Effect of Anabasine on Catecholamine Secretion From the Perfused Rat Adrenal Medulla

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Abstract

Objectives. The present study was designed to investigate the characteristic effects of anabasine on secretion of catecholamines (CA) from the isolated perfused rat adrenal gland and to establish its mechanism of adrenomedullary secretion.

Methods. The adrenal gland was isolated by a modification of the Wakade method, and perfused with normal Krebs-bicarbonate solution. The content of CA was measured using fluorometry.

Results. The perfusion of anabasine (30−300 μM) into an adrenal vein for 60 min resulted in great increases in CA secretions in a dose-dependent fashion. Upon repeated injection of anabasine (100 μM) at 120 min-intervals, CA secretion was rapidly decreased after the third injection of anabasine. However, there was no statistical difference between the CA secretory responses of both 1st and 2nd treated groups by the successive administration of anabasine at 120 min-intervals. Tachyphylaxis to the releasing effects of CA evoked by anabasine was observed by repeated administration. Therefore, in all subsequent experiments, anabasine was not administered successively more than twice at only 120 min-intervals. The CA-releasing effects of anabasine were depressed by pretreatment with chlorisondamine (selective neuronal nicotinic receptor antagonist, 1 μM), atropine (muscarinic receptor antagonist, 2 μM), nicardipine (L-type dihydropyridine Ca^{2+} channel blocker, 1 μM), TMB-8 (anti-releaser of intracellular Ca^{2+}, 30 μM), and perfusion of EGTA (Ca^{2+} chelator, 5 mM) plus Ca^{2+}-free medium. In the presence of anabasine (100 μM), the CA secretory responses induced by acetylcholine (5.32 mM), high K^+ (direct membrane-depolarizer, 56 mM), DMPP (selective neuronal nicotinic receptor agonist, 10^{-4}M), and McN-A-343 (selective muscarinic M_1 receptor agonist, 10^{-4}M) were maximally enhanced in the first 4 min. However, as time elapsed, these responses became more inhibited at later periods. Furthermore, the perfusion of nicotine (30 μM) into an adrenal vein for 60 min also caused a great increase in CA secretion, leading to peak response in the first 0−5 min period. In the presence of nicotine (30 μM), the CA secretory responses induced by acetylcholine, high K^+, DMPP and McN-A-343 were also enhanced for the first 4 min, but later reduced to less than the control release.

Conclusions. Taken together, these experimental results indicate that anabasine affects rat adrenomedullary CA secretion in a calcium-dependent fashion. This facilitatory effect of anabasine may be mediated by activation of both cholinergic nicotinic and muscarinic receptors, which is relevant to both stimulation of Ca^{2+} influx into adrenomedullary chromaffin cells and Ca^{2+} release from cytoplasmic Ca^{2+}. Anabasine may be less potent than nicotine in rat adrenomedullary CA secretion. Anabasine, in addition to nicotine, alkaloids present in tobacco smoke may be a risk factor in causing cardiovascular diseases.

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INTRODUCTION

In addition to S(−)-nicotine, several minor tobacco alkaloids [(+/−)-nornicotine, anabaseine, S(−)-anabasine, and S(−)-N-methylanabasine] are present in tobacco smoke. These alkaloids are

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found to increase fractional ^{3}H release in a concentration-dependent manner from rat striatal slices preloaded with [^{3}H]dopamine, with desensitization of this response.[1] The genus *Anisothea* plant also contains the bipiperidyl alkaloid anabasine and the bicyclic quinolizidine lupinine.[2] Anabasine (**Fig. 1**), caffeine, methylpyrrolidine and several derivatives have moderate inhibitory activity of acetylcholinesterase with I_{50} values in the range of 87–480 μM.[3]
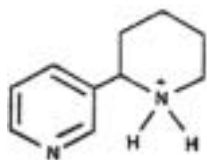

Fig. 1   Chemical structure of anabasine

In human granulosa cells, cigarette alkaloids (nicotine, cotinine and anabasine) may inhibit cellular progesterone synthesis both by inhibiting progesterone synthesis and by causing less specific toxic effects to the cell. In contrast, cigarette smoke alkaloids slightly stimulated or had no effect on estradiol production.[4] These concomitant actions of cigarette alkaloids partly explain the higher incidence of early abortion in pregnant women who smoke. Chronic treatment of mice with (−)-nicotine and anabasine increased the number of nicotinic binding sites, whereas lobeline did not affect the density of nicotinic acetylcholine (ACh) receptors.[5] Anabasine attenuated MK-801-elicited popping at a dose that did not cause clonic seizures.[6] Behaviors elicited by MK-801 in mice reflect a pharmacologically induced state of *N*-methyl-D-aspartate receptor hypofunction, which may be present in schizophrenia. Although the maximum currents generated by anabaseine and anabasine at the α_7 receptors are equivalent to that of ACh, the maximum response to nicotine was only about 65% of the ACh response. At $\alpha_4\beta_2$ receptors, the affinities and apparent efficacies of anabaseine and anabasine are much less than that of nicotine. Anabaseine, nicotine and anabasine were nearly equipotent on sympathetic (PC12) receptors, although parasympathetic (myenteric plexus) receptors are much more sensitive to anabaseine and nicotine but less sensitive to anabasine.[7] The influence of anabasine on the release of catecholamines (CA) has not been previously reported.

The present study investigated whether anabasine can modify the release of CA from the isolated perfused model of the adrenal gland. Therefore, the present study examined the effect of anabasine, a relatively selective α_7-nicotinic ACh receptor agonist, on CA secretion from the isolated perfused model of the rat adrenal gland, in comparison with the responses to nicotine, to establish the mechanism of action. The present study is the first work in which the faciltatory effect of anabasine on the CA secretion from the perfused model of rat adrenal gland was demonstrated.

MATERIALS AND METHODS

Experimental procedure

Male Sprague-Dawley rats, weighing 180 to 300 g, were intraperitoneally anesthetized with thiopental sodium (40 mg/kg). The adrenal gland was isolated by the methods described previously.[8] The abdomen was opened by a midline incision, and the left adrenal gland and surrounding area were exposed by the placement of three-hook retractors. The stomach, intestine and portion of the liver were not removed, but pushed over to the right side and covered by saline-soaked gauge pads. The urine in the bladder was removed to obtain enough working space for tying blood vessels and cannulation.

A cannula, used for perfusion of the adrenal gland, was inserted into the distal end of the renal vein after all branches of adrenal vein (if any), vena cava and aorta were ligated. Heparin (400 IU/ml) was injected into vena cava to prevent blood coagulation before ligating vessels and cannulation. A small slit was made into the adrenal cortex just opposite entrance of adrenal vein. Perfusion of the gland was started, making sure that no leakage was present, and the perfusion fluid escaped only from the slit made in adrenal cortex. Then the adrenal gland, along with ligated blood vessels and the cannula, was carefully removed from the animal and placed on a platform of a leucite chamber. The chamber was filled with water continuously circulated at 37 ± 1 °C. Animal care followed the criteria of the Animal Care Committee of the Chosun University for the care and use of laboratory animals in research.

Perfusion of adrenal gland

The adrenal glands were perfused using an ISCO

pump (WIZ Co.) at 0.31 ml/min. The perfusion was carried out with Krebs-bicarbonate solution of the following composition (mM): NaCl, 118.4; KCl, 4.7; CaCl$_2$, 2.5; MgCl$_2$, 1.18; NaHCO$_3$, 25; KH$_2$PO$_4$, 1.2; glucose, 11.7. The solution was constantly bubbled with 95% O$_2$ + 5% CO$_2$ and the final pH of the solution was maintained at 7.4−7.5. The solution contained disodium ethylenediamine tetraacetic acid (EDTA) (10 μg/ml) and ascorbic acid (100 μg/ml) to prevent oxidation of CAs.

Drug administration

Anabasine (10^{-4}M) and nicotine (3 × 10^{-5}M) were perfused into an adrenal vein for 90 min. The perfusions of 1.1-dimethyl-4-phenyl piperazinium iodide (DMPP) (10^{-4}M) for 2 min and 3-(m-chol-ro-phenyl-carbamoyl-oxy)-2-butynyl trimethyl ammonium chloride [McN-A-343] (10^{-4}M), Bay-K-8644 (10^{-5}M) and cyclopiazonic acid (10^{-5}M) for 4 min and/or a single injection of ACh (5.32 × 10^{-3}M) and KCl (5.6 × 10^{-2}M) in a volume of 0.05 ml were made into the perfusion stream via a three-way stopcock, respectively. Preliminary experiments found that upon administration of the above drugs, the secretory responses to ACh, KCl, McN-A-343, Bay-K-8644 and cyclopiazonic acid returned to preinjection level in about 4 min, whereas the responses to DMPP returned in 8 min.

Collection of perfusate

As a rule, prior to stimulation with various secretagogues, the perfusate was collected for 4 min to determine the spontaneous secretion of CA (background sample). Immediately after collection of the background sample, collection of the perfusates was continued in another tube as soon as the perfusion medium containing the stimulatory agent reached the adrenal gland. Stimulated sample was collected for 4 to 8 min. The amounts secreted in the background sample were subtracted from that secreted from the stimulated sample to obtain the net secretion value of CA, which is shown in all of the figures.

To study the effect of anabasine on the spontaneous and evoked secretion, the adrenal gland was perfused with Krebs solution containing anabasine for 90 min. The perfusate was collected for a certain period (background sample). Then the medium was changed to the one containing the secretagogue only or together with anabasine, and the perfusates were collected for the same period as for the back-ground sample. The adrenal gland perfusate was collected in chilled tubes.

Measurement of catecholamines

CA content of perfusate was measured directly by the fluorometric method of Anton and Sayre[9] without the intermediate purification with alumina for the reasons described earlier[8] using a fluorospectrophotometer (Kontron Co.). A volume of 0.2 ml of the perfusate was used for the reaction. The CA content in the perfusate of glands stimulated by secretagogues used in the present work was high enough to obtain readings several times greater than the reading of control samples (unstimulated). The sample blanks were also lowest for perfusates of stimulated and non-stimulated samples. The content of CA in the perfusate was expressed in terms of norepinephrine (base) equivalents.

Statistical analysis

Statistical differences between the control and pretreated groups were determined by the Student's t- and ANOVA-tests. A p value of less than 0.05 was considered to represent statistically significant changes unless specifically noted in the text. Values given in the text refer to means and the standard errors of the mean (S.E.M.). The statistical analysis of the experimental results used by computer program described by Tallarida and Murray.[10]

Drugs and their sources

The following drugs were used: anabasine, nicotine, acetylcholine chloride, DMPP, norepinephrine bitartrate, nicardipine hydrochloride and 3.4.5-trimethoxy benzoic acid 8-(diethylamino) octylester (TMB-8), and atropine sulfate from Sigma Chemical Co., and chlorisondamine chloride from Ciba Co., cyclopiazonic acid, McN-A-343 from RBI. Drugs were dissolved in distilled water (stock) and added to the normal Krebs solution as required except nicardipine, anabasine and nicotine. Nicardipine and anabasine were dissolved in 99.5% ethanol and nicotine in dimethylsulfoxide. Drugs were diluted appropriately (final concentration of alcohol or dimethylsulfoxide was less than 0.1%). Concentrations of all drugs used are expressed in terms of molar base.

RESULTS

Secretory effect of CA evoked by anabasine from the perfused rat adrenal gland

When the adrenal gland was perfused with oxygenated Krebs-bicarbonate solution for 60 min before the experimental protocol was initiated, the spontaneous CA secretion reached steady state. The basal CA release from the perfused rat adrenal medulla amounted to 21 ± 2 ng for 2 min from 8 adrenal glands. The releasing effects of the perfusion of anabasine at doses of 30, 100 and 300 μM for 60 min were produced in a dose-dependent fashion, as shown in **Fig. 2－upper**. The peak responses of the CA secretion were evoked at the first $0-5$ min period after the loading of anabasine at all doses, to 500 ± 54 ng $(0-5$ min$)$ at 30 μM, 713 ± 36 ng $(0-5$ min$)$ at 100 μM, and $1,090 \pm 21$ ng $(0-5$ min$)$ at 300 μM, respectively. As time elapsed, these enhanced effects were gradually inhibited to $38-52$ % of the first period $(0-5$ min$)$. In addition to $S(-)$-nicotine, several minor tobacco alkaloids $[(+/-)$-nornicotine, anabaseine, $S(-)$-anabasine, and $S(-)$-N-methylanabasine$]$ present in tobacco smoke were found to increase fractional ^{3}H release in a concentration-dependent manner from rat striatal slices preloaded with $[^3$H]dopamine, with desensitization of this response.[1] This result seems to be similar to the findings of the present work that anabasine significantly increases the CA release from the perfused rat adrenal medulla. The repetitive time-course effect of anabasine $(100 \ \mu$M$)$ infusion into the perfusion stream for 60 min at 120 min-intervals exerted significant responses of CA secretion over the background release. In 6 rat adrenal glands, the anabasine-evoked CA secretory responses were $733-333$ ng $(0-60$ min$)$ for the 1st period, and $693-320$ ng $(0-60$ min$)$ for the 2nd period at 5 min intervals, respectively. There was no statistically significant difference between the 1st and 2nd period groups, as shown **Fig. 2－lower**. The tachyphylaxis to CA-releasing effects of anabasine was not observed. However, in all subsequent experiments, anabasine was not administered more than twice at 120 min intervals.

Effects of chlorisondamine and atropine on anabasine-evoked CA secretion from the perfused rat adrenal gland
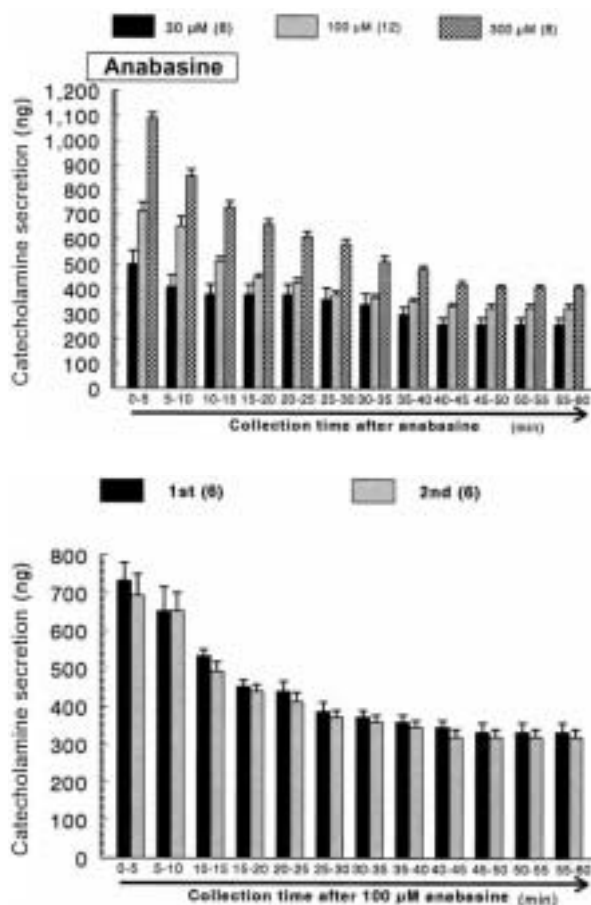
In order to examine the effect of chlorison-

Fig. 2 Effects of anabasine on secretion of catecholamines in the rat adrenal glands

Upper: Dose-dependent effects of anabasine on secretion of catecholamines from the perfused rat adrenal glands. Anabasine at concentrations of 30, 100 and 300 μM was perfused into an adrenal vein twice for 60 min at 120 min interval, respectively. Perfusion of anabasine followed perfusion with normal Krebs-bicarbonate solution for 1 hr before the experimental protocols were initiated. The data are expressed with mean $\pm$ S.E. The perfusate was collected for 60 min at 5 min-intervals. There was significant difference between each dose group. Abscissa: Time of collection (min). Ordinate: Secretion of catecholamine in ng for 5 min. The vertical columns and bars denote means and the standard errors of the corresponding means, respectively. Number in the parenthesis indicates the number of animals used in the experiments.

Lower: Time-course effects of repeated administration of anabasine on the catecholamine secretion in perfused rat adrenal glands. Anabasine (100 μM) was perfused into an adrenal vein for about 60 min at 120 min-intervals after the initiation of perfusion with normal Krebs solution. There was no significant difference between the 1st and 2nd groups.

damine, a selective nicotinic receptor antagonist, on anabasine-induced CA release, the rat adrenal gland was loaded with 10^{-6} M chlorisondamine for 60 min. In the presence of chlorisondamine, the CA outputs evoked by perfusion with anabasine $(10^{-4}$ M$)$ for 60 min amounted to $56-74\%$ of their corresponding control (100%) from 12 experiments $(\textbf{Fig. 3} - \textbf{upper})$.

Two types of muscarinic receptors $(M_1$ and $M_2)$ characterized by high or low affinity for the muscarinic antagonist pirenzepine were present in sympathetic ganglia.[11] Therefore, it would be interesting to examine the effect of atropine, muscarinic antagonist on CA release evoked by anabasine. In the present work, the CA output induced by anabasine was greatly reduced in the rat adrenal gland preloaded with 2×10^{-6} M pirenzepine. In 12 rat adrenal glands, 10^{-4} M anabasine-evoked CA-releasing responses in the presence of atropine were depressed by $50-78\%$ of their control secretions (100%), as shown in **Fig. 3** − **lower.**

Effects of perfusion of Ca^{2+}-free Krebs, nicardipine and TMB-8 on anabasine-evoked CA secretion from the perfused rat adrenal gland

The physiological release of CA and dopamine-β-hydroxylase from the perfused cat adrenal gland is dependent on the extracellular calcium concentration.[12] It was of particular interest to test whether the secretory effect induced by anabasine is also related to extracellular calcium ions. In order to test the effect of nicardipine, a L-type dihydropyridine Ca^{2+} channel blocker,[13] on the anabasine-evoked CA secretion, nicardipine $(10^{-6}$ M$)$ was loaded simultaneously along with anabasine into the adrenal gland for 60 min. In the presence of nicardipine, the CA release induced by simultaneous perfusion of anabasine $(10^{-4}$ M$)$ for 60 min was depressed to $73-83\%$ of the corresponding control response (100%) from 12 rat adrenal glands, as shown in **Fig. 4** − **upper**. Also, the adrenal gland was perfused with calcium-free Krebs solution containing 5×10^{-3} M EGTA for 60 min. In the absence of extracellular calcium, the CA-releasing responses evoked by anabasine $(10^{-4}$ M$)$ were significantly inhibited to $74-86\%$ of their corresponding control response (100%) from 12 rat glands, as shown in **Fig. 4** − **lower**.

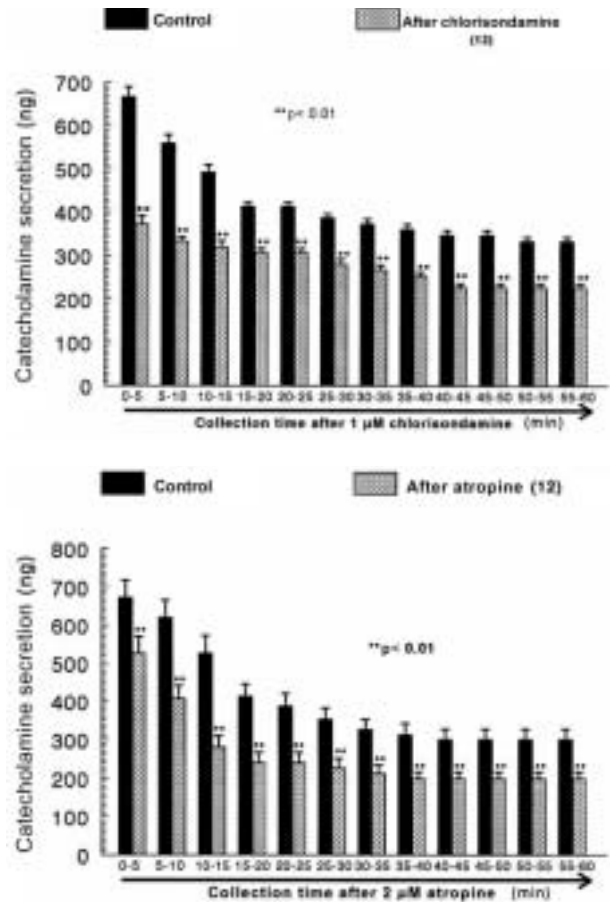Muscarinic, but not nicotinic activation causes the CA secretion independent of extracellular calci-

Fig. 3 **Effect of chlorisondamine** *(upper)* **and atropine** *(lower)* **on the secretion of catecholamine evoked by anabasine in the rat adrenal gland**

Secretion of catecholamines evoked by anabasine $(100$ μM$)$ was evoked for 60 min after perfusion of adrenal gland with Krebs solution containing 1.0 μM chlorisondamine or 2 μM atropine. "Control" and "After" indicate amounts of catecholamine released by anabasine before (Control) and after the preloading with chlorisondamine or atropine. Statistical differences were compared between amounts of catecholamines evoked by anabasine before (Control) and after the pretreatment. Other legends are the same as in Fig. 2.

um in the perfused cat adrenal glands.[14] The presence of an intracellular calcium pool is linked to muscarinic receptors, and TMB-8, an intracellular calcium antagonist, inhibits both nicotinic and muscarinic stimulation-induced CA release in the rat adrenal glands.[15] Therefore, we attempted to examine the TMB-8 on the anabasine-evoked CA secretion. In 8 rat adrenal glands, the CA secretion evoked by perfusion of anabasine $(10^{-4}$ M$)$ after loading with TMB-8 $(10^{-5}$ M$)$ for 60 min were
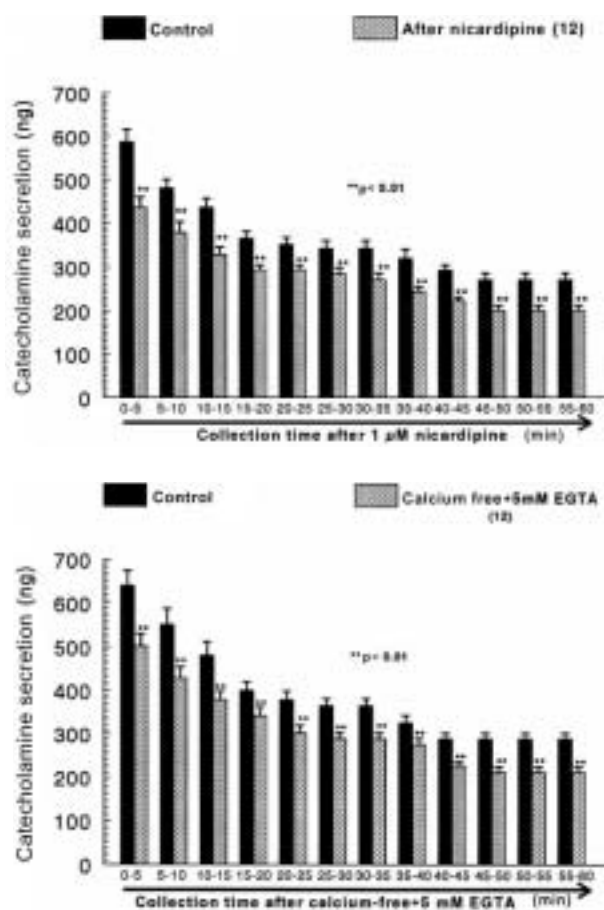
Fig. 4   **Effect of nicardipine (upper) and Ca²⁺-free Krebs-perfusion (lower) on anabasine-evoked catecholamine secretory responses in the rat adrenal gland**

Secretion of catecholamine evoked by anabasine (100 μM) was induced for 60 min following perfusion of adrenal gland with Ca²⁺-free Krebs solution containing EGTA (5 mM) or with Krebs solution containing nicardipine (1.0 μM). Other legends are the same as in Fig. 2.

greatly inhibited to 73−83 % of their corresponding control response (100 %), as shown in **Fig. 5**.

Effects of anabasine on CA secretion evoked by ACh, excess K⁺, DMPP and McN-A-343 from the perfused rat adrenal gland

Figs. 2−5 suggest that anabasine produces the CA secretion from the perfused rat adrenal medulla through cholinergic stimulation in Ca²⁺-dependent fashion. Therefore, it would be interesting to examine the effect of anabasine on the CA secretory responses evoked by ACh, high K⁺, DMPP and McN-A-343 from the isolated perfused rat adrenal
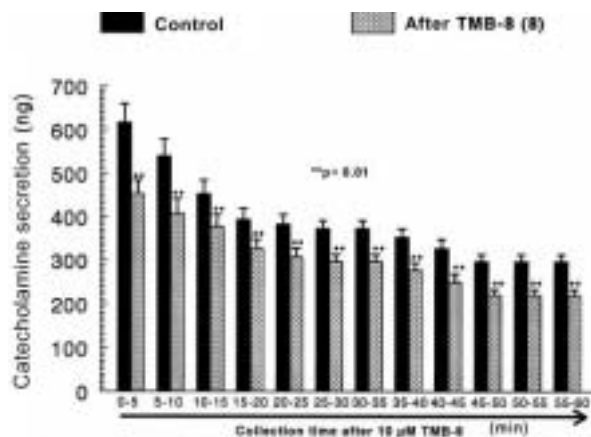

Fig. 5   **Effect of TMB-8 on catecholamine secretion evoked by anabasine in the rat adrenal gland**

TMB-8 (10 μM) was added to the perfusion stream for 60 min after obtaining the corresponding control responses of anabasine (100 μM). Other legends are the same as in Fig. 2.

glands. In order to test the effect of anabasine on cholinergic receptor-stimulated CA secretion as well as membrane depolarization-mediated secretion, 10^{-4}M anabasine was loaded into the adrenal medulla for 60 min. In the present work, ACh (5.32 mM)-evoked CA release before perfusion with anabasine was 2,568 ± 324 ng (0 − 4 min) from 12 rat adrenal glands. However, in the presence of anabasine (10^{-4}M) for 60 min, it was greatly enhanced to 297 % (0 − 4 min) of the control release (100 %) although it was rather inhibited to 72 % of the control response only at last period (60 − 64 min), as illustrated in **Fig. 6 −upper**. The direct membrane-depolarizing agent, like high potassium, sharply stimulates CA secretion. High K⁺ (56 mM)-evoked CA release in the presence of anabasine (10^{-4}M) was significantly enhanced to 123 − 300% of the control secretion (4,036 ± 421 ng, 0−4 min) from 10 glands, but was not affected in the last period (60−64 min), as shown in **Fig. 6 −lower**.

When DMPP (10^{-4}M for 2 min), a selective nicotinic receptor agonist in autonomic sympathetic ganglia, was perfused through the rat adrenal gland, a sharp and rapid increase in CA secretion was evoked. As shown in **Fig. 7 −upper**, DMPP-evoked CA release prior to the perfusion with anabasine was 2,943 ± 121 ng (0−8 min), but in the presence of anabasine (10^{-4}M) was potentiated by 109−315 % of the control. Moreover, in the presence of anabasine (10^{-4}M), the CA secretory
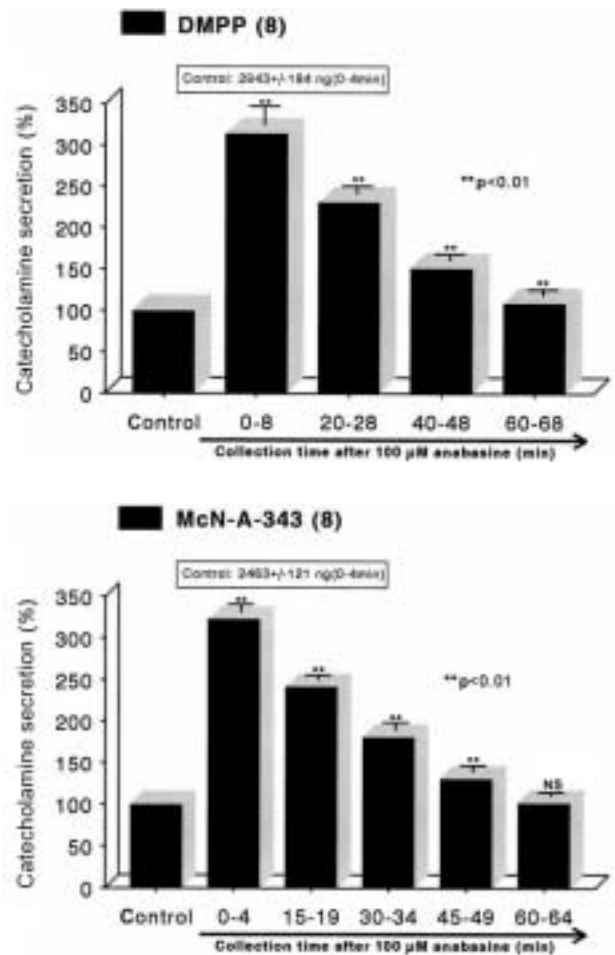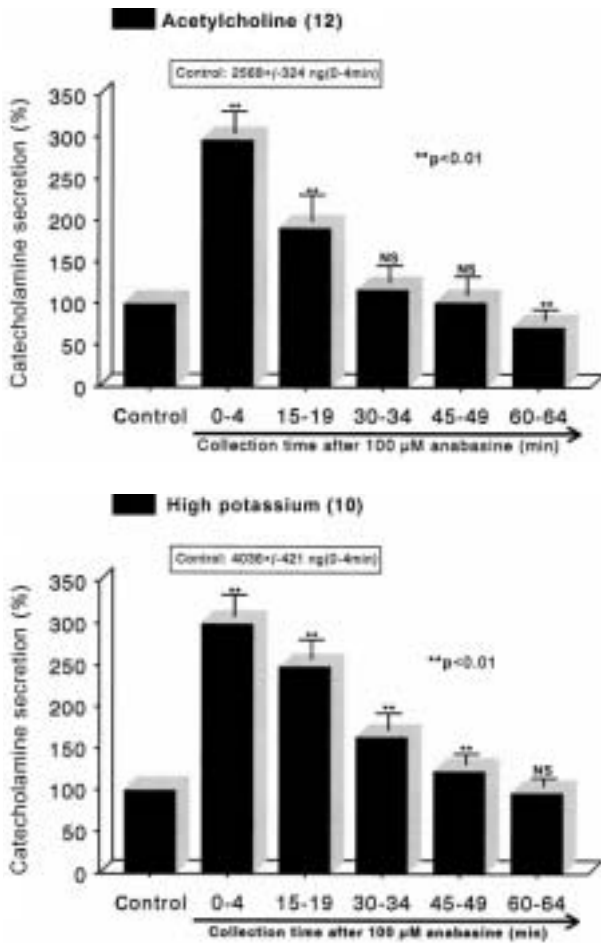
Fig. 6 Effect of anabasine-infusion on catecholamine secretion evoked by acetylcholine (*upper*) and high potassium (*lower*) in the perfused rat adrenal gland

The catecholamine secretory response evoked by acetylcholine $(5.32 \times 10^{-3} M)$ or high potassium $(5.6 \times 10^{-2} M)$ was induced simultaneously along with anabasine $(100 \mu M)$ after obtaining the control response of acetylcholine or high potassium. Statistical differences were obtained by comparing "Control" and "After" preloading with anabasine. The perfusate evoked by acetylcholine or high potassium was collected for 4 min at 15 min interval during the perfusion of anabasine. Other legends are the same as in Fig. 2.

Fig. 7 Effect of anabasine-infusion on catecholamine secretion evoked by DMPP (*upper*) and McN-A-343 (*lower*) in the rat adrenal gland

DMPP $(10^{-4} M)$ or McN-A-343 $(10^{-4} M)$ was perfused into the adrenal vein for 2 min simultaneously along with anabasine $(100 \mu M)$ perfusion after obtaining the control response of DMPP or McN-A-343. The perfusate evoked by DMPP and McN-A-343 was collected for 8 min at 20 min interval and for 4 min at 15 min interval during the perfusion of anabasine, respectively. Other legends are the same as in Figs. 2 and 6.

response evoked by McN-A-343 $(10^{-4} M$ for 4 min), a selective muscarinic M_1 receptor agonist (Hammer and Giachetti, 1982), was enhanced by $131-323 \%$ of the control secretion $(2,463 \pm 121 ng, 0-4 min)$ from 8 glands, but there was no change in the last period $(60-64 min)$, as shown in **Fig. 7 – lower**.

Secretory effect of CA evoked by nicotine from the perfused rat adrenal gland

The present work found that anabasine increases the CA secretion in the perfused rat adrenal gland as shown in **Figs. 2–6.** Therefore, in order to establish whether there is similarity in the CA secretion between nicotine and anabasine in the rat adrenal glands, it would be interesting to determine the effect of nicotine on the CA secretion in the same perfused model of the rat adrenal gland.
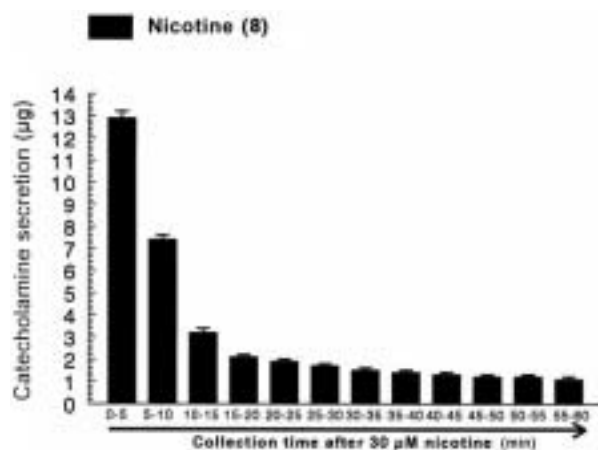
Fig. 8 Time-course effect of nicotine on secretion of catecholamines from the rat adrenal gland

Nicotine (30 μM) was perfused into the adrenal vein for 90 min. Perfusion of nicotine was made after perfusion with normal Krebs-bicarbonate solution for 1 hr before the experimental protocols were initiated. Other legends are the same as in Fig. 2.

As illustrated in **Fig. 8**, the time-course effect of nicotine (3×10^{-5} M) infusion into the perfusion stream for 60 min exerted significant responses of the CA secretion over the background release, leading to the peak release at the first period (0−5 min). In 8 rat adrenal glands, the nicotine (3×10^{-5} M)-evoked CA secretory response was 1,120−12,906 ng (0−60 min). However, it seems likely that nicotine is more potent in CA release than anabasine. Tachyphylaxis to the releasing effects of CA evoked by nicotine was observed (data not shown).

DISCUSSION

These experimental data demonstrate that anabasine causes CA secretion in a calcium-dependent fashion from the isolated perfused rat adrenal gland through activation of neuronal nicotinic ACh receptors as well as partly muscarinic ACh receptors located on the rat adrenomedullary chromaffin cells.

In general, the CA secretion is strongly stimulated by ACh. Released through stimulation of the splanchnic nerve, ACh activates the nicotinic and muscarinic receptors.[16] In bovine chromaffin cells, the cholinergic-stimulated CA secretion is mediated by the activation of nicotinic receptors associated with Na^+ channels. Admittance of Na^+ depolarizes the membrane and activates opening of voltage-dependent Ca^{2+} channels, and thus increases

$[Ca^{2+}]_i$.[17] However, in other species, for instance, the rat,[15,18,19] the cat[20] and the guinea pig,[21,22] nicotinic and muscarinic receptors seem to function in a synergic way in the release of the secretory process. In terms of these findings, in the present work, the anabasine-induced release of CA was due presumably to exocytosis of CA storage vesicles subsequent to activation of nicotinic ACh receptors in the rat adrenomedullary chromaffin cells, since it was inhibited greatly in the presence of chlorisondamine. Chlorisondamine is known to be a selective antagonist of neuronal nicotinic cholinergic receptors.[13] In support of this idea, the affinities of the three nicotinoid compounds (nicotine, anabasine and anabaseine) for rat brain membrane α-bungarotoxin binding sites and their potencies for stimulating *Xenopus* oocyte homomeric α_7 receptors, expressed in terms of their active monocation concentrations, displayed the same rank order, anabaseine > anabasine > nicotine.[7] Although the maximum currents generated by anabaseine and anabasine at α_7 receptors were equivalent to that of ACh, the maximum response to nicotine was only about 65 % of the ACh response. At $\alpha_4\beta_2$ receptors, the affinities and apparent efficacies of anabaseine and anabasine were much less than that of nicotine. Anabaseine, nicotine and anabasine were nearly equipotent on sympathetic (PC12) receptors, although parasympathetic (myenteric plexus) receptors were much more sensitive to anabaseine and nicotine but less sensitive to anabasine.[7] Based on this finding, the present result that annabasine-evoked CA secretion was inhibited by chlorisondamine indicates that anabasine can cause the CA release from the rat adrenal medulla by activation of nicotinic receptors. Moreover, anabasine, a relatively selective α_7-nicotinic ACh receptor agonist, attenuated MK-801-elicited popping at a dose that did not cause clonic seizures.[6] Abnormal promoter variants for genetic expression of the α_7-nicotinic ACh receptor polypeptide subunit, which are located on chromosome 15, have been identified in schizophrenia patients.[23] CA secretion in adrenomedullary chromaffin cells is triggered by a increase of free calcium concentration ($[Ca^{2+}]_i$) in cytoplasm. $[Ca^{2+}]_i$ quickly returns to basal values through the seizure of Ca^{2+} inside intracellular organelles and by extrusion to the extracellular environment.[16, 24] In many cell types, the ATP-dependent calcium pump, in the plasmatic membrane and in cytoplasmatic organelles, and the $Na^+/$

Ca^{2+} exchanger, in the plasmatic membrane, form the main systems of Ca^{2+} extrusion.[25-27] There is evidence that absence of extracellular Na^+/Ca^{2+} changes the direction of the ion movement by means of the Na^+/Ca^{2+} exchanger. This results in Ca^{2+} influx and in the activation of mechanisms of CA secretion in chromaffin cells of the adrenal medulla of many species.[26, 28-31]

Also, in this study, the anabasine-evoked CA secretory response was inhibited by the pretreatment with atropine, in addition to inhibition by the autonomic ganglionic blockade. This finding indicates that anabasine-evoked CA release is exerted at least partly by stimulation of muscarinic ACh receptors. Adrenal medullary cells are derived from the neural crest and share a number of physiological and pharmacological properties with postganglionic sympathetic neurons. Adrenal medullary cells abundantly express muscarinic receptors, including M_1 receptors,[32] which elicit cyclic GMP accumulation in cells.[33] There have been a number of reports that show cyclic GMP accumulation by ACh or muscarine in adrenal medullary cells.[23,34-36] Previously, muscarinic receptors were characterized in bovine adrenal medulla by radioligand binding assay with [³H] quinuclidinyl benzilate.[32] At least two distinct subtypes of muscarinic receptors were found in the adrenal medullary cells, and these receptors were predominantly composed of M_1 receptors. In view of these results, the finding of this study that anabasine-evoked CA release was inhibited by pretreatment with atropine indicates that anabasine-evoked CA secretion is mediated partly through activation of muscarinic M_1 receptor in the perfused rat adrenal gland. In the present work, anabasine-stimulated CA secretion in the perfused rat adrenal medullae in standard Krebs solution in the presence of atropine reduced to 50% of the control release. These data confirm the conclusions of other authors who showed that muscarinic stimulus is active in CA secretion in the adrenomedullary chromaffin cells of rats.[18,19]

The indispensable role of calcium in the neurosecretory process is well established. As mentioned above, calcium plays a crucial role in the depolarization-neurotransmitter release coupling process in many types of secretory cells.[37-39] Furthermore, nicotinic (but not muscarinic) stimulation also releases ACh from the chromaffin cells by a calcium-dependent mechanism.[40] The activation of nicotinic receptors stimulates the secretion of CA

by increasing Ca^{2+} entry through receptor-linked, and/or voltage-dependent Ca^{2+} channels, in perfused rat adrenal glands[18] and isolated bovine adrenal chromaffin cells.[41-43]

Cholinergic stimulus is one of the chief factors in the physiological secretion of catecholamines. The participation of nicotinic and muscarinic receptors was recorded in $[Ca^{2+}]_i$ increase either by extracellular influx or by mobilization of intracellular stores. CA secretion induced by nicotinic stimulus has a higher percentage than the muscarinic one.[44] Nicotinic stimulus increases $[Ca^{2+}]_i$ by extracellular influx, whereas muscarinic stimulus triggers the mobilization of Ca^{2+} intracellular stores.[44] Lack of extracellular Ca^{2+} does not seem to interfere with response to muscarinic stimulus.[45]

In the present study, removal of extracellular Ca^{2+} markedly depressed the secretion of CA evoked by anabasine. The pretreatment of nicardipine, a dihydropyridine derivative, and an L-type Ca^{2+} channel blocker, also blocked the anabasine-evoked CA release. The secretory effect of anabasine apparently seems to be dependent on extracellular calcium. However, in this experiment, the reason for the considerable response to anabasine in the Ca^{2+}-free Krebs plus EGTA solution remains unclear. In the presence of TMB-8, an inhibitor of the intracelluar calcium stores, the anabasine-evoked secretion of CA was greatly inhibited in the perfused adrenal gland. TMB-8 is also known to inhibit caffeine-induced $^{45}Ca^{2+}$ release from, but not its uptake by, a sarcoplasmic reticulum preparation of skeletal muscle,[46] and in isolated bovine adrenomedullary cells.[47,48] Moreover, the caffeine-evoked secretion of CA from the perfused cat adrenal gland in the absence of extracellualr calcium is also inhibited.[49] Activation of muscarinic receptors causes increase of $[Ca^{2+}]_i$ not only by extracellular influx but also by the mobilization of intracellular stores.[30] Mobilizing Ca^{2+} from intracellular reserves, muscarinic agonists stimulate CA secretion even in the absence of extracellular Ca^{2+}[22,45] or after depolarization with high extracellular K^+.[44]

Therefore, this experimental result suggests that chromaffin cells of the rat adrenal gland contain the intracellular calcium store that participates in the secretion of CA, as shown in bovine adrenal glands.[50] Such a store may not be easily depleted by the mere removal of extracellular calcium. Intracellular stores of calcium are involved in the

contraction of smooth muscle produced by nora-drenaline or ACh in Ca^{2+}-free media.[51-55]

Interestingly, in this study, the reason why the secretory responses of CA evoked by ACh, DMPP and McN-A-343 were rather depressed at the later period in the presence of anabasine (continuous infusion) is unclear, although enhanced in the initial period. In support of this idea, anabaseine and anabasine are weak partial agonists upon the $\alpha_4\beta_2$ receptor, displaying 8 and 4%, respectively, of the maximal current elicited by ACh.[7] Therefore, anabasine probably has a partial agonist activity.

In the present study, nicotinic (30 μM) initially enhanced CA secretion evoked by ACh and high K^+, but later rather inhibited the secretion with time-dependency. In the light of these findings, the mode of anabasine action is somewhat different from that of the nicotine action on CA releasing effects evoked by cholinergic stimulation as well as by membrane depolarization in the perfused rat adrenal medulla. However, this difference may be due to the concentrations of these agents (30 μM nicotine and 100 μM anabasine) used in this study. Time course effect of nicotine on CA release in the present work produced a very similar pattern to that of anabasine. In support of this idea, nicotinic action (endogenous ACh, splanchnic nerve stimulation) in CA secretion from the rat adrenal gland is largely reduced (75%) by hexamethonium alone.[18] Based on these results, it seems that there is little difference in mode of action between anabasine and nicotine at least in the rat adrenomedullary CA secretion. In terms of the finding that anabasine appears as a partial agonist in *Xenopus* oocyte,[7] the present finding that anabasine inhibited CA secretory responses evoked by cholinergic stimulation at later period after initial enhancement imply that anabasine has the properties of an antagonist at the nicotinic ACh receptors which mediate the CA secretion in adrenomedullary chromaffin cells.

In the present work, repeated administration of anabasine (10^{-4}M) at 120 min intervals in the perfused rat adrenal gland caused the CA secretory response the rapidly decrease after the third perfusion of anabasine (data not shown). Moreover, the release of CA evoked by the continuous infusion of anabasine was gradually time-dependently reduced from 10 min after the initiation of anabasine infusion in comparison with the initial period (0-5 min). Tachyphylaxis to the releasing effects of CA evoked by anabasine was observed on repeated administration. In support of this finding, the release of CA evoked by DMPP declined abruptly between the first and second periods of exposure to DMPP in isolated rabbit adrenal glands and guinea pig atria.[56] This reduction may be due to the agonist desensitization of the nicotinic receptors. In this study, the repeated perfusion of anabasine from the third application at 120 min-intervals also produced a desensitization-like effect (tachyphylaxis) between the 1st and 3rd periods. However, repetitive perfusion of DMPP in the isolated perfused rat adrenal gland did not produce any desensitization-like effect (tachyphylaxis) between the 1st and 2nd, 3rd periods.[15] From these results, the existence of different species in the CA secretion evoked by anabasine cannot be excluded.

Taken together, these experimental data suggest that anabasine can cause the secretion of CA in a calcium-dependent fashion from the isolated perfused rat adrenal gland through activation of both neuronal nicotinic ACh receptors and partly muscarinic ACh receptors located on the rat adrenomedullary chromaffin cells. Anabasine could also affect the cardiovascular system in a similar fashion to nicotine.

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