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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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9. The peptides tested for cross-reactivity with DBI antiserum were as follows: vasoactive intestinal polypeptide, several enkephalins ([Met^5], [Leu^5], [Met^5-Arg^6], [Met^5-Arg^6-Phe^7]-octapeptide), [Phe-Met-Arg-Phe-NH_2]-peptide, β-endorphin, dynorphin, substance P, somatostatin, corticotropin-releasing factor, adrenocorticotropic hormone (several fragments), α-, β-, and γ-melanocyte–stimulating hormone, neurotensin, cholecystokinin, small and large myelin basic proteins, histones, and GABA-modulin (*1*). None of these peptides competed with ^{125}I-labeled DBI up to 10^{-6}*M*. Standard DBI used for immunization and radioimmunoassay was prepared with minor modification of the method described (*3*).
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-

tential (Fig. 2A). The effects were apparent within 30 seconds of application at pulse intervals of 10 seconds and became quite steady within 5 minutes. The effects were reversible (trace d in Fig. 2A), recovery being complete after 10 to 15 minutes of washing. The prolongation of the action potential was dose-dependent, and the half-time for steady effects was reduced as the dose was increased. These effects were also seen with fraction II. The possible involvement of Ca^{2+} currents was examined further by use of the whole-cell patch-clamp method (*11*). The patch pipette was modified to permit intracellular perfusion (*12*). At the usual holding potential of −40 to −50 mV, Na^+ currents were completely inactivated, and the outward holding current flowed through the inwardly rectifying K^+ channel, I_{K1}. The transient outward K^+ channel was also inactivated at this holding potential (*13*). At the test potentials of −30 mV or greater used to produce Ca^{2+} currents, I_{K1} was negligible (*14*), and when the inward current reached its peak value in 3.0 msec or less there was no significant time-dependent outward current (I_X) either (*15*). Hence, the peak inward current measured from the zero current level was virtually the Ca^{2+} current. Atrotoxin produced a clear increase in this peak current over the range of potentials tested (Fig. 2D). The time to peak was not changed, but the rate of inactivation was faster so that the amplitude of Ca^{2+} current at 50 msec was unchanged. The increase in Ca^{2+} current was not voltage-dependent, nor was there any effect on the holding current. The steady-state inactivation of Ca^{2+} current was measured by means of a 2-second prepulse combined with a test pulse to +10 mV; it remained unchanged at an atrotoxin concentration of 10^{-6} g/ml. In these experiments, tetrodotoxin ($5 \times 10^{-5} M$) was used to block Na^+ currents. As was the case for the action potential plateau, the increase of Ca^{2+} current produced by atrotoxin was dose-dependent, and the maximum effects were observed at about 10^{-5} g/ml (Fig. 3A). The experimental points could be fitted by a one-to-one drug-receptor binding curve:

$$\%\ \text{increase of } Ca^{2+}\ \text{current} = \frac{[M]}{[M] + K_D}$$

where [M] is toxin concentration and K_D is the apparent dissociation constant of 5×10^{-7} g/ml.

A similar dose-dependent increase of Ca^{2+} currents was observed in neonatal rat ventricular cells. In these experiments, Ca^{2+} currents were isolated by suppressing Na^+ and K^+ currents.

The increase of Ca^{2+} current produced by atrotoxin was not blocked by phentolamine ($5 \times 10^{-6} M$) or propranolol ($5 \times 10^{-6} M$), so that neither α nor β adrenoceptors were involved. The increase occurred only when atrotoxin was applied to the bath. When atrotoxin was added to the intracellular solution in maximal concentrations (10^{-5} g/ml), Ca^{2+} currents were unchanged. We know that intracellular exchange had occurred because substitution of Cs^+ for K^+ intracellularly blocked I_{K1} in 30 seconds.

The specificity of the atrotoxin effects for Ca^{2+} channels was examined further by studying the actions of the compound on Na^+ currents. In these experiments, Ca^{2+} currents were suppressed by Co^{2+} substitution for Ca^{2+}. No effects on Na^+ currents were observed. As noted above, at the usual holding potentials, current was flowing through I_{K1}, and these currents were also unaffected (Fig. 2D). In adult guinea pig myocytes a small outward K^+ current, I_X, was present at 500 msec after a step increase to potentials more positive than +20 mV (*14*). This current was also unaffected by atrotoxin. We therefore concluded that the atrotoxin effects were specific for the Ca^{2+} channel. The absence of nonspecific effects was consistent with the absence of phospholipase or protease activity in the active fraction.

Atrotoxin also affected the high-affinity dihydropyridine receptor in cardiac membrane preparations. This receptor is thought to be part of the membrane Ca^{2+} channel. The inhibition of [^{3}H]nitrendipine binding to guinea pig sarcolemma by atrotoxin is shown in Fig. 3A. The half-maximal concentration for the inhibition was 2.5×10^{-6} g/ml, which gave a K_I (inhibition constant) of 6×10^{-7} g/ml when we used a K_D for nitrendipine of

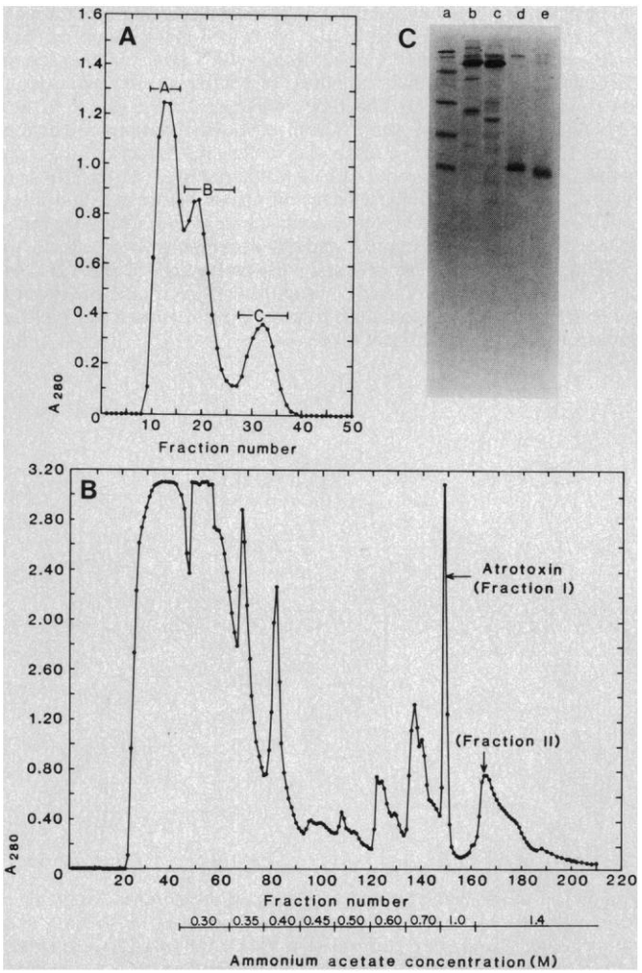

Fig. 1. Fractionation of *Crotalus atrox* venom. Lyophilized venom (500 mg) provided by the Miami Serpentarium was suspended in 7.5 ml of 0.090*M* ammonium acetate, *p*H 6.8, containing the protease inhibitors aminobenzamidine (33 m*M*), phenylmethysulfonyl fluoride (33 m*M*), pepstatin A (3.3 μg/ml), leupeptin (16 μg/ml), and aprotinin (16 μg/ml). Insoluble material was removed by centrifuging at 26,000*g* for 30 minutes at 4°C. (A) Supernatant (7.5 ml) was applied to a 1.8 by 40.5 cm Sephadex G-100 column and eluted with 0.090*M* ammonium acetate. The material eluting in the void volume (peak A), with protease inhibitors added at same concentrations as above, was applied to a 1.8 by 18 cm Bio-Rex 70 column (B) and was eluted stepwise with 0.090*M* to 2.00*M* ammonium acetate. Phospholipase A was determined by the method of Wells and Hanahan (*16*), and protease activity was assayed with Azocoll (Calbiochem-Behring). Acrylamide gels [19.5 percent acrylamide, 0.5 percent *N*,*N*-methylene-bis-acrylamide (both weight by volume)] were run as described by Laemmli (*17*). After electrophoresis the gels were stained with Coomassie Brilliant Blue (C). In lane a the molecular weight markers are: myosin (200K), β-galactosidase (116.3K), phosphorylase B (92.5K), bovine serum albumin (66.2K), ovalbumin (45K), carbonic anhydrase (31K), soybean trypsin inhibitor (21.5K), and lysozyme (14.4K). Lane b is fraction I, unreduced; lane c is fraction I, reduced; lane d is fraction II, unreduced; and lane e is fraction II, reduced.

0.1 n*M*. Scatchard analysis of [^{3}H]nitrendipine binding in the presence and absence of atrotoxin is shown in Fig. 3B. The observed change in K_D with no apparent change in B_{max} (the maximum concentration of binding sites) suggested that the inhibition was competitive. However, in the presence of atrotoxin, the rate of dissociation of [^{3}H]nitrendipine bound to guinea pig membranes in the absence of atrotoxin was 0.044 ± 0.009 min^{-1} (n = 7) and in the presence of atrotoxin was 0.072 ± 0.009 min^{-1} (n = 4). These differences analyzed by a t-test were statistically significant ($P < 0.01$). They suggested that bound atrotoxin affects the nitrendipine binding site in an allosteric fashion.

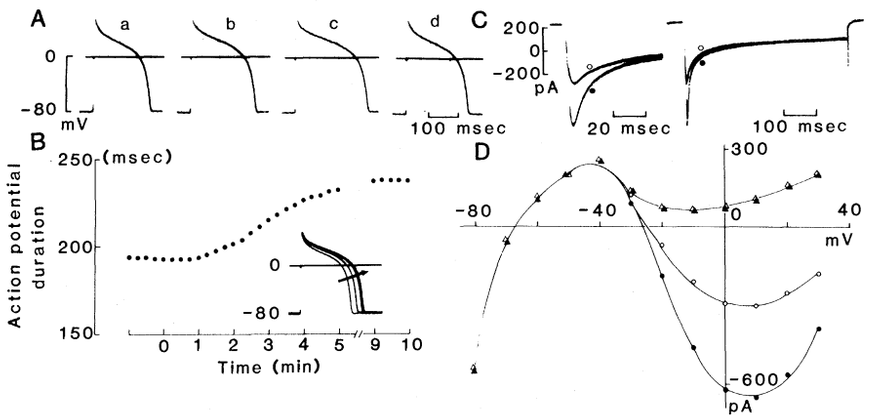

Fig. 2. Atrotoxin prolongs the action potential and increases Ca^{2+} currents. Single ventricular cells were isolated from guinea pig and neonatal rat heart as described (*18*) and were placed in a chamber (0.2 ml) which was rapidly (2 ml/min) perfused with solution containing (in millimoles per liter): NaCl, 135; KCl, 5.4; $CaCl_2$, 1.8; $MgCl_2$, 1; glucose, 10; and Hepes, 5 (*p*H 7.4). Electrode (~2 megohms) contained (in millimoles per liter): potassium aspartate, 130; EGTA, 5; ATP, 2; Hepes, 5 (*p*H 7.3). The action potential was elicited by passing a current pulse through the recording electrode. The currents were measured by the whole-cell patch-clamp method (*11*) at room temperature (20° to 22°C). (A) Effects of atrotoxin (10^{-7} g/ml) on the action potential. (Trace a) control. (Traces b and c) 3 and 5 minutes, respectively, after addition of atrotoxin. (Trace d) 5 minutes after washing. (B) The time course of change in the action potential duration measured at 80 percent repolarization. The inset shows superimposed action potentials before and 3, 5, and 10 minutes after the toxin was added. (C) Effect of atrotoxin (10^{-6} g/ml) on the Ca^{2+} currents. Ca^{2+} currents produced by a voltage step to +10 mV from a holding potential of −40 mV at 0.1 Hz. Superimposed current traces before (○) and after addition of the toxin (●). (D) Current-voltage relation of peak (circles) and steady currents (measured at 500 msec). Open symbols show control and filled symbols show effects of atrotoxin (10^{-6} g/ml). The holding potential was −40 mV, and pulses were applied at 0.1 Hz. At potentials less negative than −30 mV, which were used to produce Ca^{2+} currents, the inward rectifier current was negligible (*14*); hence peak and steady currents were measured from the zero current level rather than from the holding current level.

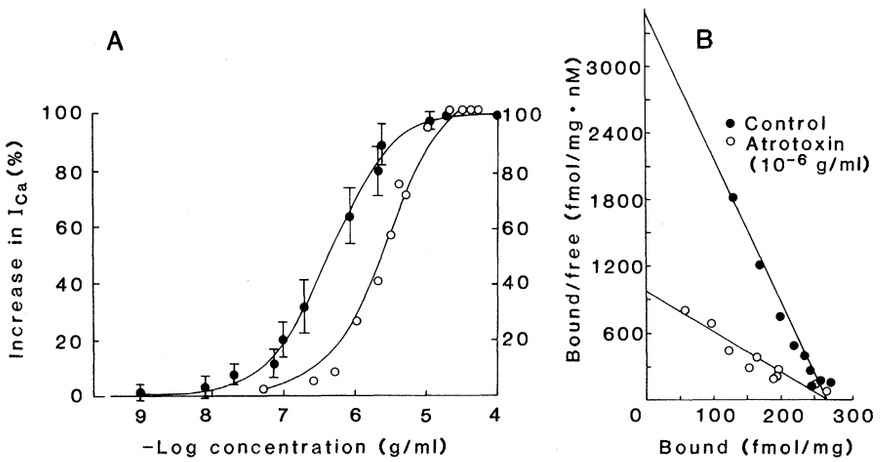

Fig. 3. The effect of atrotoxin on [^{3}H]nitrendipine binding to guinea pig membranes. Guinea pig ventricular membranes were prepared by a technique modified from that of Velema and Zaagsma (*19*). All nitrendipine binding assays were performed in 50 m*M* tris (*p*H 7.4). (A) Dose-response curve for the effect of atrotoxin on Ca^{2+} current. The percentage increase in peak Ca^{2+} currents was plotted against atrotoxin concentration. Each of these points (●) represents the mean value for four to eight cells. Vertical bars indicate standard errors. The solid line is the theoretical curve for one-to-one atrotoxin-receptor interaction with a mean effective dose of 5×10^{-7} g/ml. Also shown is the inhibition of 0.3 n*M* [^{3}H]nitrendipine binding by atrotoxin (75 μg of guinea pig membrane protein per sample). Each of these points (○) is the average of two determinations (average difference, 6.2 ± 4.9). (B) Scatchard plot of [^{3}H]nitrendipine binding in the presence (○) and absence of (●) of atrotoxin (10^{-6} g/ml). Each point is the average of two determinations.

Our results show that atrotoxin is a specific toxin for cardiac Ca^{2+} channels. In the doses used it had no effect on either the Na^+ or the K^+ channel, the two major voltage-dependent channels in these cardiac myocytes. Our isolation procedures indicated that atrotoxin is highly charged and has a molecular weight greater than 15,000 (*10*). It did not penetrate cell membranes, and its action was on the extracellular portion of the Ca^{2+} channel. The specificity, charge and large size of atrotoxin, make it a valuable tool for the isolation and characterization of Ca^{2+} channels.

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