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Change in B_{max} and K_d for [^{3}H]Flunitrazepam Observed in the Course of Washing Rat Brain Tissue with Distilled Water

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SUMMARY

A method for the removal of an endogenous inhibitor(s) of benzodiazepine receptor binding (extraction in distilled water at 20°) was used to evaluate the possible influence of this inhibitor(s) on the affinity of the benzodiazepine receptor *in vivo*. The results indicate that the presence of an inhibitor(s) in the concentration which exists in the rat brain leads to a 30-fold increase in the K_d value as compared with the results obtained *in vitro*. The endogenous inhibitor(s) appeared to be a thermostable, proteinase K-resistant substance(s) with M_r between 500 and 10,000 daltons. Repeated washings of the brain tissue with distilled water were accompanied by a decrease in the B_{max} for [^{3}H]flunitrazepam. The [^{3}H]flunitrazepam binding sites with K_d = 1.7 nM and B_{max} = 61 fmoles/mg of protein were found in the supernatant collected after the second and third washings in distilled water. The presence of diazepam and the benzodiazepine antagonist Ro 15-1788 prevented in a dose-dependent manner the decrease in the B_{max} value for [^{3}H]flunitrazepam during the tissue washing with distilled water.

INTRODUCTION

Speth *et al.* (1) discovered 100% enhancement of benzodiazepine receptor density in the brain of rats treated with diazepam 1 hr before decapitation. In studying this phenomenon we had found that it could be modeled *in vitro* by addition of diazepam to the rat brain homogenate before tissue washing (2). It was suggested that the presence of diazepam in the media during the tissue washing resulted either in elicitation of "spare" receptors or prevention of the loss of the benzodiazepine receptors. In the present work we have studied the influence of repeated washings of the brain homogenate on the K_d and B_{max} values for [^{3}H]FNZ.[1] In these experiments an inhibitory activity for the benzodiazepine specific binding was detected in the supernatant obtained after the first washing.

METHODS

Male Wistar rats (100–120 g) were decapitated and the brains were removed immediately. The brains were homogenized in 10–140 volumes of distilled water (pH 6.1) at 15° with a Virtis-45 homogenizer (30 sec, rheostat setting at 8) and washed one to four times by centrifugation (100,000 × *g* for 60 min at 20°) and rehomogenization in the same volume of distilled water. In some experiments the tissue was washed in distilled water at pH 8.5 (adjusted with sodium hydroxide) and in distilled water at 5° (pH 6.1 and 8.5). After the last centrifugation the pellets were resuspended in 50 volumes of ice-cold 50 mM Tris-HCl buffer (pH 7.3 at 20°) (Buffer T). The supernatant obtained after the first centrifugation in 15 volumes of distilled water was collected and concentrated on ultrafilters UM-05, UM-2 and UM-10 (Amicon). The filtrates and concentrates were tested for the presence of inhibitory activity for [^{3}H]diazepam specific binding. The supernatants after the second and third washings in distilled water were collected and, after adjusting the pH to 7.5 with 20 mM sodium hydroxide, concentrated 5-fold on the ultrafilter YM-30 (Amicon). The specific binding of [^{3}H]FNZ in the concentrates was determined by the method of Yousufi *et al.* (3).

In some experiments the tissue washing was performed in the presence of diazepam, Ro 15-1788, muscimol, haloperidol, and imipramine. Diazepam was either added to the homogenate once, at the beginning of the first washing at a concentration of 100–5000 nM, or all of the washings were performed at a constant drug concentration of 20–1000 nM. Other substances were present during the washing at a constant concentration of 1000 nM.

In the control experiments the brain tissue was homogenized in 50 volumes of Buffer T at 4°. The homogenate was either preincubated at 4°, 20°, or 37° for 2 hr or washed three times by centrifugation and rehomogenization in 50 volumes of Buffer T at 4° or 20° or in 50 mM Tris-citrate buffer (pH 7.1 at 20°) or in 50 mM K,Na-phosphate buffer (pH 7.3 at 20°).

Specific binding of [^{3}H]benzodiazepines was determined as described (4) for four to six concentrations of labeled ligand in the range of 0.1–13 nM in 1-ml final volume samples corresponding to 20 mg of initial tissue weight. Nonspecific binding determined in the presence

[1] The abbreviations used are: FNZ, flunitrazepam; GABA, γ-aminobutyric acid.

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TABLE 1

Effect of washing and preincubation on [^{3}H]FNZ binding to rat brain membranes

Results are expressed as means ± standard error of the mean. Kinetic parameters were calculated from Scatchard plots.

Treatment	B_{max}	K_d	No. of independent experiments
	pmoles/g tissue	*nM*	
Fresh untreated tissue	79 ± 7	1.64 ± 0.07	7
Preincubation for 2 hr in 50 volumes of Buffer T at			
4°	78	1.60	1
20°	66 ± 5	1.61 ± 0.05	2
37°	79 ± 4	1.40 ± 0.10	2
Three washings in 50 volumes of Buffer T at			
20°	89 ± 5	1.24 ± 0.06	3
Three washings in 50 volumes of H_2O at			
20°, pH 6.1	27 ± 4	1.56 ± 0.10	3
20°, pH 8.5	21 ± 3	1.49 ± 0.08	2
5°, pH 6.1	35 ± 5	1.36 ± 0.11	2
5°, pH 8.5	38	1.32	1

of 1000 nM unlabeled flunitrazepam was less than 15% of the total binding. The concentration of binding sites (B_{max}) and the dissociation constant (K_d) were determined by Scatchard analysis.

[^{3}H]Flunitrazepam (84.4 Ci/mmole was obtained from New England Nuclear Corporation (Boston, Mass.), and [^{3}H]diazepam from Amersham (Amersham, England); flunitrazepam and Ro 15-1788 were donated by Dr. W. Haefely (F. Hoffmann-La Roche & Company, Basel, Switzerland). Diazepam was obtained from Richter (Hungary).

RESULTS

Influence of tissue washing on B_{max} value. Preincubation and washing in any of the buffers used did not result in significant changes in the [^{3}H]FNZ B_{max} (Table 1). At the same time the following results were obtained after washing with distilled water at 20°: The B_{max} after one washing in 10–20 volumes of distilled water did not change, but after two washings in 10–20 volumes and one washing in 40–100 volumes of distilled water it decreased to 20% of the initial value after four washings in 40 volumes (Fig. 1). Additional washings abolished [^{3}H]FNZ specific binding. The decrease in the B_{max} for [^{3}H]FNZ was observed also after washing in distilled water at 5° (Table 1). Preincubation of water-washed membranes in Buffer T at 0° or 20° for 1 hr did not lead to any increase in [^{3}H]FNZ specific binding.

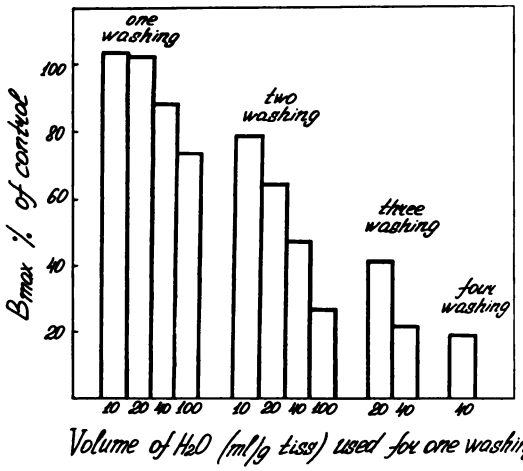

FIG. 1. *Influence of brain tissue washings in distilled water at 20° on [^{3}H]FNZ B_{max}*

B_{max} values are expressed as percentage of B_{max} in fresh homogenate (79 ± 7 pmoles/g of tissue). Each value is the mean of at least three experiments. The standard error of the mean was less then 10% of the mean values.

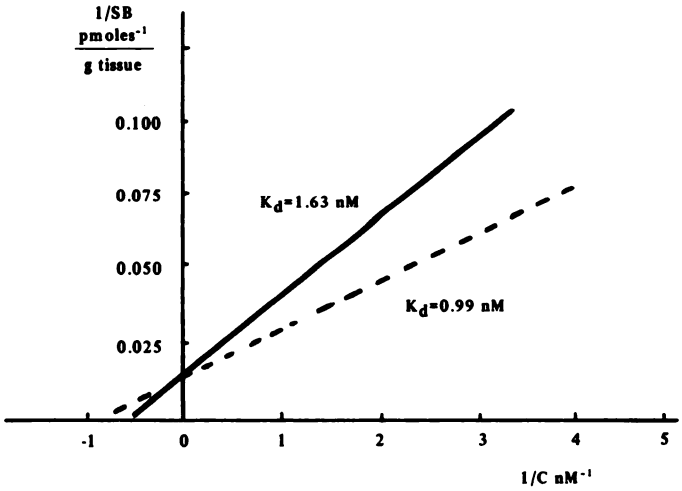

FIG. 2. *Double-reciprocal plots of specific binding (SB) produced by various concentrations of [^{3}H]FNZ (C) in fresh homogenate (——) and after a single washing in 12 volumes (milliliters per gram of tissue) of distilled water at 20° (- - -)*

The experiment was repeated three times with similar results.

Influence of tissue washing on K_d value. After one washing in 10–100 volumes of any of the buffers used, the K_d value for [^{3}H]FNZ was not significantly changed. One washing in 10–20 volumes of distilled water at 20° gave a 40% decrease in the K_d value (Fig. 2; $p < 0.001$ compared with the washing in buffer). Further increase in the number of washings and volume of distilled water or buffer used was accompanied by a gradual increase in K_d up to 1.62 mM after four washings in distilled water, and to 1.98 nM after six washings in 40 volumes of Buffer T (data not shown).

Effect of drugs on decrease in B_{max} during washing with distilled water. Introduction of diazepam into the washing media prevented the [^{3}H]FNZ B_{max} decrease observed in the course of washing with distilled water (Fig. 3; Table 2). This effect was proportional to the diazepam concentration in the media (Table 2) and occurred both when the drug was introduced into the media just once in the first wash and when all of the tissue washings were performed at a constant diazepam concentration (Table 2). However, in the second case the "protection" of benzodiazepine receptors by diazepam was more effective. Ro 15-1788 was even more effective than diazepam as a "protector" for benzodiazepine receptors at the concentration of 1000 nM (Table 2). The presence of muscimol, haloperidol, and imipramine during the washing was without any effect.

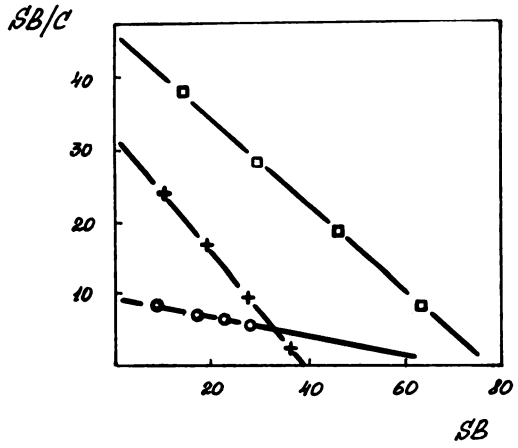

FIG. 3. *Diazepam prevents disappearance of the benzodiazepine receptor during washing of brain tissue in distilled water at 20°*

Scatchard plots of [^{3}H]FNZ specific binding. *SB*, specific binding of [^{3}H]FNZ in picomoles per gram of tissue; *C*, concentration of free [^{3}H] FNZ (nanomolar); □, fresh homogenate; +, homogenate washed twice in 50 volumes of distilled water at 20°; ○, washed as above but in the presence of diazepam at a concentration of 1000 nM.

Identification of solubilized [^{3}H]FNZ binding sites in distilled water supernatant. Specific [^{3}H]FNZ binding was detected in the concentrates from the supernatants obtained after the second and third centrifugations of rat brain tissue in 30–40 volumes of distilled water (see Methods) and was proportional to the protein concentration up to 3 mg/ml (data not shown). The binding sites were not retained by 0.22 μm pore size GS filters (Millipore) or by GF/B filters in the absence of polyethylene glycol 6000 and bovine γ-globulin. Scatchard analysis of [^{3}H]FNZ binding at different concentrations (0.3–10 nM) gave a K_d value of 1.7 nM and a B_{max} of 61 fmoles/mg of protein. The distilled water-solubilized [^{3}H]FNZ binding sites were apparently more stable at pH 7.5–8.2 (the half-life was approximately 12 hr at 4°) and were unstable at low pH; specific binding disappeared during 5 min at pH 5.0. In the supernatants obtained in Buffer T no specific binding of [^{3}H]FNZ was observed under the same conditions.

TABLE 2

Effect of various drugs on disappearance of the benzodiazepine receptor during tissue washing in distilled water at 20°

The tissue was washed three times in 40 volumes of distilled water in the presence of drug. The drug was removed by two additional washings in 40 volumes of Buffer T at 4°. B_{max} for [^{3}H]FNZ was determined as described under Methods and expressed as percentage of control (nonwashed homogenate). Values are means ± standard error of the mean.

Drug	B_{max}
	% of control
None	24 ± 4
Diazepam	
100 nM	35 ± 3
250 nM	45 ± 6
1000 nM	68 ± 5
Ro 15-1788, 1000 nM	71 ± 10
Muscimol, 1000 nM	26 ± 3
Haloperidol, 1000 nM	24 ± 2
Imipramine, 1000 nM	26 ± 2
Diazepam, added once at the beginning of washing to the concentration of	
1000 nM	40 ± 5
5000 nM	58 ± 7

TABLE 3

Effect of supernatant obtained after rat brain homogenate centrifugation in 15 volumes of distilled water at 20° on [^{3}H] diazepam specific binding

The supernatant was concentrated 5 times on various ultrafilters. Filtrate or concentrate (0.5 ml) was added to 0.6 ml of membrane suspension (10 mg of initial tissue), and specific binding of [^{3}H]diazepam (final concentration 12 nM) was determined as described under Methods.

Addition to membranes	[^{3}H]Diazepam specific binding	% Inhibition
	cpm · 10^{-3}	
None	54.92	0
UM-05 (500 M_r cutoff)		
Filtrate	52.20	4
Concentrate	12.92	76
UM-2 (2,000 M_r cutoff)		
Filtrate	53.38	3
Concentrate	30.73	44
UM-10 (10,000 M_r cutoff)		
Filtrate	43.36	21
Concentrate	31.14	43
Initial supernatant	46.25	16

Water extraction of the endogenous inhibitor of benzodiazepine specific binding. Addition of supernatant obtained after the first washing of rat brain homogenate in 15 volumes of distilled water at 20° to the incubation mixture resulted in decreased [^{3}H]diazepam specific binding in a dose-dependent manner (data not shown). The endogenous inhibitor present in this supernatant appeared to be a thermostable substance (95°, 15 min) with M_r between 2,000 and 10,000 daltons (Table 3). The ability of the inhibitor to decrease the [^{3}H]diazepam specific binding was not changed when the incubation mixture was assayed according to the method of Yousufi *et al.* (3), i.e., in the presence of polyethylene glycol 6000 and γ-globulin. This finding, supported by the ability of the inhibitor to decrease the [^{3}H]FNZ binding to the solubilized binding sites described above, indicates that the inhibitor itself cannot bind the ^{3}H-ligand; i.e., it is not a pseudo-competitive inhibitor. We could not detect any inhibitory activity in the supernatants obtained after the second and third washings of rat brain tissue in distilled water and after washing in all of the buffers used. Inhibitory activity with similar properties was also detected in bovine kidney and brain.

DISCUSSION

In the present work it is shown that one washing of brain tissue in 10–20 volumes of distilled water at 20° resulted in a 40% decrease in the [^{3}H]FNZ K_d value. Since under these conditions no change in the B_{max} was observed (Fig. 2), it is possible to suppose that the removal of a competitive inhibitor for [^{3}H]FNZ binding takes place, and one may attempt to evaluate its concen-

tration in intact brain tissue. With that end in view, let us introduce the following designations: C_i, inhibitor concentration in the initial tissue homogenate; K_i, its inhibition constant; K^0_d, authentic constant of [^{3}H]FNZ dissociation; and K'_d, its apparent constant of dissociation, which is observed in the presence of a putative competitive inhibitor. Taking into consideration that in the case of competitive inhibition, $K'_d = K^0_d(1 + C_i/K_i)$, the authentic constant may be presented as

$$K^0_d = \frac{K'_d}{1 + C_i/K_i} \quad (1)$$

Assuming that one washing in 12 volumes of distilled water diminishes inhibitor concentration 12-fold, it is possible to determine from the relationship described by Eq. 1 the K^0_d values at two concentrations of a competitive inhibitor: C_i, concentration in the initial homogenate, and $C_i/12$, concentration after the washing in 12 volumes of distilled water.

$$K^0_d = \frac{K'_{d1}}{1 + C_i/K_i} \quad (2)$$

$$K^0_d = \frac{K'_{d2}}{1 + C_i/12K_i} \quad (3)$$

Necessary for the solution of this system of equations the apparent constants K'_{d1} and K'_{d2} were determined experimentally and appeared to be 1.63 nM and 0.99 nM, respectively (Fig. 2). Solving the system of Eqs. 2 and 3 we obtain: $C_i/K_i = 0.64$.

Taking into consideration that the protocol for the specific binding measurements includes dilution of the hypothetical inhibitor 50 times (see Methods), the observed value has to be increased 50 times. The C_i/K_i value for intact rat brain tissue becomes equal to 32. In two other experiments values of C_i/K_i obtained for rat brain (without pons-medulla) were equal to 31 and 38. This method may be useful for the evaluation of the endogenous inhibitor concentration in the samples of tissue. Our preliminary data indicate, for example, that the C_i/K_i value in the rat cerebellum is equal to 19.

The presence in the brain of an endogenous competitive inhibitor of benzodiazepine receptors with a C_i/K_i value of approximately 30 may explain the constraint on the ability of benzodiazepines to interact with their receptor in brain as postulated by Speth *et al.* (4).

The ultrafiltration studies indicate that the M_r of the supposed endogenous inhibitor is more than 500 and less than 10,000 (Table 3). Thus this inhibitor is not equal to inosine, hypoxanthine, and β-carboline, which were recently isolated from brain as a possible benzodiazepine receptor ligand (5, 6). It is interesting that the endogenous peptide which inhibits [^{3}H]diazepam specific binding described by Masotti *et al.* (7) also has an M_r of more than 1800. Attempts now in progress to characterize the properties of the endogenous inhibitor fraction reveal its apparent heterogeneity; the inhibitory activity was also resistant to proteinase K treatment (10 mg/ml, 37°, 2 hr).

Intensive washing of the tissue resulted in an increased [^{3}H]FNZ K_d, possibly due to the removal of endogenous GABA (8, 9).

During repetitive tissue washing in distilled water the B_{max} for [^{3}H]FNZ decreased to nondetectable levels. At the same time, in the supernatants obtained during this washing, [^{3}H]FNZ binding sites were described which, according to commonly used criteria (not sedimented during 1 hr at 100,000 × *g* and permeable through 0.22 μM pore size filters), represent soluble benzodiazepine receptor molecules. Approximately 25–30% of the binding sites lost from the membranes were recovered in the distilled water supernatant [i.e., they are detectable with the method of Yousufi *et al.* (3)]. Our preliminary data indicate that distilled water-solubilized benzodiazepine receptors are of a central type, because clonazepam is a more potent inhibitor of [^{3}H]FNZ binding than is diazepam. When intensively prewashed, triply frozen-thawed tissue was used it was observed that the B_{max} for [^{3}H] muscimol and the sensitivity of the membrane-bound benzodiazepine receptor to GABA stimulation also decreased after further repetitive washings with distilled water.[2] These data led us to speculate that benzodiazepine receptors solubilized in our experiments represent a part of the GABA-benzodiazepine receptor complex. Whether this complex or only benzodiazepine binding sites are solubilized with distilled water remains unknown.

The supposition that tissue proteases and hydrolases can contribute to the disappearance of membrane-bound benzodiazepine receptors during washings with distilled water is not supported by the data presented in this and a previous paper (2). However, this possibility cannot be excluded. The main arguments against this supposition are as follows: (a) The action of tissue-degrading enzymes should be more pronounced as the concentration of the tissue in the homogenate increases, and the activity of degrading enzymes should decrease as the number of tissue washings increases (if these enzymes are not membrane-bound); in our experiments the opposite correlation was observed (Fig. 1). (b) The existence of degrading enzymes which are completely inactivated in the presence of 50 mM Tris or phosphate buffers and active in distilled water at the same pH range appears unlikely.

Our experiments show that, independently of pH (from 6.1 to 8.5) and temperature (from 5° to 25°), the decrease in [^{3}H]FNZ B_{max} can be observed during washing in distilled water. Preliminary data indicate also that repetitive washings in 5 and 10 mM Tris-HCl buffer (pH 7.5 at 20°) lead to a decrease in the B_{max} for [^{3}H]FNZ. These results led us to propose that the main condition which leads to the disappearance of benzodiazepine binding sites from the membranes during washing is the low ionic strength of the washing medium. Solubilization of external membrane proteins under similar conditions is well established (10).

The decrease in the [^{3}H]FNZ B_{max} after repetitive washings with distilled water as described in the present paper is inhibited in the presence of diazepam, the benzodiazepine antagonist Ro 15-1788, and flunitrazepam (2) but not by other drugs (muscimol, haloperidol, and imipramine) (Table 3; Fig. 2). These observations may be interpreted as an indication of some "protection" by

[2] A. Ya. Korneyev and M. I. Factor, unpublished observations.

the ligand of benzodiazepine receptors from solubilization in the course of washing with distilled water. In experiments by Gavish and Snyder (11) it was also observed that diazepam protects benzodiazepine receptors from heat inactivation. These data suggest that ligand binding transforms the receptor into a more rigid (stable) conformation.

In conclusion, the present paper describes a procedure for removal of an endogenous inhibitor(s) of the benzodiazepine receptor from brain tissue. The possible influence of this inhibitor(s) on the pharmacological action of benzodiazepines was evaluated. It was shown that solubilization of [^{3}H]FNZ binding sites takes place during repetitive tissue washings in distilled water. This method of solubilization may be useful in further studies of benzodiazepine receptor properties inasmuch as it avoids the use of detergents and additionally it is sensitive to the functional condition of the benzodiazepine receptor: solubilization is prevented in the presence of the ligand.

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