumazenil was also found not to alter the basal glutamate levels. One plausible explanation for this selective response of benzodiazepines could be the activity-dependent nature of effective GABA synaptic responses (Thompson and Gahwiler, 1989; Mohler, 1998; Jackson et al., 1999). Drugs like tiagabine and vigabatrin that positively modify the amplitude of GABA-mediated inhibitory post-synaptic potentials (IPSPs) were more effective when IPSPs were evoked by trains of high frequency stimuli, rather than when elicited by a single stimulus (Jackson et al., 1999, 2000).

We conclude that under the present experimental conditions, diazepam-elicited anticonvulsant effect and attenuation of glutamate elevation in pilocarpine-induced seizures are mediated at the level of benzodiazepine-$GABA_A$ receptor complex, which may involve the modulation of both pre- and post-synaptic sites of the receptor complex. The effects of diazepam on pilocarpine-induced augmentation of glutamate, and not on the basal glutamate levels, suggest activity-dependent enhancement of GABAergic inhibition.

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