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The binding of [^{3}H]-diazepam to guinea-pig ileal longitudinal muscle and the *in vitro* inhibition of contraction by benzodiazepines

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1 The longitudinal muscle-myenteric plexus strip preparation of the guinea-pig ileum was used to study the binding of [^{3}H]-diazepam and the effect of benzodiazepines on its contraction.

2 Scatchard analysis of binding indicated a single class of binding sites with $K_D = 43$ nM and $B_{max} = 229$ fmol/mg protein. Binding was of peripheral type based on the much greater binding affinity of Ro5–4864 as compared to clonazepam. Binding of [^{3}H]-diazepam reached equilibrium at 10 min and dissociated rapidly ($T_{1/2} = 1.3$ min). The K_D derived from the rate constants agreed with that from the Scatchard analysis.

3 Benzodiazepines produced a dose-dependent decrease in the electrically induced contractions of the longitudinal muscle strip, but their potencies in this effect did not correlate with their binding affinities.

4 Diazepam antagonized the contractions of the longitudinal muscle strip induced by K^+, Ca^{2+}, histamine and carbachol. The inhibition of Ca^{2+}-induced contractions was reversed by increasing the concentration of Ca^{2+} in the medium.

Introduction

Benzodiazepines (Bzds) have long been used for their anxiolytic, anti-convulsant, muscle relaxant and sedative-hypnotic properties (Zbinden & Randall, 1967; Randall, Schallek, Sternbach & Ning, 1974; Denber, 1975), although their mechanism of action remains unclear. A major advance was achieved, however, with the discovery of specific and high affinity Bzd receptors in the brain that appear to mediate at least some of the actions of these compounds (Mohler & Okada, 1977; Squires & Braestrup, 1977; Macherer, Kochman, Bierschenk & Bremner, 1978). The identification of these receptors in turn led to many intensive and fruitful investigations into the biochemical and cellular aspects of the Bzd effects (for review see Tallman, Paul, Skolnick & Gallager, 1980).

Aside from the receptors in the brain, there is another class of pharmacologically distinguishable Bzd binding sites found mainly in peripheral tissues such as lung, liver and kidney (Squires & Braestrup, 1977). These sites do not seem to be involved in the central actions of Bzds and consequently have been largely ignored. Recently, however, they have been identified in a wide variety of tissues and cells, including mast cells (Taniguchi, Wang & Spector, 1980), platelets (Wang, Taniguchi & Spector, 1980), lymphocytes (Wang, Taniguchi, Sugiura & Spector, 1981), heart (Davies & Huston, 1981; Taniguchi, Wang & Spector, 1982) and numerous neuronal and non-neuronal cell lines (Syapin & Skolnick, 1979; Wang, Morgan & Spector, 1982). Despite the detailed characterization of these sites, their functional significance remains an enigma. To the long list of tissues that possess the peripheral type Bzd sites, we now add the longitudinal smooth muscle-myenteric plexus layer of the guinea-pig ileum. Furthermore, the electrically or pharmacologically induced contractions of the longitudinal muscle is inhibited by Bzds, although there does not appear to be a correlation between this inhibition and the binding of the compounds to the peripheral type binding sites.

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Methods

Preparation of ileal longitudinal muscle

Hartley guinea-pigs (300–500 g were stunned and exsanguinated. The ileum, except for 10 cm at either end, was removed and the longitudinal muscle-myenteric plexus layer dissected out as described by Rang (1964). A strip of ileum 5–10 cm long was inserted over a 0.1 ml glass pipette mounted on a stand. The longitudinal muscle layer was separated from the underlying tissue by gentle stroking with cotton-tipped sticks. The strip was then mounted on hooks in a jacketed 5 ml organ bath containing bicarbonate-buffered Krebs solution at 37°C and bubbled with a mixture of 95% O_2 and 5% CO_2. The composition of the Krebs solution was (mM): NaCl 113.0, KCl 4.7, $CaCl_2$ 2.5, KH_2PO_4 1.2, $MgSO_4$ 1.2, $NaHCO_3$ 25.0 and dextrose11.5. Strips were field-stimulated by two platinum electrodes placed at the top and bottom of the bath. Square wave pulses of 2 ms duration and supramaximal voltage (60 V) were delivered at a frequency of 0.1 Hz from a pulse generator (S48 Stimulator, Grass Intrument Co.). Contractile responses were recorded isometrically on a Polygraph (Model 7, Grass Instrument Co.) via a force-displacement transducer. To induce contractions with Ca^{2+}, the longitudinal muscle strip was first equilibrated with Ca^{2+}-free medium for 60 min and washed twice with the same medium. KCl (50 mM) was then added to maximally induce the K^+ component of contraction and partially depolarize the muscle strip so that the Ca^{2+}-dependent portion of the concentration could be studied independently of the K^+ effects. When the KCl-induced contraction reached a plateau, Ca^{2+} (400 μM to 10 mM) was added to induce further contractions.

Binding studies

To prepare a crude membrane fraction for binding studies the longitudinal muscle strips were homogenized for 20 s at maximum speed in 50 mM $NaKPO_4$ buffer (pH 7.4) using a Polytron (Brinkmann Instruments). The homogenate was centrifuged at 49,000 *g* for 10 min at 4°C. The pellet was washed once and resuspended in 5 volumes of buffer. Aliquots of the membrane fraction (0.2 to 1 mg of protein) were incubated with 3.6 nM [^{3}H]-diazepam for 15 min at 0°C in a total volume of 250 μl. Assays were terminated by filtration and counted in Aquasol (New England Nuclear) as previously described (Taniguchi

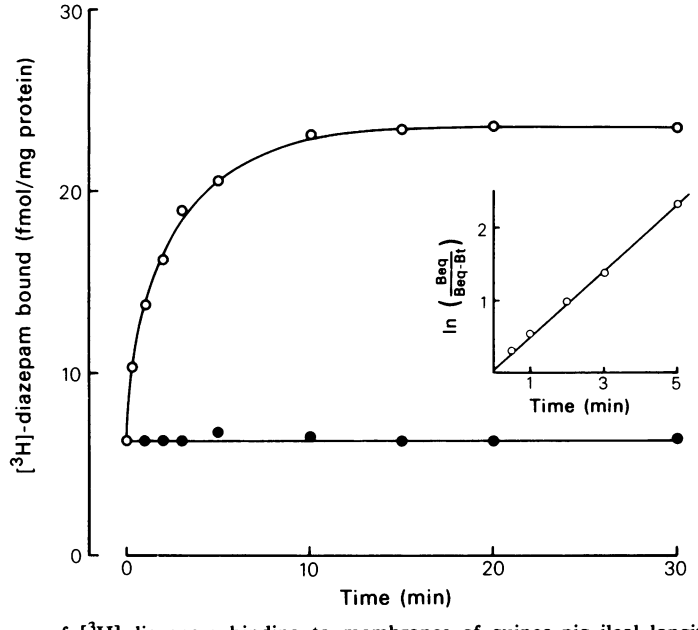

Figure 1 Time-course of [^{3}H]-diazepam binding to membranes of guinea-pig ileal longitudinal muscle. A representative experiment is shown, with open circles (○) as specific binding and closed circles (●) as non-specific binding. The experiment was repeated 3 times with similar results. The inset shows a regression line that is determined by least-squares fit ($r = 0.99$). Beq = fmol specifically bound [^{3}H]-diazepam at equilibrium, and Bt = fmol specifically bound [^{3}H]-diazepam at time t. K_{obs} is the slope of this line and is 0.58. $k+1$ can be calculated from the equation $k+1 = (K_{obs} - k\text{-}1)/[^3H]$-diazepam, where k-1 is the rate constant of dissociation (see Figure 2) and [^{3}H]-diazepam is the concentration of labelled ligand (3.6 nM).

et al., 1980). Protein was determined b the method of Lowry, Rosebrough, Farr & Randall (1951).

Materials and drugs

[^{3}H]-diazepam (76.8 Ci/mmol) was obtained from New England Nuclear. Acetylcholine, (±)-adrenaline, atropine, carbachol, 2-Cl-adenosine, cimetidine, diphenhydramine, DL-γ-aminobutyric acid (GABA), hexamethonium, histamine, morphine, (±)-noradrenaline, phentolamine, (±)-propranolol, pyrilamine and 5-hydroxytryptamine (5-HT) were purchased from Sigma Chemical Company. The benzodiazepine compounds and levallorphan were provided by Hoffmann-La Roche, Inc. The code numbers refer to the following compounds: Ro22-8349, 5-(2,4-dichlorophenyl)-1,3-dihydro-1-methyl-2H-1,4-benzodiazepin-2-one; Ro5-5115, 5-(4-chlorophenyl)-1-methyl-3H-1,4-benzodiazepin-2(1H)-one; Ro5-6669, 7-chloro-5-(4-methoxyphenyl)-1-methyl-3H-1, 4-benzodiaze-pin-2 (1H)-one; Ro5-4864; 7-chloro-5(4-chlorophenyl)-1, 3-dihydro-1-methyl-2H-1,4-ben-zodiazepin-2-one; Ro5-6993, 7-chloro-1-ethyl-5-(*p*-chlorophenyl)-3H-1, 4-benzodiazepin-2(1H)- one; Ro5-6945, 7-chloro-1-allyl-5-(*p*-chloro-phenyl)-3H-1,4-benzodiazepin-2(1H)-one. All drug concentrations were calculated as the free base. All chemicals used were of reagent grade and obtained through commercial sources.

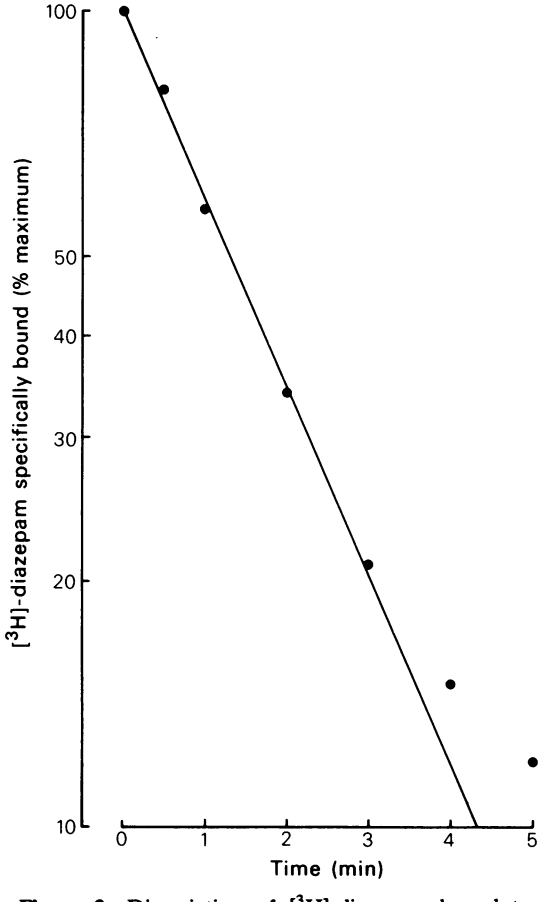

Figure 2 Dissociation of [^{3}H]-diazepam bound to membranes of guinea-pig ileal longitudinal muscle. A typical experiment is shown in which the membranes were incubated with [^{3}H]-diazepam as described in the Methods and after 15 min at 0°C, unlabelled diazepam was added to the incubation medium to yield a final concentration of 10 μM (time 0). Specifically bound counts were determined at the indicated times and expressed as a percentage of specific counts bound at time 0. The experiment was repeated three times with similar results.

Kinetics of [^{3}H]-diazepam binding

The binding of [^{3}H]-diazepam to guinea-pig ileal longitudinal muscle membrane was time-dependent,

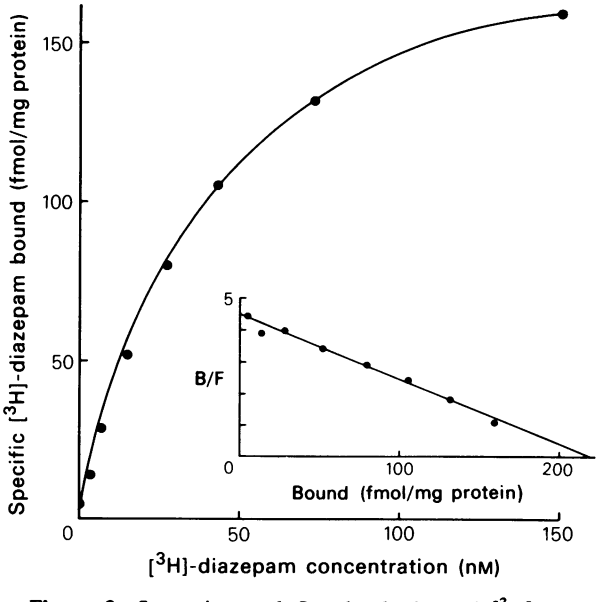

Figure 3 Saturation and Scatchard plots of [^{3}H]-diazepam binding to guinea-pig ileal longitudinal muscle membranes. Membranes were incubated with increasing concentrations of [^{3}H]-diazepam (1.4 to 150 nM) as described in the Methods. Results from a typical experiment are shown. The experiment was repeated 4 times with similar results. The inset shows the Scatchard plot where bound (B) = fmol specifically bound [^{3}H]-diazepam per mg protein, free (F) = concentration of [^{3}H]-diazepam present in the incubation medium. The regression line ($r = 0.98$) indicates a K_D of 50 nM and a B_{max} of 217 fmol/mg protein.

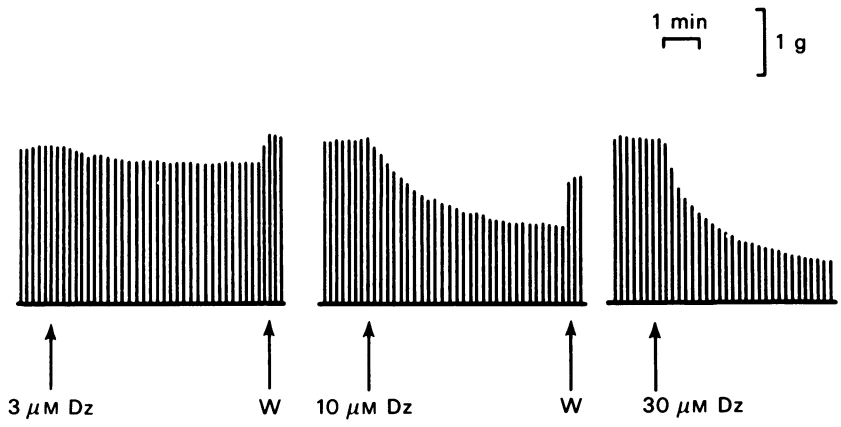

Figure 4 Inhibition of electrically induced contractions of guinea-pig ileal longitudinal muscle strip by diazepam (Dz). The strip was stimulated as described in the Methods and Dz was added to the bath to achieve the indicated final concentrations. The strip was washed with fresh Krebs medium as indicated by W.

achieving equilibrium after 10 min of incubation (Figure 1), with a rate constant of association ($k+1$) of 1.4×10^7 M^{-1} min^{-1}. Dissociation of bound [^{3}H]-diazepam was rapid and obeyed first order kinetics, with $T_{\frac{1}{2}} = 1.3$ min (Figure 2) and a rate constant of dissociation ($k-1$) of 0.53 min^{-1}, which when combined with $k+1$ in the equation $K_D = k\text{-}1/k+1$ (Bennett, 1978) yields a K_D value of 37.9 nM.

Saturability and specificity of [^{3}H]-diazepam binding

The specific binding of [^{3}H]-diazepam was saturable (Figure 3). Scatchard analysis revealed a single class of binding sites with an apparent equilibrium dissociation constant (K_D) of 43 ± 4 nM ($n = 5$). This value is in close agreement with the K_D calculated from the rate constants. The maximum amount of [^{3}H]-diazepam bound (B_{max}) was 229 ± 5 fmol/mg protein ($n = 5$). Specific binding of [^{3}H]-diazepam increased linearly with increasing protein concentration (0.2 to 1.2 mg) of the crude membrane fraction from the longitudinal muscle (data not shown). The binding was specific in that it was not affected by 10 μM of each of the following substances: (±)-adrenaline,

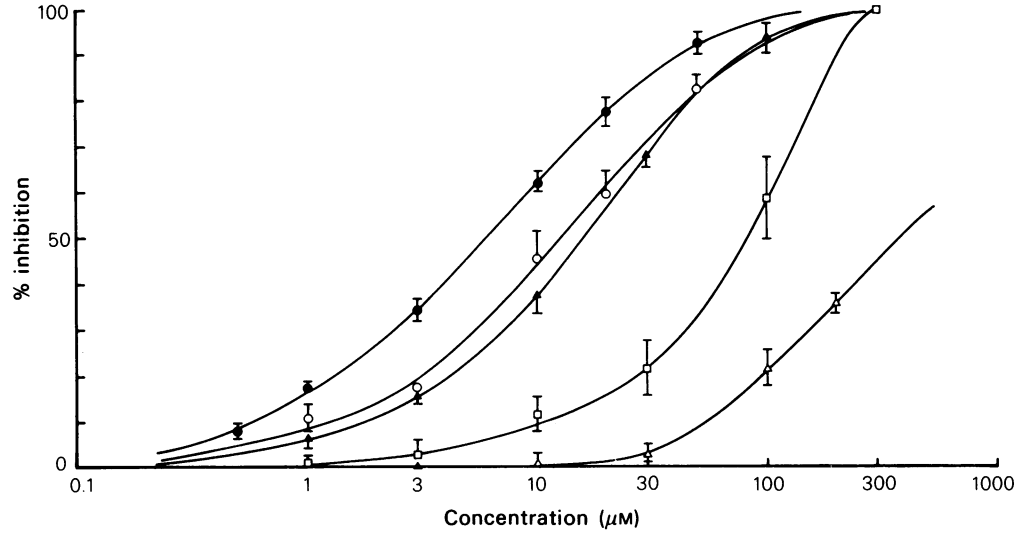

Figure 5 Dose-response curves for five benzodiazepines in inhibiting electrically induced contractions of the guinea-pig ileal longitudinal muscle strip. The strips were stimulated as described in the Methods. Each point is the mean of 3 experiments done on different strips. (●): Ro5-5115, (○): diazepam, (▲): Ro5-4864, (□): chlordiazepoxide, (△): clonazepam.

Table 1 ED_{50} values for various benzodiazepines in inhibiting contractions of ileal longitudinal muscle strips as compared to their IC_{50} values as competitiors for [^{3}H]-diazepam binding sites in longitudinal muscle membranes

Benzodiazepines	*ED_{50}-inhibition of contraction* (μM)*	*IC_{50}-binding**
Ro22-8349	4	16 nM
Ro5-5115	6	94 nM
Ro5-6669	8	10 nM
Diazepam	13	113 nM
Ro5-4864	15	14 nM
Ro5-6993	19	9 nM
Chlordiazepoxide	80	> 10 μM
Ro5-6945	100	2 nM
Clonazepam	400	> 0 Z0M

*Each result represents the mean of at least 3 experiments.

(±)-noradrenaline, (±)-propranolol, phentolamine, 5-HT, histamine, diphenhydramine, acetylcholine, atropine, carbachol, hexamethonium, morphine, levallorphan, DL-γ-aminobutyric acid (GABA),2-Cl-adenosine, pyrilamine and cimetidine (data not shown).

Binding of [^{3}H]-diazepam was of the peripheral type since Bzds selective for the CNS sites competed very weakly, if at all, for the binding while peripheral selective compounds were very potent displacers of [^{3}H]-diazepam. Clonazepam, for example, is selective for the brain sites and failed to displace [^{3}H]-diazepam binding to longitudinal muscle membrane (IC_{50} > 10 μM). Ro5-4864, on the other hand, is

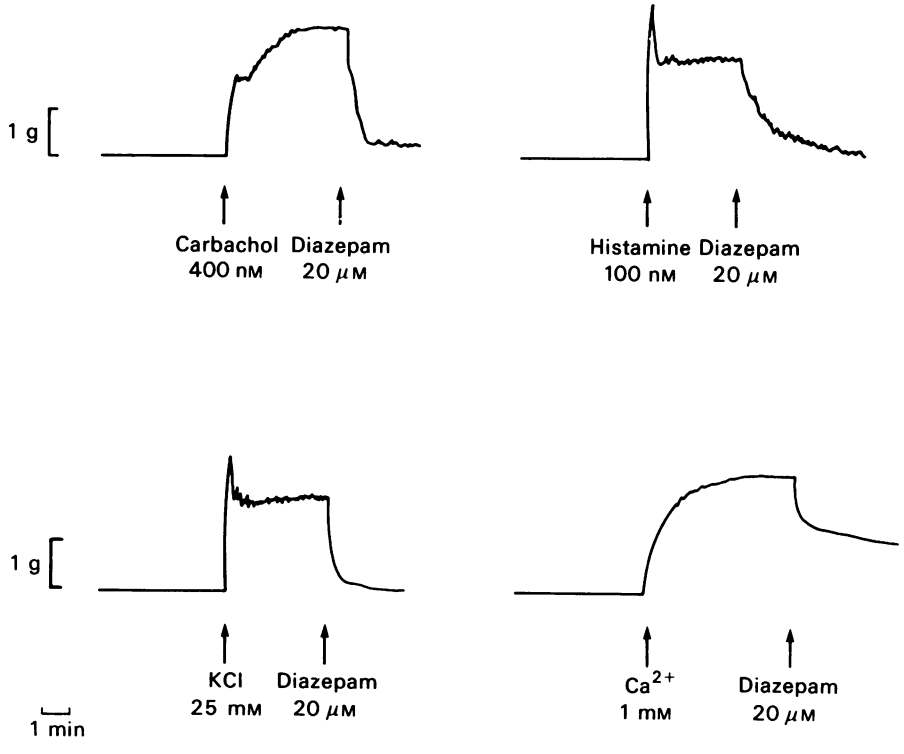

Figure 6 Inhibition by diazepam of guinea-pig ileal longitudinal muscle strip contractions induced by carbachol, histamine, KCl and Ca^{2+}. The agents, in concentrated solutions, were diluted in the organ bath containing the strip to achieve the indicated final concentrations. When the resulting contractions reached a plateau, diazepam was added to a final concentration of 20 μM. For Ca^{2+}-induced contractions see Methods for further details.

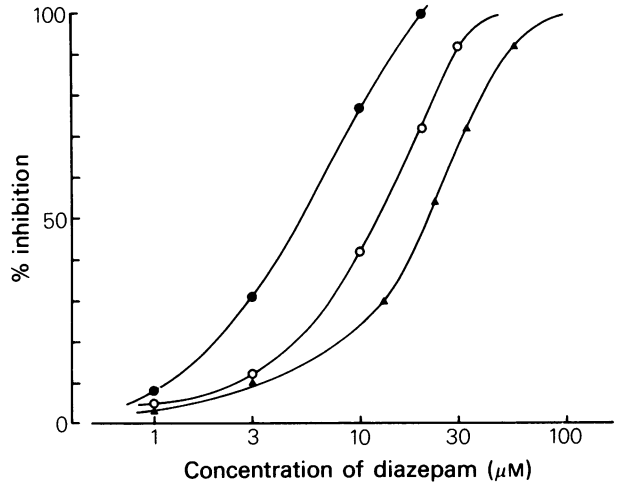

Figure 7 Dose-response curves for diazepam in inhibiting Ca^{2+}-induced contractions of the guinea-pig ileal longitudinal muscle strip. The strips were stimulated by Ca^{2+} as described in the Methods and the effect of various doses of diazepam was studied. (●): 400 μM Ca^{2+}; (○): 4 mM Ca^{2+}, (▲): 10 mM Ca^{2+}.

selective for peripheral sites and displaced [^{3}H]-diazepam at IC_{50} of 14 ± 2 nM ($n = 5$).

Effect of benzodiazepines on contractile responses of the longitudinal muscle

The electrically induced contractions of the ileal longitudinal muscle strip were dose-dependently inhibited by diazepam (Figure 4). The inhibition was apparent within 1 min after application of the drug and was reversed by washing the strip with fresh drug-free buffer. Other Bzd compounds, at various doses, showed similar effects, and the log dose-response curves for several representative compounds are presented in Figure 5. The half-effective doses (ED_{50}) determined from these curves were ranked with their respective binding constants (Table 1). There was no apparent correlation between the two parameters.

Diazepam also inhibited contraction of the strip induced by carbachol, histamine, KCl and Ca^{2+} (Figure 6) in a dose-dependent manner. The log dose-response curve for the inhibition by diazepam of Ca^{2+}-induced contraction is shown in Figure 7. When the Ca^{2+} concentration in the medium is increased, the dose-response curve for diazepam is progressively shifted to the right in a parallel manner, resulting in an increase in the ED_{50} of diazepam.

Discussion

The peripheral type Bzd binding sites have been identified in a myriad of tissues, cell types and cultured cell types. To date only a few tissues and cell lines examined do not possess these sites; among them are red blood cells (unpublished observation), skeletal muscle and crude homogenate of rat small intestine (Braestrup & Squires, 1977). Since most of the Bzd sites in the ileum are located in the longitudinal muscle layer, using the whole ileum to study binding in effect dilute the concentration of binding sites on a per mg protein basis. This would explain the inability of Braestrup & Squires to demonstrate peripheral Bzd sites in the ileum. The Bzd sites in the ileal longitudinal muscle layer are of the peripheral type based on the potencies of selective central or peripheral Bzd ligands in displacing [^{3}H]-diazepam binding, and are very similar in terms of affinity, binding kinetics and ligand specificity to that reported for other tissues and cells.

While the peripheral type Bzd sites are widely distributed, there is currently no known physiological function associated with them. Indeed, there is very little knowledge of any biological effects of Bzds apart from their CNS effects. In the guinea-pig ileal longitudinal muscle preparation, however, there is not only the presence of these binding sites but also an inhibition by the Bzds of electrically stimulated contractions of the longitudinal muscle strip. This inhibition is dose-dependent, reversible and appears to be postsynaptic in nature since the Bzds can inhibit contractions induced by various ions, carbachol and histamine. Furthermore, the inhibition by diazepam of Ca^{2+}-induced contractions is partially reversed by increasing concentrations of Ca^{2+} in the bath, suggesting an involvement of Ca^{2+} in the inhibitory effect of diazepam.

Finally, the inhibitory action of the Bzds is probably a pharmacological effect independent of and dissociated from the binding sites, as there is no correlation between the binding affinities of various Bzd ligands and their potencies in inhibiting the contractions of the longitudinal muscle strip. This is similar to the situation in the platelets, where they are peripheral type Bzd binding sites and the uptake of 5-HT is inhibited by the Bzds (unpublished results), but the two phenomena appear to be unrelated, again because of a lack of correlation. It appears then, that the physiological role of the peripheral type Bzd binding remains undefined and requires further study.

Results

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