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Effects of GYKI 52466 and some 2,3-benzodiazepine derivatives on hippocampal in vitro basal neuronal excitability and 4-aminopyridine epileptic activity

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Abstract

In order to determine whether the anticonvulsant effect of 2,3-benzodiazepines is also displayed in a model of in vitro epilepsy, such as the ''epileptiform'' hippocampal slice, we studied the effects of 2,3-benzodiazepine 1-(4-aminophenyl)-4-methyl-7,8-methylenedioxe-5*H* 2,3-benzodiazepine hydrochloride (GYKI 52466) and some new 2,3-benzodiazepine derivatives on CA1 basal neuronal excitability and on CA1 epileptiform burst activity produced by 4-aminopyridine in rat hippocampal slices. The results showed that GYKI 52466 affected basal neuronal excitability as evidenced by its influence on the magnitude of the CA1 orthodromic-evoked field potentials. 2,3-Benzodiazepines showed their antiepileptic effect also in an in vitro model of experimental epilepsy. The effects of the new 2,3-benzodiazepine derivatives suggest that the methylenedioxidation in positions 7 and 8 of the 2,3-benzodiazepine ring is the main structural modification for the antiepileptic effect of 2,3-benzodiazepines to take place.

Keywords: GYKI 52466; 2,3-Benzodiazepine; Anticonvulsant; Epilepsy; Hippocampus; Brain slice; Epileptiform bursting

1. Introduction

A large body of evidence supports the opinion that the antiepileptic effect of some anticonvulsants may be mediated by interference with excitatory amino acid neurotransmission (Meldrum, 1985). A group of drugs with anticonvulsant effects, recently hypothesized to interact with excitatory amino acid neurotransmission, is represented by 2,3-benzodiazepines (Rogawsky, 1993). These compounds are derivatives of tofisopam, a 2,3-benzodiazepine that exerts different pharmacological effects from those of conventional 1,4-benzodiazepines such as diazepam. In fact, even when tofisopam displays anxiolytic effects, it does not produce myorelaxant or sedative or anticonvulsant effects. Furthermore, tofisopam does not affect classical benzodiazepine recognition sites (Rogawsky, 1993). However, further studies on 2,3-benzodiazepines have evidenced that a derivative compound termed GYKI 52466 (1-(4-aminophenyl)-4-methyl-7,8-methylenedioxy-5*H* 2,3-benzodiazepine hydrochloride), displays, unlike tofisopam, anticonvulsant and myorelaxant effects, but does not affect classical benzodiazepines recognition sites (Tarnawa et al., 1990). Electrophysiological studies with rat cortical slices have recently demonstrated that GYKI 52466 was an antagonist of excitatory amino acid neurotransmission. Specifically, the drug antagonizes the excitatory effects produced by glutamate and quisqualate, but not those produced by *N*-methyl-D-aspartate (NMDA) (Ouardouz and Durand, 1991). More recently, using the ''whole cell recording'' technique, it has been shown that GYKI 52466 is a highly selective, non-competitive antagonist of the excitatory effects of α-amino-3-hydroxy-5 methyl-4-isoxazolepropionic acid (AMPA) in rat hippocampal neuronal cultures (Donevan and Rogawsky, 1993; Paternain et al., 1995).

According to the role of excitatory amino acids in the genesis of neuronal injury, GYKI 52466 exerts, like the (non-) NMDA receptor antagonist, 6-cyano-7-nitroquinoxaline 2,3-dione (CNQX), neuroprotective effects, preventing the hippocampal neuronal death due to global ischemia in rats (Le Peillet et al., 1992). GYKI 52466

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displays a wide spectrum of antiepileptic effects in in vivo models of experimental epilepsy. It inhibits electrical, chemical or reflex seizures. Systemically administered, GYKI 52466 prevents convulsions due to maximal electroshock at dosages also affecting motor coordination in the rotarod test. The drug appears more potent against reflex seizures, inhibiting sound-induced convulsions in DBA/2 mice at doses that do not affect motor coordination (Chapman et al., 1991). Furthermore, GYKI 52466 prevents convulsions caused by kainic acid or 4-aminopyridine with a higher efficacy than the non-NMDA receptor antagonists, DNQX or CNQX (Rogawsky, 1993).

Since in vivo studies have shown the efficacy of 2,3-benzodiazepines in antagonizing convulsions caused by 4-aminopyridine (Rogawsky, 1993), the effects of GYKI 52466 were studied on the CA1 epileptiform burst activity induced by 4-aminopyridine in rat hippocampal slices. Similar extracellular electrophysiological changes were induced by treatment of hippocampal slices with various epileptogenic agents with different mechanisms of action. In particular, while the in vitro models of epileptiform activity produced by kainic acid or magnesium-free solution are dependent on the postsynaptic stimulation of non-NMDA and NMDA receptors, respectively, the model of 4-aminopyridine presents a complex genesis. The epileptogenic agent is a blocker of $K_{\pm}$ conductances, specifically K^+ IA currents (Voskuyl and Albus, 1985). The inhibition of neuronal membrane repolarization produced by the blockage of these conductances elicits an increase in postsynaptic inhibitory and excitatory potentials (Perreault and Avoli, 1991). Furthermore, 4-aminopyridine increases the release of neurotransmitters including γ-aminobutyric acid (GABA), glutamate and noradrenaline (Versteeg et al., 1995). Therefore, it has been hypothesized that this model of experimental epilepsy has a presynaptic genesis that depends on the increased release of glutamate caused by the excitation of afferent axons. In agreement with this hypothesis, drugs capable of blocking the neurotransmitter release, e.g., the Ca^{2+} channel antagonist, verapamil, inhibit the 4-aminopyridine epileptiform burst activity (Frank et al., 1988).

Finally, in this paper, we studied the in vitro antiepileptic effects of some new 2,3-benzodiazepines, analogues of tofisopam, in order to determine which structural modifications of the 2,3-benzodiazepine ring are necessary for antiepileptic activity.

2. Materials and methods

2.1. Slice preparation

Male Wistar rats, weighing 200–300 g, were killed by decapitation under ether anesthesia, the skull was opened and the hippocampus was rapidly removed. Hippocampal slices 450 μm thick were cut with a tissue chopper (McIlwain) and immediately placed into an interface-type recording chamber, where they were constantly perfused at a rate of 12 ml/min with an artificial cerebrospinal fluid saturated with 95%–5% of O_2–CO_2. The artificial cerebrospinal fluid had the following composition: 122 mM NaCl, 0.4 mM KH_2PO_4, 3 mM KCl, 1.2 mM $MgSO_4$, 25 mM $NaHCO_3$, 1.3 mM $CaCl_2$, 10 mM glucose (pH 7.3). The temperature of the perfusion chamber was maintained at 33 ± 1°C. A period of 60–90 min elapsed between the preparation of the slices and the recording session.

2.2. Recording session

Orthodromic field potentials were recorded with 3M NaCl-filled glass microelectrodes (5–10 MΩ) in the CA1 area of the stratum radiatum (dendritic field potentials) or stratum pyramidale (somatic field potentials) after electrical stimulation (0.1 Hz, 70 μs, 200–400 μA) by means of a bipolar tungsten steel electrode positioned in the stratum radiatum. Electrical potentials, in particular the amplitude of the somatic population spike (measured from the beginning to the maximum of the negative deflexion of the field potential) or the slope of the dendritic or somatic excitatory postsynaptic potential (EPSP) (measured from the beginning to the maximum of the negative or positive deflexion of the field potential) were amplified, recorded on tape (Racal 4DS), digitized at 10 kHz and analyzed on averaged (five consecutive recordings) traces. Before beginning the application of drugs, a stable field potential was achieved within 30–60 min. At the beginning of each experiment, a stimulus–response curve, i.e., complete input/output curves, was recorded. As the aim of these experiments was to compare the effects of the test drugs on basal neuronal excitability and on in vitro epileptogenesis, the electrical field potentials were set at supramaximal stimulation in order to evoke a full-blown population spike. In addition, paired-pulse stimulation at 15-ms intervals was performed on the set responses. Experiments in which the amplitude of the PS was lower than 2 mV were not considered. Before beginning drug treatment, 60 min was allowed to elapse in order to obtain a stable (max 0.2 mV, −1 ms changes between stimulations) field potential.

The extracellular-evoked dendritic orthodromic field potential consisted of a negative-going wave (EPSP, 1–2 mV, 3–4 ms). The extracellular-evoked somatic orthodromic field potential consisted of a negative population spike (PS, 2–4 mV, 2–3 ms) with a latency of 3–4 ms from the artifact of the stimulus, superimposed on a positive-going wave (EPSP, 1–2 mV, 3–4 ms). Paired-pulse stimulation at 15-ms intervals produced a secondary or conditioned evoked PS with the same characteristics as the primary evoked potential but of a higher magnitude. Thus, under control conditions, there was a small degree of paired-pulse facilitation or synaptic facilitation.

2.3. Experimental procedure

In the first experimental series, the effects of a 30-min perfusion with the drugs were analyzed on CA1 basal neuronal excitability as evidenced by their influence on the CA1-evoked dendritic and somatic orthodromic PS. In addition, experiments with paired-pulse stimulation, at 15-ms interpulse intervals, were also done to evaluate the influence of the drugs on the secondary or conditioned CA1-evoked somatic orthodromic PSs and their effects were evaluated on the CA1 recurrent inhibition (Dingledine and Laengton, 1980; Sagratella et al., 1991, 1993). These results are expressed as percent changes of the CA1 EPSP and PS amplitude with respect to pre-drug values.

In the second experimental series, the effects of a 30-min perfusion with the drugs were analyzed on the CA1-evoked somatic epileptiform burst activity produced by 100 μM 4-aminopyridine. The epileptogenic agent alone or in combination with the anticonvulsant drug was directly added to the perfusion solution, and after 30 min of perfusion, their effects were evaluated on the duration of CA1 extracellular epileptiform burst activity. This was measured from the first to the last population spike with an amplitude higher than 0.2 mV (Sagratella et al., 1987; Domenici et al., 1994).

In the third experimental series, we studied the effects of some new 2,3-benzodiazepines. The synthesis of these tofisopam-related compounds was performed according to a previous report (Gatta et al., 1985). All these compounds have an aromatic group, differently substituted in position 1, and two methoxilic groups (compounds of the A series) or a methylendioxy group (compounds of B series) in positions 7 and 8 of the benzodiazepine ring. The benzenic ring is substituted at position 2 with a Cl or a nitro group ($C_{16}H_{11}ClN_2O_2$ compound B_2; $C_{18}H_{17}N_3O_4$ compound A2) and at position 4 with a Cl or a F ($C_{18}H_{17}ClN_2O_2$ compound A1; $C_{16}H_{11}ClN_2O_2$ compound B1; $C_{16}H_{11}FN_2O_2$ compound B3). The chemical structure of the new 2,3-benzodiazepines is reported in Fig. 1. The effects of a 30-min perfusion with the new 2,3-benzodiazepine derivatives was analyzed on the CA1-evoked somatic orthodromic epileptiform burst activity produced by 4-aminopyridine (100 μM). The epileptogenic agent alone or in combination with the 2,3-benzodiazepine derivatives was directly added to the perfusion solution, and after 30 min of perfusion, their effects were evaluated on the duration of CA1 extracellular epileptiform burst activity.

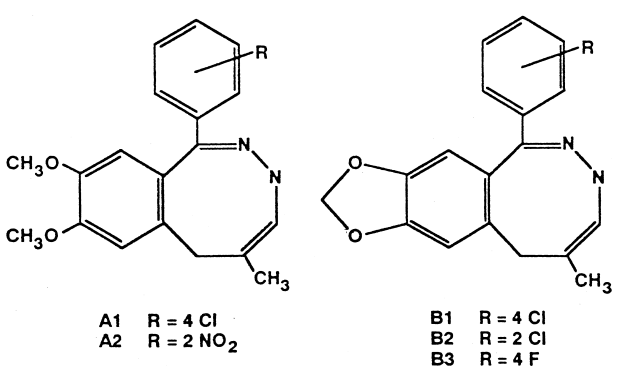

Fig. 1. Chemical structures of some new 2,3-benzodiazepine derivatives. The figure shows, on the right, the chemical structure of a tofisipam-like compound and, on the left, the chemical structure of a GYKI 52466-like compound. Below this, it is indicated which modifications of *R* of the benzoic ring were necessary to obtain the new 2,3-benzodiazepine derivatives.

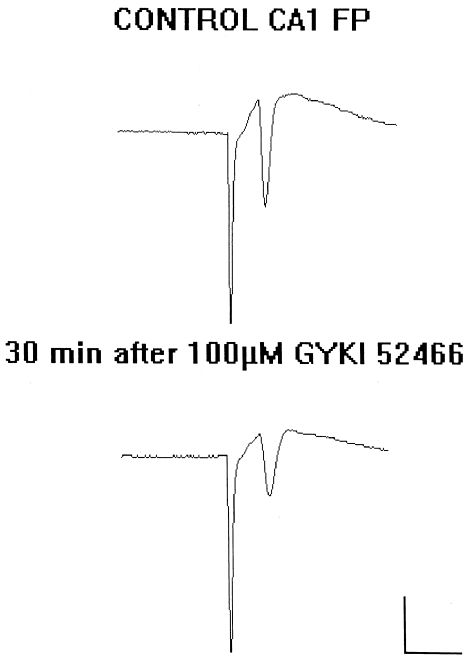

Fig. 2. Effects of GYKI 52466 on CA1 basal neuronal excitability in rat hippocampal slices. Top: Control CA1 somatic extracellular field potential (FP). Bottom: The drug produced within 30 min a reduction of the magnitude of the somatic orthodromic EPSP and population spike. Calibrations: 5 mV, 5 ms.

2.4. Drugs

4-Aminopyridine and GYKI 52466 were dissolved directly in the artificial cerebrospinal fluid. The new 2,3-benzodiazepines were prepared in dimethyl sulfoxide (DMSO) and then added to the artificial cerebrospinal fluid (the final DMSO concentration was 0.1%). Preliminary experiments have shown that 0.1% DMSO did not affect control CA1 population spikes ($N = 6$), and that 0.1% DMSO did not significantly affect the duration of CA1 epileptiform burst activity in response to 100 μM 4-aminopyridine ($N = 3$). Concentration ranges were 50–100 μM for the tested 2,3-benzodiazepines.

2.5. Statistical analysis

Unpaired Student's *t*-test was performed on the means of the values of the duration of the CA1 extracellular

Table 1
Effects on basal neuronal excitability
Effects of 30-min perfusion of slices with the drugs on the amplitude of the dendritic EPSP and of the primary somatic EPSP and of the primary (PSA) and secondary (2PSA) population spikes at intermediate levels of paired-pulse stimulation.
Abbreviations: R = registration; d = dendritic; s = somatic; A = significantly different from GYKI 52466 50 μM ($P < 0.05$ according to unpaired Student's *t*-test).

Drugs	Concentration (μM)	*N*	R	EPSP	PSA	2PSA
				(% changes ± S.E.M from pre- drug)		
GIKI 52466	50	5	d	−10±7	–	–
GIKI 52466	100	5	d	−65±12 A	–	–
GYKI 52466	50	10	s	−15±8	−20±13	−15±9
GYKI 52466	100	10	s	−45±18 A	−63±12 A	–

epileptiform burst activity after the epileptogenic agents or the epileptogenic agents plus the anticonvulsant drugs. A *P* value of < 0.05 was considered a significant difference.

3. Results

3.1. Effects on basal neuronal excitability

Slice perfusion with 50–100 μM GYKI 52466 ($N = 10$) produced a dose-dependent significant ($P < 0.05$) reduction of the magnitude of the dendritic and somatic CA1 EPSP slope and of the population spike within 30 min (Fig. 2, Table 1). The effect disappeared within 30–60 min after withdrawing the drug from the perfusion solution. Slice perfusion with 50 μM GYKI 52466 ($N = 5$) failed to produce within 30 min significant changes in the magnitude of the secondary population spike produced by paired-pulse stimulation, but not of the primary population spike (Table 1).

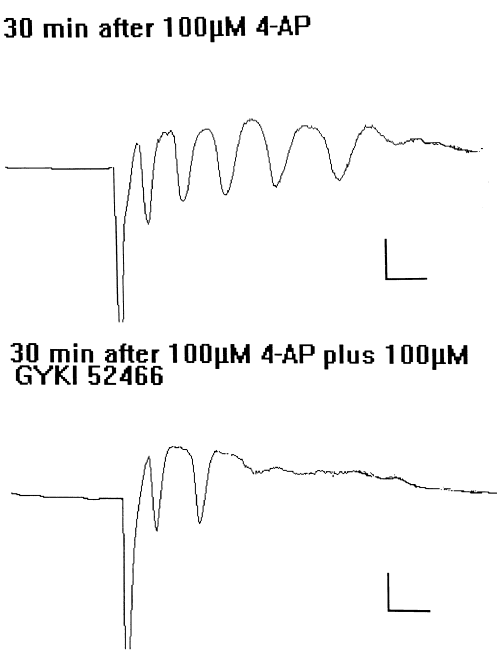

Fig. 3. Antiepileptic effects of 2,3-benzodiazepines. Top: Epileptiform burst activity produced by 4-aminopyridine (4AP). Bottom: Antiepileptic effects of GYKI 52466. Calibrations: 5 mV, 5 ms.

Table 2
Effects of 2,3 benzodiazepines on 4-aminopyridine epileptic activity
The influence of some 2,3-benzodiazepine derivatives on the burst activity duration of 4-aminopyridine-induced epileptiform bursting. Abbreviation: 4-AP = 4-aminopyridine.

Drugs	Concentration (μM)	*N*	Epileptiform bursting duration (means ± S.E.M)
4-AP	100	8	23.3±1.6
4-AP + GYKI 52466	100 + 50	5	9.7±2.1*
4-AP + GYKI 52466	100 + 100	5	6.8±1.8**
4-AP + A1	100 + 100	4	27.0±8.0
4-AP + A2	100 + 100	4	31.0±7.0
4-AP + B1	100 + 100	4	22.5±8.5
4-AP + B2	100 + 100	6	7.6±2.1**
4-AP + B3	100 + 100	6	14.6±5.0*

*Significantly different from controls ($P < 0.05$ in agreement with unpaired Student's *t*-test).
**Significantly different from controls ($P < 0.05$ in agreement with unpaired Students' *t*-test).

3.2. Effects on CA1 epileptiform burst activity

Slice perfusion with 100 μM 4-aminopyridine ($N = 8$) produced within 30 min the development of an epileptiform-evoked burst in 100% of experiments. This activity was characterized by an increase in activity in the magnitude of the primary PS and by the appearance of several additional PSs that prolonged the duration of the evoked potential from 2–3 to 10–30 ms (Fig. 3). In our hands, 4-aminopyridine did not produce spontaneous field potentials. In this case, evoked potentials were not disturbed by the preceding of spontaneous activity (Voskuyl and Albus, 1985; Perrault and Avoli, 1991).

Slice perfusion with 50–100 μM GYKI 52466 together with 100 μM 4-aminopyridine ($N = 10$) produced within 30 min a significant ($P < 0.05$), concentration-dependent

reduction of the duration of 4-aminopyridine-induced epileptiform burst activity as compared to the effect of slice perfusion with the epileptogenic agent alone (Fig. 3, Table 2).

Slice perfusion with A1 or A2 or B1 (up to 100 μM) together with 100 μM 4-aminopyridine ($N = 12$) failed to produce within 30 min any significant modification of the duration of 4-aminopyridine-induced epileptiform burst activity (Table 2).

Slice perfusion with 100 μM B2 or 100 μM B3 together with 100 μM 4-aminopyridine ($N = 12$) produced within 30 min a significant ($P < 0.05$) decrease in the duration of 4-aminopyridine-induced epileptiform burst activity (Table 2).

4. Discussion

Biochemical and morphological studies have demonstrated that the CA1 Schaffer collateral–pyramidal cell interconnections are glutamatergic synapses (Fleck et al., 1993). Furthermore, electrophysiological intracellular studies have revealed that the CA1 EPSP is dependent on the activation of excitatory amino acid receptors of the non-NMDA subtype, while the influence of NMDA receptors is partial (Collingridge and Lester, 1989). As reported for the non-NMDA antagonist, CNQX (Collingridge and Lester, 1989), the 2,3-benzodiazepine, GYKI 52466, reduced the magnitude of the extracellular CA1 PS and thus, CA1 basal neuronal excitability. In addition, GYKI 52466 failed to modify the small degree of synaptic facilitation present under control conditions as evidenced by the lack of effect on the control paired-pulse facilitation. The classical 1,4-benzodiazepines, which potentiate GABAergic neurotransmission, are reported to shift the control paired-pulse facilitation into paired-pulse inhibition (Ashton and Wauquier, 1985). Thus, the lack of effect of the 2,3-benzodiazepines on paired-pulse facilitation indicates that the depressive activity of the drugs does not depend on an increase of GABAergic neurotransmission, as it does with 1,4-benzodiazepines. All in all, the data on basal neuronal excitability demonstrated that GYKI 52446, like the non-NMDA receptor antagonist, CNQX (Collingridge and Lester, 1989), affected the neurotransmission mediated by non-NMDA receptors in the hippocampal CA1 area, suggesting an inhibitory influence of 2,3-benzodiazepines on these receptors.

The 2,3-benzodiazepine, GYKI 52466, is reported to display a wide spectrum of anticonvulsant actions in vivo (Chapman et al., 1991) and also antiepileptic effects in vitro. The drug has been found to be effective in a concentration-dependent manner in presynaptic models of in vitro epilepsy produced by the potassium blocker, 4-aminopiridine (see Section 1). The different effects of NMDA and non-NMDA antagonists in the 4-aminopyridine in vitro epileptiform model have been reported. In particular, only non-NMDA receptor antagonists are reported to block the interictal spontaneous or evoked 4-aminopyridine epileptic activity (Gean, 1990; Avoli et al., 1996). This suggests that the epileptiform activity produced by 4-aminopyridine, which is evoked by an increased release of glutamate, is mainly dependent on the activation of non-NMDA receptors, as it is in the kainic acid or AMPA model. Thus, the inhibitory effects of GYKI 52466 in the 4-aminopyridine model further confirm the influence of the drug on glutamatergic neurotransmission mediated by non-NMDA receptors.

On the whole, the data suggest that GYKI 52466 affects both basal neuronal activity and the genesis of orthodromic epileptiform burst activity, as does the non-NMDA receptor antagonist, CNQX (Hwa and Avoli, 1991).

A structure–activity relationship study was also performed with some new 2,3-benzodiazepines related to tofisopam. The results showed that compounds from A series, without the methylenedioxy group in positions 7 and 8 of the 2,3-benzodiazepine ring, failed, in contrast to B2 and B3 compounds, to antagonize the CA1 epileptiform burst activity evoked by 4-aminopyridine. The data suggest that the methylendioxidation in positions 7 and 8 is the main structural change responsible for the antiepileptic effect of 2,3-benzodiazepines. The structure of the aromatic group in position 1 of the benzodiazepine ring was also important for the antiepileptic effect of the drugs. In fact, the substitution of the aminic group of GYKI 52466 with Cl at position 4 of the phenyl group blocked the antiepileptic effect (compound B1). This effect persisted when F replaced the aminic group (compound B3). Conversely, structural modifications at position 4 of the phenyl group were not essential for the antiepileptic effect of 2,3-benzodiazepines. In fact, compound B2, with a Cl at position 2 of the phenyl group, also had an antiepileptic effect.

Some data in the literature on several derivatives of GYKI 52466 demonstrated that structural modifications at position 3 of the benzodiazepine ring enhance the biological activity of 2,3-benzodiazepines (Tarnawa et al., 1993; Donevan et al., 1994). The present data show that structural modifications within the phenyl group, at position 1, are also important for the antiepileptic effect of 2,3-benzodiazepines.

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