2006년 **2**월
박사학위논문

Influence of Anabasine on Catecholamine Secretion from the Perfused Rat Adrenal Medulla

조선대학교 대학원

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이 논문을 의학박사 학위신청논문으로 제출함.

2005 년 10 월 일

조선대학교 대학원

의학과

정민규

정 민 규의 박사학위논문을 인준함

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2005 년 12 월 30 일

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Anabasine이 흰쥐 관류부신수질에서 카테콜아민 분비작용에 미치는 영향

정 민 규

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Anabasine은 담배에 니코틴과 함께 함유되어 있는 minor alkaloid성분의 하나이며, 부교감신경(장근신경총) 수용체에 대해서는 anabaseine 및 nicotine에 비해서 감수성이 약하지만 교감신경 수용체에서 니코틴 및 anabaseine과 동등한 효력을 갖는 것으로 알려져 있다 (Kem 등, 1997). 본 연구에서는 흰쥐 적출 관류부신에서 카테콜아민(CA) 분비작용에 대한 anabasine의 특성을 검색하고 작용기전을 규명코자 시도하여 얻어진 연구결과는 다음과 같다.

Anabasine (30 ~ 300 μM)은 부신정맥 내로 60분간 주입시 흰쥐부신수질로부터 용량의존적으로 뚜렷한 카테콜아민(CA) 분비작용을 나타내었다. Anabasine (100 μM)을 120분 간격으로 반복 투여하면 2번째 투여 이후에는 anabasine의 CA 분비작용은 급격히 감소하였다. 그러나, anabasine의 CA 분비작용은 첫번째 와 두번째 투여에서 통계학적인 차이가 없었다. 이와 같이 anabasine의 반복투여로 anabasine의 CA 분비작용에 대한 반응급강현상(tachyphylaxis)을 관찰 할 수 있었다. 따라서 이후 모든 실험에서 anabasine은 120분 간격으로 2회 이상 연속 투여하지 않았다. Anabasine의 CA 분비작용은 atropine (항무스카린제, 2 μM),

chlorisondamine (자율신경절 니코틴수용체차단제, 1 μM), nicardipine (L형 디히드로피리딘계 칼슘통로차단제, 1 μM) 및 TMB-8 (세포내 저장고에서 칼슘유리 억제제, 30 μM)의 전처치 및 Ca^{2+}-free+ 5mM EGTA (칼슘킬레이터)함유 Krebs 액의 관류에 의해서 현저히 억제되었다. 더욱이, anabasine (100 μM)존재 하에서 아세틸콜린(ACh, 5.32 mM)), 고칼륨 (직접적인 막탈분극제, 56mM), DMPP (선택적인 자율신경절 니코틴수용체 작동제, 100 μM)및 McN-A-343 (선택적인 무스카린성 M_1-수용체 작동제, 100 μM)에 의한 CA 분비작용은 첫 4분간은 현저히 증가되었으나 그 이후에는 시간의 경과에 따라서 점진적으로 억제됨이 관찰되었다. Bay-K-8644 (L형 디히드로피리딘계 칼슘통로개방제, 10 μM) 및 cyclopiazonic acid (세포내 저장고에서 Ca^{2+}-ATPase억제제, 10 μM)에 의한 CA 분비작용도 anabasine (100 μM)의 처치 하에서 첫 4분간은 증가되었으나 그 이후에는 시간 의존적으로 점차 감소되었다. 니코틴은 부신정맥 내로 60분간 주입시 뚜렷한 카테콜아민(CA) 분비작용을 나타내었으며 첫 5분간에서 최고 분비반응을 나타내었다. 또한, 니코틴(30 μM) 존재 하에서 ACh, 고칼륨, DMPP 및 McN-A-343에 의한 CA 분비작용은 첫 4분간은 현저히 증가되었으나 그 이후에는 시간의 경과에 따라서 대조치 이하로 오히려 억제되었다.

이상의 연구결과를 종합하여 보면, anabasine은 흰쥐 적출 관류 부신수질에서 칼슘 의존적으로 카테콜아민 분비작용을 나타내었다. 이와 같은 분비작용은 흰쥐 부신수질의 크롬친화세포에 존재하는 니코틴 수용체 및 일부 무스카린 수용체의 활성화를 통해서 나타나며, 이는 칼슘 유입 및 세포질내 칼슘유리증가와 관련이 있는 것으로 사료된다. Anabasine은 또한 니코틴에 비해 흰쥐 부신수질에서 CA분비작용에 대한 효력이 낮은 것으로 생각된다.

I. 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 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 (Dwoskin et al., 1995). The genus *Anisothea* plant also contains the bipiperidyl alkaloid anabasine and the bicyclic quinolizidine lupinine (Van Wyk et al., 1991). Anabasine, caffeine, methylpyrrolidine and several derivatives have moderate inhibitory activity of acetylcholinesterase (AChE) with I_{50} values in the range of 87-480 μM (Karadsheh et al., 1991).

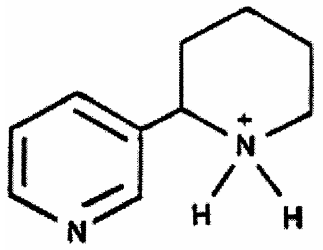

Fig. 1 Chemical structure of anabasine.

In human granulosa cells, it was suggested that cigarette alkaloids (nicotine, cotinine and anabasine) 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 (Gocze et al., 1999). These concomitant actions of cigarette alkaloids partly explain the higher incidence of early abortion in pregnant women

who smoke. Bhat et al. (1991) have earlier reported that chronic treatment of mice with (-)-nicotine and anabasine increased the number of nicotinic binding sites, whereas lobeline did not affect the density of nAChRs. It has been shown that anabasine attenuated MK-801-elicited popping at a dose that did not cause clonic seizures (Mastropaolo et al., 2004). Behaviors elicited by MK-801 in mice reflect a pharmacologically-induced state of NMDA receptor hypofunction (NRH), which has been proposed to exist in schizophrenia. Although the maximum currents generated by anabaseine and anabasine at α7 receptors were equivalent to that of ACh, the maximum response to nicotine was only about 65% of the ACh response. At α4β2 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 (Kem et al., 1997). The influence of anabasine on the release of catecholamines (CA) has not been previously reported. The purpose of the present study is to investigate whether anabasine can modify the release of CA from the isolated perfused model of the adrenal gland. Therefore, the present study was carried out to examine the effect of anabasine, a relatively selective α7-nicotinic ACh receptor agonist , on CA secretion from the isolated perfused model of the rat adrenal gland, in comparison with the responses to nicotine, and to establish the its 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.

II. MATERIALS AND METHODS

Experimental procedure

Male Sprague-Dawley rats, weighing 180 to 300 grams, were intraperitoneally anesthetized with thiopental sodium (40 mg/kg). The adrenal gland was isolated by the methods described previously (Wakade, 1981). 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 and urine in bladder was removed in order to obtain enough working space for tying blood vessels and cannulations.

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 cannulations. 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 continuously circulated with water heated at 37 $\pm$1 ^{o}C (Fig. 2).

Perfusion of adrenal gland

The adrenal glands were perfused by means of ISCO pump (WIZ Co.) at a rate of 0.31 ml/min. The perfusion was carried out with Krebs-bicarbonate solution of

following composition (mM): NaCl, 118.4; KCl, 4.7; $CaCl_2$, 2.5; $MgCl_2$, 1.18; $NaHCO_3$, 25; KH_2PO_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 EDTA (10 μg/ml) and ascorbic acid (100 μg/ml) to prevent oxidation of CAs.

Drug administration

Anabasine (10^{-4} M) and nicotine (3 x 10^{-5} M) were perfused into an adrenal vein for 90 min. The perfusions of DMPP (10^{-4} M) for 2 minutes and McN-A-343 (10^{-4} M), Bay-K-8644 (10^{-5} M) and cyclopiazonic acid (10^{-5} M) for 4 minutes and/or a single injection of ACh (5.32 x 10^{-3} M) and KCl (5.6 x 10^{-2} M) in a volume of 0.05 ml were made into perfusion stream via a three-way stopcock, respectively. In the preliminary experiments, it was found that upon administration of the above drugs, secretory responses to ACh, KCl, McN-A-343, Bay-K-8644 and cyclopiazonic acid returned to preinjection level in about 4 min, but the responses to DMPP 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 the 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's was collected for 4 to 8 min. The amounts secreted in the background sample have been 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. Then, the perfusate was collected for a certain period (background sample). Then the medium was changed to the one containing the secretagogue or along with anabasine, and the perfusates were collected for the same period as that for the background sample. The adrenal gland's perfusate was collected in chilled tubes.

Measurement of catecholamines

CA content of perfusate was measured directly by the fluorometric method of Anton and Sayre (Anton and Sayre, 1962) without the intermediate purification alumina for the reasons described earlier (Wakade, 1981) using fluorospectrophotometer (Kontron Co., Milano, Italy). A volume of 0.2 ml of the perfusate was used for the reaction. The CA content in the perfusate of stimulated glands by secretagogues used in the present work was high enough to obtain readings several folds 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

The statistical difference between the control and pretreated groups was 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 was made by computer program described by Tallarida and Murray (1987).

Drugs and their sources

The following drugs were used: anabasine, nicotine, acetylcholine chloride, 1.1-dimethyl-4-phenyl piperazinium iodide (DMPP), norepinephrine bitartrate, nicardipine hydrochioride and 3.4.5-trimethoxy benzoic acid 8-(diethylamino) octylester (TMB-8), and atropine sulfate from Sigma Chemical Co., U.S.A., and chlorisondamine chloride from Ciba Co., U.S.A., cyclopiazonic acd, (3-(m-cholro-phenyl-carbamoyl-oxy)-2-butynyl trimethyl ammonium chloride [McN-A-343] from RBI, U.S.A.). 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 DMSO. They were diluted appropriately (final concentration of alcohol or DMSO was less than 0.1 %). Concentrations of all drugs used are expressed in terms of molar base.

III. RESULTS

The 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 experimental protocol is 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 galnds. The releasing effects to the perfusion of anabasine at doses of 30, 100 and 300 μM for 60 min are produced in a dose-dependent fashion, as shown in Fig. 3 and Table 1. The peak responses of the CA secretion were evoked at the first 0-5 min period after the loading of anabasine at all doses. They were 500±54 ng (0-5 min) at 30 μM, 713±36 ng (0-5 min) at 100 μM, and 1090±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) are present in tobacco smoke is 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 (Dowskin et al., 1995). This result seems to be similar to the findings of the present work that both anabasine significantly increases the CA release from the perfused rat adrenal medulla. The repetitive time-course effect of anabasine (100 μM) infusion into the perfusion stream for 60 min at 120 min-interval 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 interval, respectively. There was no statistically significant difference between 1st and 2nd period groups, as shown Fig. 4 and table 2. 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 interval.

Effects of atropine and chlorisondamine on anabasine-evoked CA secretion from the perfused rat adrenal gland

In order to examine the effect of chlorisondamine, a selective nicotinic receptor antagonist, on anabasine-induced CA release, the rat adrenal gland was loaded with 10^{-6} M chlorisondamine for 60min was introduced. 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 (Fig. 5 and Table 3).

Hammer and Giachetti (1982) demonstrated that 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. 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 x 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 ~ 93% of their

control secretions (100%), as shown in Fig. 6 and table 3.

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

It has been found that the physiological release of CA and dopamine-β-hydroxylase from the perfused cat adrenal gland is dependent on the extracellular calcium concentration (Dixon et al., 1975). It was of particular interest to test whether the secretory effect induced by anabasine is also related to extracellular calcium ions. Thus, the adrenal gland was perfused with calcium-free Krebs solution containing 5 x 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. 7 and Table 4.

In order to test the effect of nicardipine, a L-type dihydropyridine Ca^{2+} channel blocker (Hardman et al., 1995), on the anabasine-evoked CA secretion, nicardipine (10^{-6} M) was loaded simultaneously along with anabasine into the adrenal gland for 60min. In the presence of nicardipine, the CA releases induced by simultaneous perfusion of anabasine (10^{-4} M) for 60 min was depressed to 73 ~ 83% of their corresponding control response (100%) from 12 rat adrenal glands, as shown in Fig. 8 and table 4. Fig. 8 illustrates the inhibitory effect of nicardipine on the CA secretory responses evoked by anabasine.

It has been reported that muscarinic, but not nicotinic activation causes the CA secretion independent of extracellular calcium in the perfused cat adrenal glands (Nakazato et al., 1988). It is also found that the presence of an intracellular

calcium pool is linked to muscarinic receptors, and that TMB-8, an intracellular calcium antagonist, inhibits both nicotinic and muscarinic stimulation-induced CA release in the rat adrenal glands (Lim and Hwang, 1991). Therefore, it was 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 greatly inhibited to 73 ~ 83% of their corresponding control response (100%), as shown in Fig. 9 and Table 4.

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

In figures 2~8, It is suggested that anabasine produces the CA secretion from the perfused rat adrenal medulla through cholinergic stimulation in Ca^{2+}-dependent fashion. Therefore, it would be interesting to examine effect of anabasine on the CA secretory responses evoked by ACh, high K^+, DMPP and McN-A-343 from the isolated perfused rat adrenal 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 2568 ± 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. 10 and Table 5). It has been found that 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 (12108 ± 1040 ng, 0-4 min) from 10 glands, but there was not affected at last period (60-64 min), as shown in Fig. 11 and Table 5.

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. 12 and table 5, DMPP-evoked CA release prior to the perfusion with anabasine was 9275 ± 672 ng (0-8 min), while in the presence of anabasine (10^{-4} M), it was potentiated by 109 ~ 315% of the control. Moreover, in the presence of anabasine (10^{-4} M), the CA secretory 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 (7951 ± 244 ng, 0-4 min) from 8 glands, but there was no change at last period (60-64 min), as shown in Fig. 13 and Table 5.

Effects of anabasine on CA secretion evoked by Bay-K-8644 and cyclopiazonic acid from the perfused rat adrenal glands

It has been found that Bay-K-8644 is a selective L-type calcium channel activator, which causes positive inotropy and vasoconstriction in isolated tissues and intact animals (Schramm et al., 1982; Wada et al., 1985) and enhances basal Ca^{2+} uptake (Garcia et al., 1984) and CA release (Lim et al., 1992). Therefore, it was of interest to determine the effects of anabasine on Bay-K-8644-stimulated CA secretion from the isolated perfused rat adrenal glands. In the absence of anabasine, Bay-K-8644 (10^{-5} M) given into the perfusion stream produced CA secretion of 128 ±

10 ng (0-4 min). However, in the presence of anabasine (10^{-4} M), Bay-K-8644-stimulated CA secretion was significantly increased by 133 ~ 340% of the corresponding control secretion, but it was not affected at last period (60-64 min), as shown in Fig. 14 and table 14.

Cyclopiazonic acid, a mycotoxin from *Aspergillus* and *Penicillium*, has been described as a highly selective inhibitor of Ca^{2+}-ATPase in skeletal muscle sarcoplasmic reticulum (Goeger & Riley, 1989; Seidler et al., 1989). It may be extremely valuable pharmacological tool for investigating intracellular Ca^{2+} mobilization and ionic current regulated by intracellular calcium (Suzuki et al., 1992). When cyclopiazonic acid (10^{-5} M) was given into the perfusion stream, the CA secreted from the gland amounted to 139 ± 11 ng for 0-4 min. However, as shown in Fig. 15 and table 6, the pretreatment with anabasine (10^{-4} M) potentiated cyclopiazonic acid (10^{-5} M)-evoked CA secretion by 108 ~ 339% of the control response.

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

In the present work, it was found that anabasine increases the CA secretion in the perfused rat adrenal gland as shown in Fig. 3 ~ 15 and table 1 ~ 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.

As illustrated in Fig. 16 and table 7, 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, this nicotine (3 x 10^{-5} M)-evoked CA secretory responses were 1120 ~ 12906 ng (0 ~ 60 min). However, it seems likely that nicotine is more potent in CA release that anabasine. The tachyphylaxis to releasing effects of CA evoked by nicotine was not observed (data not shown). However, in all subsequent experiments, nicotine was not administered more than twice at 120 min-intervals.

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

It has also been found that, in this study, nicotine (3 x 10^{-5} M) greatly increased the CA secretory response. Therefore, it was of interest to determine the effect of nicotine on CA secretion evoked by ACh, high K^+, DMPP and McN-A-343 from the isolated perfused rat adrenal glands. In the present experiment, ACh (5.32 mM)-evoked CA release, before perfusion with nicotine, was 3358 ± 339 ng (0-4 min). In the presence of the nicotine (3 x 10^{-5} M) for 60 min, ACh-evoked CA release initially increased to 153% of the control in the first period (0-4 min). As time elapsed this CA release was gradually reduced to 58% of the control (Fig. 17 and Table 8). High K^+ (56 mM)-evoked CA release, in the presence of the nicotine, was maximally enhanced to 255% of the control secretion (12014 ± 1140 ng, 0-4 min) for the first 4 min period. The CA release of only the last period (96 ± 15%, 60-64 min) returned to the control level, as shown in Fig. 18 and table 8. In 9 rat adrenal glands, DMPP (10^{-5} M) perfused into the

adrenal gland evoked the CA secretion of 8537 ± 659 ng (0-8 min) before loading with nicotine. Following perfusion with the nicotine, it was maximally diminished to a maximum of 19% of the corresponding control release in time-dependent fashion (Fig. 19 and Table 8). Moreover, in the presence of the nicotine, McN-A-343-evoked CA secretory responses were also time-dependently inhibited to 22% of the control secretion (6581± 244 ng, 0-4 min) from 8 glands, as shown in Fig. 20 and table 8.

IV. DISCUSSION

These experimental data demonstrate that anabasine causes the 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 the stimulation of the splanchnic nerve, it activates nicotinic and muscarinic receptors (Yamaguchi, 1992). 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 an increase of $[Ca^{2+}]]_i$ (Yamanara et al., 1986). However, in other species, for instance, the rat (Wakade and Wakade, 1983; Malhotra et al., 1988: Lim and Hwang, 1991), the cat (Abad et al., 1992) and the guinea pig (Misbahuddin and Oka, 1988; Nassar-Gentina et al., 1997), 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 (Hardman et al., 1995). In support of this idea, Kem and his colleagues (1997) have reported that 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 α7 receptors, expressed in terms of their active monocation concentrations, displayed the same rank order, anabaseine>anabasine> nicotine. Although the maximum currents generated by anabaseine and anabasine at α7 receptors were equivalent to that of ACh, the maximum response to nicotine was only about 65% of the ACh response. At α4β2 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 (Kem et al., 1997)**.** 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, it has been shown that anabasine, a relatively selective α7-nicotinic ACh receptor agonist, attenuated MK-801-elicited popping at a dose that did not cause clonic seizures (Mastropaolo et al., 2004). Abnormal promoter variants for genetic expression of the α7-nicotinic ACh receptor polypeptide subunit, which are located on chromosome 15, have been identified in schizophrenia patients (Leonard et al., 2002). The CA secretion in adrenomedullary chromaffin cells is triggered by a concentration increase of free calcium ($[Ca^{2+}]_i$) in cytoplasm. $[Ca^{2+}]_i$ quickly returns to basal values through the seizure of Ca^{2+} inside intracellular organelles and by its extrusion to extracellular environment (Burgoyne, 1984; Yamaguchi, 1992). 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 (Kaczorowski et al., 1984; Kao and Cheung, 1990; Chen et al., 1992). There evidence that absence of extracellular Na^{+}/Ca^{2+} changes the direction of the ion movement by means of 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 (Liu and Kao, 1990; Kao and Cheung, 1990; Duarte et al., 1994; Warashina and Fujiwara, 1995; Tokumura et al., 1998).

Also, in this study, the anabasine-evoked CA secretory response was inhibited by pretreatment with atropine, in addition to the inhibition by 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 (Yamanaka et al., 1986), which elicit cyclic GMP accumulation in cells (Yanagihara et al., 1979). There have been a number of reports that show cyclic GMP accumulation by ACh or muscarine in adrenal medullary cells (Schneider et al., 1979; Yanagihara et al., 1979; Derome et al., 1981; Lemaire et al., 1981). Previously, Yamanaka et al. (1986) characterized muscarinic receptors in bovine adrenal medulla by radioligand binding assay with [^{3}H]quinuclidinyl benzilate. They showed that at least two distinct subtypes of muscarinic receptors exist in the adrenal medullary cells, and these receptors are 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. This data confirm conclusions of other authors who showed that muscarinic stimulus has an active participation in the CA secretion in adrenomedullary chromaffin cells of rats (Wakade and Wakade, 1983; Malhotra et al., 1988).

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 (Douglas, 1968; Schulz and Stolze, 1980; Williams, 1980). Furthermore, it has been found that nicotinic (but not muscarinic) stimulation also releases ACh from the chromaffin cells by a calcium-dependent mechanism (Mizobe and Livett, 1983). 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 (Wakade and Wakade, 1983) and isolated bovine adrenal chromaffin cells (Kilpatrick et al., 1981; 1982; Knight and Kesteven, 1983).

Cholinergic stimulus is one of the chief factors in the physiological secretion of catecholamines. The participation of nicotinic and muscarinic receptors was recorded in a $[Ca^{2+}]_i$ increase either by extracellular influx or by mobilization of intracellular stores. Yamagami et al. (1991) indicated that CA secretion induced by nicotinic stimulus has a higher percentage than the muscarinic one. The same authors hold that nicotinic stimulus increases $[Ca^{2+}]_i$ by extracellular influx, whilst muscarinic stimulus triggers the mobilization of Ca^{2+} intracellular stores. Lack of

extracellular Ca^{2+} doesn't seem to interfere with response to muscarinic stimulus (Harish et al., 1987).

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 is 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 (Chiou and Malagodi, 1975), and in isolated bovine adrenomedullary cells (Mibahuddin et al., 1985; Sasakawa et al., 1984). Moreover, it has been shown that the caffeine-evoked secretion of CA from the perfused cat adrenal gland in the absence of extracellualr calcium is also inhibited (Yamada et al., 1988). Activation of muscarinic receptors causes increase of $[Ca^{2+}]_i$ not only by extracellular influx but also by the mobilization of intracellular stores (Warashina and Fujiwara, 1995). Mobilizing Ca^{2+} from intracellular reserves, muscarinic agonists stimulate CA secretion even in the absence of extracellular Ca^{2+} (Harish et al., 1987; Nassar-Gentina et al., 1997) or after depolarization with high extracellular K^+ (Yamagami et al., 1991).

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 (Baker and Knight, 1978).

Such a store may not be easily depleted by the mere removal of extracellualr calcium. Some investigators (Bozler, 1969; Ohashi et al., 1974; Casteels and Raeymaeker, 1979; Malagodi and Chiou, 1974; Takahara et al., 1990) reported that intracellular stores of calcium have been shown to play some role in the contraction of smooth muscle produced by noradrenaline or ACh in Ca^{2+} -free media.

Interestingly, in this study, the reason why the secretory responses of CA evoked by ACh, DMPP and McN-A-343 were rather depressed at later period in the presence of anabasine (continuous infusion) is unclear, although they were enhanced at initial period. In support of this idea, it has been shown that anabaseine and anabasine were weak partial agonists upon the α4β2 receptor, displaying 8 and 4%, respectively, of the maximal current elicited by ACh (Kem et al., 1997). Therefore, bearing these facts in mind, it is felt that anabasine 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, it suggests that the mode of anabasine's action is some different from that of nicotine's 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 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 with that of anabasine. In support of this idea, it has been found that nicotinic action (endogenous ACh, splanchnic nerve stimulation) in CA secretion from the rat adrenal gland was largely reduced (75%)

by hexamethonium alone (Wakade and Wakade, 1983). 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 finding that anabasine appears as a partial agonist in the *Xenopus* oocyte (Kem et al., 1997), the data obtained here that anabasine inhibited CA secretory responses evoked by cholinergic stimulation at later period after initial enhancement imply that it has the properties of an antagonist at the nicotinic ACh receptors which mediate the CA secretion in adrenomedullary chromaffin cells.

In the present work, upon the repeated administration of anabasine (10^{-4}M) at 120min intervals in the perfused rat adrenal gland, the CA secretory response rapidly decreased 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, Collet and Story (1984) found that 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. This reduction may be due to the agonist desensitization of the nicotinic receptors. In this study, the repeated perfusion of anabasine from the third treatment of it at 120 min intervals, also produced a desensitization-like effect (tachyphylaxis) between the 1st and 3rd periods. However, Lim and Hwang (1991) have found that the 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. From these results, the existence of

different species in the CA secretion evoked by anabasine can not 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.

V. SUMMARY

In addition to S(-)-nicotine, several minor tobacco alkaloids ((+/-)-nornicotine, anabaseine, S(-)-anabasine, and S(-)-N-methylanabasine) are present in tobacco smoke. It has been shown that 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 (Kem et al., 1997). 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. The perfusion of anabasine (30 ~ 300 μM) into an adrenal vein of for 60 min resulted in great increases in CA secretions in a dose-dependent fashion. Upon the repeated injection of anabasine (100 μM) at 120 min-intervals, CA secretion was rapidly decreased after third injection of anabasine. However, there was no statistical difference between CA secretory responses of both 1st and 2nd treated groups by the successive administration of anabasine at 120 min-intervals. Tachyphylaxis to releasing effects of CA evoked by anabasine was observed by the repeated administration. Therefore, in all subsequent experiments, anabasine was not administered successively more than twice only 120 min-intervals. The CA-releasing effects of anabasine were depressed by pretreament with chlorisondamine (a selective neuronal nicotinic receptor antagonist, 1 μM), atropine (a muscarinic receptor antagonist, 2 μM), nicardipine (L-type dihydropyridine Ca^{2+} channel blocker, 1 μM), TMB-8 (an anti-releaser of

intracellular Ca^{2+}, 30 μM), and the perfusion of EGTA (a Ca^{2+} chelater, 5 mM) plus Ca^{2+}-free medium. In the presence of anabasine (100 μM), the CA secretory responses induced by ACh (5.32 mM), high K^{+} (a direct membrane-depolarizer, 56 mM), DMPP (a selective neuronal nicotinic receptor agonist, 10^{-4} M), and McN-A-343 (a selective muscarinic M_1-receptor agonist, 10^{-4} M) were maximally enhanced at 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 at first 0-5 min period. In the presence of nicotine (30 μM), the CA secretory responses induced by ACh, high K^{+}, DMPP and McN-A-343 were also enhanced for first 4 min, but later rather reduced to less than control release.

Taken together, these experimental results indicate that anabasine causes the rat adrenomedullary CA secretion in a calcium-dependent fashion. It is suggested that this facilitatory effect of anabasine may be mediated by activation of both cholinergic nicotinic and muscarinic receptor, which is relevant to both stimulation of the Ca^{2+} influx and Ca^{2+} release from cytoplasmic Ca^{2+}. It also seems that anabasine might be less potent in rat adrenomedullary CA secretion than nicotine.

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