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Purification and Characterization of a Bungarotoxin Polypeptide Which Blocks Nicotinic Receptor Function in Primary Culture of Adrenal Chromaffin Cells

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SUMMARY

In primary cultures of bovine chromaffin cells, commercially available preparations of α-bungarotoxin inhibit the acetylcholine (ACh)- or nicotine-evoked release of endogenous catecholamines. The potency of different lots of α-bungarotoxin is not related to the α-bungarotoxin peptide content but to that of another peptide (termed P-4 bungarotoxin) present as an impurity in the α-bungarotoxin preparations. P-4 Bungarotoxin was isolated and purified to homogeneity by high-pressure liquid chromatography (HPLC). Homogeneity was established by a variety of means, including polyacrylamide gel electrophoresis, HPLC, end carboxy group analysis and NH_2-terminal amino acid sequence. Purified P-4 bungarotoxin contains approximately 121 amino acid residues, and it is different in its amino composition, molecular weight, and amino acid sequence from α-bungarotoxin and β-bungarotoxin. P-4 Bungarotoxin ($IC_{50} \simeq 1$ nM) blocked the ACh-induced release of endogenous catecholamines but failed to block the KCl-induced catecholamine release. Although P-4 bungarotoxin is endowed with phospholipase A_2 activity, its effect on ACh-evoked catecholamine release persists when the phospholipase activity is blocked (99.9%) by treatment of the toxin with *p*-bromophenacyl bromide. P-4 Bungarotoxin may represent a useful tool with which to study nicotinic receptor function in sympathetic and central nervous system neurons.

INTRODUCTION

α-Bungarotoxin is a peptide purified from *Bungarus multicintus* venom that specifically blocks nicotinic receptor function by binding irreversibly to these recognition sites. The curare-mimetic action of this toxin is well-documented in the electric organ of certain fishes and eels and in the skeletal neuromuscular junction of mammalian and nonmammalian species (1–3). However, conflicting results exist on the ability of α-bungarotoxin to block nicotinic receptors in central nervous system and in peripheral ganglia (4). Although it has been consistently reported that α-bungarotoxin binds robustly to neurons of sympathetic ganglia (4), brain homogenates (4), clonal cell lines derived from rat pheochromocytoma (5), and to chromaffin cells of adrenal medulla (6, 7), a blockade of nicotinic receptor function in vertebrate neuronal elements with this toxin is controversial (5, 8–12) (for a review see ref. 4).

For example, we observed that commercially available lots of α-bungarotoxin (0.1 μM or higher) reduce the catecholamine release induced by nicotine or ACh[2] (7) in primary cultures of cow adrenal medulla chromaffin cells. However, in the same cell preparation, Trifaro and Lee (13) and Kilpatrick *et al.* (14) failed to prevent the ACh- or nicotine-evoked release of [^{3}H]NE by using 0.125 or 1 μM α-bungarotoxin, respectively. One possible explanation for such discrepancy could be found in the method employed to measure catecholamine release. In fact, we recently observed that the release of [^{3}H]NE is less sensitive to the inhibitory effect of α-bungarotoxin than is the release of endogenous catecholamines (15). Alternatively, the discrepancy between the results of Trifaro and Lee (13) and Kilpatrick *et al.* (14) and our results may reflect the use of α-bungarotoxin samples with different potency on the nicotinic receptors of the adrenal cells. To analyze the latter possibility, we have compared the effect of different α-bungarotoxin preparations by directly measuring their potencies on the amount of endogenous NE and E released by the adrenal

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[2] The abbreviations used are: ACh, acetylcholine; NE, norepinephrine; E, epinephrine; Hepes, 4-(2-hydroxyethyl)-1-piperazineethansulfonic acid; HPLC, high-pressure liquid chromatography; SDS, sodium dodecyl sulfate; TFA, trifluoroacetic acid.

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chromaffin cells. We report that α-bungarotoxin preparations from different commercial sources inhibit the ACh- or nicotine-induced release of catecholamines with different potencies. The potency is related to the presence of a variable amount of an active peptide (termed P-4 bungarotoxin) which obviously is not the α-bungarotoxin peptide. We believe that the presence of variable amounts of this peptide in α-bungarotoxin preparations is responsible for some of the contrasting results reported in the literature.

MATERIALS AND METHODS

Cell preparation and catecholamine release studies. The chromaffin cells were isolated from bovine adrenal medulla following the method described by Kilpatrick *et al.* (14), and they were placed in 24-well plastic dishes (Costar). Each well contained approximately 350,000 cells in 1 ml of Hepes (5 mM)-buffered (pH 7.4) Dulbecco's modified Eagle's medium (GIBCO) supplemented with 10% calf serum (GIBCO), penicillin (100,000 units/liter) (Pfizer), gentamycin (40 mg/liter) (Shering), nystatin (50,000 units/liter), (Squibb), and 5 μM fluorodeoxyuridine (Sigma). The cells were maintained in culture for 5–7 days before being used. A more detailed account of the cell preparation has been already reported by us (16).

To study catecholamine release, Dulbecco's modified Eagle's medium was replaced with 500 μl of Locke's solution (pH 7.4 at 37°) containing 154 mM NaCl, 5.6 mM KCl, 3.6 mM $NaHCO_3$, 2.3 mM $CaCl_2$, 5.6 mM glucose, and 5 mM Hepes. The cells were washed once more in Locke's solution and were incubated at 37° for 15 min with or without the drug to be tested. The final reaction volume was 500 μl, and the catecholamine release was induced by the addition of different concentrations of nicotine or ACh in the presence of 10 μM eserine. Following incubation for a specified period, 100 μl of the incubation medium were aspirated and added to 100 μl of 0.8 N $HClO_4$. The wells were then dried and the cells were resuspended in 0.6 ml of 0.4 N $HClO_4$ to extract the catecholamines. NE and E were separated and quantified with HPLC (16), using a reverse-phase column (ODS-10; 250 mm × 4 mm; Bio-Rad) and a glassy carbon electrochemical detector (TL-5 Bio-Analytical system). The effect of drugs on catecholamine secretion was studied by running triplicate or quadruplicate samples.

HPLC for α-bungarotoxin analyses. The analyses were carried out using a Spectrophysics liquid chromatography system equipped with the SP 8700 solvent delivery system using a reverse-phase ODS-10 Bio-Rad column. Peptides were detected by monitoring absorbance at 210 nm or by colorimetric reaction using the method of Lowry *et al.* (17).

SDS gel electrophoresis. An apparatus manufactured by Bio-Rad (Model 220) was employed in these experiments, using polyacrylamide gels 2.8 mm thick. SDS gel electrophoresis was run according to the method of Laemmeli (18), with minor modifications. The gel was calibrated using a "low molecular weight" protein calibration kit from Pharmacia. Coomassie brilliant blue G was used to stain the proteins.

Amino acid analysis. The protein was hydrolyzed in 6 N HCl for 20 hr at 110° under vacuum. Analysis was performed on a Hitachi 835 automatic amino acid analyzer.

COOH-terminal amino acid analysis. The COOH-terminal amino acid residue was identified by the hydrozinolysis method (19) and was confirmed by the carboxypeptidase A method (20).

Amino acid sequence analysis. The purified lyophilized toxin (1 nmole) was dissolved in 30 μl of 1% SDS in water. The SDS was recrystallized twice from hot ethanol. The samples were loaded on a gas-phase sequenator (21) and submitted to NH_2-terminal sequence analysis by automated Edman degradation. Phenylthiohydantoin-derivatized amino acids were identified by HPLC on an IBM cyano column. Details on identification of phenylthiohydantoin-derivatized amino acids and standard chromatograms have been described (22).

Phospholipase A_2 activity. Phospholipase A_2 activity was measured at 37° using the method described by Hirata *et al.* (23). [1-^{14}C]Phosphatidylcholine-L-dipalmitoyl-2-palmitoyl (specific activity 52 mCi/mmole) was used as substrate. The reaction mixture contained, in a total volume of 22 μl, 0.2 mM radioactive substrate, 0.8% Nonidet P-40, 50 mM Tris-HCl (pH 8), 2 mM Ca^{2+}, and different amounts (from 0.01 to 1 μg) of P-4 bungarotoxin. The reaction was carried out for 5 min and stopped by the addition of 80 μl of ethanol.

The product of the reaction (free [^{14}C]palmitoic acid) was separated from the original material by silica gel G thin-layer chromatography using chloroform/methanol/water (65:25:4, v/v) as solvent. For example, in the absence and presence of 0.1 μg of P-4 bungarotoxin, 261 and 5206 cpm were obtained, respectively.

p-Bromophenacyl bromide treatment. P-4 Bungarotoxin (100 μg) was incubated in 2000 μl of reaction mixture containing 1960 μl of 50 mM Tris-HCl (pH 8) and 40 μl of 34 mM *p*-bromophenacyl bromide in acetone. The reaction mixture was incubated for different time periods at 30°. The reaction was stopped by acidifying the sample to pH 3. Excess reagent and products were purified on a Bio-Gel P-2 column (1 × 10 cm) equilibrated with 0.1 N acetic acid. Samples without P-4 bungarotoxin or with P-4 bungarotoxin but without *p*-bromophenacyl bromide were run as controls.

Phrenic diaphragm preparation. Isolated rat phrenic nerve hemidiaphragms were suspended in a 15-ml bath containing Krebs solution (pH 7.4). The preparation was constantly bubbled with 95% O_2 and 5% CO_2 at 32°. The phrenic nerve was passed through a pair of electrodes and stimulated with supramaximal 5-V rectangular wave pulses of 1-msec duration at a frequency of 0.2 Hz. The preparations were allowed

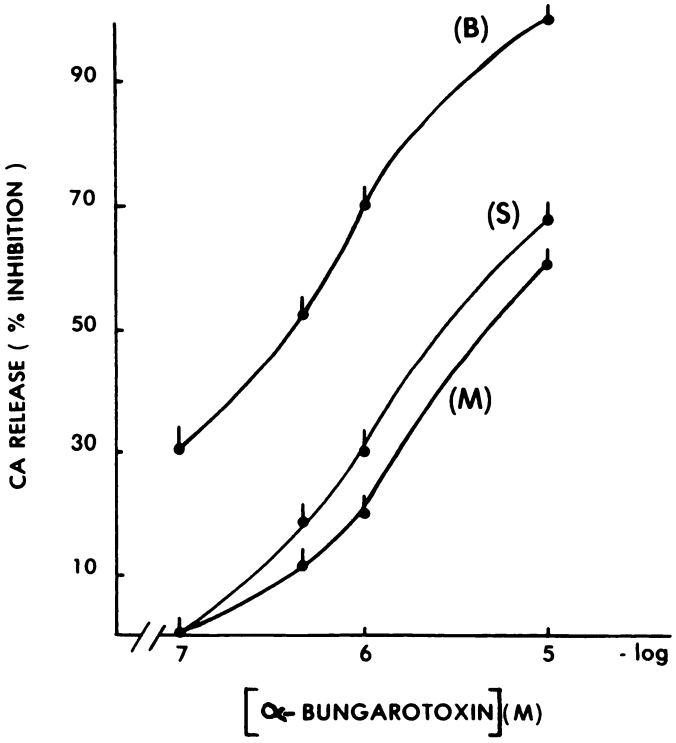

FIG. 1. *Dose-response curve for inhibition of ACh-stimulated catecholamine release by different commercially available preparations of α-bungarotoxin*

Boehringer (*B*), Sigma (*S*), or Miami Serpentarium (*M*) α-bungarotoxin was added to the cell 15 min before the addition of ACh + eserine. There was no effect of the different α-bungarotoxin preparations on the total catecholamine content or on the basal catecholamine release. Values are the average and range of quadruplicate determinations. In this cell preparation, 10 μM ACh + eserine caused, in a 15-min incubation, an 18 ± 0.7% release of the endogenous catecholamine content. Release of endogenous catecholamines in the absence of ACh was 2.5 ± 0.1% of the total content. Each incubation well contained approximately 3 × 10^5 cells (see Materials and Methods). In control cells the total catecholamine content was 45 nmoles/10^6 cells.

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to stabilize for 30 min. When neuromuscular transmission was completely blocked, the muscle was stimulated directly with 50-V 0.4-msec duration pulses to determine whether the muscle was still capable of contracting.

RESULTS

Effect of different preparations of α-bungarotoxin on catecholamine release. All different preparations of α-bungarotoxin we have tested inhibited ACh-evoked release of endogenous catecholamines from adrenal cells in culture (see Fig. 1). However, there was up to a 10-fold difference in the concentration required to inhibit by 50% the release of catecholamines. Among the different α-bungarotoxin preparations tested, the most potent batch (Boehringer Lot 1190407; IC_{50} approximately 1 μM) was used to compare its effects on spontaneous, nicotine-, ACh-, and KCl-evoked release of NE and E. Under control conditions at 37°, cells kept for 5–7 days in culture possessed slightly more E than NE. A small percentage of the endogenous catecholamines (approximately 2%) was found to be released in the incubation medium (Table 1). Boehringer Lot 1190407 α-bungarotoxin in concentrations up to 10 μM failed to influence the spontaneous release of NE and E. ACh evoked a dose-related release of endogenous and NE and E (Table 1). A common feature of all of the experiments is that the net percentage of endogenous NE released by ACh or nicotine was 1.2- to 1.6-fold higher than that of E. Boehringer Lot 1190407 α-bungarotoxin inhibited with almost equal potency the ACh- or nicotine-evoked release of NE and E (Table 1). However, it failed to block the KCl-induced NE and E release (Table 1). The effect of α-bungarotoxin was maintained unabated even when the toxin was washed out before the addition of ACh or nicotine.

Identification of a bungarotoxin peptide (P-4 bungarotoxin) which blocks nicotinic receptor function of adrenal chromaffin cells. In order to study whether the ability of α-bungarotoxin to block ACh-induced catecholamine release from adrenal cells was linked to the authentic α-bungarotoxin peptide or to another toxin peptide, an active preparation of α-bungarotoxin (Boehringer Lot 1190407) was applied to a Bio-Sil ODS-10 Bio-Rad reverse-phase HPLC column. The column was equilibrated with 0.1 M NaH_2PO_4 + 0.2% H_3PO_4 (pH 2.5) and was eluted with a 0–60% acetonitrile gradient.

As indicated in Fig. 2, at last four UV-absorbing peaks were separated and termed P-1, P-2, P-3, and P-4 bungarotoxin, respectively. When these peaks were tested for their ability to block ACh- or nicotine-induced catecholamine release (see Fig. 3), only P-4 bungarotoxin blocked the response. Considering the molecular weight of these peptides (see legend to Fig. 3), the IC_{50} for P-4 bungarotoxin was calculated to be around 1 nM. P-2, P-1, and P-3 bungarotoxins were ineffective up to concentrations of 1 μM. The inhibition of ACh-evoked catecholamine release by P-4 bungarotoxin also persisted unchanged when the toxin was removed from the incubation medium 1 hr before the addition of ACh. In contrast, the inhibitory effect was reversed in cells exposed to P-4 bungarotoxin for 15 min and then maintained without toxin for 12 hrs. The effect of P-4 bungarotoxin was related to its peptide structure, because it was abolished by preincubation of the toxin with trypsin or pronase (data not shown). P-2 and P-4 bungarotoxin (1 μg) inhibited rat diaphragm twiches induced by electrical stimulation of the phrenic nerve but did not inhibit that induced by direct muscle stimulation. The effect of P-4 bungarotoxin was reversed by a 1-hr washing, whereas the effect of the P-2 toxin persisted after extensive washing.

Since P-4 bungarotoxin is the only bungarotoxin pep-

TABLE 1

Effect of Boehringer lot 1190407 α-bungarotoxin on acetylcholine-, nicotine-, and KCl-induced catecholamine release

Each incubation well contained approximately 3 × 10^5 cells. Cells were incubated with ACh + 10μM eserine, nicotine, or KCl for 15 min at 37°. Each value is the mean ± standard error of four different samples. Results similar to the one reported in the table were obtained in three different experiments. α-Bungarotoxin (α-BTX) added to the cells 15 min before the addition of ACh or nicotine failed to change NE and E release or NE and E cell content in control preparations.

Cell Treatment	Catecholamines						
	Released		Cell content		% Released		
	NE	E	NE	E	NE	E	NE + E
	ng/well		*ng/well*				
Control	34 ± 4	33 ± 3	1330 ± 5	1920 ± 29	2.5	1.6	2.1 ± 0.18
ACh, 5 μM	178 ± 13	199 ± 11	1310 ± 38	1980 ± 11	12	9.1	10.5 ± 0.8
ACh, 5 μM + α-Btx, 1 μM	110 ± 5	111 ± 10	1350 ± 10	1990 ± 15	7.5	5.2	6.4 ± 0.5*
ACh, 50 μM	337 ± 8	302 ± 5	1130 ± 32	1780 ± 41	23	14	19 ± 0.5
ACh, 50 μM + α-Btx, 1 μM	191 ± 9	206 ± 7	1280 ± 15	1860 ± 20	13	9.9	12 ± 0.4*
Nicotine, 50 μM	320 ± 10	310 ± 8	1110 ± 16	1870 ± 23	22	15	17.5 ± 1.0
Nicotine, 50 μM + α-Btx, 1 μM	190 ± 7	170 ± 5	1300 ± 18	1920 ± 25	13	8.1	10 ± 0.9*
KCl, 56 mM	250 ± 10	271 ± 8	1220 ± 13	1850 ± 15	17	13	15 ± 0.8
KCl, 56 mM + α-Btx, 1 μM	245 ± 11	270 ± 12	1280 ± 10	1820 ± 18	16	13	14 ± 0.6

*$p < 0.01$ when cells treated with ACh (or nicotine) + Boehringer Lot 1190407 α-bungarotoxin were compared with cells treated with the same concentration of ACh (or nicotine).

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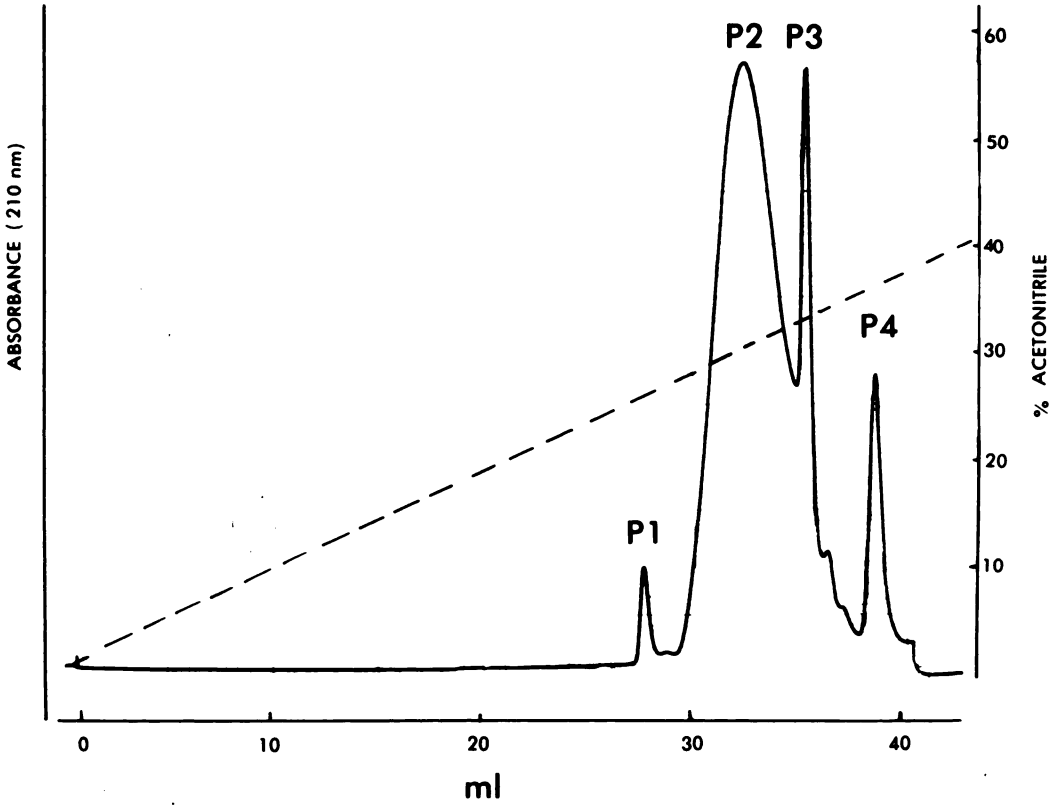

FIG. 2. *Reverse-phase HPLC of 500 μg of α-bungarotoxin (Boehringer Lot 1190407)*

The conditions were as follows: Bio-Sil ODS-10 column (4 × 250 mm), flow rate 1 ml/min, temperature 20°. After sample application, the column was washed for 30 min with starting buffer (0.1 M NaH_2PO_4 + 0.2% H_3PO_4, pH 2.5). Peptides were eluted with a gradient from 100% starting buffer to 70% acetonitrile/30% starting buffer. Four major UV-absorbing peaks termed P-1, P-2, P-3, and P-4 were eluted from the column. Fractions of 1 ml were collected, neutralized, lyophilized, and analyzed for their effect on catecholamine release (see Fig. 3) or rat phrenic diaphragm twitches.

tide that powerfully inhibits nicotinic function in adrenal chromaffin cells, the material was further purified and characterized. Purity was established by analyzing P-4 bungarotoxin on HPLC with a Bio-Rad ODS-10 column using either an isocratic mobile phase [70% 0.1 M NaH_2PO_4 + 0.1% H_3PO_4 (pH 2.5) + 30% acetonitrile] or 0.1% TFA in water as a mobile phase and 0–70% 0.1% TFA/acetonitrile as gradient. As shown in Fig. 4, using 25 μg of protein, a single UV-absorbing peak was observed under both HPLC conditions studied. Figure 5 indicates that the P-4 bungarotoxin is homogeneous by SDS gel electrophoretic analysis. NH_2-terminal amino acid analysis indicated that asparagine was the only amino acid terminal (Table 2). COOH-terminal amino acid analysis using hydrazinolysis and the carboxypeptidase A method was adopted as further criteria for purity of P-4 bungarotoxin. This analysis showed that the COOH terminus was free and that glycine was the only amino acid residue. The amino acid composition of P-4 bungarotoxin in comparison to that of α- or β-bungarotoxin [reported by Lee (3)] is shown in Table 2. The molecular weight of P-4 bungarotoxin was estimated to be approximately 15,000 on the basis of the amino acid composition (Table 2) and 17% SDS polyacrylamide gel electrophoresis (Fig. 5). The NH_2-terminal amino acid sequence of the purified P-4 bungarotoxin was determined for the first 55 residues. Only one sequence was detectable, and it is reported in Table 2. The sequence represents a single polypeptide chain, because yield at the first step was 80–90%. This high initial yield eliminates the possibility that a polypeptide with a blocked NH_2 terminus could be present in amounts stoichiometric with P-4 bungarotoxin. In addition, contaminating sequences were not present at detectable levels (less than 3%). P-4 Bungarotoxin can be easily extracted and purified from the complete snake venom using the HPLC technique. Starting with 50 mg of crude venom, we can purify approximately 500 μg of P-4 bungarotoxin.

Effect of P-4 bungarotoxin on ACh-evoked catecholamine release. P-4 Bungarotoxin inhibited in a dose-dependent manner the ACh-evoked catecholamine release (see Fig. 6), but failed to inhibit KCl-induced catecholamine release (data not shown). One intriguing observation was that the P-4 bungarotoxin fraction possessed phospholipase activity (see Fig. 7). We then decided to treat P-4 bungarotoxin with *p*-bromophenacyl-bromide in order to block its phospholipase activity. When the product of this reaction was applied to HPLC using a phosphate buffer-acetonitrile gradient or a TFA-acetonitrile gradient, no signal was detected in the position of the original P-4 material, suggesting that all of the original material was transformed. In addition, the phospholipase activity was decreased by more than 99% (see Fig. 7). In spite of the marked decrease in phospho-

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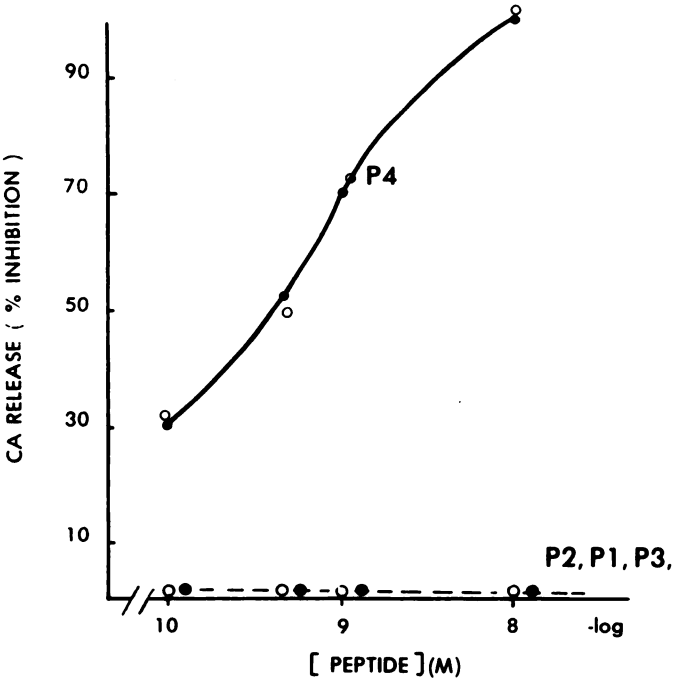

FIG. 3. *Inhibition of ACh- and nicotine-induced catecholamine release by P-1, P-2, P-3, and P-4 bungarotoxins separated by HPLC*

P-1, P-2, P-3, and P-4 were added to the cells 15 min before ACh + eserine or nicotine. Conditions of the experiments were identical with those of Fig. 1 and Table 1. The catecholamines released by 7.5 μM ACh (●——●) were 15% after subtraction of basal release (2.1% of the total content). Those released by 7.5 μM nicotine (O——O) were 16%. In control cells the catecholamine content was 40 nmoles/10^6 cells. The molecular weights of the four bungarotoxin peaks were estimated by 17% SDS slab gel electrophoresis and calculated to be around 8,000 for P-1, P-2, and P-3, and 15,000 for P-4 bungarotoxin, respectively.

lipase activity, the P-4 bungarotoxin/*p*-bromophenacyl derivative was still fully capable of blocking ACh-evoked release of catecholamines (Fig. 8).

DISCUSSION

Using as starting material commercially available preparations of α-bungarotoxin or native *Bungarus multicintus* venom, a 15,000 M_r toxin has been purified to homogeneity. This toxin has been partially sequenced and termed P-4 bungarotoxin. P-4 Bungarotoxin in the nanomolar concentration range acts as an apparent competitive inhibitor of nicotinic receptor stimulation of primary cultures of cow adrenal chromaffin cells.

The P-4 toxin is present in a small percentage (1–10%) in both native snake venom and α-bungarotoxin preparations. Thus, it could be suggested that P-4 bungarotoxin is a precursor of α-bungarotoxin. However, by comparing the amino acid composition of P-4 bungarotoxin and α-bungarotoxin (Table 2) and the amino acid sequence of the two toxins (Table 2 and refs. 24 and 25), it is evident that the α-bungarotoxin peptide is not contained in the P-4 molecule. The NH_2-terminal sequence of the first 10 residues of P-4 bungarotoxin (Asn-Leu-Tyr-Gln-Phe-Lys-Asn-Met-Ile) is highly homologous with that of various snake venom toxins endowed with phopholipase activity (24, 25). Moreover, the sequence of P-4 bungarotoxin from the amino acid residues 1–55 is identical with the sequence of phospholipase A from *B. multicintus* venom (26) and shows a high degree of homology with the A chain of β_1-bungarotoxin (27). Although P-4 bungarotoxin, like β-bungarotoxin, possesses phopholipase activity, it is apparently different from the β-bungarotoxins because it has a smaller mo-

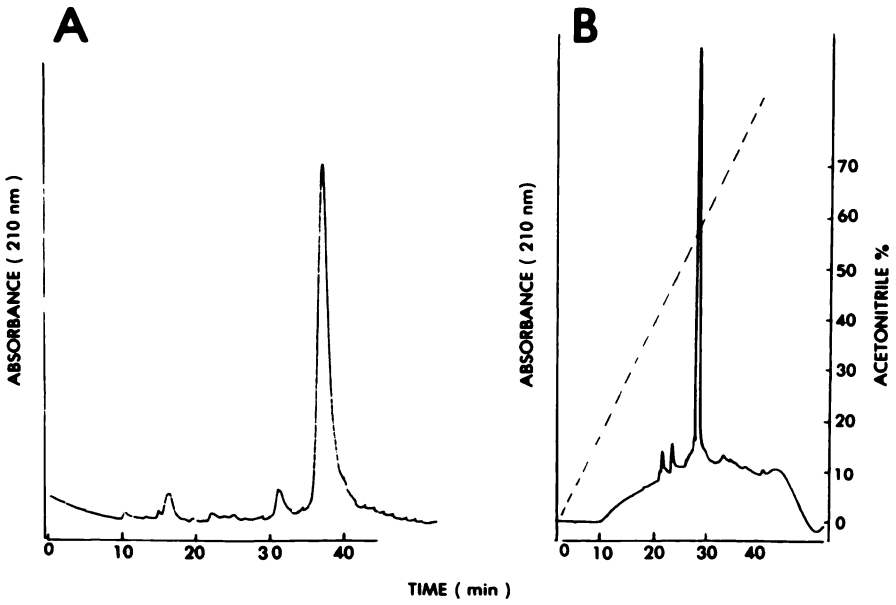

FIG. 4. *HPLC analysis of purified P-4 bungarotoxin*

P-4 Bungarotoxin obtained from reverse-phase HPLC (Fig. 2) was tested for purity under two different HPLC conditions. A. A Bio-Sil ODS-10 column was developed with 70% (0.1 M NaH_2PO_4/0.2% H_3PO_4) + 30% acetonitrile under isocratic conditions (flow rate 0.5 ml/min), and 25 μg of P-4 peptide were applied to the column. B. A Bio-Sil ODS-10 column was equilibrated with 0.1% TFA in water. The peptide was eluted with a 35-min linear gradient of 0.1% TFA/acetonitrile from 0% to 70% (– – –) (flow rate 1 ml/min), and 25 μg of P-4 peptide were applied to the column.

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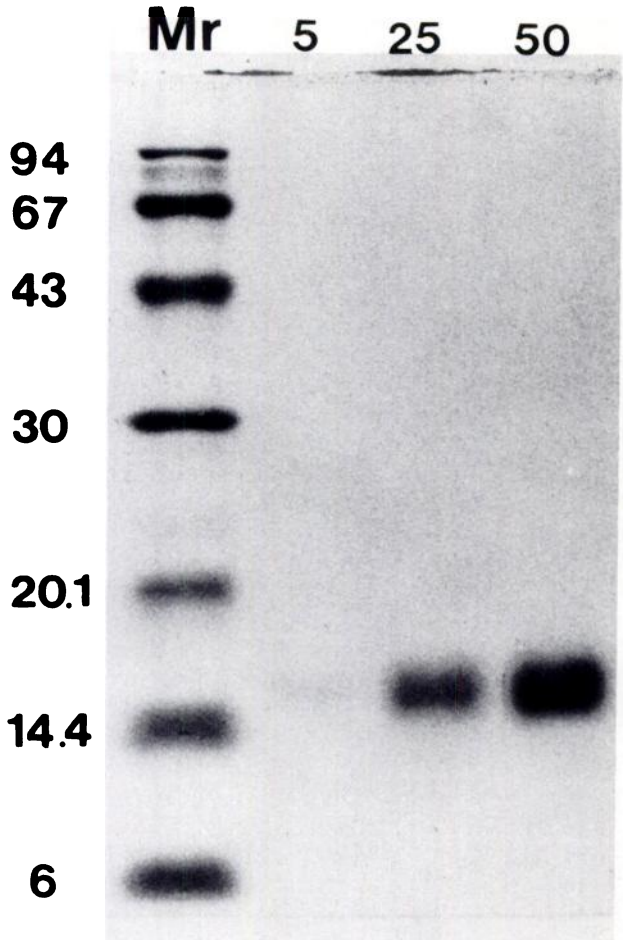

FIG. 5. *SDS/17% polyacrylamide gel electrophoresis*

P-4 Bungarotoxin obtained from reverse-phase HPLC (Fig. 2) was desalted on a Bio-Gel P_2 column (0.5 × 30 cm) equilibrated with 0.1 N acetic acid. The amount of protein applied to each lane is given in micrograms on top of the gel. The molecular weight markers (M_r, labeled × 10^{-3}) used were phosphorylase *b* (94,000), bovine serum albumin (67,000), ovalbumin (43,000), carbonic anhydrase (30,000), soybean trypsin inhibitor (20,100), α-lactalbumin (14,400), and insulin (6,000). Coomassie brilliant blue G was used to stain the protein.

lecular weight and a different amino acid composition (see Table 2). However, since β-bungarotoxins have not been completely characterized, it cannot be excluded that P-4 bungarotoxin represents the product of transformation of one of them. A toxin with an NH_2-terminal amino acid sequence similar to that of P-4 bungarotoxin was recently isolated from α-bungarotoxin preparations.[3]

A number of laboratories have reported that α-bungarotoxin binds specifically to mammalian neuronal membranes which contain ACh receptors (4–8). We have previously reported that [^{125}I]α-bungarotoxin binds with high affinity (2–3 nM) to intact adrenal chromaffin cells and to membranes of adrenal medulla (7). This binding was prevented by 0.1 mM *d*-tubocurare or 0.1 mM nicotine. Therefore, the evidence that α-bungarotoxin was also capable of preventing ACh-evoked release of catecholamines (Fig. 1) could have suggested that α-bungarotoxin binding sites were associated with nicotinic receptor sites in adrenal chromaffin cells. However, such inference is at variance with strong evidence indicating that α-bungarotoxin binding sites may be different from the ACh receptors in P_{12} cells, a tumor cell line originat-

[3] C. Gotti, personal communication.

TABLE 2

Amino acid composition of P-4 Bungarotoxin in comparison with the amino acid composition of α- and β-bungarotoxin

Initial (1–55) NH_2-terminal amino acid sequence of P-4 bungarotoxin: N L Y Q F K N M I V C A G T R P W I G Y V N Y G C Y C G A G G S G T P V D E L D R C C Y V H D N C Y G E A E K.

Residue	Relative no. of residues		
	P-4 Bungarotoxin[a]	α-Bungarotoxin[b]	β-Bungarotoxin[b]
Asx	13	4	22
Thr	6	7	11–12
Ser	6	6	6
Glx	7	5	12
Pro	8	8	8
Gly	13	4	16
Ala	14	5	11
Cys	7	10	19–20
Val	6	5	4
Met	1	1	2
Ile	7	2	8
Leu	4	2	7
Tyr	11	2	13
Phe	5	1	6
His	2	2	5
Lys	6	6	13
Arg	5	3	14
Trp[c]			
Total	121	73	179

[a] These values represent the mean of amino acid analyses on two different sample preparations.

[b] These values are from Lee (3).

[c] Tryptophan was destroyed during analyses.

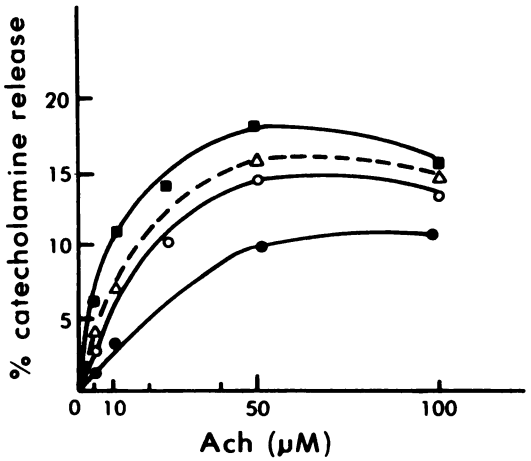

FIG. 6. *Antagonism of ACh-evoked release of endogenous catecholamines by P-4 bungarotoxin*

Various concentrations of acetylcholine were tested in the absence (■——■) and presence of 0.5 nM (Δ- - -Δ), 1 nM (○——○) and 5 nM (●——●) P-4 bungarotoxin. The total catecholamine content was 45 nmoles/10^6 cells. Basal release was 1.9% of total. P-4 bungarotoxin was added 15 min before ACh + eserine. Each point represents the mean of one experiment in which triplicate samples were assayed. The variation was less than 10% of the mean.

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ing from adrenal medulla. In P_{12} cells, antibodies prepared against electric eel nicotinic receptors failed to cross-react with the α-bungarotoxin binding component, although they blocked ACh receptor function (5, 11).

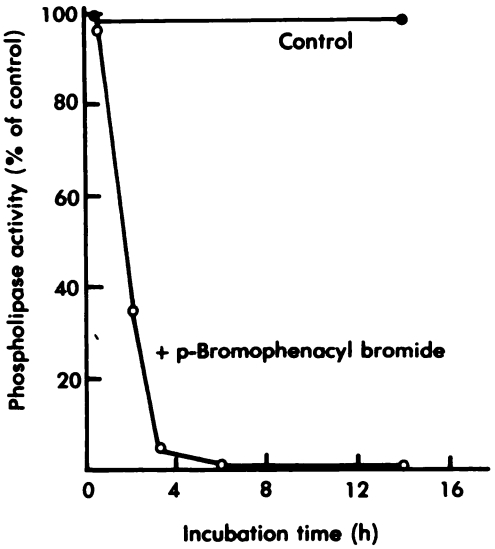

FIG. 7. *Time course of inactivation of phospholipase activity of P-4 bungarotoxin by p-bromophenacyl bromide treatment*

Purified P-4 bungarotoxin peptide (100 μg) was incubated for the time indicated with *p*-bromophenacyl bromide under the conditions described under Materials and Methods. One hundred per cent activity is the phospholipase activity of P-4 bungarotoxin fraction without (●) the addition of *p*-bromophenacyl bromide. In the absence and presence of 0.4 μg of P-4 bungarotoxin, 300 ± 25 (n = 3) and 26,000 ± 150 (n = 3) cpm were obtained respectively. *Open circles* (○) represent the phospholipase activity after incubation for different time periods with *p*-bromophenacyl bromide. After 6 hr of incubation with the inhibitor, 285 ± 30 and 469 ± 52 (n = 3) cpm were obtained in the absence and presence of P-4 bungarotoxin, respectively. The phospholipase activity remaining was less than 0.1%. At 6 hr the toxin had completely reacted with *p*-bromophenacyl bromide as indicated by the disappearance of the UV-absorbing peak from HPLC.

Furthermore, we observed that the IC_{50} of commercially available preparation of α-bungarotoxin to inhibit ACh or nicotine-evoked catecholamine release from adrenal cells varied by more than 10-fold depending on the lot of α-bungarotoxin. This variability in α-bungarotoxin preparations that were similar in their potency to displace [^{125}I]α-bungarotoxin binding and in their potency to block neuromuscular transmission prompted us to examine whether authentic α-bungarotoxin or another toxin present as a variable contaminant in the preparation was responsible for the action on the adrenal medulla cells.

Therefore, commercially available preparations of α-bungarotoxin were analyzed by HPLC. This technique not only allowed us to obtain a highly purified α-bungarotoxin peptide (P-2 fraction of Fig. 2) but also to purify to homogeneity three other toxin fragments. Using this technique, we discovered that the ability of different lots of α-bungarotoxins to block ACh-evoked catecholamine release from the adrenal cells is not related to the α-bungarotoxin peptide but to the presence of the P-4 bungarotoxin peptide. This polypeptide is apparently different from bungarotoxin 3.1 and 3.3 of Ravdin *et al.* (28, 29) because it has a larger molecular weight, but it could have some similarities to the 15,000 M_r peptide isolated by Quick and Lamarca (10). P-4 Bungarotoxin in the nanomolar concentration range not only blocks the acetylcholine-induced catecholamine release in adrenal cells (its effect is slowly reversible) but, similar to the Quick and Lamarca peptide (10), also blocks ganglionic transmission in frog sympathetic ganglia reversibly.[4] However, P-4 bungarotoxin (1 μg/ml) blocked reversibly the neuromuscular transmission, whereas the 15,000 M_r toxin of Quick and Lamarca (10) was ineffective at the neuromuscular junction up to a concentration of 1 μg/ml.

[4] L. Saiani, H. Kageyama, B. M. Conti-Tronconi, and A. Guidotti, manuscript in preparation.

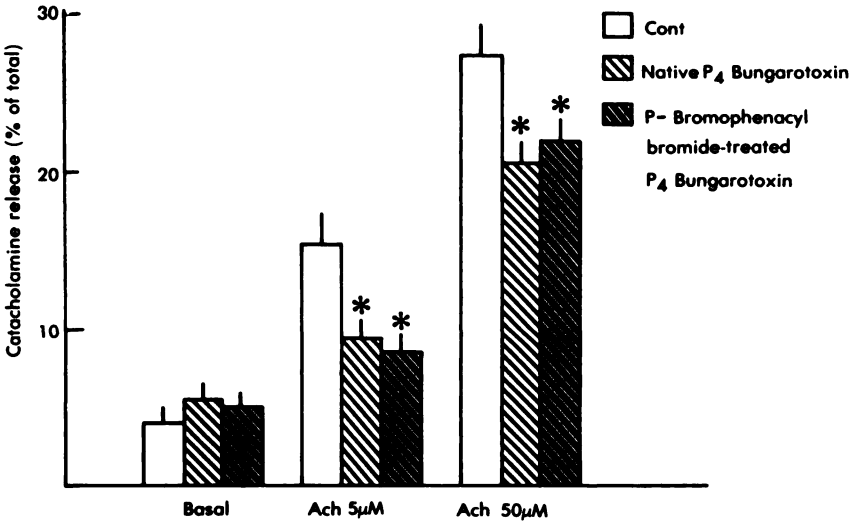

FIG. 8. *Effect of native and p-bromophenacyl bromide-modified P-4 bungarotoxin on the release of catecholamines evoked by acetylcholine*

Native P-4 bungarotoxin (1 nM) or modified P-4 bungarotoxin (1 nM) (see Fig. 7) was incubated with the cells 15 min before addition of acetylcholine + eserine. Each value represents the mean ± standard error of triplicate determinations. Native or modified P-4 bungarotoxin at the concentration used failed to change the total content of NE and E in the cells. The catecholamine content in these cells was 48 nmoles/10^6 cells. The basal release was 2.5% of the total.

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Blockade of ACh-induced catecholamine release from adrenal medulla can occur through several mechanisms. P-4 Bungarotoxin could be acting at one of the several steps intervening between binding of ACh to the receptor and the secretion of catecholamines, hence it could act on sites different from the ACh receptor. However, there is only a limited number of possibilities, because KCl-evoked catecholamine release is insensitive to the action of even higher concentrations of P-4 bungarotoxin. The toxin could be activating an endogenous acetylcholinesterase. This possibility also seems unlikely, because the effect of ACh is consistently being studied in the presence of 10 μM eserine. Moreover, similar effects of P-4 bungarotoxin were observed when catecholamine release was induced with nicotine (see Fig. 3).

Another possible mode of action of P-4 bungarotoxin could be linked to the phospholipase activity that we have found to be associated with the peptide. It has been postulated, for example, that β-bungarotoxin, which also possesses phospholipase activity, reduces cholinergic transmission at least in part by decreasing the amount of ACh released from presynaptic nerve terminals (30). This effect of β-bungarotoxin can be prevented when the phospholipase activity is blocked by treating the toxin with *p*-bromophenacyl bromide (30). In the case of P-4 bungarotoxin, however, we can show that inhibition of phospholipase activity (more than 99.9%) does not abate the P-4 bungarotoxin inhibition of ACh-induced catecholamine release.

Therefore, the results suggest that P-4 bungarotoxin may be acting by blocking ACh-nicotinic receptors in cultures of bovine chromaffin cells. Since P-4 bungarotoxin is effective in a low concentration range and its effect persists for at least 1 hr when it is removed from the medium, it is suggested that P-4 bungarotoxin binds tightly to some sites of the nicotinic receptor. It is reasonable to believe that P-4 bungarotoxin may turn out to be a relevant agent with which to study nicotinic receptor function in adrenal chromaffin cells. A method to obtain high specific activity labeled P-4 bungarotoxin without loss of its biological activity is currently under study.

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