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Micromolar-affinity benzodiazepine receptors regulate voltage-sensitive calcium channels in nerve terminal preparations

(receptor binding/diazepam/anticonvulsants/Ca^{2+} uptake/synaptosomes)

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ABSTRACT **Benzodiazepines in micromolar concentrations significantly inhibit depolarization-sensitive Ca^{2+} uptake in intact nerve-terminal preparations. Benzodiazepine inhibition of Ca^{2+} uptake is concentration dependent and stereospecific. Micromolar-affinity benzodiazepine receptors have been identified and characterized in brain membrane and shown to be distinct from nanomolar-affinity benzodiazepine receptors. Evidence is presented that micromolar, and not nanomolar, benzodiazepine binding sites mediate benzodiazepine inhibition of Ca^{2+} uptake. Irreversible binding to micromolar benzodiazepine binding sites also irreversibly blocked depolarization-dependent Ca^{2+} uptake in synaptosomes, indicating that these compounds may represent a useful marker for identifying the molecular components of Ca^{2+} channels in brain. Characterization of benzodiazepine inhibition of Ca^{2+} uptake demonstrates that these drugs function as Ca^{2+} channel antagonists, because benzodiazepines effectively blocked voltage-sensitive Ca^{2+} uptake inhibited by Mn^{2+}, Co^{2+}, verapamil, nitrendipine, and nimodipine. These results indicate that micromolar benzodiazepine binding sites regulate voltage-sensitive Ca^{2+} channels in brain membrane and suggest that some of the neuronal stabilizing effects of micromolar benzodiazepine receptors may be mediated by the regulation of Ca^{2+} conductance.**

Benzodiazepines (BZs) are important therapeutic agents with precise cellular and molecular activities, which have been the object of intense investigation. Specific BZ binding sites have been described that bind BZs in the nanomolar concentration range (1–3). Nanomolar-affinity BZ receptors (nM BZRs) have been linked on the molecular level with the γ-aminobutyric acid (GABA) receptor and the chloride ion channel. Furthermore, nM BZR binding has been correlated with some of the pharmacological effects of the BZs, including inhibition of pentylenetetrazol-induced seizure activity, muscle relaxant properties, and impairment of mouse rotorod performance. In addition, studies from this laboratory (4) have identified and characterized a distinct class of specific BZ binding sites that bind BZs in the micromolar concentration range, designated micromolar-affinity BZ receptors (μM BZRs). Binding to μM BZRs was readily distinguished from nM BZR binding on the basis of kinetic and pharmacological properties (4). In addition, μM BZR binding potency correlates with BZ inhibition of maximal electric-shock-induced seizure activity but not against inhibition of pentylenetetrazol-induced seizures or other effects correlating with nM BZR binding. Thus, it was suggested that μM BZRs may play a role in mediating some of the neuronal stabilizing effects of the BZs (4). To more completely elucidate the pharmacological properties of the BZs, it is important to determine the specific cellular mechanism(s) regulated by μM BZRs.

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Voltage-sensitive Ca^{2+} channels have been demonstrated in several neuronal preparations (5–7) and in synaptosomes (8, 9), and they have been characterized by the pattern of inhibition by Mn^{2+} (9, 10), Co^{2+} (6, 10, 11), and high concentrations of verapamil (9, 10). It has been observed that micromolar concentrations of diazepam (DZ) and other BZs inhibit depolarization-dependent Ca^{2+} uptake in synaptosomal preparations (12–15), suggesting that this inhibitory effect on Ca^{2+} uptake may be mediated by μM BZRs. In this study, we provide evidence that BZ inhibition of voltage-dependent Ca^{2+} channels in synaptosomes is regulated by BZ interaction with a population of μM BZ binding sites. Micromolar BZs were shown to completely inhibit depolarization-dependent Ca^{2+} uptake, and BZ inhibition was additive with that of known Ca^{2+} channel antagonists. Thus, BZs act as Ca^{2+} channel blockers in intact synaptosomal preparations.

MATERIALS AND METHODS

Materials. [^{3}H]Clonazepam (CNZ), [^{3}H]flunitrazepam (FNZ), and [^{3}H]diazepam (DZ) (each at 25–50 Ci/mmol; 1 Ci = 37 GBq) were the gift of W. E. Scott (Hoffmann–LaRoche). $^{45}Ca^{2+}$ (≈1 Ci/mmol) was obtained as the chloride salt from New England Nuclear. Nitrendipine and nimodipine were provided by A. Scriabine (Miles Institute of Preclinical Pharmacology). All BZs were donated by Hoffmann–LaRoche.

Tissue Preparation. Whole brains were removed from female Sprague–Dawley rats (100–150 g) by the rapid-kill technique in which time from decapitation to homogenization was <20 sec (16). Brains were homogenized in a 10% solution of 0.32 M sucrose/1 mM EDTA/10 mM Tris·HCl, pH 7.4/0.3 mM phenylmethylsulfonyl fluoride ($PhMeSO_2F$) at 4°C. Membranes were prepared from P_2 (17,000 × *g* pellet) by subjection to osmotic shock and repeated washing (4 times) in 20 mM Tris·HCl, pH 7.4/1 mM $MgCl_2$/0.3 mM $PhMeSO_2F$, as described (4). Synaptosomes were prepared from P_2 by a modification of the discontinuous Ficoll flotation method of Booth and Clark (17). Synaptosomes harvested from the 6%–10% Ficoll interface were then thoroughly washed in isotonic buffer of the following composition: 136 mM NaCl/5.0 mM KCl/1.2 mM $MgCl_2$/1.2 mM $CaCl_2$/2.4 mM NaH_2PO_4/10 mM glucose/20 mM Tris·HCl (pH 7.4).

BZ Binding. Binding of [^{3}H]CNZ, [^{3}H]FNZ, and [^{3}H]DZ to nM BZRs and μM BZRs was carried out as described (4). Briefly, membranes (2–5 mg/ml) were incubated for 1 hr at 4°C in the presence of a 1:20 or 1:40 dilution of the radioligand. Total and nonspecific binding were determined in the absence and presence of excess unlabeled drug. Binding was assayed by filtration over Whatman GF/B filters attached to suction. Filters were washed twice with 5 ml of ice-cold buffer, and the radioactivity was determined in a Beckman

Abbreviations: BZ, benzodiazepine; nM BZR, nanomolar benzodiazepine receptor; μM BZR, micromolar benzodiazepine receptor; GABA, γ-aminobutyric acid; DZ, diazepam; CNZ, clonazepam; FNZ, flunitrazepam; $PhMeSO_2F$, phenylmethylsulfonyl fluoride; TTX, tetrodotoxin.

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LS 2800 scintillation counter.

Photoaffinity labeling was conducted with nitro-containing BZs (FNZ and CNZ) using shortwave ultraviolet illumination. Membranes or synaptosomes were equilibrated for 1 hr at 4°C (as described above) with 600 μM drug. The reaction mixtures were then exposed uniformly, at various times, to shortwave ultraviolet light (Mineralight C81) at a distance of 5 cm at 4°C. Maximal labeling was achieved in 30 min. The membranes or synaptosomes were then washed twice with 20 vol of the appropriate buffer to remove any unbound drug. Control experiments routinely performed included membranes illuminated in the absence of drug and membranes equilibrated with drug but not illuminated.

Calcium Flux Measurements. Accumulation of $^{45}Ca^{2+}$ in synaptosomes was according to the methods of Blaustein (9) as conducted in this laboratory (12, 14, 18). Synaptosomes (0.6–1.0 mg/ml) were kept at 4°C in isotonic buffer until they were preequilibrated with ethanol or the appropriate drug (1:20 or 1:40 dilution) for 5 min at 37°C in a shaking water bath. Accumulation of $^{45}Ca^{2+}$ in control and depolarized synaptosomes was initiated with the addition of the radioisotope contained in an equal volume (usually 0.5 ml) of either normal isotonic buffer (5 mM K^+, final concentration) or an isotonic high K^+ buffer (70 mM K^+, final concentration). Accumulation continued at 37°C for 1 min until the reaction was terminated by the addition of ice-cold isotonic buffer containing EGTA (7 mM, final concentration). Samples were immediately filtered over Whatman GF/B filters and washed twice with 4 ml of ice-cold buffer. Efflux studies were performed on synaptosomes equilibrated with $^{45}Ca^{2+}$ under both control and depolarized conditions. Equilibrated samples were then diluted 1:25 with Ca^{2+}-free isotonic buffer (containing 71 mM Na^+, 70 mM K^+, and 7 mM EGTA) to promote efflux, and aliquots were removed at various intervals. The filters were then assayed and the specific activity of $^{45}Ca^{2+}$ in the incubation mixtures (usually 1–4 nCi/nmol) was then determined. Depolarization-dependent $^{45}Ca^{2+}$ uptake was defined as the difference between $^{45}Ca^{2+}$ present in control (low K^+) and depolarized (high K^+) synaptosomes.

RESULTS

Micromolar BZ Binding and Inhibition of Ca^{2+} Uptake. Binding of [^{3}H]DZ to brain membranes shows a double saturation curve (Fig. 1). Binding saturates in nanomolar and micromolar ranges, corresponding to binding of nM BZRs and μM BZRs. A Scatchard plot of the binding data is consistent with the existence of two pharmacologically distinct receptor populations (4). In contrast, the concentration curve of DZ inhibition of synaptosomal Ca^{2+} uptake is not biphasic. DZ shows no observable inhibition in the nanomolar concentration range, but substantial effects are evident in micromolar concentrations (Fig. 1). The inhibition is concentration dependent and parallels μM BZR binding. It has been previously demonstrated that the binding affinity of nM BZRs (19, 20), but not μM BZRs (4), was enhanced by GABA or muscimol. However, we found that GABA and muscimol, at concentrations maximally potentiating binding to nM BZRs, did not potentiate the inhibitory effects of BZs on voltage-sensitive Ca^{2+} uptake. These observations suggest that DZ inhibition of Ca^{2+} uptake is associated with μM BZR binding and not nM BZR binding.

Kinetics of DZ Inhibition of Synaptosomal Ca^{2+} Uptake. The accumulation of Ca^{2+} in nerve terminal preparations is a time-dependent phenomenon (Fig. 2). The presence of depolarizing concentrations of K^+ caused a dramatic increase in accumulation at all time intervals examined. The values (5–10 nmol of Ca^{2+} per mg of protein) for $^{45}Ca^{2+}$ uptake in control and depolarized synaptosomes were comparable to those observed previously (9). DZ (150 μM) had little effect on low K^+ uptake. However, DZ (150 μM) had substantial

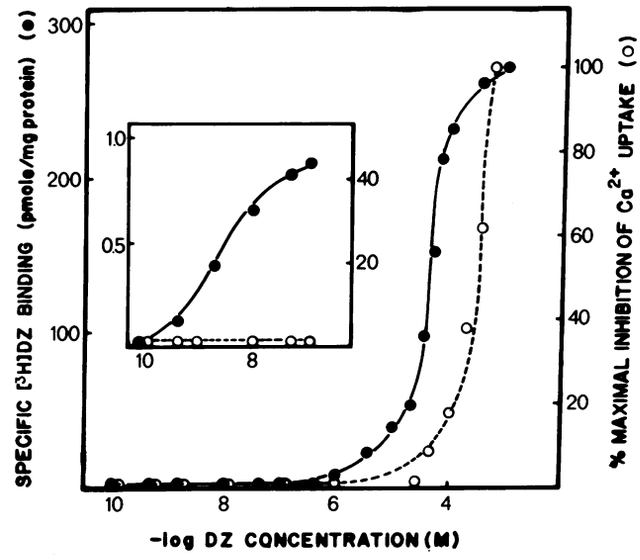

FIG. 1. Comparison of the saturation curve for [^{3}H]DZ membrane binding and DZ inhibition of high K^+-induced Ca^{2+} uptake. [^{3}H]DZ binding (●) was carried out by filtration assay with 300 μg of brain membrane protein. Data represent specific binding and are the mean of 10 determinations. DZ inhibition of $^{45}Ca^{2+}$ uptake (○) was determined by quantitation of high K^+-stimulated synaptosomal $^{45}Ca^{2+}$ uptake in the presence or absence of DZ at various concentrations. Data represent the mean of 12 determinations. (*Inset*) Expansion of [^{3}H]DZ binding curve and DZ inhibition of Ca^{2+} uptake at low DZ concentrations.

effects on stimulated synaptosomes, causing a 26.9% inhibition of high K^+ uptake at 1 min.

When studied under the conditions described above, Ca^{2+} accumulation represents the composite of two opposing parameters: Ca^{2+} influx and Ca^{2+} efflux (21). To determine the effects of BZs on Ca^{2+} influx and efflux, we examined Ca^{2+} fluxes under previously established conditions favoring each parameter individually. Fig. 3*A* describes Ca^{2+} uptake studies carried out at short time intervals prior to reaching equilibrium, thus favoring Ca^{2+} influx over Ca^{2+} efflux. DZ (150

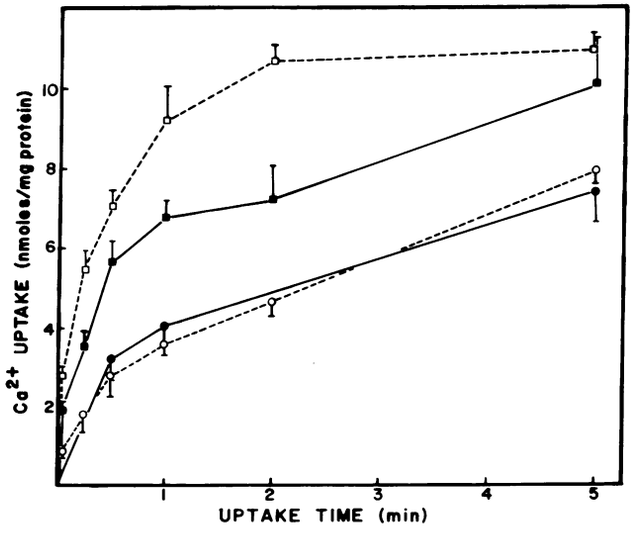

FIG. 2. Effects of DZ on synaptosomal Ca^{2+} uptake. Data represent $^{45}Ca^{2+}$ uptake into synaptosomes in the presence of low (5 mM) K^+ (○) and high (70 mM) K^+ (□). The effect of DZ (150 μM) on Ca^{2+} uptake in the presence of low K^+ (●) and high K^+ (■) is also illustrated. Values represent the mean of 8 determinations and are representative of 3 experiments (error bars represent SEM).

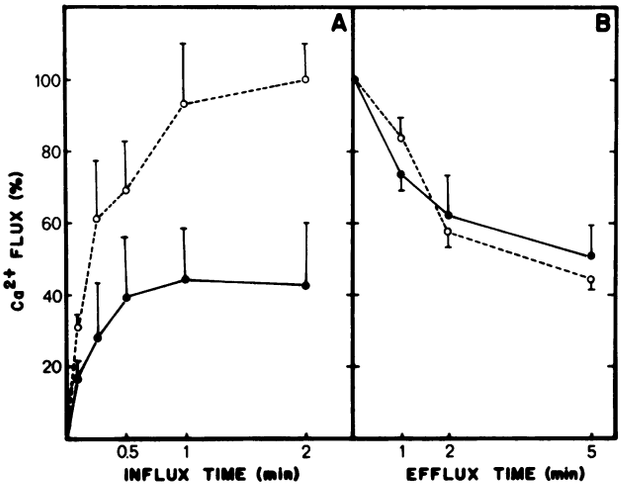

FIG. 3. Effect of DZ on synaptosomal Ca^{2+} influx and efflux. (*A*) High K^+-induced $^{45}Ca^{2+}$ influx was determined in the presence (●) and absence (○) of DZ (150 μM). The data are expressed as percent of maximal uptake (n = 8; mean ± SEM). (*B*) Efflux of $^{45}Ca^{2+}$ from prelabeled synaptosomes was measured in the presence (●) or absence (○) of DZ (150 μM). The data are expressed as percent of zero time control (n = 3; mean ± SEM).

μM) inhibition of depolarization-dependent Ca^{2+} uptake was 46.6% at 5 sec and 53.7% at 15 sec. In contrast, using prelabeled ($^{45}Ca^{2+}$) synaptosomes to study Ca^{2+} efflux, DZ (150 μM) had no significant effect on Ca^{2+} efflux at time intervals up to 5 min (Fig. 3*B*). The results indicate that, under standard experimental conditions, the inhibition of Ca^{2+} uptake observed in the presence of DZ (Figs. 1 and 2) is due to inhibition of Ca^{2+} influx and not to stimulation of Ca^{2+} efflux.

Characterization of BZ Inhibition of Ca^{2+} Uptake. The inhibition of depolarization-induced Ca^{2+} uptake is characteristic of a variety of BZs. We observed concentration-dependent inhibition of Ca^{2+} uptake with DZ, CNZ, chlordiazepoxide, and medazepam (Fig. 4*A*). A similar pattern of inhibition was found with flunitrazepam (FNZ), Ro5-5345, oxazepam, nitrazepam, Ro5-5807, and Ro21-3981 (data not shown).

Inhibition of Ca^{2+} uptake by the stereoisomeric pair B10(+)(Ro11-6893)/B10(−)(Ro11-6896) demonstrates that

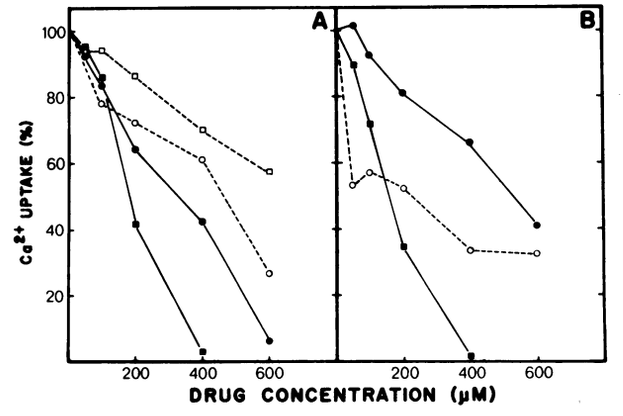

FIG. 4. BZ inhibition of Ca^{2+} uptake. (*A*) Inhibition of K^+-dependent $^{45}Ca^{2+}$ uptake in the presence of chlordiazepoxide (□), clonazepam (○), DZ (●), and medazepam (■). The data represent the mean of 8 determinations expressed as percent of maximal $^{45}Ca^{2+}$ uptake in the absence of drug. (*B*) Inhibition of K^+-induced $^{45}Ca^{2+}$ uptake by the peripheral BZ, Ro5-4864 (■), and by the stereoisomeric pair, B10(+) (active) (○) and B10(−) (inactive) (●). The data give the mean of 6 determinations and are expressed as percent of control in the absence of drug.

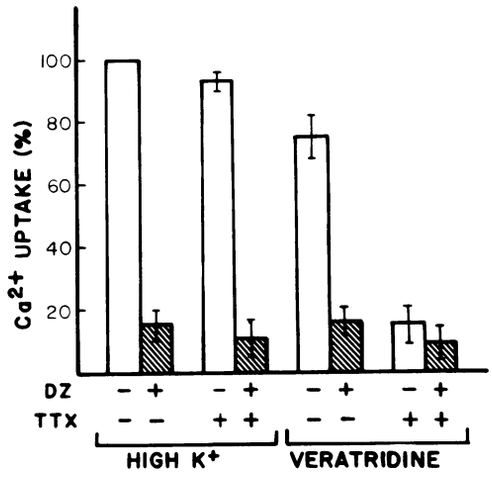

FIG. 5. Effect of DZ (0.6 mM) on high K^+- and 75 μM veratridine-stimulated Ca^{2+} uptake in the presence or absence of TTX (0.4 μM). The data are expressed as percent of maximal Ca^{2+} uptake in the presence of high K^+ and represent the mean ± SEM of 8 determinations.

this BZ inhibition is a stereospecific phenomenon (Fig. 4*B*). B10(+) was substantially more potent at low concentrations (50 μM) than was the less active isomer B10(−). One of the most potent BZs examined for inhibition of Ca^{2+} uptake was Ro5-4864 (Fig. 4*B*). The potency of Ro5-4864 in this assay is comparable to that of DZ. This observation would not be expected if BZ inhibition is mediated by the nM BZR, in which Ro5-4864 is less potent than DZ by a factor of 11,000 (3); however, it is consistent with mediation by the μM BZR in which Ro5-4864 and DZ are approximately equipotent (4).

BZs Act as Calcium Channel Antagonists. Blaustein and coworkers have demonstrated that high K^+, directly depolarizing synaptosomes, and veratridine, a depolarizing agent that acts by inducing Na^+ uptake, stimulate Ca^{2+} uptake through voltage-sensitive Ca^{2+} channels (9, 10). Tetrodotoxin (TTX), a potent Na^+ channel blocker, was used to further characterize this system, because it inhibits the effects of veratridine, but not high K^+, on Ca^{2+} uptake (9). We used these techniques to demonstrate that the BZs inhibit depolarization-dependent Ca^{2+} uptake. BZs inhibited Ca^{2+} uptake stimulated by depolarization, induced by either high K^+ or veratridine (Figs. 2 and 5). TTX blocked Ca^{2+} uptake in response to veratridine, but it did not influence high K^+-induced Ca^{2+} uptake (Fig. 5).

Calcium influx due to synaptosomal depolarization occurs predominantly through voltage-sensitive Ca^{2+} channels (9–11), but it may also be via TTX-sensitive voltage-dependent Na^+ channels (6, 7, 11). To determine whether BZs inhibit Ca^{2+} uptake by blocking Ca^{2+} and/or Na^+ channels, we studied the effects of DZ on high K^+-induced Ca^{2+} uptake in the presence of a concentration of TTX that was shown to block veratridine-induced depolarization (Fig. 5). High K^+-induced Ca^{2+} uptake and its inhibition by DZ were not significantly affected by TTX (Fig. 5). Since maximal concentrations of DZ inhibited high K^+-induced Ca^{2+} uptake in the presence of TTX by >85%, these results indicate that the majority of BZ-inhibited Ca^{2+} uptake is not passing through the TTX-sensitive Na^+ channels.

Voltage-sensitive Ca^{2+} channels in synaptosomes and other neuronal preparations are blocked by Mn^{2+} (9, 10), Co^{2+} (6, 10, 11), and only by high concentrations of verapamil, nimodipine, and nitrendipine (9, 10, 22, 23). Under our experimental conditions (Table 1), all four Ca^{2+} channel antagonists characteristically blocked depolarization-induced Ca^{2+} uptake in synaptosomes. Millimolar concentrations of Mn^{2+}

Table 1. Comparison of the effects of BZs and Ca^{2+} channel antagonists on K^+-induced synaptosomal Ca^{2+} uptake

Agent	K^+-induced Ca^{2+} uptake, % inhibition
DZ (0.2)	56 ± 5.3
DZ (0.6)	95 ± 4.6
Ro5-4864 (0.2)	66 ± 2.4
Aff. lab. FNZ (0.6)	73 ± 9.2
Mn^{2+} (1.0)	63 ± 4.1
Mn^{2+} (10)	93 ± 3.3
Mn^{2+} (1.0) and DZ (0.2)	86 ± 4.8
Co^{2+} (0.5)	55 ± 5.1
Co^{2+} (5.0)	88 ± 2.6
Co^{2+} (0.5) and DZ (0.2)	84 ± 3.2
Verapamil (0.5)	45 ± 4.3
Nitrendipine (0.6)	55 ± 3.6
Nimodipine (0.6)	45 ± 2.9

Synaptosomes were incubated for 5 min at 37°C with various test agents. Values give the percent inhibition of high K^+-stimulated $^{45}Ca^{2+}$ uptake and represent the mean ± SEM of 6 determinations. Concentrations (mM) are in parenthesis. Aff. lab., affinity labeled.

and Co^{2+} inhibited high K^+-induced Ca^{2+} uptake by >88%. Micromolar concentrations of DZ also blocked Ca^{2+} uptake by >95%. In addition, BZ inhibition was additive with that of Mn^{2+} and Co^{2+}. These results indicate that BZs act as Ca^{2+}-channel antagonists in nerve terminal preparations.

Irreversible μM BZ Binding and Inhibition of Ca^{2+} Uptake. Nitro-containing BZs, such as CNZ and FNZ, have been shown to interact irreversibly with their binding sites as a consequence of ultraviolet irradiation (24, 25). Specific irreversible BZ binding to brain membranes saturated in the μM range (Fig. 6*A*) and paralleled the μM BZR binding curve. Photoaffinity labeling of the intact synaptosomes with [^{3}H]FNZ demonstrated that the majority of irreversible binding was associated with the synaptosomal membrane.

Photoaffinity-labeled synaptosomes that were subsequently washed free of unbound BZ showed markedly decreased voltage-dependent Ca^{2+} uptake in comparison to control preparations (Fig. 6*B*). This effect of membrane-bound BZs on Ca^{2+} influx occurred in the absence of unbound FNZ in the reaction medium. Furthermore, in preliminary studies, the Ca^{2+}-channel antagonist, nitrendipine, effectively inhibited the [^{3}H]FNZ photoaffinity labeling of the μM BZ binding sites. These results indicate that the Ca^{2+}-channel antagonist properties of the BZs are mediated by the interaction of these compounds with a population of membrane μM BZ binding sites.

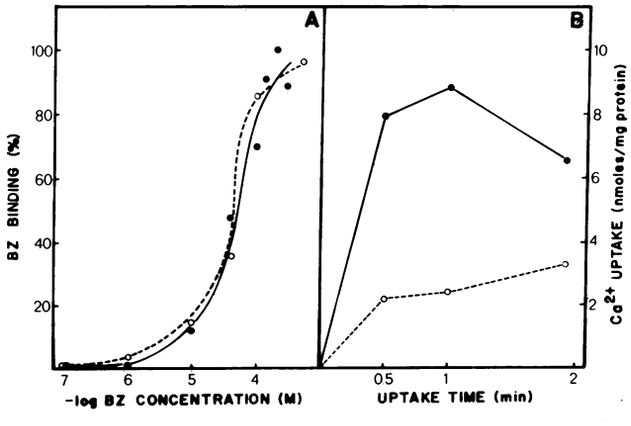

FIG. 6. (*A*) Comparison of the saturation curves for specific [^{3}H]DZ membrane binding (○) and irreversible specific [^{3}H]FNZ binding (●). The data give the mean of 6 determinations and represent percent of maximal binding. (*B*) High K^+-induced $^{45}Ca^{2+}$ uptake in control (●) and FNZ irreversibly labeled synaptosomes (○). The data give the mean of 4 determinations.

DISCUSSION

The results demonstrate the BZs in μM concentrations inhibit depolarization-dependent Ca^{2+} uptake in intact synaptosomal preparations. BZ inhibition of Ca^{2+} uptake was stereospecific and was shown by several criteria to be mediated by the μM BZRs and not by nM BZRs. The BZ concentration curve for μM BZR binding closely paralleled the curve for inhibition of Ca^{2+} uptake. When nM BZR binding was saturated, no observable inhibition of Ca^{2+} uptake was seen. DZ and Ro5-4864 were essentially equipotent in blocking Ca^{2+} uptake and μM BZR binding; however, Ro5-4864 is less potent than DZ in binding to the nM BZR by a factor of >11,000. Furthermore, GABA and muscimol, which potentiated BZ binding to the nM BZR, had no potentiating effect on μM BZR binding or BZ inhibition of Ca^{2+} uptake. Irreversible binding of FNZ and CNZ to the synaptosomal membrane μM BZ binding sites irreversibly blocked voltage-dependent Ca^{2+} uptake. These results suggest that a major physiological function of μM BZ binding sites is the regulation of voltage-sensitive Ca^{2+} uptake.

The BZ inhibition of depolarization-dependent Ca^{2+} uptake was shown to be identical to the inhibition by Ca^{2+} channel antagonists. In neuronal and synaptosomal preparations, Ca^{2+} channels have been characterized by their inhibition by Mn^{2+} and Co^{2+} (6, 9–11) and by high micromolar concentrations of verapamil, nimodipine, and nitrendipine (9, 10, 22, 23). Under our experimental conditions, the BZs acted in the same fashion as these Ca^{2+}-channel antagonists. The observation that Mn^{2+}, Co^{2+}, and DZ can individually produce a complete inhibition of voltage-dependent Ca^{2+} uptake also indicates that the BZs are acting as Ca^{2+}-channel antagonists. Furthermore, recent experiments in this laboratory have shown that Mn^{2+}, Co^{2+}, and nitrendipine block the irreversible binding of BZs to specific μM BZ binding sites. These results demonstrate that the BZs act as Ca^{2+}-channel antagonists and may interact with the same membrane sites as Mn^{2+} and Co^{2+}. Thus, the BZs may serve as powerful markers for identifying the molecular components of the Ca^{2+} channel. However, further studies are required to determine whether BZs bind to a membrane-bound regulator of the voltage-sensitive Ca^{2+} channel or to the channel itself.

Plasma concentrations of DZ that produce clinical effects in rats and humans range from 0.05 to 50 μM (26–28), and the brain levels of DZ and other BZs were shown to be 2–8 times higher than corresponding blood concentrations in the mouse, rat, and guinea pig (29, 30). Thus, plasma and brain concentrations are more than adequate to maximally saturate nM BZRs, and at higher doses, can reach micromolar concentrations that would allow them to bind to a significant proportion of the μM BZRs (30). Many of the clinical effects of the BZs have been correlated with binding to the nM BZR (1–3); however, some of the effects of the BZs on seizure activity and neuronal stabilization do not correlate with nM BZR binding. The ability of acutely administered intravenous DZ to rapidly control generalized tonic–clonic seizures and status epilepticus (31) only lasts 20–40 min after injection, corresponding to the high (>10 μM) blood and brain levels of DZ reached for the first 20–40 min after intravenous administration. At these high DZ concentrations (>10 μM), the drug is effective in treating epilepsy in humans (31), inhibiting maximal electric-shock-induced seizures in animals (13), and preventing kindling in rats (32). At submicromolar concentrations, DZ is effective in binding to nM BZRs but has no significant clinical effects on maximal electric-shock-induced seizures, kindling, or tonic–clonic seizures in humans (13, 29–32). These clinical effects require micromolar

levels of the BZs that could produce significant μM BZR binding and inhibition of voltage-sensitive Ca^{2+} uptake. These observations suggest that some of the stabilizing effects of BZs on neuronal tissue in humans and animals may be mediated by interaction with μM BZRs and subsequent regulation of Ca^{2+} conductances.

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