Biochemical Pharmacology, Vol. 44, No. 9, pp. 1779–1785, 1992.
Printed in Great Britain.

0006-2952/92 $5.00 + 0.00

INHIBITION OF NICOTINIC ACETYLCHOLINE RECEPTOR-MEDIATED SECRETION AND SYNTHESIS OF CATECHOLAMINES BY SEA URCHIN TOXIN IN CULTURED BOVINE ADRENAL MEDULLARY CELLS

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(*Received* 9 *December* 1991; *accepted* 17 *July* 1992)

Abstract—We previously reported the partial purification and characterization of a toxic substance (sea urchin toxin) isolated from the pedicellariae of the sea urchin *Toxopneustes pileolus* (Nakagawa and Kimura, *Jpn J Pharmacol* **32**: 966–968, 1982). In the present study, we examined the effect of sea urchin toxin on catecholamine secretion and synthesis in cultured bovine adrenal medullary cells. Sea urchin toxin inhibited the secretion of catecholamines stimulated by carbachol and nicotine but not by veratridine or a high concentration of K^+. The toxin inhibited the carbachol-evoked influx of $^{22}Na^+$ and $^{45}Ca^{2+}$ at concentrations similar to those for catecholamine secretion. The inhibition of catecholamine secretion by sea urchin toxin was not overcome by increasing the concentration of carbachol. Preincubation of cells with the toxin caused a time-dependent inhibition in the secretion stimulated by carbachol even when the toxin was removed from the incubation medium. The toxin suppressed catecholamine synthesis and tyrosine hydroxylase activity in carbachol-stimulated cells. In addition, sea urchin toxin inhibited [^{3}H]phencyclidine binding to adrenal medullary cells whereas it did not alter cyclic GMP accumulation caused by muscarine. Further purified fractions from sea urchin toxin by concanavalin A affinity column chromatography also inhibited carbachol-evoked secretion of catecholamines. These results suggest that sea urchin toxin inhibits carbachol-enhanced secretion and synthesis of catecholamines by suppression of nicotinic acetylcholine receptor-mediated Na^+ influx and subsequent Ca^{2+} influx in cultured adrenal medullary cells.

Previous studies have demonstrated that the crude extracts isolated from the globiferous pedicellariae of the toxopneustid sea urchins produce various pharmacological effects such as local paralysis, spasmogenesis, cardiotoxicity and active materials releasing action [1–3]. Recently, we reported that a toxic fraction partially purified from the pedicellariae of the sea urchin *Toxopneustes pileolus* causes a release of histamine from rat mast cells and antagonizes acetylcholine-induced contraction of frog rectus abdominis [4–6].

Adrenal medullary cells are paraneurons of neural crest origin and share many physiological and pharmacological properties with postganglionic sympathetic neurons. Stimulation of nicotinic acetylcholine receptors in adrenal medullary cells causes a rapid secretion of catecholamines [7–9] with a simultaneous increase in catecholamine synthesis [10]; the influx of Ca^{2+} is essential to trigger these responses. The increase in catecholamine synthesis is associated with an activation of tyrosine hydroxylase that catalyzes the rate-limiting step in the biosynthesis of catecholamines [11, 12]. Muscarinic acetylcholine receptors, on the other hand, mediate cyclic GMP accumulation without causing catecholamine secretion in bovine adrenal medulla [13].

Recently, several investigators have demonstrated that cultured adrenal medullary cells have at least three distinct types of ionic channels: (1) nicotinic acetylcholine receptor-associated ion channels responsible for carbachol-induced Na^+ influx [9, 14, 15], (2) voltage-dependent Na^+ channels responsible for veratridine-induced Na^+ influx [9, 14, 15] and (3) voltage-dependent Ca^{2+} channels [14–17] which are activated by a high concentration of K^+ (high K^+). Our previous studies suggest that either carbachol-induced Na^+ influx or veratridine-induced Na^+ influx contributes to Ca^{2+} influx via voltage-dependent Ca^{2+} channels whereas high K^+ gates voltage-dependent Ca^{2+} channels without Na^+ influx [14, 15].

In the present study, we examined whether the toxic fraction (sea urchin toxin) partially purified from the pedicellariae of the sea urchin *T. pileolus* would affect the secretion and synthesis of catecholamines in cultured bovine adrenal medullary cells. To investigate the site of action of sea urchin toxin, we further examined the effect of the toxin on the ionic channels described above and on muscarinic receptor-mediated cyclic GMP accumulation.

MATERIALS AND METHODS

Materials. The drugs were obtained from the

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following sources: Eagle's minimum essential medium, Nissui Seiyaku; calf serum, Nacalai Tesque; carbachol, veratridine, and collagenase, Sigma; L-[U-^{14}C]tyrosine (514 mCi/mmol) and $^{45}CaCl_2$ (0.5 to 2.0 Ci/mmol), Amersham International; and ^{22}NaCl (6–17 Ci/mmol), [^{3}H]phencyclidine (50 Ci/mmol) and L-[1-^{14}C]tyrosine (53.4 mCi/mmol), New England Nuclear. Non-radioactive phencyclidine was a gift from Dr. Toshitaka Nabeshima, Department of Hospital Pharmacy, Nagoya University School of Medicine.

Partial purification of sea urchin toxin. Sea urchin toxin was partially purified from the pedicellariae of the sea urchin *T. pileolus* as reported previously [4]. Briefly, the pedicellariae were removed from five sea urchins and extracted with H_2O. The crude extract was fractionated by ammonium sulfate (30–65% saturation) and subjected to gel filtration on a Sephadex G-200 column (2.5 × 65 cm). Two major peaks of protein were obtained as Peak I fraction and Peak II fraction. The peak II fraction was used as sea urchin toxin in the present study. The biological activity (U) of the toxin was expressed as protein concentration which caused a contraction of longitudinal smooth muscle of isolated guinea-pig ileum equivalent to 50% of a 40 mM KCl-induced contraction. Total protein of the Peak II fraction was 32.0 mg and its specific activity was 2000 U/mg protein. In some experiments, sea urchin toxin was dissolved in 20 mM Tris–HCl buffer (pH 7.4) containing 0.4 M NaCl and applied on a concanavalin A-Sepharose column (1.0 × 10 cm). An unretained fraction was recovered as pre-concanavalin A fraction (tube numbers 2–8). Glycoproteins were eluted by 50 mM methyl-α-D-mannoside and two fractions were obtained: concanavalin A-A fraction (tube numbers 21–24) and concanavalin A-B fraction (tube numbers 25–30).

Isolation of adrenal medullary cells and primary culture. Adrenal medullary cells were isolated by collagenase digestion of slices of bovine adrenal medulla as described previously [13]. The cells were suspended in Eagle's minimum essential medium containing 10% calf serum and antibiotics, and cultured (4 × 10^6 cells/dish, 35 mm Falcon) at 37° in 5% CO_2–95% air [18].

Secretion of catecholamines. Oxygenated Krebs–Ringer phosphate (KRP)* buffer was used throughout. It was composed as follows (mM): NaCl, 154; KCl, 5.6; $MgSO_4$, 1.1; $CaCl_2$, 2.2; NaH_2PO_4, 0.85; Na_2HPO_4, 2.15; glucose, 10; and 0.5% bovine serum albumin, adjusted to pH 7.4. The secretion of catecholamines was examined, as reported previously [13]. The cells were incubated at 37° for 5 min with or without various secretagogues and sea urchin toxin. Catecholamines secreted into the medium were adsorbed to aluminium hydroxide and estimated by the ethylenediamine condensation method [19], using a fluorescence spectrometer (Hitachi model 650-10S) with an excitation wavelength of 420 nm and an emission of 540 nm.

Influx of $^{22}Na^+$ and $^{45}Ca^{2+}$. Influx of $^{22}Na^+$ and $^{45}Ca^{2+}$ was measured as reported previously [14]. The cells were incubated with 1.5 μCi of ^{22}NaCl or 1.5 μCi of $^{45}CaCl_2$ at 37° for 5 min in 1.0 mL of KRP buffer in the presence or absence of test compounds. $^{22}Na^+$ and $^{45}Ca^{2+}$ in the cells were counted by a Beckman LS-7000 liquid scintillation counter.

Measurement of cyclic GMP. Cells were preincubated at 37° for 10 min with 3-isobutyl-1-methylxanthine (IBMX) (0.3 mM) and then incubated for another 5 min with or without test compounds in the presence of IBMX (0.3 mM). After aspiration of the medium, the cells were rapidly scraped in ice-cold 7% trichloroacetic acid and centrifuged. The supernatant fraction was applied to an ion exchange column (AG 50W × 4, H^+ type, 0.8 × 7 cm) [13]. Cyclic GMP was assayed using a cyclic GMP assay kit (Amersham International).

Synthesis of [^{14}C]catecholamines. The cells were incubated with L[U-^{14}C]tyrosine (20 μM, 2.35 × 10^6 dpm) at 37° for 20 min in 2 mL of KRP buffer in the presence or absence of carbachol and sea urchin toxin. The cells were scraped in 0.4 M perchloric acid. After extraction, [^{14}C]catechol compounds were isolated from [^{14}C]tyrosine by aluminum hydroxide adsorption. [^{14}C]Catecholamines formed were separated by ion-exchange chromatography on a Duolite C-25 column (H^+ type, 0.4 × 7 cm) [18] and counted by a liquid scintillation counter.

Activity of tyrosine hydroxylase. The cells (4 × 10^6) were incubated at 37° for 3 min in the presence or absence of carbachol and sea urchin toxin. The cells were homogenized in 30 mM potassium phosphate buffer (pH 6.8), containing 50 mM NaF and 1 mM EDTA. After centrifugation at 20,000 *g* for 10 min, the supernatant was applied to a Sephadex G-25 column (0.9 × 3.1 cm). Tyrosine hydroxylase activity in the effluent was measured by a modified decarboxylase-coupled assay [20, 21]. The specific activity of the enzyme was expressed in nanomoles of $^{14}CO_2$ formed per minute per milligram of protein. Protein was determined according to the method of Bradford [22].

[^{3}H]Phencyclidine binding to adrenal medullary cells. Isolated cells (5 × 10^6) were incubated with or without sea urchin toxin (15–75 μg/mL) at 4° for 45 min in 1 mL of KRP buffer in the presence of 4.3 μM [^{3}H]phencyclidine, as described previously [23]. The binding assay was performed in the absence (total binding) and presence (nonspecific binding) of 1 mM unlabeled phencyclidine, and the specific binding was calculated as the difference between total and nonspecific binding.

Statistics. All values are expressed as means ± SD. Statistical analysis was carried out using Student's *t*-test.

RESULTS

Effect of sea urchin toxin on the secretion of catecholamines evoked by various secretagogues in cultured adrenal medullary cells. Sea urchin toxin (125 μg/mL) had little effect on the basal secretion of catecholamines from cultured cells (Fig. 1). Stimulation of acetylcholine receptors by carbachol and nicotine caused the secretion of catecholamines

* Abbreviations: IBMX, 3-isobutyl-1-methylxanthine; and KRP, Krebs–Ringer phosphate.

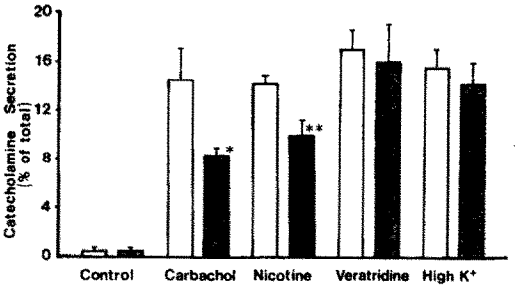


Fig. 1. Effects of sea urchin toxin on catecholamine secretion induced by various secretagogues in cultured bovine adrenal medullary cells. Cells were incubated with or without carbachol (0.3 mM), nicotine (0.1 mM), veratridine (0.1 mM), high K^+ (56 mM K^+) or sea urchin toxin (125 μg/mL) at 37° for 5 min. In high K^+ solution, NaCl was reduced to maintain the isotonicity of the medium; other constituents were the same as those in KRP buffer. Sea urchin toxin was present (hatched columns) or absent (open columns). Catecholamines secreted into the medium were measured (see Materials and Methods) and expressed as a percentage of total catecholamines (total catecholamines as norepinephrine plus epinephrine in the cells = 45–50 μg/4 × 10^6 cells). Each column shows the mean of 4 experiments and the vertical bar represents the SD. Key: (*) $P < 0.001$ compared with carbachol alone; and (**) $P < 0.05$ compared with nicotine alone.

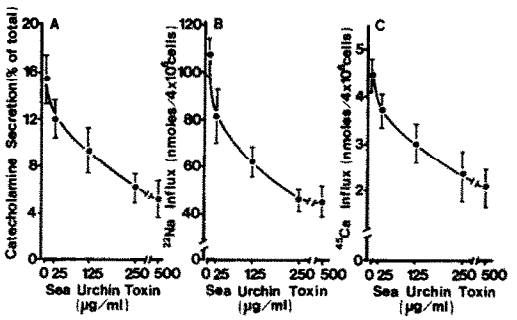


Fig. 2. Concentration–response curves of sea urchin toxin for the inhibition of catecholamine secretion (A), $^{22}Na^+$ influx (B) and $^{45}Ca^{2+}$ influx (C) caused by carbachol. In (A), cells were incubated with carbachol (0.3 mM) and various concentrations of sea urchin toxin at 37° for 5 min. Catecholamines secreted into the medium were measured as described in the legend of Fig. 1. In (B) and (C), cells were incubated at 37° for 5 min with 1.5 μCi of $^{22}NaCl$ or $^{45}CaCl_2$ in 1.0 mL of KRP buffer in the presence of carbachol (0.3 mM) and various concentrations of sea urchin toxin. The influx of $^{22}Na^+$ or $^{45}Ca^{2+}$ was measured (see Materials and Methods), and is expressed as nmol/4 × 10^6 cells, being calculated from its initial specific radioactivity in the incubation medium. Data are means ± SD of 4–6 experiments.

(14.4 and 14.2% of total catecholamines, respectively). Sea urchin toxin (125 μg/mL) significantly inhibited carbachol- and nicotine-evoked secretion of catecholamines (8.4 and 10.1% of total catecholamines). Veratridine, an activator for voltage-dependent Na^+ channels [24], and high K^+ also produced the secretion of catecholamines (17 and 15.6% of total catecholamines). Sea urchin toxin (125 μg/mL), however, did not affect the stimulatory effect of veratridine and high K^+ on the secretion of catecholamines (16 and 14.2% of total catecholamines, respectively).

Concentration–response curves of sea urchin toxin for the inhibition of catecholamine secretion, $^{22}Na^+$ influx and $^{45}Ca^{2+}$ influx evoked by carbachol. Since sea urchin toxin inhibited the stimulatory effect of carbachol on catecholamine secretion (Fig. 1), the inhibitory site of sea urchin toxin on carbachol-evoked secretion of catecholamines was examined. As shown in Fig. 2A, the toxin inhibited carbachol-evoked secretion of catecholamines in a concentration-dependent manner (25–250 μg/mL). The toxin also inhibited the carbachol-evoked influx of $^{22}Na^+$ and $^{45}Ca^{2+}$ at concentrations similar to those for catecholamine secretion (Fig. 2, B and C).

Inhibitory mode of sea urchin toxin on carbachol-induced secretion of catecholamines. We investigated whether the toxin competes with carbachol for the nicotinic receptors. The inhibitory effect of the toxin, however, was not overcome by increasing the concentration of carbachol (Fig. 3A). Double-reciprocal plot analysis demonstrated an uncompetitive type of inhibition with respect to carbachol (data not shown).

Next, we examined the reversibility of inhibition

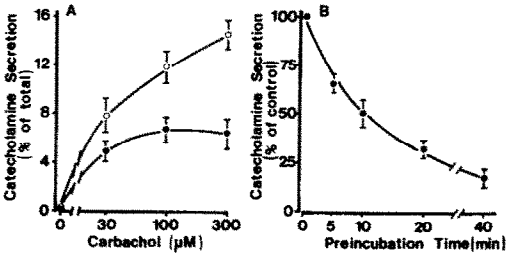


Fig. 3. Characterization of the inhibitory effect of sea urchin toxin on carbachol-evoked secretion of catecholamines. (A) Cells were stimulated at 37° for 5 min with various concentrations of carbachol in the presence (●) or absence (○) of sea urchin toxin (250 μg/mL). Catecholamines secreted were measured as described in the legend of Fig. 1. Data are means ± SD of 4 experiments. (B) Cells were preincubated with or without sea urchin toxin (125 μg/mL) at 37° for the indicated period and washed four times with 1.0 mL of KRP buffer, and then stimulated for another 5 min with carbachol (0.3 mM) in the absence of sea urchin toxin. Catecholamine secretion was measured. Catecholamine secretion caused by carbachol from cells preincubated without sea urchin toxin (control) (catecholamines secreted = 7.2 ± 0.4 μg) was assigned a value of 100% and each data point is expressed as a percentage of that value. Data are means ± SD of 4 experiments.

of catecholamine secretion by the toxin. Preincubation of cells with sea urchin toxin produced a time-dependent inhibition in carbachol-evoked secretion even after the toxin was removed from the incubation medium (Fig. 3B).

Table 1. Effect of sea urchin toxin on basal and carbachol-stimulated [^{14}C]catecholamine synthesis and tyrosine hydroxylase activity

	[^{14}C]Catecholamine synthesis (dpm × 10^{-4}/4 × 10^6 cells/20 min)	(%)	Tyrosine hydroxylase activity (nmol/min/mg)	(%)
Control	5.92 ± 0.02	100	1.38 ± 0.05	100
Sea urchin toxin	6.05 ± 0.37	102	1.25 ± 0.09	90
Carbachol	13.45 ± 0.25	228	3.88 ± 0.30	281
Carbachol + sea urchin toxin	7.82 ± 0.02*	132	2.60 ± 0.24†	188

[^{14}C]Catecholamine synthesis: cells were incubated at 37° for 20 min with or without carbachol (0.3 mM) and sea urchin toxin (250 μg/mL) in 2.0 mL of KRP buffer containing L-[U-^{14}C]tyrosine (20 μM, 2.35 × 10^6 dpm). [^{14}C]Catecholamines formed were measured as described in Materials and Methods. Data are means ± SD of four experiments. Tyrosine hydroxylase activity: cells were incubated for 3 min with or without carbachol (0.3 mM) and sea urchin toxin (250 μg/mL). Tyrosine hydroxylase activity in a soluble fraction was assayed *in vitro* (see Materials and Methods). Data are means ± SD of 3–4 experiments.

* $P < 0.001$ compared with carbachol.

† $P < 0.01$ compared with carbachol.

Table 2. Competition of [^{3}H]phencyclidine binding by unlabeled phencyclidine and sea urchin toxin

	[^{3}H]Phencyclidine binding (%)
Phencyclidine	
5 μM	80.7 ± 2.1
100 μM	37.8 ± 3.0
400 μM	15.3 ± 4.5
Sea urchin toxin	
15 μg/mL	80.0 ± 5.0
30 μg/mL	60.7 ± 20.0
75 μg/mL	39.3 ± 7.7

In the absence or presence of the indicated drugs, dispersed cells (5 × 10^6) were incubated with 4.3 μM [^{3}H]-phencyclidine at 4° for 45 min and [^{3}H]phencyclidine bound to the cells was counted. In parallel experiments, nonspecific binding was determined in the presence of 1 mM unlabeled phencyclidine and specific binding was obtained by subtracting nonspecific binding from total binding. [^{3}H]Phencyclidine specific binding (70.9 ± 18.9 pmol/5 × 10^6 cells) was assigned a value of 100%; data are expressed as a percentage of [^{3}H]-phencyclidine specific binding. Data are means ± SD from 3–4 experiments.

Table 3. Effect of sea urchin toxin on muscarine-stimulated cyclic GMP accumulation

	Cyclic GMP (pmol/4 × 10^6 cells)	
	Control	Muscarine
None	0.255 ± 0.033	0.560 ± 0.092
Atropine	ND*	0.304 ± 0.099
Sea urchin toxin	0.256 ± 0.112	0.663 ± 0.074

Cells were preincubated with IBMX (0.3 mM) at 37° for 10 min and then were incubated for another 5 min with or without *dl*-muscarine (0.1 mM), sea urchin toxin (250 μg/mL) or atropine (4 μM). Cyclic GMP in the cells was measured as described in Materials and Methods. Data are means ± SD of 3–4 experiments.

* Not determined.

Effect of sea urchin toxin on [^{14}C]catecholamine synthesis and tyrosine hydroxylase activity stimulated by carbachol. Our previous report [25] has demonstrated that in rat pheochromocytoma PC12 cells 12-*O*-tetradecanoylphorbol 13-acetate or forskolin phosphorylates and activates tyrosine hydroxylase via activation of protein kinase C or cyclic AMP-dependent protein kinase, respectively. To investigate the influence of sea urchin toxin on cellular protein kinases such as cyclic AMP-dependent protein kinase or protein kinase C, we examined the effects of sea urchin toxin on the synthesis of [^{14}C]catecholamines and the activity of tyrosine hydroxylase. Sea urchin toxin (250 μg/mL), however, did not affect the basal synthesis of [^{14}C]-catecholamines or the activity of tyrosine hydroxylase (Table 1). The toxin inhibited the carbachol-stimulated synthesis of [^{14}C]catecholamines and the carbachol-evoked activation of tyrosine hydroxylase.

Effect of sea urchin toxin on [^{3}H]phencyclidine binding to adrenal medullary cells. A previous study has shown that phencyclidine inhibits nicotinic acetylcholine receptor-associated ionic channels in skeletal muscle and Torpedo electric organ [26]. Since sea urchin toxin inhibited carbachol- and nicotine-evoked secretion of catecholamines, we examined the effect of the toxin on [^{3}H]phencyclidine binding to adrenal medullary cells. Sea urchin toxin as well as phencyclidine attenuated the specific binding of [^{3}H]phencyclidine to the cells in a concentration-dependent manner (Table 2).

Effect of sea urchin toxin on muscarine-increased cyclic GMP accumulation in the cells. We studied the effect of sea urchin toxin on muscarinic acetylcholine receptor-mediated cyclic GMP accumulation. Muscarine caused an approximately 2.2-fold

increase in cyclic GMP content (Table 3). The stimulatory effect of muscarine was almost abolished by atropine, an antagonist of muscarinic acetylcholine receptors. Sea urchin toxin, however, did not affect muscarine-stimulated cyclic GMP accumulation.

Effects of further purified fractions on carbachol-evoked catecholamine secretion. We further purified the sea urchin toxin by concanavalin A-Sepharose column chromatography and obtained three fractions. Pre-concanavalin A fraction (tube numbers 2–8) was not retained in the affinity column, whereas concanavalin A-A fraction (tube numbers 21–24) and concanavalin A-B fraction (tube numbers 25–30) were retained and eluted by methyl-α-D-mannoside (Fig. 4A). We examined the effects of the three fractions on carbachol-evoked secretion of catecholamines. As shown in Fig. 4B, these two fractions of concanavalin A-A and A-B (75 μg/mL) inhibited carbachol-evoked secretion. The pre-concanavalin A fraction (80 μg/mL) also slightly inhibited the secretion.

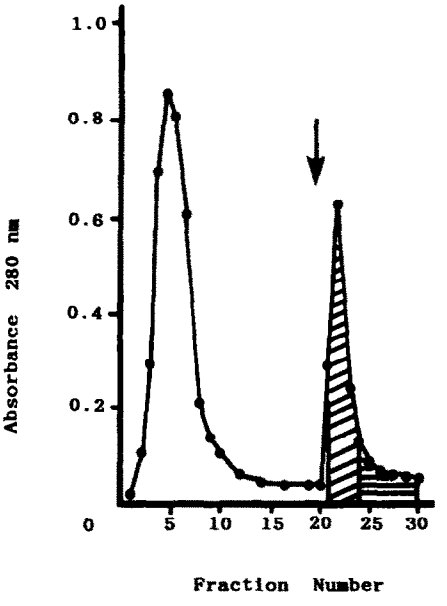
DISCUSSION

Inhibitory effect of sea urchin toxin on nicotinic receptor-mediated catecholamine secretion but not muscarinic receptor-mediated cyclic GMP accumulation. In bovine adrenal medullary cells, nicotinic acetylcholine receptors are involved in the secretion of catecholamines [8, 13] and muscarinic acetylcholine receptors are involved in cyclic GMP production [13]. In the present study, sea urchin toxin inhibited nicotinic receptor-mediated secretion of catecholamines (Fig. 1) but did not affect muscarinic receptor-mediated cyclic GMP accumulation (Table 3). These results indicate that sea urchin toxin antagonizes nicotinic receptor-mediated secretion caused by carbachol.

Sea urchin toxin inhibition of carbachol-induced catecholamine secretion by interference with Na^+ influx via nicotinic receptor-associated ion channels. Sea urchin toxin had little effect on catecholamine secretion stimulated by either veratridine or high K^+ (Fig. 1), suggesting that the toxin, at least in the concentration used, does not inhibit voltage-dependent Na^+ channels and voltage-dependent Ca^{2+} channels. On the other hand, the toxin inhibited carbachol-evoked influx of $^{22}Na^+$ at concentrations similar to those for $^{45}Ca^{2+}$ influx and catecholamine secretion (Fig. 2). The result suggests that sea urchin toxin inhibits carbachol-evoked secretion primarily by interfering with Na^+ influx via nicotinic receptor-associated ion channels. The inhibitory effect of the toxin was not reversed by increasing the concentration of carbachol (Fig. 3A), suggesting that the toxin does not compete with carbachol at the nicotinic receptors. The inhibitory effect of the toxin on catecholamine secretion seems to be irreversible and dependent on the preincubation time with the toxin (Fig. 3B).

The precise mechanism or site of action of sea urchin toxin remains to be determined. In bovine adrenal medullary cells, we previously reported that phencyclidine binds to two distinct classes of the sites which are functionally linked to a nicotinic receptor–ion channel complex and to voltage-

A



B

	Catecholamine Secretion (% of total)
None	0.3 ± 0.1
Carbachol	13.2 ± 2.4
+ Pre-Con A	10.4 ± 1.4
+ Con A-A	6.9 ± 1.2
+ Con A-B	6.2 ± 1.5

Fig. 4. Effects of further purified fractions on catecholamine secretion. (A) Isolation of sea urchin toxin by concanavalin A-Sepharose column chromatography. Sea urchin toxin (the Peak II fraction of Sephadex G-200 column) was applied on a concanavalin A-Sepharose column (1 × 10 cm). An unretained fraction was recovered as pre-concanavalin A fraction (tube numbers 2–8). Glycoproteins were eluted by 50 mM methyl-α-D mannoside in 20 mM Tris–HCl buffer (pH 7.4). Two fractions were obtained: A fraction (tube numbers 21–24) and B fraction (tube numbers 25–30). An arrow shows the elution by 50 mM methyl-α-D mannoside. (B) Effects of three fractions on carbachol-evoked secretion. Cultured cells were preincubated at 37° for 10 min with or without pre-concanavalin A fraction (80 μg/mL), concanavalin A-A fraction or concanavalin A-B fraction (75 μg/mL). After preincubation, cells were incubated with carbachol (0.3 mM) for another 5 min in the presence or absence of each fraction. Catecholamine secretion was measured and presented as a percentage of total catecholamines (48 ± 3 μg/4 × 10^6 cells). Data are means ± SD of 3–6 experiments. Key: Pre-Con A, pre-concanavalin A fraction (tube numbers 2–8); Con A-A, concanavalin A-A fraction (tube numbers 21–24); Con A-B, concanavalin A-B fraction (tube numbers 25–30).

dependent Na^+ channels [23]. In the present study, the specific binding of [^{3}H]phencyclidine was concentration-dependently inhibited by sea urchin toxin (Table 2). Since sea urchin toxin at the concentrations used in this experiment did not affect voltage-dependent Na^+ channels (Fig. 1), it suggests the possibility that the toxin acts at the site or near the site of phencyclidine binding on nicotinic acetylcholine receptor–ion channel complex in adrenal medullary cells. The present results may be consistent with our previous report that sea urchin toxin inhibits acetylcholine-induced contraction of frog skeletal muscle [6].

We also cannot rule out the possibility that sea urchin toxin interacts with a site(s) distinct from the nicotinic receptor–ion channel complex. Several neurotoxins such as botulinum toxin [27] and tetanus toxin [28] are reported to inhibit catecholamine secretion by interfering with exocytotic processes in the cells. Furthermore, there are pertussis toxin-sensitive or -insensitive GTP binding proteins which may modulate catecholamine secretion in adrenal medullary cells [29]. Since sea urchin toxin may interact with GTP binding proteins or the cellular exocytotic processes described above, further studies would be expected.

Inhibitory effect of sea urchin toxin on the synthesis of [^{14}C]*catecholamines stimulated by carbachol.* Although the molecular mechanism(s) for the stimulation of catecholamine synthesis or the activation of tyrosine hydroxylase is not well established, Ca^{2+}-dependent or cyclic AMP-dependent phosphorylation plays an important role in these responses [21, 25, 30, 31]. In the present study, sea urchin toxin did not affect by itself the basal synthesis of catecholamines and the activity of tyrosine hydroxylase, suggesting that the toxin has little effect on the phosphorylation of tyrosine hydroxylase. Sea urchin toxin, however, inhibited catecholamine synthesis and tyrosine hydroxylase activity stimulated by carbachol. Since stimulation of nicotinic receptors by carbachol causes Na^+ influx and subsequent Ca^{2+} influx which enhances catecholamine synthesis or tyrosine hydroxylase activity [14, 18], the toxin seems to attenuate the stimulation by carbachol of catecholamine synthesis probably by suppressing Na^+ influx via nicotinic receptor-associated ion channels and subsequent Ca^{2+} influx in the cells.

Further purification and characterization of sea urchin toxin. We further purified the sea urchin toxin by concanavalin A affinity column chromatography and obtained three fractions. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis showed that the concanavalin A-A fraction contained at least three glycoproteins (mol. wt = 29.5, 18–19 and 17 kDa) and the concanavalin A-B fraction had two of the three glycoproteins (mol wt. = 18–19 and 17 kDa) (unpublished observations). These two fractions of concanavalin A-A and A-B more strongly inhibited carbachol-evoked secretion than did the pre-concanavalin A fraction (Fig. 4B). We are currently purifying these glycoproteins in concanavalin A fractions by high performance liquid chromatography and investigating the precise mechanism of the toxin on catecholamine secretion. The toxin may be a useful tool for studies on nicotinic acetylcholine receptor–ionic channel complex.

In conclusion, we have demonstrated that sea urchin toxin inhibits nicotinic receptor-mediated secretion and synthesis of catecholamines by inhibiting the influx of Na^+ and Ca^{2+} in cultured adrenal medullary cells.

Acknowledgements—The authors would like to thank Dr. Toshitaka Nabeshima, Department of Hospital Pharmacy, Nagoya University School of Medicine, for his gift of non-radioactive phencyclidine. We also thank Ms. Yumiko Toyohira for her expert assistance in the isolation of bovine adrenal medullary cells and in the preparation of the line figures and Ms. Mie Yamamoto for typing this manuscript.

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