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CHANGES IN BENZODIAZEPINE RECEPTOR LIGAND AFFINITY IN THE PRESENCE OF 4,5,6,7-TETRAHYDROISOXAZOLO-(5,4-c-)-PYRIDIN-3-OL (THIP)

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KEY WORDS: highly specific GABA agonists; benzodiazepine binding; benzodiazepine receptor ligands.

At least three groups of ligands of benzodiazepine (BD) receptors have now been described [3]. These groups of ligands, depending on their pharmacological activity, can be differentiated into agonists, opposite agonists, and antagonists [3, 8]. Many experiments to study radioligand binding have shown that GABA agonists or barbiturates increase the affinity of receptors for agonists of BD-receptors [1, 3-7, 11] and reduce affinity for inverse agonists; affinity for antagonists of BD-receptors (Ro 15-1788, CGS 8216), moreover, is unchanged [3]. It has been suggested that the ability of GABA, muscimol, or barbiturates to increase or decrease the affinity of BD receptor ligands may reflect the pharmacological activity of these substances [3, 5, 7].

A group of highly specific agonists of $GABA_A$ receptors has recently been described [9, 10], including 4,5,6,7-tetrahydroisoxazolo-(5,4-c-)-pyridin-3-ol (THIP), isoguvacine, and piperidine-4-sulfonic acid which, unlike GABA and muscimol, do not increase BD binding [2, 9, 10]. In the absence of chloride ions at 0°C, moreover, these substances inhibited BD binding and antagonized the stimulating action of muscimol or GABA [2, 9, 10]. The inhibitory action of THIP or isoguvacine was manifested best if binding took place at 0°C with unwashed (intact) membranes in Tris-HCl buffer of low molarity [2, 10]. These effects were explained on the grounds that THIP possesses a mixed agonistic-antagonistic action or through its effect on a special population of GABA receptors linked with BD receptors [2, 9, 10].

In this paper we give data showing that BD receptor ligands change their affinity in the presence of THIP, and that the shift of affinity induced by THIP can be used to predict the activity of these substances in vitro.

EXPERIMENTAL METHOD

The forebrain of adult male Wistar rats was homogenized (1100 rpm, 10 passages) in 50 volumes of 25 mM Tris-HCl buffer in a glass homogenizer with Teflon pestle (Braun Melsungen). The homogenate was diluted to 600 volumes (w/v) and used directly in the binding experiments. Aliquots of the homogenate in a final volume of 1 ml were incubated with 1.25-1.35 nM ^{3}H-flunitrazepam (^{3}H-FNZ, 74-83 Ci/mmole, Amersham International, England) at 0°C for 1 h with corresponding concentrations of the substances and in the presence or absence of THIP (100-200 μM). Nonspecific binding was determined as the difference between binding in the absence and presence of 5 μM diazepam. At the end of incubation the bound and free radioligand was separated by filtration through GF/B (Whatman, England) filters. The filters were washed twice with 5 ml

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of buffer and placed in flasks containing dioxan scintillator. The flasks were kept in darkness for 4-6 h, after which radioactivity in them was counted in an LS-6800 counter (Beckman, USA). In each experiment six to ten concentrations of displacing substances were used. Each experiment consisted of a displacement curve in the absence and in the presence of THIP. Inhibiting concentrations (IC_{50}) were determined by the use of Hill's graphs and by the method of least mean squares. To construct a Schatchard plot membranes in a final volume of 0.5 ml were incubated with six concentrations (from 0.25 to 32 nM) of ^{3}H-FNZ. Maximal density of binding sites and the dissociation constant (K_d) were determined by linear regression analysis.

THIP was generously provided by the Lundbeck Company, unlabeled BD and Ro 15-1788 by Hoffman LaRoche, CGS 8216 by Ciba Geigy, and ethyl-β-carboline-3-carboxylate (β-CCE) by Dr. A. Korneev.

EXPERIMENTAL RESULTS

THIP inhibited binding of ^{3}H-FNZ with intact membranes in proportion to its concentration (Fig. 1). The maximum effect was observed in a concentration of 0.5-1, when specific binding of ^{3}H-FNZ was inhibited by 70%.

The inhibitory action of THIP depended on the concentration of the Tris-HCl buffer. In buffer of low molarity this effect was stronger and reached a maixmum in the 5 mM buffer (Fig. 2). Incidentally, specific binding of ^{3}H-FNZ in the absence of THIP was unchanged over the range of concentrations of buffer from 5 to 100 mM.

Specific binding of ^{3}H-FNZ in the absence and presence of THIP (Scatchard analysis) showed that inhibition of binding was caused by a decrease of affinity and a decrease in the density of binding sites (Fig. 3). Agonists of BD receptors, on incubation in the presence of THIP, reduced their own affinity, which was reflected by a decrease in K_i of these substances (Table 1). The BD receptor antagonists Ro 15-1788 and CGS 8216 did not change their affinity in the presence of THIP. β-CCE, which is a BD receptor agonist with weak negative pharmacological activity, caused a small increase of affinity in the presence of THIP, and the shift of THIP was greater than unity.

The results of this investigation confirm earlier observations that THIP inhibits binding of ^{3}H-FNZ with intact membranes and that the inhibitory action of THIP depends on the concentration of the substance in the incubation medium and on the molarity of the Tris-HCl buffer.

TABLE 1. Inhibitory Concentrations (IC_{50}) and Inhibition Constants (K_i) of BD Receptor Ligands in the Absence and Presence of THIP (100 μM) in 25 mM Tris-HCl Buffer at 0°C

Substance	−THIP		+ THIP		$\frac{K_i(-THIP)}{K_i(+THIP)}$
	IC_{50}	K_i	IC_{50}	K_i	
Diazepam (n = 4)	9,4±1,2	4,6	19,2±2,1**	12,3	0,37
Nitrazepam (n = 5)	5,6±0,2	2,7	9,8±1,3*	6,3	0,42
Flunitrazepam (*n*=4)	1,4±0,5	0,7	2,2±0,2*	1,4	0,5
Medazepam (n = 5)	495±43	242	1704±511**	1091	0,22
Chlordiazepoxide (*n*=4)	514±21	251	889±12**	569	0,44
Ro 15-1788 (*n*=3)	1,58±0,11	0,85	1,53±0,09	0,97	0,88
CGS 8216 (*n*=3)	0,40±0,09	0,20	0,35±0,10	0,22	0,91
β-CCE (*n*=7)	1,78—0,01	0,9	1,30—0,1*	0,69	1,30

Legend. K_i was determined by the equation $K_i = \frac{IC_{50}}{1 + \frac{I}{K_d}}$. *P < 0.05; **P < 0.01.

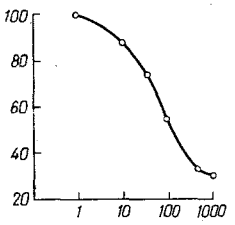

Fig. 1

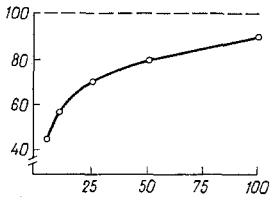

Fig. 2

Fig. 1. Effect of increasing concentrations of THIP on binding of ^{3}H-FNZ with intact membranes at 0°C in 25 mM Tris-HCl buffer. Abscissa, concentration of THIP (in μM); ordinate, specific binding of ^{3}H-FNZ (in %).

Fig. 2. Dependence of inhibitory effect of THIP on ^{3}H-FNZ binding on molarity of Tris-HCl buffer. Abscissa, concentration of Tris-HCl buffer (in $M \cdot 10^{-3}$); ordinate, specific binding of ^{3}H-FNZ (in % of control, in absence of THIP, taken as 100%).

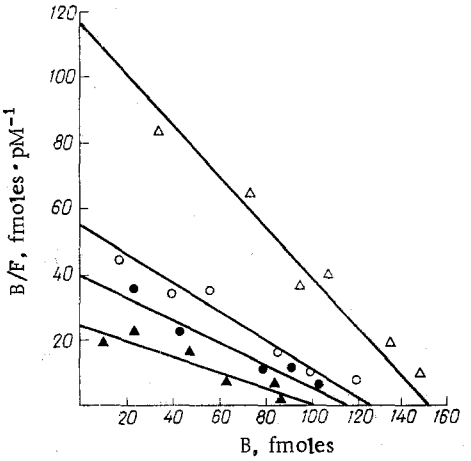

Fig. 3. Saturation kinetics of binding of ^{3}H-FNZ with intact rat brain membranes in 25 mM Tris-HCl buffer, between Scatchard coordinates, in absence and presence of THIP. Empty triangles — control; empty circles — THIP (100 μM); filled circles — THIP (200 μM); filled triangles — THIP (500 μM).

In contrast to previous investigations the present experiments showed that inhibition of binding of ^{3}H-FNZ is caused by a decrease of affinity and of the density of binding sites. These results are evidence that the shift of affinity induced by THIP can be used to predict the pharmacological activity of BD receptor ligands. Data on the change in affinity of opposite agonists of BD receptors are not given in this paper, but the preliminary results of our experiments with methyl-6,7-dimethoxy-4-ethyl-β-carboline-3-carboxylate (DMCM) showed that its affinity was increased in the presence of THIP. The use of the THIP-induced shift has certain advantages over the GABA or barbiturate shift, for this method does not require prolonged washing of the membranes. The mechanism by which THIP inhibits binding of ^{3}H-FNZ is not clear. Previous experiments showed that the action of THIP on ^{3}H-FNZ binding depends strictly on the experimental conditions. If well washed membranes were used, THIP did not change ^{3}H-FNZ binding at 0°C, or strengthened it only weakly, but it antagonized the action of muscimol [2, 10, 11]. If ^{3}H-FNZ binding was determined at a higher temperature (30 or 37°C) in the presence of chloride ions, THIP, like GABA or muscimol, increased ^{3}H-FNZ binding. On the basis of these data the inhibitory action of THIP can be explained by its shifted agonistic-antagonistic effect [2, 10, 11]. In fact, if ^{3}H-FNZ binding is carried out with intact membranes at 0°C, THIP can reduce binding due to antagonists toward endogenous GABA present in the incubation medium. Consequently, affinity of BD receptor ligands in the presence of THIP

also is reduced in the opposite direction compared with changes in the presence of GABA. In that case, in the presence of THIP only a change in K_d ought to be observed. However, saturation analysis in the presence of THIP revealed a decrease not only of affinity, but also of density of the receptors, which cannot be explained by antagonistic relations between GABA and THIP. It is likewise not clear why the inhibitory action of THIP is exhibited only in a buffer of low molarity.

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MUSCARINIC AGONISTS HAVE NO EFFECT ON SPONTANEOUS QUANTUM TRANSMITTER RELEASE FROM FROG MOTOR NERVE ENDINGS

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The study of the mechanisms regulating transmitter release from nerve endings through the intervention of presynaptic autoreceptors is of great interest [2, 5, 14]. Both spontaneous and evoked quantal acetylcholine secretion in the neuromuscular junction of warm-blooded animals and in synapses of amphibians is modified by the action of cholinomimetics through activation of receptors of the nicotine type [7-11, 13]. There is evidence in the literature that both nicotinic and muscarinic receptors may participate in the mechanism of this effect. However, reduction of the frequency of miniature end-plate potentials (MEPP) under the influence both of carbachol (CCh), a mimetic which can activate both nicotinic and muscarinic receptors, and also purely muscarinic agonists, namely muscarine and metacholine, has been described, so that the authors cited could postulate the existence of receptors of muscarinic type on frog motor nerve endings [12]. There is also evidence that inhibition of spontaneous transmitter secretion in frogs takes place in the presence of the nicotine mimetic suberyldicholine [4], whereas the muscarine antagonist atropine does not abolish the decrease in frequency of MEPP induced by CCh [1]. In the present investigation, to remove the contradictions in the question of the existence of muscarinic receptors on frog motor nerve endings, the effect of several substances, differing essentially in their chemical structure, but possessing high muscarinomimetic activity, was tested on spontaneous quantal acetylcholine release.

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