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# Diazepam-Binding Inhibitor: A Neuropeptide Located in Selected Neuronal Populations of Rat Brain

Abstract. *An endogenous polypeptide of rat brain has been identified that is capable of displacing 1,4-benzodiazepines and the esters of the 3-carboxylic acid derivatives of β-carbolines from their specific synaptic binding sites. This polypeptide was termed diazepam-binding inhibitor (DBI). Previous studies have shown that DBI injected intraventricularly in rodents elicits "proconflict" responses and antagonizes the "anticonflict" action of benzodiazepines. An antiserum to this peptide, directed toward an immunodeterminant near its amino terminus, makes it possible to detect, measure, and study the neuronal location of this peptide in rat brain. In the rat cerebral cortex, DBI immunoreactivity is located in neurons that are not GABAergic (GABA, γ-aminobutyric acid); in the cerebellum and hippocampus, however, it might be present also in GABAergic neurons.*

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Anxiety can be lessened or precipitated in humans by various chemicals that bind with high affinity to a specific recognition site for benzodiazepines. The occupancy of these sites by various derivatives of 1,4-benzodiazepines facilitates GABAergic (GABA, γ-aminobutyric acid) transmission, enhances the threshold to convulsions elicited by drugs that impair GABAergic transmission, prolongs the opening bursts of chloride ion channels elicited by GABA, and relieves episodes of anxiety in humans (*1*). In contrast, occupancy of these sites by esters of β-carboline 3-carboxylic acid negatively modulates GABAergic transmission, lowers the convulsion threshold to drugs that impair GABAergic transmission, depresses chloride ion conductance evoked by GABA, and precipitates panic anxiety in humans (*2*). The question then arises as to whether the benzodiazepine recognition sites are physiologic receptor sites or drug acceptors. In demonstrating that a drug recognition site has physiological significance, it is relevant to show that an endogenous ligand exists. Identification of such a compound may provide a new approach to studies of the molecular mechanisms operative in brain function. Identifying this ligand may also help to characterize the biochemical abnormalities associated with symptoms, such as anxiety, convulsions, and sleep disorders, that are ameliorated by benzodiazepine treatment.

From homogenates of rat and human brain, we have isolated and purified to homogeneity a neuropeptide that displaces $^3$H-labeled β-carboline 3-carboxylic acid (median inhibitory concentration, 1 μ*M*) bound to high-affinity recognition sites in crude synaptic membranes (*3*). This peptide was termed diazepam-binding inhibitor (DBI) to indicate its ability to displace specific ligands from benzodiazepine recognition sites (*3*). DBI has 104 amino acid residues and contains two identical octadecapeptide chains with the following amino acid sequence: $NH_2$-QATVGDVNTDRPGLLDLK-OH(*4*). DBI probably functions as a precursor of this octadecapeptide, which might act as an endogenous ligand of benzodiazepine recognition sites. DBI belongs to a new class of brain polypeptides that presumably takes part in the regulation of GABAergic transmission. The sequence of 52 of the 104 amino acid residues of the DBI structure is as follows: (52)TQPTDEEMLFIYSHFKQATVGDVNTDRPGLLDLKGKXIMKTYVEKVEELKKKY-OH(104). This sequence does not resemble any known mammalian neuropeptide (*3*).

When injected intraventricularly into rats subjected to the "conflict-punishment" behavioral model proposed by Vogel *et al.* (*5*), DBI antagonizes the "anticonflict" action of diazepam and facilitates "conflict" behavior; that is, it enhances the shock-induced suppression of drinking in thirsty animals by lowering the threshold for the response (*3*). These actions of DBI are antagonized by the imidazobenzodiazepine RO 15 1788 (*3*), which is a specific benzodiazepine antagonist. This suggests that the brain contains a specific ligand for benzodiazepine recognition sites that is operative in GABA receptor modulation in a manner similar to esters of β-carboline 3-carboxylic acid and is capable of facilitating the onset of conflict in the Vogel model. We infer that a modulation of specific GABAergic synapses might be operative because DBI added to primary cultures of spinal cord neurons decreases the

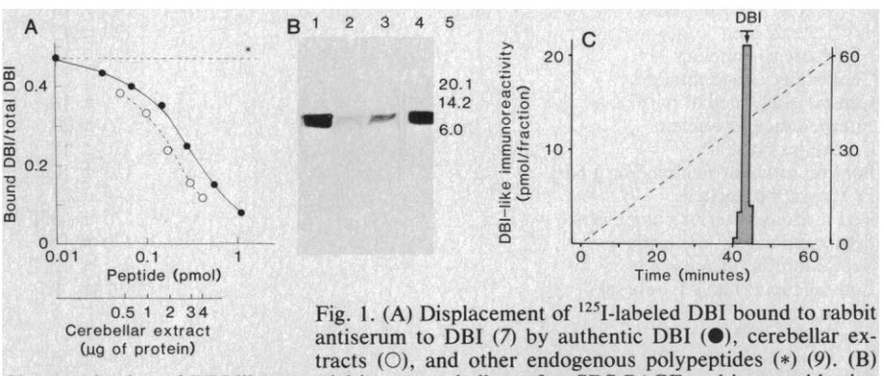


Fig. 1. (A) Displacement of $^{125}$I-labeled DBI bound to rabbit antiserum to DBI (*7*) by authentic DBI (●), cerebellar extracts (○), and other endogenous polypeptides (*) (*9*). (B) Characterization of DBI-like material in rat cerebellum after SDS-PAGE and immunoblotting with DBI antiserum. Cerebellar extracts were subjected to 15 percent SDS-PAGE (*3*) and blotted onto nitrocellulose paper. The paper was probed with antiserum (1:1000) and then visualized by the peroxidase method (*14*). (Lane 1) Standard DBI (10 μg). (Lane 2) 1*N* acetic acid cerebellar extract (24 μg of protein). (Lane 3) 0.1 percent SDS cerebellar extract (100 μg of protein). The sample was homogenized in 0.1 percent SDS–3 percent β-mercaptoethanol and 15 percent glycerol in 50 m*M* tris buffer (*p*H 7.4), boiled for 5 minutes, and centrifuged at 48,000*g* for 20 minutes. The supernatant was applied to the gel. (Lane 4) Comparative staining of DBI (10 μg) by Coomassie blue after SDS-PAGE. (Lane 5) Molecular weight markers (in thousands). (C) Characterization of DBI-like immunoreactivity in rat cerebellar extract after reversed-phase HPLC. Acetic acid cerebellar extract (~300 μg of protein) was chromatographed on a reversed-phase HPLC column equilibrated with 0.1 percent TFA-$H_2O$ (TFA, trifluoroacetic acid). The column was developed with a 0 to 60 percent linear gradient of 0.1 percent TFA-acetonitrile over 60 minutes at a flow rate of 2 ml/min. Fractions were lyophilized before the radioimmunoassay.

duration of chloride ion channel opening bursts elicited by GABA (*6*). We now describe our immunohistochemical study of the distribution and neuronal location of DBI in rat brain.

Rabbits were immunized with purified DBI in Freund's complete adjuvant as described (*7*). The antiserum had a high affinity for rat brain DBI; under standard radioimmunoassay conditions, as little as 0.05 pmol of authentic DBI could be detected with the antiserum diluted 1:10,000 and DBI labeled with $^{125}I$ by the Bolton-Hunter technique. The antiserum specificity was established according to the following criteria: (i) the antiserum did not cross-react with several other brain neuropeptides (Fig. 1A); (ii) brain extracts prepared with acetic acid or 0.1 percent sodium dodecyl sulfate (SDS), chromatographed by 15 percent SDS–polyacrylamide gel electrophoresis (PAGE), and then electroblotted on nitrocellulose paper evinced a single band of DBI-like immunoreactivity migrating with characteristics identical to those of standard DBI (Fig. 1B); and (iii) acetic acid extracts of brain containing 100 to 1000 μg of protein, purified by gel chromatography with G-50 (1 by 70 cm) and G-75 (1 by 70 cm) columns, or by reversed-phase high-performance liquid chromatography (HPLC) on a μBondapak $C_{18}$ column (7.8 mm by 30 cm) (Fig. 1C), produced a single peak of DBI-like immunoreactivity. The chromatographic retention volume of this peak was identical to that of authentic DBI.

To characterize this antiserum with regard to the immunogenic determinant, we obtained DBI fragments and tested their immunoreactivity. Because DBI contains two methionine residues, cleavage of DBI by cyanogen bromide (CNBr) and subsequent separation of the digested material by HPLC yielded three fragments. The carboxyl terminal (CNBr-3) and intermediate (CNBr-2) fragments did not react with the DBI antibody, whereas the CNBr-1 fragment, which contains the amino-terminal part of DBI (*8*), evinced DBI-like immunoreactivity. Because the sequencing of the CNBr-1 fragment was prevented by a block of the DBI amino terminus, we performed a tryptic digestion of CNBr-1 with subsequent purification by HPLC with a reversed-phase column and carried out immunogenic characterization of the fragments with the DBI antibody. This procedure revealed that the antigenic determinant is associated with a peptide fragment with a blocked amino terminus. In addition, one fragment with "proconflict" action was obtained that produced an amino acid sequence similar to that of the octadecapeptide described earlier (*4*). Because two copies of this octadecapeptide were detected (one in CNBr-1 and one in CNBr-2), this fragment could be the endogenous ligand for benzodiazepine recognition sites.

Table 1. Regional distribution of DBI-like immunoreactivity in rat brain. Rats were decapitated and brains were rapidly removed and frozen on dry ice. Various brain regions were identified and dissected by means of the stereotaxic coordinates of Paxinos and Watson (*16*). Coronal sections of the brain (500 μm thick) were obtained in a cryostat at −10°C, and brain structures were punched with a stainless steel needle (inner diameter, 1 mm) as described (*17*). Samples were extracted by sonication in 200 volumes of 1*N* acetic acid and centrifuged at 10,000*g* for 2 minutes. Portions of the supernatant were lyophilized and used for the radioimmunoassay. Proteins were determined as described (*18*) in the acid suspension of various brain regions. Each value (picomoles per milligram of protein) is the mean ± standard error for at least five rats.

| Brain region | DBI-like immunoreactivity |
|---|---|
| Hypothalamus | |
| Arcuate nucleus | 350 ± 24 |
| Ventral medial nucleus | 190 ± 14 |
| Supraoptic nucleus | 170 ± 14 |
| Anterior nucleus | 155 ± 12 |
| Dorsal medial nucleus | 150 ± 20 |
| Medial preoptic area | 145 ± 18 |
| Lateral area | 120 ± 13 |
| Posterior nucleus | 120 ± 16 |
| Central periaqueductal gray | 185 ± 15 |
| Central gray, medial part | 175 ± 18 |
| Interpeduncular nucleus | 170 ± 20 |
| Cerebellar cortex | 170 ± 12 |
| Pontine reticular nucleus, oral part | 150 ± 16 |
| Dorsal raphe nucleus | 140 ± 25 |
| Bed nucleus anterior commissure | 125 ± 8.0 |
| Septohippocampal nucleus | 120 ± 15 |
| Septofimbrial nucleus | 115 ± 12 |
| Lateral septal nucleus, dorsal | 95 ± 9.0 |
| Hippocampus | |
| CA2 to CA | 100 ± 11 |
| Dentate gyrus | 109 ± 6.3 |
| Substantia nigra | 105 ± 10 |
| Medial and lateral habenular nucleus | 100 ± 7.0 |
| Olfactory tubercle | 89 ± 6.0 |
| Globus pallidus | 71 ± 8.1 |
| Ventroposterior thalamic nucleus | 68 ± 5.4 |
| Nucleus accumbens | 60 ± 8.0 |
| Caudate putamen | 57 ± 7.2 |
| Cortex | |
| Frontal | 45 ± 3.6 |
| Parietal | 57 ± 8.1 |
| Occipital | 44 ± 9.0 |
| Pituitary | |
| Anterior | 12 ± 1.0 |
| Posterior | 60 ± 9.0 |

Using DBI as a reference standard, we established that the DBI-like material detected in brain by the radioimmunoassay (*9*) was practically unchanged by a paraformaldehyde perfusion of this tissue or focused microwave fixation of the brain (*10*). The highest content of DBI-like immunoreactivity in rat brain (Table 1) was in the arcuate nucleus of the hypothalamus; the next highest contents (in descending order) were in the ventral medial, supraoptic, and other hypothalamic nuclei, the periaqueductal gray, and the cerebellar cortex. Much lower concentrations were found in the nucleus accumbens, caudate and putamen, and various cortical regions. The lowest amounts of DBI were detected in the anterior pituitary. The content of DBI in the arcuate nucleus was about seven times higher than the content in the anterior pituitary (Table 1).

Using the DBI antiserum (1:40,000) coupled with the peroxidase reaction, we detected intense immunoreactivity in axons and cell bodies of many brain regions (Fig. 2); only a few cell bodies were visualized in the brains of untreated rats, and most of these were visualized only after the rats were injected with colchicine (70 μg intracerebroventricularly 48 hours before the animals were killed; Fig. 2, D, E, and G).

As expected from the radioimmunoassay (Table 1), a dense network of immunoreactivity was detected in many hypothalamic nuclei (Fig. 2A), particularly in the arcuate nucleus (Fig. 2A), the corpus amygdaloideus (Fig. 2A), the hippocampus (Fig. 2, A to D), and the

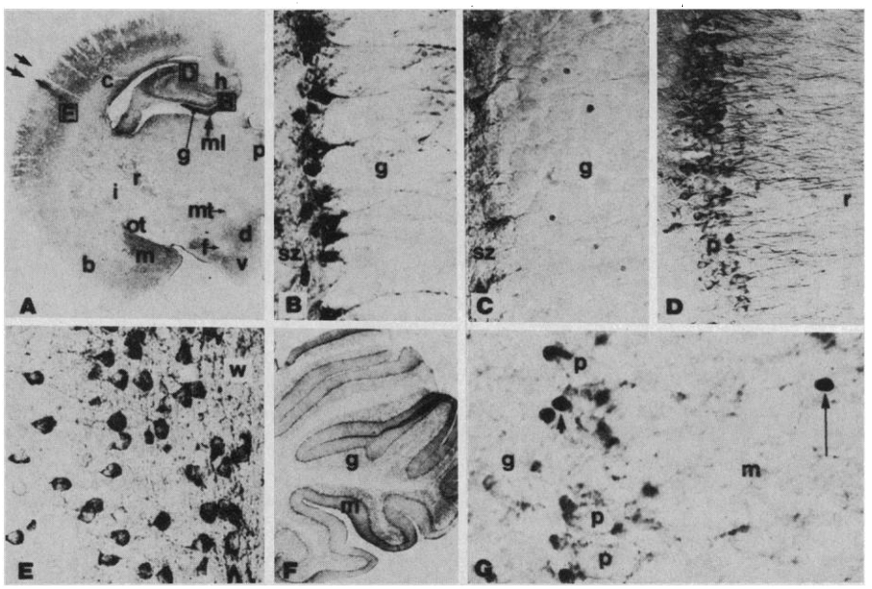


Fig. 2. DBI immunostaining in various telencephalic regions (A to E) and in the cerebellum (F to G) (*15*). (A) Low magnification of a coronal section of normal rat brain at approximately the interaural 6.2-mm level (*16*). Note the high density of DBI immunoreactivity in the medial amygdaloid nucleus (m), dorsomedial (d) and ventromedial (v) hypothalamic nuclei, paraventricular thalamic nucleus (p), reticular thalamic nucleus (r), and various areas of the hippocampus (h) [for example, in the molecular layer (ml) of the dentate gyrus]. Also note the moderate density of DBI immunoreactivity in the basal amygdaloid nucleus (b) and in the cerebral cortex, except in the outer layer (arrows indicate the dura). Low or virtually no DBI immunoreactivity is seen in the corpus callosum (c), internal capsule (i), fornix (f), mamillothalamic tract (mt), granular layer (g) of the dentate gyrus, and optic tract (ot). Squares with capital letters correspond to the areas with high magnification in (B), (D), and (E) (×15). (B) DBI-positive cells and fibers in the dentate gyrus of the hippocampus of rat brain. The DBI-positive cells are localized in the subgranular zone (sz), but no staining is associated with granular cells (g) (×450). (C) Same area as in (B) taken from rats receiving kainic acid (3.7 nmol intracerebroventricularly) 6 days before. Note the disappearance of DBI-immunoreactive cells and fibers in the subgranular zone (sz) and in the granular layer (g). The tissue sections in (B) and (C) were processed under the same incubation and staining conditions (×450). (D) DBI-positive cells in the pyramidal cell layer of the CA1 area of the hippocampus after rats were treated with colchicine (70 μg intracerebroventricularly) 2 days before. The axons of pyramidal cells (p) containing immunoreactivity project to the stratum radiatum (r) (×225). (E) DBI-positive cells in the VI layer of the parietal cortex in colchicine-treated animals (w, white matter) (×400). (F) Low magnification of rat cerebellum, showing DBI immunoreactivity in the molecular (m) and granular (g) layers (×15). (G) High magnification of cerebellum from a rat given colchicine, showing DBI-containing Golgi neurons (short arrow) in the granular layer (g) and around unstained Purkinje cells (p) and the stellate cells (long arrow) in the molecular layer (m) (×450).

cerebellum (Fig. 2, F and G). Immunoreactivity was less dense in the median eminence, corpus striatum, cortex, and thalamic nuclei (Fig. 2A). Although DBI-immunoreactive neurons are densely and uniformly distributed in most of the amygdala and diencephalic nuclei, the distribution pattern of DBI in hippocampus, cortex, and cerebellum is characteristic.

In the hippocampus (Fig. 2B), DBI immunoreactivity was dense, even without colchicine treatment, in a palisade of cells beneath the granular cell layer in an area where GABA-containing basket neurons are predominant (*11*). DBI-positive axons or dendrites originating from these cells project across the granular cell layer and terminate in the molecular layer. The pyramidal cells in hippocampi of untreated rats did not stain; however, in rats receiving an intraventricular injection of colchicine, these cells became immunoreactive (Fig. 2D). Rat hippocampal neurons are extremely sensitive to the neurotoxic action of the potent convulsant kainic acid (*12*). Intracerebroventricular injections of kainic acid (3.7 nmol) 6 days before the animals were killed resulted in a complete disappearance of pyramidal cells from the stratum granulosum of the CA3 area of the hippocampus. This lesion was associated with a complete disappearance of DBI-immunoreactive fibers and cell bodies from the hilus of the area dentata (Fig. 2, B and C), but staining in other hippocampal areas was still present.

In the cortex (Fig. 2A), DBI immunoreactivity was virtually absent in the external cortical layer and was very weak in the white matter; in contrast, after colchicine treatment, immunoreactivity was dense in cells of cortical layers 5 and 6 (Fig. 2E). In the cerebellum, DBI immunoreactivity was dense in fibers of the molecular layer (Fig. 2, F and G). The Purkinje cells were not stained, but their cell bodies were densely innervated by DBI-positive axons and were also surrounded by small immunoreactive cells (basket or Bergman cells or both; Fig. 2G). In the granular layer of the cerebellum, neurons containing DBI (presumably Golgi neurons) were sparse (Fig. 2G). The localization of DBI in this area of the brain is similar to the reported immunocytochemical localization of GABA (*11*).

Taken together, these findings suggest that DBI-containing neurons may be present with GABA-containing neurons in distinct brain areas; however, we have not yet provided direct evidence that DBI and GABA are colocalized. The relatively low density of DBI-containing neurons in brain structures such as the striatum and substantia nigra, which are rich in GABA-containing neurons, suggests that not all GABAergic cells may contain DBI. Moreover, pyramidal cells in the hippocampus and cerebral cortex, which presumably do not contain GABA, show strong DBI immunoreactivity in colchicine-treated rats.

In conclusion, we have shown that DBI, a putative precursor of a new family of brain peptides, elicits a proconflict response (*4*) when injected intraventricularly into rats and is located in neurons of various hypothalamic nuclei and of the cerebral, cerebellar, and limbic cortices. In agreement with its proposed role as precursor, DBI can be visualized in neuronal cell bodies when its axonal transport is inhibited by colchicine. This new family of neuropeptides may have a physiologic role in regulating the onset of behavioral patterns typical of conflict situations, including anxiety, convulsions, and perhaps aggression. Because a DBI-like peptide was also detected in human brain (*13*), its role in the regulation of physiologic and pathologic behavioral patterns in humans can now be investigated.

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6. Application of micromolar concentrations of DBI to spinal cord neurons in primary cultures has an effect on the GABA-induced chloride current similar to that of β-carboline 3-carboxylate (J. Bormann *et al.*, *Regul. Peptides*, in press).
7. Antisera to DBI were obtained in four rabbits (2.5 kg in body weight) by intradermal injection of 200 μg of purified DBI in 2 ml of complete Freund adjuvant. The injection was repeated after 1 month, and blood was taken from the animals 10 days after the last injection. The antiserum with the highest antibody titer was used for the radioimmunoassay; this was carried out in polypropylene tubes. Standard DBI, other polypeptides, or brain extracts were incubated with the antiserum (diluted 1:10,000) and with DBI labeled with $^{125}I$ by the Bolton-Hunter technique (specific activity, ~200 Ci/mmol) in 0.25 ml of 0.05*M* sodium phosphate buffer (*p*H 7.4) containing 0.2*M* NaCl, 12.5 mg of bovine serum albumin, and 0.3 mg of calf thymus histone type II. The incubation was carried out at 4°C for 15 hours or longer. The $^{125}I$-labeled DBI bound to the antibody was separated from free $^{125}I$-labeled DBI by adding 0.75 mg of protein A in 300 μl of 50 m*M* tris and 2 m*M* $MgCl_2$ (*p*H 8).
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10. The content of DBI in the cerebellum was 170 ± 12 pmol per milligram of protein in controls (mean ± standard error of the mean), 150 ± 15 pmol per milligram of protein in rats perfused with paraformaldehyde, and 165 ± 18 pmol per milligram of protein in animals exposed to microwaves ($n$ = 5).
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13. DBI was extracted from human brain (cortex and cerebellum) obtained post mortem by means of a procedure identical to that used to purify DBI from rat brain (*3*). Human DBI showed migration on SDS-PAGE, amino acid sequence, amino acid composition, and chromatographic characteristics on HPLC that were similar to those of rat DBI. Human DBI cross-reacts with antibodies to rat DBI. The distribution of DBI in human postmortem brains, measured by means of antibodies to human DBI, parallels that in rats; DBI is also present in human spinal fluid (from 0.5 to 1 pmol per 1 ml).
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15. The brains were perfused with 4 percent formaldehyde plus 0.1 percent glutaraldehyde for 20 minutes and then were fixed with the same solution for 2 to 3 hours. The brain slices (20 to 30 μm thick) were cut with a vibratome and then stained by the peroxidase-antiperoxidase method [L. A. Sternberger, *Immunocytochemistry* (Wiley, New York, 1979), pp. 104–169] after blocking the endogenous peroxidase activity with a 0.5 percent peroxide solution. The optimum dilution of DBI antiserum was 1:40,000. Preabsorption of the antiserum with DBI (5 n*M*) completely abolished the appearance of immunoreactivity; absorption of the antiserum with the neuropeptides listed in (*9*) did not decrease the DBI immunoreactivity.
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# Atrotoxin: A Specific Agonist for Calcium Currents in Heart

Abstract. *A specific label for voltage-dependent calcium channels is essential for the isolation and purification of the membrane protein that constitutes the calcium channel and for a better understanding of its function. A fraction of* Crotalus atrox *that increases voltage-dependent calcium currents in single, dispersed guinea pig ventricular cells was isolated. In the doses used, neither sodium nor potassium currents were changed. The fraction was active in the absence of detectable phospholipase or protease activity, and the active component, designated atrotoxin, produced its effect rapidly and reversibly. The effect was produced by extracellular but not intracellular application of the agent. The increase in $Ca^{2+}$ current was blocked by the $Ca^{2+}$ channel blockers cobalt and nitrendipine. The active fraction completely blocked specific [$^3$H]nitrendipine binding to guinea pig ventricular membrane preparations. The inhibition of nitrendipine binding by atrotoxin was apparently via an allosteric mechanism. Thus atrotoxin was shown to bind to the $Ca^{2+}$ channel and to act as a specific $Ca^{2+}$ channel agonist.*

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The use of certain members of the dihydropyridine class of organic molecules that block or increase voltage-dependent $Ca^{2+}$ currents has increased our understanding of calcium channels (*1*). These agents bind with a high affinity to membrane preparations from excitable tissues that contain $Ca^{2+}$ channels, but their usefulness as $Ca^{2+}$ channel labels has some limitations: (i) they produce large amounts of nonspecific binding in radiolabeled dihydropyridine binding assays; (ii) in many tissues, they bind with lower affinity to a second site (*2*); (iii) in some tissues such as brain, they have little or no demonstrable pharmacological effect (*3*); and (iv) at doses associated with binding to the low-affinity sites, $Na^+$ channels (*4*) and $K^+$ channels (*5*) are also blocked. These complications prompted us to search for other labels without these undesirable qualities. Agents that are likely to be membrane-impermeant seemed particularly attractive. Toxins that may act on voltage-dependent $Ca^{2+}$ currents have been described (*6, 7*), but a direct demonstration was made in only one instance (*7*) and the toxins used, veratridine and batrachotoxin, acted on $Na^+$ channels as well.

A protein of molecular weight 10,000 from *Crotalus atrox* venom depresses myocardial activity (*8*), and a number of small basic proteins with myotoxic activity have been isolated from rattlesnake venoms (*9*). Our original purpose was to fractionate *Crotalus atrox* venom, purify the proteins, and characterize the effects of these proteins on voltage-dependent $Ca^{2+}$ channels. In the process of examining the separated fractions, we isolated a fraction that blocked high-affinity [$^3$H]nitrendipine binding to guinea pig ventricular membranes and that specifically and reversibly increased voltage-dependent $Ca^{2+}$ currents in isolated myocytes.

Two different fractions were isolated by gel filtration on a Sephadex G-100 column (Fig. 1A) followed by ion exchange on a Bio-Rex 70 column (Fig. 1B) (*10*). Both of these fractions increased $Ca^{2+}$ currents and were subsequently shown to block [$^3$H]nitrendipine binding. Fraction I (atrotoxin) had no detectable phospholipase or protease activity, was not dialyzable, and was destroyed by heating for 2 minutes at 100°C. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis of this fraction (Fig. 1C, lane b) showed that the major polypeptide component had a molecular weight of 56,000. Other polypeptides were present in smaller amounts. Which of these polypeptides was responsible for the activity is not yet known. The second fraction (fraction II) had a major polypeptide with an apparent molecular weight of 15,000 (Fig. 1C, lane d). This fraction had some phospholipase activity (6.0 $\mu eq \cdot min^{-1} \cdot mg^{-1}$) and was therefore not characterized further. For comparison, the enzyme activity of the purified phospholipase $A_2$ of *Crotalus atrox* (Calbiochem-Behring) was 190 $\mu eq \cdot min^{-1} \cdot mg^{-1}$. In support of the conclusion that the activity of the atrotoxin was not due to contaminating phospholipase, the purified phospholipase $A_2$ from *Crotalus atrox* venom inhibited rather than activated $Ca^{2+}$ currents in intact myocytes.

When added to normal Tyrode solution, atrotoxin prolonged the action potential of isolated guinea pig ventricular myocytes and elevated the plateau without changing the resting membrane po-