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Inhibition of Ca^{2+} conductance in identified leech neurons by benzodiazepines

(Ca^{2+} channels/electrophysiology/Ca^{2+} channel blockers/benzodiazepine receptors)

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ABSTRACT **Benzodiazepines (BZs) in micromolar concentrations inhibit Mn^{2+}- and Co^{2+}-sensitive regenerative divalent cation potentials, which are revealed in the presence of tetraethylammonium ion, in leech nociceptive neurons (N cells). This BZ effect is reversible and dose-dependent. The BZs, like Mn^{2+} and Co^{2+}, inhibit the maximum rate of depolarization ($\dot{V}_{max}$) and duration of divalent cation potentials at concentrations that do not significantly affect resting membrane potential or $\dot{V}$max of the Na^+-dependent action potential. Ultraviolet-induced BZ binding to micromolar-affinity sites in ganglia and isolated cells irreversibly blocks Ca^{2+} conductance in neurons without significantly affecting resting membrane potentials. BZ binding studies with leech neuronal membrane show saturable, specific binding in the micromolar concentration range that was similar to BZ binding to synaptosomal membrane fractions. The apparent K_d obtained from the micromolar-affinity BZ binding curve for leech ganglionic membrane preparations agrees well with the apparent K_i estimated from the dose–response curve measuring BZ inhibition of $\dot{V}_{max}$ of the divalent cation potentials. These findings indicate that BZs act like Ca^{2+}-channel antagonists in intact neuronal preparations and are consistent with the hypothesis that BZ binding to micromolar-affinity receptors modulates voltage-gated Ca^{2+} channels.**

Benzodiazepines (BZs) in micromolar concentrations inhibit voltage-dependent Ca^{2+} uptake in mammalian nerve terminal preparations (1–3). In synaptosomes, BZs act like the classical Ca^{2+}-channel blockers Mn^{2+} and Co^{2+} (4). Voltage-sensitive Ca^{2+} uptake in synaptosomes is regulated by micromolar-affinity BZ binding sites and not by the nanomolar-affinity BZ receptors that have been implicated in the regulation of Cl^- conductances (4).

To determine the electrophysiological effects of BZs on Ca^{2+} conductance, we studied the actions of these compounds on identified neurons in the leech *Macrobdella decora*. Long-lasting regenerative Ca^{2+} potentials, revealed by blocking outward K^+ currents, have previously been characterized for leech neurons (5–7) and for neurons in several other invertebrate and vertebrate preparations (8). Nanomolar-affinity BZ receptors do not exist in the leech (9) or other closely related annelids (10). However, micromolar-affinity BZ binding sites, as identified and characterized in rat brain membrane (11), were not studied in the leech, even though behavioral effects of BZs were described in these animals at high BZ concentrations (9).

Here we show that Mn^{2+}- and Co^{2+}-sensitive regenerative divalent cation potentials, which are revealed in the presence of tetraethylammonium ion (Et_4N^+) (5–7) in identified leech nociceptive neurons (N cells) (12) are inhibited by micromolar concentrations of several BZs, and we provide evidence that suggests that BZ inhibition of voltage-dependent Ca^{2+} conductance is regulated by BZ interaction with a population of micromolar-affinity BZ membrane binding sites.

MATERIALS AND METHODS

Materials. [^{3}H]Clonazepam (CNZ) (43 Ci/mmol, 1 Ci = 37 GBq) and all other BZs were gifts of W. E. Scott and P. F. Sorter of Hoffman-LaRoche. Leeches of the species *Macrobdella decora* were obtained from commercial suppliers and maintained in spring water at 15°C.

Electrophysiology. Experiments in leech ganglia were performed on the medial and lateral N cells (13), which were identified in normal leech Ringer's solution (120 mM NaCl/2 mM $CaCl_2$/4 mM KCl/10 mM glucose/10 mM Tris Cl, pH 7.4) by their size, position, and electrical parameters, as described by Nicholls and Baylor (12). The intracellular recording techniques used in this study have been described in detail (6, 12). To facilitate equilibration of the drugs, the connective capsules overlaying ganglia were removed. Divalent cation potentials were elicited in Na^+-free Et_4N^+/Ringer's solution (1–3 mM $SrCl_2$ or $CaCl_2$/4 mM KCl/25 mM Et_4NCl/220 mM sucrose/10 mM glucose/10 mM Tris Cl, pH 7.4) after cell identification in normal Ringer's solution. Permanent recording was done with a PDP-8 computer system which digitized the traces at 23 kHz. The maximum rates of depolarization ($\dot{V}_{max}$) of the Na^+-dependent action potentials of N cells were analyzed by a computer subroutine written for this purpose (14, 15). The $\dot{V}_{max}$ of the divalent cation potentials were determined by differentiation of the action potential with an analog circuit having a time constant of 1 msec. Due to the low solubility of BZs in aqueous media, stock solutions of these drugs were made in absolute ethanol and then diluted to final concentration in Na^+-free Et_4N^+/Ringer's solution. The ethanol content of the experimental Ringer's solution did not exceed 0.5%. Control applications showed that this ethanol concentration had no detectable effect on the $\dot{V}_{max}$, duration, or amplitude of the divalent cation potentials.

The K_i for BZ inhibition of $\dot{V}_{max}$ of the Sr^{2+} potentials was estimated from the data points. From this value the theoretical reverse Langmuir curve (16, 17) was computed from the equation $\dot{V}'_{max}/\dot{V}_{max} = 1/1 + ([BZ]/K_i)$, where $\dot{V}'_{max}$ is the maximal rate of depolarization in the presence of drug. In the case of the normalized $\dot{V}'_{max}/\dot{V}_{max}$ values, the SD values do not include those of the control (before drug) values.

BZ Binding to Intact Ganglia. For comparative purposes, electrophysiological and binding studies with whole ganglia and BZs were conducted under the same conditions. Isolated nerve cords containing four ganglia each were placed in normal Ringer's solution (2 ml) in a small Petri dish. Aliquots

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Abbreviations: BZ, benzodiazepine; CNZ, clonazepam; DZ, diazepam; MDZ, medazepam; Et_4N^+, tetraethylammonium; $\dot{V}_{max}$, maximum rate(s) of depolarization; N cell, nociceptive neuron.

(20 μl) of absolute ethanol containing drug were added to initiate binding, and the dish was equilibrated for 5 min at room temperature. For electrophysiological studies, the ganglia were incubated at 4°C with 400 μM CNZ (or, for binding studies, with 1 μM [^{3}H]CNZ) in the presence or absence of UV illumination. Following UV exposure, the CNZ solutions were removed and the ganglia were washed with several changes of normal Ringer's solution over a period of 2 hr. The preparations were then either examined electrophysiologically or used for quantitation of [^{3}H]CNZ binding. To determine [^{3}H]CNZ binding, individual ganglia were dissected, homogenized in 100 μl of normal Ringer's solution per ganglion, and suction-filtered on Whatman GF/B filters, which then were used for quantitation of membrane-bound [^{3}H]CNZ by liquid scintillation counting. For studies of binding to cells within the ganglia, each labeled and washed ganglion was fixed with 4% (wt/vol) paraformaldehyde for 5 min. The cell bodies and neuropil region were microdissected free from the outer connective capsule. This neuron-enriched preparation was solubilized in 1% (wt/vol) $NaDodSO_4$, and incorporation of [^{3}H]CNZ was measured by liquid scintillation counting of the dissolved preparation.

BZ Membrane Binding Studies. Isolated nerve cords from 20 leeches were homogenized in 20 mM Hepes/1 mM $MgCl_2$/0.3 mM phenylmethylsulfonyl fluoride, pH 7.4. A synaptosomal membrane fraction (20,000 × *g* pellet) from rat brain was prepared (4) and washed in Hepes buffer. Specific [^{3}H]CNZ binding in both preparations (0.5–1.0 mg of protein/ml) was carried out by the photoaffinity-labeling technique described previously (4). Binding assays were conducted at 4°C in plastic microtiter plates so that all reaction mixtures could be uniformly exposed to UV irradiation. Aliquots (5 μl) of [^{3}H]CNZ and 5 μl of ethanol with or without nonradioactive BZ were first added to each microtiter plate well. Then 90 μl of ganglionic or synaptosomal preparation was added to each well to initiate binding. The radioligand and nonradioactive CNZ were equilibrated for 1 hr, subjected to UV illumination (10 min), and then filtered and washed on Whatman GF/B filters attached to suction (4).

RESULTS

BZ Inhibition of Divalent Cation Potentials. BZs reversibly inhibited Ca^{2+} potentials recorded from lateral and medial N cells (13) in Na^+-free, Et_4N^+-containing solutions; the inhibition was dose-dependent. It is well substantiated that such potentials are mediated by specific Ca^{2+} channels and that Sr^{2+} or Ba^{2+} can substitute for Ca^{2+} as a current carrier through the channel in leech neurons (6) as well as in other neurons (for review see ref. 8). In the present study, we used Sr^{2+} as the current carrier because it produced the most stable recording conditions and because there is evidence that Sr^{2+} in leech neurons may be less effective than Ca^{2+} in activating Ca^{2+}-dependent K^+ conductances, $\bar{g}_{K,Ca}$ (18, 19).

Long-lasting (seconds) action potentials of constant $\dot{V}_{max}$, duration, and amplitude in the nociceptive neurons were obtained after ≈5 min incubation in Na^+-free, Sr^{2+}/Et_4N^+/Ringer's solution. The $\dot{V}_{max}$ of the potentials was dependent on extracellular divalent cation concentration and was 7.4 ± 0.8 V/sec (n = 32) in 1 mM Sr^{2+}. The potentials were found to be stable for up to 30 min at a stimulus frequency of one per min. The potentials were reversibly blocked by Mn^{2+} and Co^{2+}, and the size of the overshoot varied logarithmically with $[Ca^{2+}]_o$ and $[Sr^{2+}]_o$: 28.5 mV per 10-fold change in divalent cation concentration. The $\dot{V}_{max}$, duration, and amplitude of the regenerative Sr^{2+} potentials of medial N cells were resistant to 50 μM tetrodotoxin, a dose which effectively abolished the Na^+-dependent action potential of these cells (20). This indicates that the current underlying the prolonged cation action potential is not passing through the Na^+ channel but through a distinct Ca^{2+} conductance.

In the majority of our experiments, we used medazepam (MDZ) because it was the most water-soluble BZ and was one of the most potent inhibitors of synaptosomal Ca^{2+} influx (4). As shown in Fig. 1 and Fig. 2*A*, MDZ decreased $\dot{V}_{max}$ and the duration of the Sr^{2+} action potential in a dose-dependent manner. In the concentration range used in this study, the BZs did not significantly affect the resting membrane potential or the input resistance of the cells in either normal or Na^+-free Sr^{2+}/Et_4N^+/Ringer's solution. Throughout the experiments, resting membrane potentials varied less than ±3 mV.

The dose–response curve for MDZ inhibition of $\dot{V}_{max}$ of the divalent cation potentials (Fig. 1*C*) could be well-fitted by a reverse Langmuir adsorption isotherm for bimolecular reactions (16, 17). The apparent K_i determined from the experiments was 100 μM. This value represents an approximation of the true K_i, because it is derived from an indirect measurement of the Sr^{2+} conductance $\bar{g}_{Sr}$ and, as discussed by Ritchie and Rogart (21), the actual K_i value is likely to be somewhat lower. The BZ inhibition of the duration of the potentials was also dose-dependent (Fig. 2*A*), and we used this parameter for measuring reversibility and relative potency of the drugs. Reversibility of the BZ action on the Sr^{2+} conductance is shown for CNZ in Fig. 2*C* and Fig. 4. Although the blocking effect of the drug was detectable within 1 min of application, reversibility occured at a slower rate (Fig. 2*C*). After prolonged washing (1–2 hr), the drug effect on $\dot{V}_{max}$ was almost completely reversed (Fig. 4). To determine whether MDZ was representative of other BZs, we

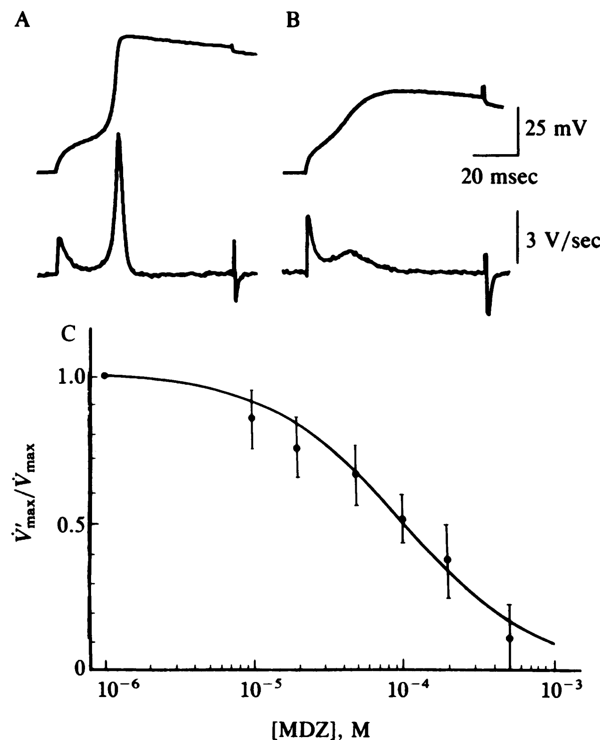

FIG. 1. MDZ inhibition of $\dot{V}_{max}$ of the regenerative Sr^{2+} potentials in medial N cells. (*A*) The rising phase of the Sr^{2+} potential (upper trace) with the differentiated waveform (lower trace) in Na^+-free Sr^{2+}/Et_4N^+/Ringer's solution without drug. (*B*) The rising phase of the Sr^{2+} potential (upper trace) with the differentiated waveform (lower trace) in Na^+-free Sr^{2+}/Et_4N^+/Ringer's solution with 500 μM MDZ. (*C*) Dose–response curve for the effect of MDZ on $\dot{V}_{max}$. $\dot{V}'_{max}$, $\dot{V}_{max}$ in the presence of the drug. Data points represent the mean of 10–15 determinations ± SD. From the data points, an apparent K_i of 100 μM was estimated; from this value, the theoretical Langmuir curve (solid line) was computed (see *Materials and Methods*).

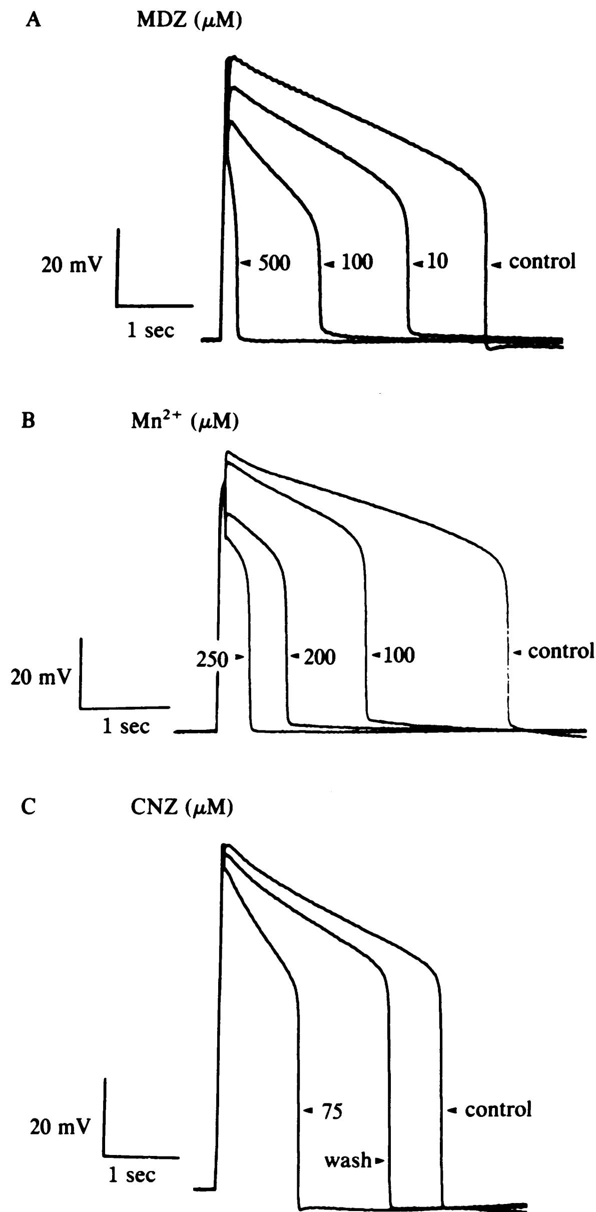

FIG. 2. Effects of BZs and Mn^{2+} on Sr^{2+} potentials in lateral N cells. (*A*) Dose-dependent inhibition by MDZ of the regenerative Sr^{2+} potential. All potentials were recorded from the same cell at steady state after 3 min incubation at the indicated drug concentrations. (*B*) Dose-dependent inhibition by Mn^{2+} of the regenerative Sr^{2+} potential. All potentials were recorded as described in *A*. (*C*) Reversible action of CNZ. The Sr^{2+} potential was inhibited by 75 μM CNZ. The wash condition shown was obtained 10 min after drug removal.

tested the actions of CNZ, diazepam (DZ), and Ro 5-4864 on the neurons. As shown in Fig. 3, these compounds also inhibited the regenerative Sr^{2+} potentials in the micromolar concentration range. The effects of the BZs on potentials carried by Ca^{2+} paralleled those obtained for the Sr^{2+} potentials. We obtained a complete and reversible block of Ca^{2+}-dependent potentials with DZ at a concentration of 400 μM.

The inhibitory effect of BZs on the voltage-dependent Ca^{2+} conductance was not due to a nonspecific, local anesthetic-like effect. At 200 μM MDZ, which produced 70% inhibition (Fig. 1*C*) of $\dot{V}_{max}$ of the Sr^{2+} potential, the $\dot{V}_{max}$ of the Na^{+}-dependent action potential showed no statistically significant change (control, 67.5 ± 5.6 V/sec; MDZ, 63.9 ± 4.6 V/sec; mean ± SD, n = 12). These results suggest that

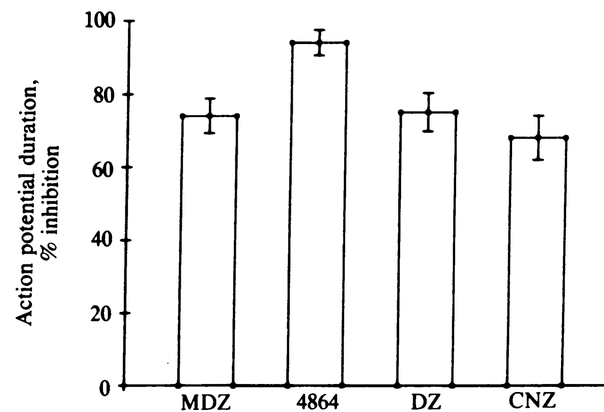

FIG. 3. The relative potency of MDZ, Ro 5-4864, DZ, and CNZ in inhibiting the duration of the Sr^{2+} potential. Determinations were carried out at a 75 μM concentration of each drug. For each drug, the mean of 3 or 4 determinations ± SD is shown.

the BZ effect may be selective for the divalent cation conductance, but additional studies are necessary to rule out an effect on other ionic conductances in this BZ concentration range.

Comparison of Mn^{2+} and BZ Inhibitions of Divalent Cation Potentials. Further evidence indicating that BZs act like Ca^{2+}-channel antagonists in leech neurons is provided by comparison of the effects of Mn^{2+} and BZs on this preparation. Mn^{2+} is a Ca^{2+}-channel blocker in most vertebrate and invertebrate preparations (for review, see ref. 8), including leech preparations (6). The pattern of Mn^{2+} inhibition (Fig. 2*B*) of the divalent cation potentials was the same as that of inhibition by the BZs (Fig. 2*A*), and both Mn^{2+} and BZs in micromolar concentrations completely blocked the Sr^{2+} potential. Furthermore, both Mn^{2+} and BZs caused a marked decrease in the size of the undershoot of the Na^{+}-dependent action potential, presumably due to a decrease in the contribution of Ca^{2+}-dependent K^{+} conductance, resulting from diminished Ca^{2+} entry (18, 22). The close similarity of BZ and Mn^{2+} actions on leech neurons is consistent with the view that the BZs inhibit the same Ca^{2+} conductance as does Mn^{2+}.

Irreversible Inhibition of Ca^{2+} Conductance by Photoactivated BZs. CNZ, a nitro-containing BZ, has been shown to interact irreversibly with its binding sites in neuronal membranes after UV irradiation (4, 23, 24). Irreversible micromolar-affinity CNZ binding to synaptosomal membrane by this method was shown to inhibit depolarization-dependent Ca^{2+} uptake (4). In isolated leech ganglia, we examined the effect of UV-induced CNZ binding on both the resting membrane potential and $\dot{V}_{max}$ of the Sr^{2+} potential in the lateral N cell (Fig. 4). Treatment of the neurons with UV light (20 min) did not significantly affect the resting membrane potential or the $\dot{V}_{max}$ of the divalent cation potential. In contrast, UV illumination (20 min) in the presence of 400 μM CNZ, followed by extensive washing to remove unbound CNZ, virtually abolished the divalent cation potential in these cells (Fig. 4) without affecting the resting membrane potential. The inhibition was irreversible, despite prolonged washing with drug-free solutions for >3 hr. In the absence of UV irradiation, incubation of the ganglia under identical conditions with 400 μM CNZ, followed by prolonged washing, did not significantly modify $\dot{V}_{max}$ of the divalent cation potential or the resting membrane potential of the cells. These results indicate that the block of the divalent cation potentials resulted from UV-activated binding of CNZ to micromolar-affinity sites on the N cell.

Irreversible Micromolar-Affinity BZ Membrane Binding Sites. UV-activated [^{3}H]CNZ binding to intact ganglia was

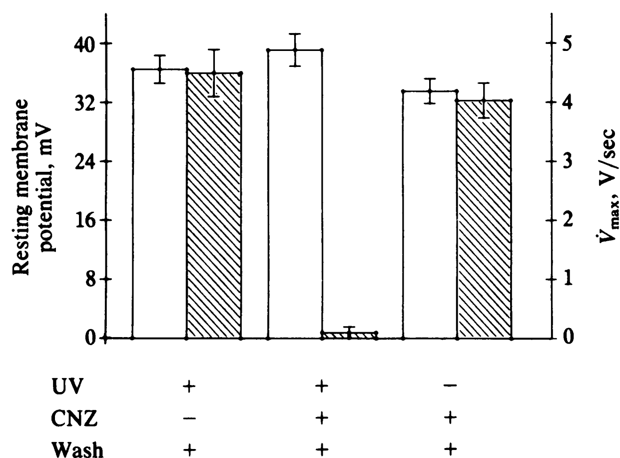

FIG. 4. Irreversible inhibition of Ca^{2+} conductance by photoactivated CNZ. Potentials were recorded from lateral N cells after CNZ (400 μM) binding and prolonged washing of the ganglia to remove unbound CNZ. Resting membrane potentials (open bars) and $\dot{V}_{max}$ (hatched bars) are shown for three conditions: UV without CNZ, UV with CNZ, and CNZ without UV. The data represent the means of 10–15 determinations ± SEM for each condition.

studied under conditions identical to those used to study the electrophysiological effects of CNZ on N cells. The ganglia were incubated with [^{3}H]CNZ with or without UV irradiation, washed for 3 hr with normal leech Ringer's solution, and assayed for [^{3}H]CNZ binding to whole ganglion membrane. Membrane isolated from labeled ganglia showed considerable irreversible [^{3}H]CNZ binding due to UV irradiation (0.01 pmol/μg of protein). Thus, under conditions where Ca^{2+} conductances were irreversibly inactivated, significant binding of [^{3}H]CNZ to ganglionic membrane was observed.

To determine whether irreversibly bound [^{3}H]CNZ was associated with neurons, ganglia were incubated with [^{3}H]CNZ with or without UV irradiation, rinsed free of unbound drug, and fixed with paraformaldehyde. The cell and neuropil region from the ganglion then were removed from the outer connective capsule and assayed for [^{3}H]CNZ binding. The neuron-enriched cell fraction contained >70% of the total recovered bound [^{3}H]CNZ in whole ganglia. These data and the data from whole ganglia membrane binding studies strongly suggest that micromolar-affinity CNZ binding sites are present in the membrane of cells within the leech ganglion.

We further quantitated UV-activated [^{3}H]CNZ binding to whole ganglion membrane by using preparations made from pooled ganglia. The BZ binding curve (Fig. 5A) in leech neuronal membranes demonstrates saturable, specific micromolar-affinity CNZ binding. A Scatchard plot (Fig. 5B) of the [^{3}H]CNZ binding data suggests a single class of binding sites with an apparent K_d of 35 μM and a binding capacity (B_{max}) of 814 pmol/mg of membrane protein. This apparent K_d agrees well with the apparent K_i of 100 μM obtained from the electrophysiological experiments with MDZ. The BZ binding curve in the leech closely parallels the BZ binding curve obtained in synaptosomes (Fig. 5A). The pharmacological properties of this binding site in leech are similar to those of the micromolar-affinity BZ receptor in rat brain (K_d for diazepam, 45 μM; B_{max}, 360 pmol/mg; ref. 11) but are clearly unrelated to those of the nanomolar-affinity BZ receptor in rat brain (K_d for diazepam, 3.5 nM; B_{max}, 0.9 pmol/mg; ref. 23).

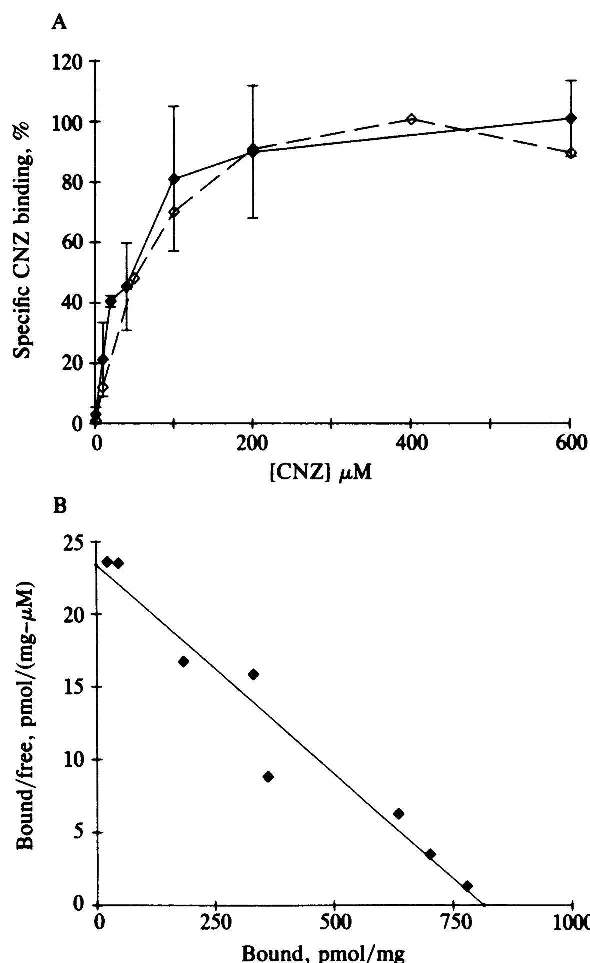

FIG. 5. UV-induced specific [^{3}H]CNZ binding in leech neuronal preparations. (A) Comparison of UV-induced specific [^{3}H]CNZ binding in leech neuronal preparations (◆) and rat crude synaptosomes (◇). The leech data represent specific (total minus nonspecific) binding ± SD ($n \geq 6$). The synaptosome data represent the means of 10 determinations; SDs were omitted for clarity. (B) Scatchard plot of leech neuronal [^{3}H]CNZ binding. The binding data yield a single line ($r = 0.974$) with a K_d of 35 μM and a B_{max} of 814 pmole/mg of membrane protein.

DISCUSSION

The divalent cation conductance channel in leech has the same properties as Ca^{2+} channels described in other preparations from several invertebrate and vertebrate phyla (6, 8, 25). In this report, we have shown that BZs reversibly inhibit this voltage-gated Ca^{2+} conductance in identified leech neurons in a dose-dependent manner, indicating that the BZs act as Ca^{2+}-channel blockers. BZs, like Mn^{2+}, inhibited the divalent cation potentials at concentrations that did not significantly affect resting membrane potential or $\dot{V}_{max}$ of the Na^+-dependent action potential. These findings suggest that the BZs are not acting as local anesthetics, since representative local anesthetics are known to decrease $\dot{V}_{max}$ of the Na^+-dependent action potential and affect the resting membrane potentials in leech N cells (15, 26), as they do in a variety of excitable membranes (27).

BZs are important therapeutic agents with specific binding sites with K_ds in the nanomolar (28, 29) and micromolar (4, 11) concentration ranges. These two classes of BZ receptors have been shown to be distinct by both pharmacological and physiological criteria (4, 11). Leech neurons are of particular interest in studying BZ receptors because behavioral effects of BZs have been observed (9) in this species, yet nanomolar-affinity BZ receptors do not exist in leech or other annelids (9, 10). In this report, we have demonstrated the presence of micromolar-affinity BZ binding sites in leech neuronal membrane obtained from whole ganglia. In experiments

in which neurons were isolated from the outer connective capsule after [^{3}H]CNZ labeling, 70% of BZ binding was associated with the neuronal fraction. The micromolar-affinity BZ binding site in leech membrane had essentially the same pharmacological properties as the micromolar-affinity BZ receptor characterized in rat brain, which has been shown to regulate voltage-sensitive Ca^{2+} channels in nerve terminal preparations (4). The apparent K_d of the micromolar-affinity binding site in the leech also correlated well with the electrophysiologically determined apparent K_i for BZ inhibition of divalent cation conductance. These results are consistent with the hypothesis (4) that micromolar-affinity BZ binding sites regulate voltage-sensitive Ca^{2+} channels and that these sites thus may play a role in modulating neuronal excitability (11).

Plasma concentrations of BZs that produce clinical effects in humans and rats range from 0.05 to 50 μM (30–32), with corresponding brain levels being 2–8 times higher (30–32). These concentrations of BZs are sufficient to significantly inhibit Ca^{2+} conductances in leech neurons, suggesting that some of the neuroactive effects of the BZs may be mediated by micromolar-affinity BZ binding sites that regulate Ca^{2+} conductance.

Characterization of the effects of the BZs on the Ca^{2+} conductance by intracellular recording in identified neurons directly documents the effects of these compounds on neuronal function. Furthermore, irreversible CNZ binding to enriched neuronal membrane preparations suggests a relationship between the interaction of the BZs with the cellular membrane and the regulation of the function of the Ca^{2+} channel. Although the data from this study do not allow us to exclude the possibility that BZs have effects on other ion conductances, the BZ effect on Ca^{2+} conductances, at concentrations that do not affect the resting membrane potential or the $\dot{V}_{max}$ of the Na^+-dependent action potential, suggests that BZs at these concentrations may selectively affect the Ca^{2+} channel. Thus, BZs may serve as important tools in the study of Ca^{2+} conductances and may provide a marker for subsequent characterization of the molecular components of the membrane receptors responsible for the effects of these compounds on the voltage-gated Ca^{2+} channel.

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