MOLECULAR PHARMACOLOGY, 25:379–383

Mechanism of Palytoxin-Induced [^{3}H]Norepinephrine Release from a Rat Pheochromocytoma Cell Line

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Received March 14, 1983; Accepted December 7, 1983

SUMMARY

Palytoxin, isolated from the zoanthid *Palytoha* species, is one of the most potent marine toxins. Palytoxin (1 nM–1 μM) caused a release of [^{3}H]norepinephrine from clonal rat pheochromocytoma cells in a concentration-dependent manner. This releasing action of palytoxin was markedly inhibited or abolished by Co^{2+} or Ca^{2+}-free medium, but was not modified by tetrodotoxin. The release of [^{3}H]norepinephrine induced by a low concentration (30 nM) of palytoxin was abolished in sodium-free medium and increased as the external Na^+ concentrations were increased from 3 to 100 nM, but the release induced by a high concentration (1 μM) was unaffected by varying the concentration of external Na^+ from 0 to 100 mM. The release of [^{3}H]norepinephrine induced by both concentrations of palytoxin increased with increasing Ca^{2+} concentrations from 0 to 3 mM. Palytoxin caused a concentration-dependent increase in ^{22}Na and ^{45}Ca influxes into pheochromocytoma cells at concentrations of 0.1 nM–10 nM and 1 nM–1 μM, respectively. The palytoxin-induced ^{45}Ca influx was markedly inhibited by Co^{2+}, whereas the palytoxin-induced ^{22}Na influx was not affected by tetrodotoxin. These results suggest that in pheochromocytoma cells the [^{3}H]norepinephrine release induced by lower concentrations of palytoxin is primarily brought about by increasing tetrodotoxin-insensitive Na^+ permeability across the cell membrane, whereas that induced by higher concentrations is mainly caused by a direct increase in Ca^{2+} influx into them.

INTRODUCTION

PTX,[1] isolated from the zoanthid *Palythoa* species (1), is one of the most potent marine toxins known. Recently, the molecular structure of the toxin was determined fully (2), and the molecular weight of the compound was calculated to be 2677. Many pharmacological studies have shown that PTX induces contractions in smooth muscles (3) and has inotropic actions in heart muscles (4–7). PTX also produces membrane depolarization in intestinal smooth (8, 9), skeletal (5, 6, 9), and heart muscles (6, 7), myelinated fibers (10), and spinal cord (11). Furthermore, PTX has an ability to release NE from adrenergic nerve endings of the guinea pig vas deferens (12). In spite of the availability of information concerning the stimulating action of PTX on muscles, there remains a lack of understanding of the underlying mechanisms involved.

It has been demonstrated that a pheochromocytoma cell line derived from a rat pheochromocytoma established by Green and Tishler (13) can synthesize, store, release, and take up neurotransmitters such as NE, dopamine, and acetylcholine (13–15). These cells have been used extensively as a model system for studying catecholamine metabolism in adrenergic neurons. The present study was undertaken to define the mode of the NE-releasing action of PTX using pheochromocytoma cells.

[1] The abbreviations used are: PTX, palytoxin; NE, norepinephrine: PC12h, rat pheochromocytoma cell line: TTX, tetrodotoxin: Hepes, 4-(2-hydroxyethyl)-1-piperazineethane sulfonic acid.

EXPERIMENTAL PROCEDURES

Materials. PTX, isolated from *Palythoa tuberculosa*, was donated by Prof. Y. Hirata, of Meijo University, and Dr. D. Uemura, of Shizuoka University. This toxin was dissolved in distilled water at concentrations of 10 nM–10 mM and kept frozen as stock solutions. Monazomycin was donated by Prof. N. Ōtake, of the University of Tokyo. Dulbecco's modified Eagle's medium and horse serum were purchased from Gibco (Grand Island, N. Y.). Newborn calf serum was purchased from Nakashibetsu Serum Center, Mitsubishi-Kasei Chemical Industry Co., Ltd. (Tokyo). The following materials were obtained from the companies indicated: $^{45}CaCl_2$, Amersham; ^{22}NaCl and (−)-[7-^{3}H]NE (specific activity, 46.5 Ci/mmole), New England Nuclear Corporation (Boston, Mass.); TTX, Sankyo (Tokyo); A-23187 (divalent cation ionophore) and dicetyl phosphate, Sigma Chemical Company (St. Louis, Mo.); monensin (carboxylic ionophore), Eli Lilly (Indianapolis, Ind.); and lecithin, Merck & Company (Rahway, N. J.). All other chemicals were reagent grade.

Culture of pheochromocytoma cells. PC12h cells were kindly supplied by Dr. H. Hatanaka, of Mitsubishi-Kasei Institute of Life Sciences. These cells were subcloned by Dr. Hatanaka (16) from pheochromocytoma cells established by Drs. Green and Tishler (13). The PC12h cells were maintained in Dulbecco's modified Eagle's medium containing 5% heat-inactivated horse serum. Two days before the experiments,

0026-895X/84/030379-05$02.00/0

the cells were subcultured on a polylysine-coated Falcon dish (35 mm) at a density of 1.0×10^6 cells/dish.

Incubation media. The normal assay medium had the following composition (millimolar): NaCl, 130; KCl, 5.4; $CaCl_2$, 1.8; $MgSO_4$, 0.8; glucose, 5.5; ascorbic acid, 1.2; and Hepes, 50 (pH 7.3). For Ca^{2+}-free solution, $CaCl_2$ was omitted. Low- and high-concentration Ca^{2+} solutions were composed of normal medium in which $CaCl_2$ was reduced or increased, respectively. When Na^+-free or low-concentration Na^+ solution was used, NaCl was replaced with isotonic sucrose.

Measurement of [^{3}H]NE release. Experiments to determine [^{3}H]NE release from PC12h cells were performed as described previously (17). The cells were washed with the assay medium and incubated at 37° for 60 min in 1.0 ml of assay medium containing 0.25 μCi (specific activity, 46.5 Ci/mmole) of [^{3}H]NE. Following the incubation, the cells were washed twice with the fresh medium and then incubated at 37° with 1.0 ml of [^{3}H]NE-free medium for an additional 60 min. Cultures were then washed three times and incubated for 1-min periods with 1.0 ml of the assay medium. The incubated medium was collected from the dishes by careful suction and poured directly into scintillation vials. After two 1-min intervals, the cells were stimulated by adding PTX to the various test condition media. The incubated media were collected into scintillation vials for determination of released [^{3}H]NE. At the end of the experiment, the cells were solubilized with 1.0 ml of 1% Triton X-100, and 0.1 ml of the solubilized cell medium was assayed for radioactivity. Radioactivity was determined by the use of toluene-Triton scintillation mixture. The amount of [^{3}H]NE released in the medium was expressed as the percent of total [^{3}H]NE stored in the cells at the beginning of each period.

Isolation of [^{3}H]NE. [^{3}H]NE released from PC12h cells by PTX was isolated according to the method of Greene and Rein (15), using columns of alumina and Dowex 50-X4 (200- to 400-mesh) (Na^+).

Measurement of $^{22}Na^+$ and $^{45}Ca^{2+}$ influxs. Experiments to determine $^{22}Na^+$ and $^{45}Ca^{2+}$ influxes were performed according to the modified method of Catterall and Nirenberg (18). The culture medium was removed and replaced with the assay medium (1.0 ml/dish). Measurement of influx was initiated by exposure to assay medium (0.7 ml/dish) containing 1.0 μCi of $^{22}Na^+$ or 1.0 μCi of $^{45}Ca^{2+}$, or that supplemented with PTX. Following the desired incubation period at 37°, the radioactive assay medium was aspirated, and then the cells were washed promptly with fresh medium four times. The washed cells were collected into test tubes using 0.2 ml of water and 0.2 ml of 0.2 N NaOH. The radioactivity of $^{22}Na^+$ of the combined cell suspension was determined with a gamma counter (Beckman, Gamma 8000). The amount of influx $^{22}Na^+$ was calculated as nanomoles per milligram of cell protein. For determination of $^{45}Ca^{2+}$, 0.3 ml of the suspension was transferred to scintillation vials containing 6.0 ml of toluene-Triton scintillation mixture, and the radioactivity was counted with a liquid scintillation spectrometer (Packard, Model 3385).

Measurement of lactate dehydrogenase. Assay of lactate dehydrogenase activity was carried out according to the method of Takahashi *et al.* (17). After two 1-min exposures to PTX (1 μM), the incubated medium was collected from the dish and stored at 0°. The cells were removed from the dish and lysed by sonication for 10 sec. Determination of lactate dehydrogenase activity was initiated by addition of the aliquots of the incubated medium or the cell lysate to 3.0 ml of reaction mixture containing 300 μmoles of sodium phosphate buffer (pH 7.0), 1.0 μmole of sodium pyruvate, and 0.35 μmole of NADH. Reactions were measured at a wavelength of 340 nm using a Hitachi 100-50 recording spectrophotometer.

Assay of ionophore activity on light scattering changes in rat liver mitochondria. Mitochondria were prepared from livers of male rats as described by Johnson and Lardy (19) and were suspended in 0.25 M sucrose solution (5 mM Tris-HCl, pH 7.4). A portion (30 μl) of this suspension (40 mg of mitochondrial protein) was added to the reaction mixture (3 ml) containing 150 mM sucrose, 3 mM Tris-ATP, 5 mM $MgCl_2$, 20 mM Tris-acetate (pH 7.4), and 30 mM NaCl or 30 mM $CaCl_2$. According to the method of Graven *et al.* (20), mitochondrial swelling induced by drugs was monitored at 25° by measuring the turbidity changes in the suspension at 515 nm.

Measurement of ionophore activity on liposomes. Liposomes containing lecithin and dicetyl phosphate were prepared by the method of Inoue (21). The molar ratio of lecithin:dicetyl phosphate in liposomes was 1:0.1. Liposomes containing 67 mM Ca^{2+} and 50 mM Na^+ were incubated with 300 mM glucose solution (5 mM Tris-HCl, pH 7.4) with or without PTX. The permeated ions were removed by dialysis of the glucose solution for 6 hr at room temperature. The remaining ions in liposomes were measured using an atomic absorption spectrophotometer (Varian, AA-175).

Protein assay. Protein was determined as described by Bradford (22), using bovine plasma albumin as the standard.

RESULTS

[^{3}H]NE release. PTX (1 nM–1 μM) caused a concentration-dependent release of [^{3}H]NE from PC12h cells (Fig. 1). The maximal response was about 30% of total preloaded [^{3}H]NE and was obtained with concentrations of PTX in the 100 nM–1 μM range. Figure 2 shows the stimulated release of [^{3}H]NE from PC12h cells during consecutive 1-min exposures to 30 nM PTX. The rate of release attained a maximum within the first 1 min and gradually decreased thereafter. About 90% of the radioactive materials released from PC12h cells by PTX (1 μM) was recovered in the NE fraction. The effects of Co^{2+} (10 mM) and TTX (1 μM) on the PTX (30 nM)-induced [^{3}H]NE release from PC12h cells were tested. The release of [^{3}H]NE by PTX at 30 nM and 1 μM was inhibited by 40% and 43%, respectively, by treatment with Co^{2+} and was nearly abolished by incubation with Ca^{2+}-free solution. However, the release was not affected by TTX. The external concentration of Na^+ in the Hepes medium was varied from 0 to 130 mM. The removal of external Na^+ nearly abolished the release of [^{3}H]NE induced by PTX at 30 nM. As shown in Fig. 3, the release of [^{3}H]NE induced by PTX (30 nM) increased propor-

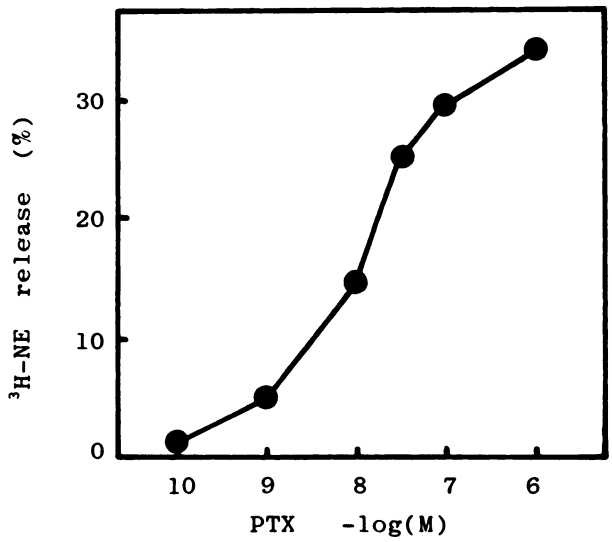

FIG. 1. *Dose-response curve for PTX in [^{3}H]NE release from PC12h cells*

PC12h cells preloaded with [^{3}H]NE were exposed for 2 min to the assay medium containing various concentrations of PTX. The amount of [^{3}H]NE released in the medium is expressed as percentage of total [^{3}H]NE stored in the cells. Each point represents the mean of results from duplicate culture dishes.

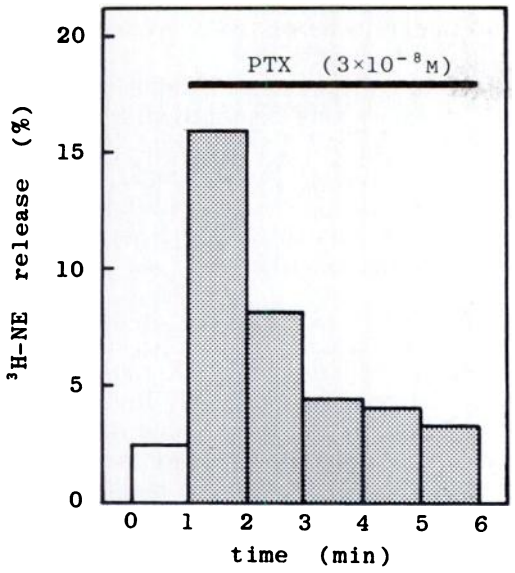

FIG. 2. *Time course of [³H]NE release induced by PTX from PC12h cells*

After a 2-min preincubation, PC12h cells preloaded with [³H]NE were incubated with a PTX-containing solution. The assay medium was changed at 1-min intervals. The amount of [³H]NE released in the medium is expressed as percentage of total [³H]NE stored in the cells. Each point represents the mean of results from duplicate culture dishes.

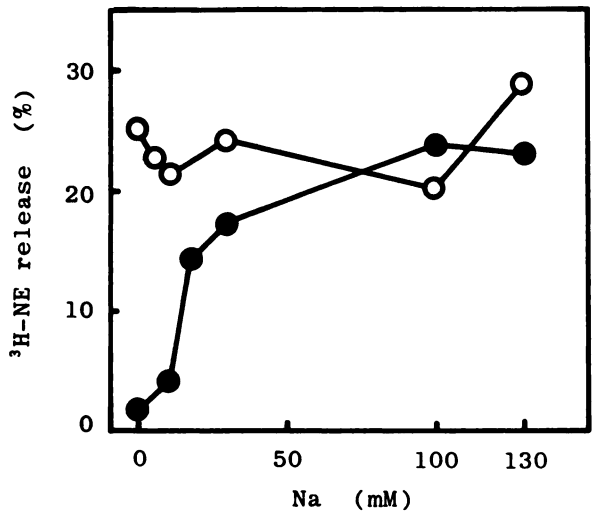

FIG. 3. *Effect of varying the concentration of external Na^+ on [³H]NE release induced by PTX from PC12h cells*

PTX was added after a 2-min incubation in desired concentrations of Na^+. Measurements of [³H]NE released were made 2 min after the application of 30 nM (●) or 1 μM (○) PTX. The amount of [³H]NE released in the medium is expressed as percentage of total [³H]NE stored in the cells. Each point represents the mean of results from triplicate culture dishes.

tionately with increasing external Na^+ concentrations up to 25 mM, and attained a maximum around 100 mM. However, the amount of [³H]NE release induced by 1 μM PTX was not changed by varying the Na^+ concentration from 0 to 130 mM. On the other hand, as shown in Fig. 4, the [³H]NE release induced by PTX (30 nM and 1 μM) increased in a linear fashion as the external Ca^{2+} concentration was increased from 0 to 2 mM and attained the maximum around 10 mM Ca^{2+}.

^{22}Na and ^{45}Ca influxes. The concentration-response

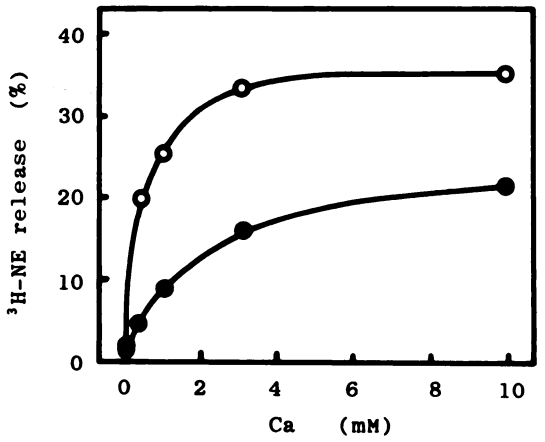

FIG. 4. *Effect of varying the concentration of external Ca^{2+} on [³H]NE release induced by PTX from PC12h cells*

PTX was added after a 2-min incubation in desired concentrations of Ca^{2+}. Measurements of [³H]NE released were made 2 min after application of 30 nM (●) or 1 μM (○) of PTX. The amount of [³H]NE released in the medium is expressed as percentage of total [³H]NE stored in the cells. Each point represents the mean of results from triplicate culture dishes.

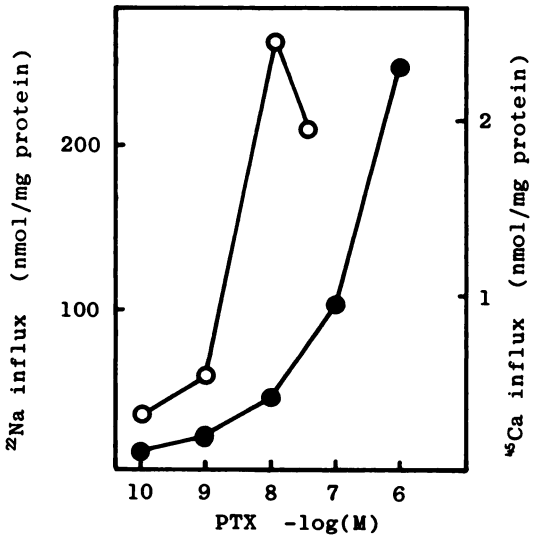

FIG. 5. *Concentration-response curve for PTX in ^{22}Na and ^{45}Ca influxes into PC12h cells*

PC12h cells were incubated for 2 min in the assay medium containing desired concentrations of PTX and 1 μCi of ^{22}Na (○) or ^{45}Ca (●). Each point represents the mean of results from duplicate culture dishes.

curve for the PTX-stimulated ^{22}Na influx into PC12h cells is shown in Fig. 5. PTX caused a concentration-dependent increase in ^{22}Na influx at concentrations above 0.1 nM. The maximal response to PTX was obtained with a concentration of 10 nM. The time course of ^{22}Na influx induced by PTX (30 nM) into PC12h cells is shown in Fig. 6. The influx of ^{22}Na increased linearly for 6 min and then reached a plateau. ^{22}Na influx induced by PTX (30 nM and 100 nM) was not modified by treatment with TTX (500 nM).

As shown in Fig. 5, ^{45}Ca influx induced by PTX was increased with increasing PTX concentrations from 1 nM to 1 μM. Figure 7 shows the time course of ^{45}Ca influx into PC12h cells after addition of PTX (30 nM). PTX-

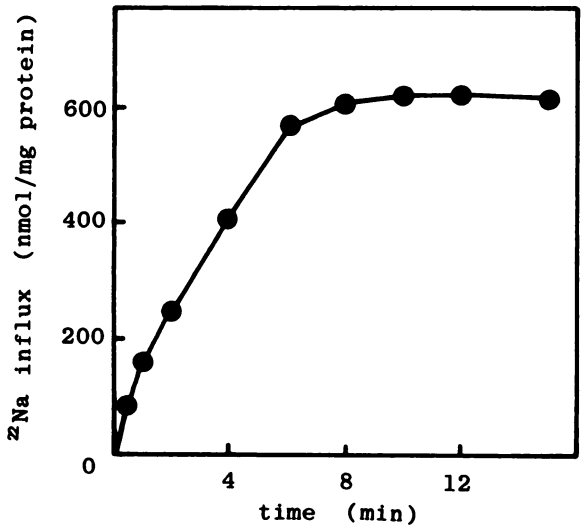

FIG. 6. *Time course of ^{22}Na influx into PC12h cells induced by PTX*
PC12h cells were incubated for desired periods in the assay medium containing 1 μCi of ^{22}Na and 30 nM PTX. Each point represents the mean of results from triplicate culture dishes.

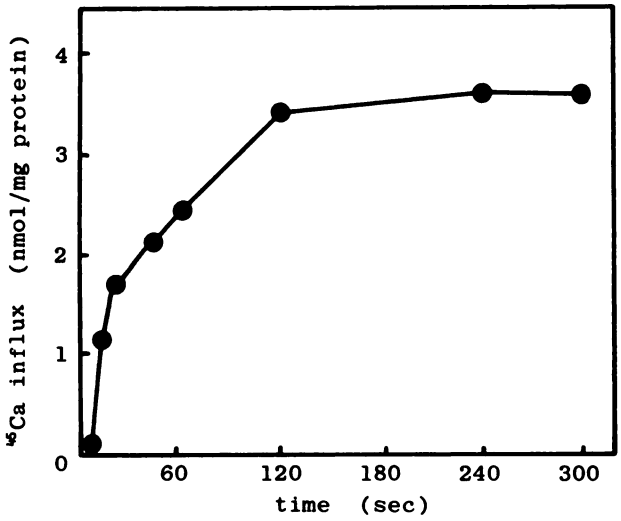

FIG. 7. *Time course of ^{45}Ca influx into PC12h cells induced by PTX*
PC12h cells were incubated for indicated periods in the assay medium containing 1 μCi of ^{45}Ca and 30 nM PTX. Each point represents the mean of results from triplicate culture dishes.

induced ^{45}Ca influx was rapidly increased and then reached a plateau 2 min after application of PTX. The PTX (100 nM)-induced ^{45}Ca influx was inhibited by approximately 40% in the presence of Co^{2+} (10 mM), but was only slightly (by 7%) decreased in Na-free medium.

Lactate dehydrogenase. After a 2-min incubation with PTX (1 μM), only 2% of the total cell lactate dehydrogenase content was released from PC12h cells.

Ionophore activity. Addition of monazomycin (1 μM) to mitochondrial suspensions caused a downward deflection of the light-scattering trace. The downward deflection is associated with mitochondrial swelling. After treatment with monazomycin, the cumulative application of monensin (10 nM to 100 nM) induced an upward deflection of the light-scattering trace in a concentration-dependent manner, whereas PTX (10 nM to 1 μM) had no effect on the turbidity.

The effects of PTX, monensin, and A23187 on the ion content of liposomes were examined after treatment with these drugs for 1 hr. After treatment with monensin (1 μM) and A23187 (50 μM), the Na^+ and Ca^{2+} contents of liposomes decreased by 85% and 40%, respectively, as compared with the control. PTX (1 μM), however, did not alter the content of either ion.

DISCUSSION

In the present experiments PTX caused a profound increase in the release of [^{3}H]NE from PC12h cells. Lactate dehydrogenase, a cytoplasmic marker, was not released during the period of [^{3}H]NE release by a high concentration of PTX (1 μM). The radioactive material released from PC12h cells by PTX was confirmed to be [^{3}H]NE. From these results, it is suggested that PTX-induced NE release from PC12h cells does not occur by cell lysis but by a secretion process. The release of [^{3}H]NE induced by a low concentration (30 nM) of PTX was nearly abolished by removal of Na^+ or Ca^{2+} from the medium, and was markedly inhibited by Co^{2+}. This effect of PTX was dependent upon the concentrations of external Na^+ or Ca^{2+}. The assay of ^{22}Na and ^{45}Ca influxes into PC12h cells indicated that PTX (0.1 nM–30 nM) markedly increased ^{22}Na entry and significantly elevated ^{45}Ca entry. Furthermore, electrophysiological studies by other investigators have indicated that PTX induces membrane depolarization of frog sartorius (5), guinea pig papillary muscle (6), and frog myelinated fibers (10). Depolarization-secretion coupling in neurons is considered to be mediated by the influx of Ca^{2+} through voltage-sensitive Ca channels in the cell membranes (23–25). These observations probably suggest that lower concentrations of PTX induce membrane depolarization, resulting from an increasing Na^+ permeability across the cell membrane, and that this depolarization leads to an increase in Ca^{2+} influx into PC12h cells, which occurs through voltage-dependent Ca^{2+} channels, resulting in [^{3}H]NE release.

On the other hand, further increases in the concentration of PTX up to 100 nM–1 μM caused an additional release of [^{3}H]NE from PC12h cells and influx of ^{45}Ca. The release of [^{3}H]NE by PTX at 1 μM was inhibited or abolished by Co^{2+} or in Ca^{2+}-free solution and was dependent upon external Ca^{2+}. However, this effect of PTX was still observed in the absence of Na^+. It has been reported that iontophoresis of Ca^{2+} into the presynaptic terminals of giant synapses of squid triggers transmitter release without depolarization, indicating that an increase in the intracellular free Ca^{2+} concentration is sufficient to cause a transmitter release (26). These observations suggest that high concentrations of PTX are able to cause a sufficient increase in Ca^{2+} influx into PC12h cells to induce a marked increase in the release of [^{3}H]NE from them without external Na^+. This may be the reason why high concentrations of PTX cause a Na^+-independent release of [^{3}H]NE from PC12h cells. On the basis of these data, it is suggested that the [^{3}H]NE release induced by higher doses of PTX may be due

primarily to an increase in the permeability of the cell membrane to Ca^{2+}, although it is partially attributed to an increase in the Na^+ membrane permeability.

There have been many reports indicating that TTX has little or no effect on contractions of muscles (6, 8), depolarization of excitable membranes (5, 10, 11), or NE release from adrenergic nerves (12) induced by PTX. Also, in the present experiments, both the PTX-induced [^{3}H]NE release and $^{22}Na^+$ influx were abolished by Na^+-free medium, but were not affected by TTX. These observations suggest that the PTX-induced increase in Na^+ permeability of the PC12h cell membrane is due to activation of TTX-insensitive Na^+ channels. It has been reported that ionophores form complexes with a variety of cations and transport them across cell or artificial membranes (27, 28). In rat pheochromocytoma cells, monensin, an ionophore for monovalent cations, and A23187, a divalent-cation ionophore, have been shown to cause a marked release of catecholamine (18, 29, 30). In order to clarify the mechanism of action of PTX on ion transport across the cell membrane, the ionophore activities of PTX were examined using membranes of mitochondria and liposomes. Monensin and A23187 showed powerful Na^+ and Ca^{2+} ion-transporting activities, respectively, whereas PTX, even at high concentrations, had no ionophoretic activity. On the basis of these results, it is concluded that the mechanism of action of PTX in increasing Na^+ permeability is clearly distinct from that of ionophores. Also, these results lead to the concept that PTX, at lower concentrations, acts on specific sites, probably TTX-resistant Na^+ channels, which regulate the permeability of the cell membrane to Na^+.

ACKNOWLEDGMENTS

We are gratefully indebted to Prof. Y. Hirata, Meijo University, and Dr. D. Uemura, Shizuoka University, for kindly supplying palytoxin, and to Prof. N. Ōtake, University of Tokyo, for kindly donating monazomycin. We are also grateful to Dr. H. Hatanaka, Mitsubishi-Kasei Institute of Life Sciences, for kindly supplying PC12h cells.

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