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Mechanism of Epibatidine-Induced Catecholamine Secretion in the Rat Adrenal Gland

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The present study was attempted to investigate the characteristics of epibatidine on secretion of catecholamines (CA) from the isolated perfused model of the rat adrenal gland, and to establish the mechanism of action. Epibatidine (3×10^{-8} M) injected into an adrenal vein produced a great inhibition in secretory response of CA from the perfused rat adrenal gland. However, upon the repeated injection of epibatidine (3×10^{-8} M) at 15 min-intervals, CA secretion was rapidly decreased after second injection of epibatidine. However, there was no statistical difference between CA secretory responses of both 1st and 2nd periods by the successive administration of epibatidine at 120 min-intervals. Tachyphylaxis to releasing effects of CA evoked by epibatidine was observed by the repeated administration. Therefore, in all subsequent experiments, epibatidine was not administered successively more than twice only 120 min-intervals. The epibatidine-induced CA secretion was markedly inhibited by the pretreatment with atropine, chlorisondamine, pirenzepine, nicardipine, TMB-8, and perfusion of Ca^{2+}-free Krebs solution containing EGTA, while was not affected by diphenhydramine. Moreover, the CA secretion evoked by ACh for 1st period (0~4 min) was greatly potentiated by the simultaneous perfusion of epibatidine (1.5×10^{-8} M), but followed by time-dependently gradual reduction after 2nd period. The CA release evoked by high potassium (5.6×10^{-8} M) for 1st period (0~4 min) was also enhanced by the simultaneous perfusion of epibatidine, but those after 2nd period were not affected. Taken together, these experimental data suggest that epibatidine causes catecholamine secretion in a calcium dependent fashion from the perfused rat adrenal gland through activation of neuronal cholinergic (nicotinic and muscarinic) receptors located in adrenomedullary chromaffin cells. It also seems that epibatidine-evoked catecholamine release is not relevant to stimulation of histaminergic receptors.

Key Words: Epibatidine, Catecholamine secretion, Nicotinic action, Adrenal gland

INTRODUCTION

(±)-Epibatidine, [exo-2-(6-chloro-3-pyridyl)-7-aza-bicyclo-(2.2.1)] heptane, is a natural toxin first isolated from the skin of the Ecuadorian poison frog *Epipedonates triclor* (Spande et al, 1992). It has been found that epibatidine displays potent analgesic activity in both tail flick assay in rats and mice and in the hot plate assay in mice (Qian et al, 1993; Badio & Daly, 1994; Sullivan et al, 1994). This analgesic activity of epibatidine was not blocked by the opioid receptor antagonist naloxone (Spande et al, 1992) and was later shown to be blocked by the nicotinic acetylcholine (ACh) receptor antagonist mecamylamine, suggesting that epibatidine might be a nicotinic ACh receptor agonist (Qian et al, 1993; Badio & Daly, 1994). It has been shown that (±) epi-

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batidine (3～300 ηM) caused a concentration-and calcium-dependent release of [^{3}H]-dopamine from striatal slices and [^{3}H]-norepinephrine release from hippocampal and thalamic slices of the rat. This (±)-epibatidine-induced neurotransmitter release was inhibited in all three regions by mecamylamine (Sacaan et al, 1995). These results demonstrate that (±)-epibatidine displays nicotinic ACh receptor agonist activity in the rat CNS and that certain effects are mediated via nicotinic receptors, as demonstrated by effects on sodium influx in cultured PC-12 cells expressing different subtypes of nicotinic receptors (Badio & Daly, 1994). In pheochromocytoma PC-12 cells, containing a ganglionic-type nicotinic receptors (Daly et al, 1991), (+)- and (−)-epibatidine had comparable potencies, with EC50 values of 72 ηM and 111 ηM, respectively. The epibatidines were about 200～300 fold more potent than (−)-nicotine in PC-12 cells (Badio & Daly, 1994). This response was effectively blocked by both d-tubocurarine and mecamylamine, demonstrating the involvement of a ganglionic-type nicotinic receptor. Moreover, mecamylamine, a ganglionic nicotinic antagonist and a general noncompetitive nicotinic antagonist, does antagonize nicotinic-induced analgesia (Martin et al, 1990), indicating the involvement of nicotinic receptors. It has been also shown that mecamylamine also blocked epibatidine-induced analgesia (Badio & Daly, 1994). Krause & his colleagues (1997) have reported that epibatidine-evoked CA release from the norepinephrine fraction of bovine chromaffin cells is 30～40% higher than from the epinephrine fraction and initial exposure to 50 ηM epibatidine reduced release induced by a second exposure to the drug. There was cross-desensitization between epibatidine and nicotine and the release of norepinephrine was inhibited more than that of epinephrine. Sullivan et al (1994) have demonstrated that (±)-epibatidine is a potent nicotinic ACh receptor agonist in vitro and in vivo with differential activity to evoke responses elicited by putative subtypes of nicotinic ACh receptors.

However, up to now, there is no report about effect of epibatidine on release of CA from the isolated perfused adrenal gland model. In the present study, it was attempted to examine the effect of epibatidine on secretion of catecholamines from the isolated perfused rat adrenal gland and to establish the mechanism of its action.

METHODS

Experimental animals

Mature male Sprague-Dawley rats, weighing 180～300 grams, were anesthetized with ether. 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 placing 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. Heparine (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±1°C.

Perfusion of adrenal gland

The adrenal glands were perfused by means of a peristaltic ISCO pump (WIZ Co. USA) 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 CA.

Drug administration

Perfusion of epibatidine (3×10^{-8} M) for 1 min or single injection of ACh (5.32×10^{-3} M) and high K^+

(5.6×10^{-2} M) in a volume of 0.05 ml were made into perfusion stream via a three way stopcock. In the preliminary experiments it was found that upon administration of the above drugs, secretory responses to ACh returned to preinjection level in 4 min and epibatidine in 12~16 min, respectively. Generally, the adrenal glands were perfused with normal Krebs solution for about one hour before the experimental protocols are initiated.

Collection of perfusate

As a rule, prior to stimulation with epibatidine, samples were collected (4 min) to determine the spontaneous secretion of CA ("background sample"). Immediately after the collection of the "background sample", collection of the perfusate was continued in another tube as soon as the perfusion medium containing epibatidine reached the adrenal gland. Each perfusate was collected for 12~16 min at 4 min-interval. The amounts secreted in the "background sample" have been subtracted from those secreted form the "stimulated sample" to obtain the net secretion value of CA, which is shown in all of the figures and tables.

To study the effect of a test agent on the spontaneous and drug-evoked secretion, the adrenal gland was perfused with Krebs solution containing the agent for 20 min. Then, the perfusate was collected for a specific time period ("background sample"), and then the medium was changed to the one containing the test agent and the perfusate were collected for the same period as that for the "background sample". The adrenal perfusate was collected in chilled tubes.

Measurement of catecholamines

CA content of perfusate was measured directly by the fluorometric method of Anton & Sayre (1962) without the intermediate purification alumina for the reasons described earlier (Wakade, 1981), using fluorospectrophotometer (Kontron Co., 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 epibatidine was high enough to obtain readings several-fold 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 norpinephrine (base) equivalents.

Statistical analysis

The statistical significance between groups was determined by utilizing the Student's *t*-test. A P-value of less than 0.05 was considered to represent statistical significant changes unless specifically noted in the text. Values given in the text refer to means with standard errors of the mean (S.E.M.). The statistical analysis of the present experimental results was made by computer program of statistics described previously by Tallarida & Murray (1987).

Drugs and their sources

The following drugs were used: (±)-epibatidine, acetylcholine chloride, norepinephrine bitartrate, nicardipine hydrochioride and 3.4.5-trimethoxy benzoic acid 8-(diethylamino) octylester (TMB-8), pirenzepine hydrochloride and diphenhydramine hydrochloride from Sigma Chemical Co., USA, and chlorisondamine chloride from Ciba Co., USA. Drugs were dissolved in distilled water (stock) and added to the normal Krebs solution as required. Concentrations of all drugs used are expressed in terms of molar base.

RESULTS

The secretory effect of CA evoked by epibatidine

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 23 ±1 ng for 2 min from 8 experiments. The releasing effects to the initial perfusion of epibatidine (3×10^{-8} M) for 1 min are shown in Fig. 1. Time-dependent effect of epibatidine (3×10^{-8} M) infusion into the perfusion stream for 1 min at 120 min-interval exerted significant responses of CA secretion over the background release. In 8 rat adrenal glands, this epibatidine-evoked CA secretory responses were 508 ±71 ng (0~4 min), 371±62 ng (4~8 min), 259± 40 ng (8~12 min), 132±21 ng (12~16 min) and 49±10 ng (16~20 min) for 1st period, and 502±83 ng (0~4 min, ns), 316±70 ng (4~8 min, ns), 217± 54 ng (8~12 min, ns), 129±32 ng (12~16 min, ns)

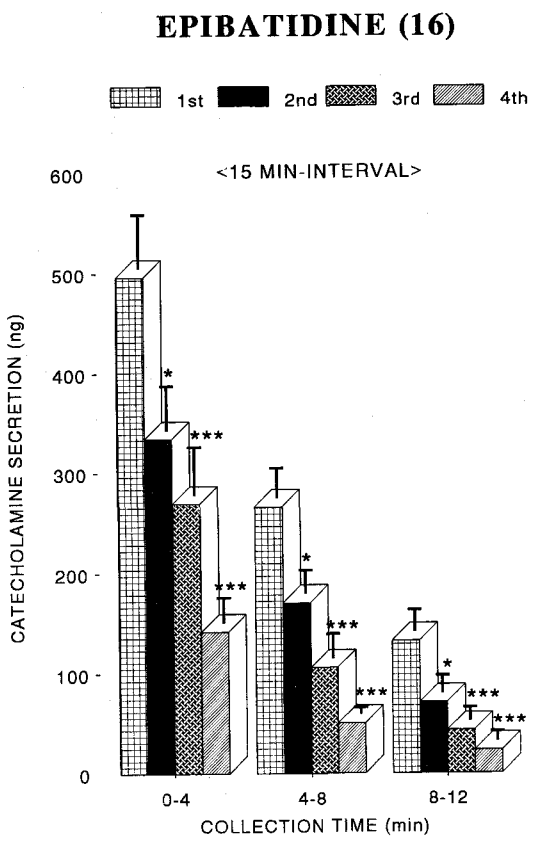

Fig. 1. Time-dependent effect of epibatidine on secretion of catecholamines (CA) from the perfused rat adrenal glands. Epibatidine (3×10^{-8} M) was perfused into an adrenal vein twice for 1 min at 120 min-interval. Perfusion of epibatidine was made after perfusion with normal Krebs-bicarbonate solution for one hour before the experimental protocols were initiated. The data are expressed with mean±S.E. from 8 rat adrenal glands. The perfusate was collected for 20 min at 4 min-intervals. The statistical significance was compared between the 1st group and 2nd group. Abscissa: Time of collection (min). Ordinate: secretion of CA in ng for 4 min. The vertical columns and bars denote means and the standard errors of the corresponding means, respectively. Number in the upper parenthesis indicates the number of animals used in the experiments. ns: Statistically nonsignificant.

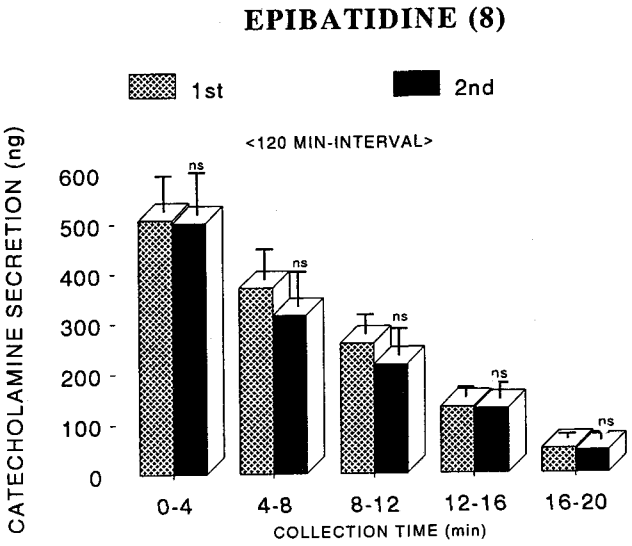

Fig. 2. The effect of repetitive administration of epibatidine on CA secretion in the perfused rat adrenal glands. Epibatidine (3×10^{-8} M) was perfused into an adrenal vein for 1 min four times at the 15 min-interval after the beginning of perfusion with normal Krebs solution. These results were obtained from 16 rat adrenal glands. The statistical significance was compared between the 1st group and 2nd~4th group. Other legends are the same as in Fig. 1. *: $P<0.05$, ***: $P<0.01$. ns: Statistically nonsignificant.

and 45±14 ng (16~20 min, ns) for 2nd period, respectively. There was no difference in CA secretion between 1st and 2nd periods. These observations are largely consistent with that described previously (Krause et al, 1997). It has been shown that epibatidine-evoked CA release from the norepinephrine fraction of bovine chromaffin cells is 30~40% higher than from the epinephrine fraction and initial exposure to 3×10^{-8} M epibatidine reduced release induced by a second exposure to the drug.

In order to examine the tachyphylaxis to releasing effects of CA evoked by epibatidine, epibatidine at the concentration of 3×10^{-8} M was given into the perfusion stream for 1 min four times consecutively at 15 min-intervals, respectively. In 16 rat adrenal glands, the CA secretions in response to each perfusion of epibatidine were significantly reduced from after 2nd infusion as compared with those of 1st infusion as shown in Fig. 2. Therefore, in all subsequent experiments, epibatidine was administered only twice at 120 min-interval.

Effect of chlorisondamine, pirenezepine and diphenhydramine on epibatidine-evoked CA secretion

In order to clarify the effect of chlorisondamine, a selective nicotinic receptor antagonist, on epibatidine-induced CA release, the rat adrenal gland was preloaded with 10^{-6} M chlorisondamine for 20 min before epibatidine was introduced. In the presence of chlorisondamine effect, the CA outputs evoked by perfusion with epibatidine (3×10^{-8} M) for 1 min

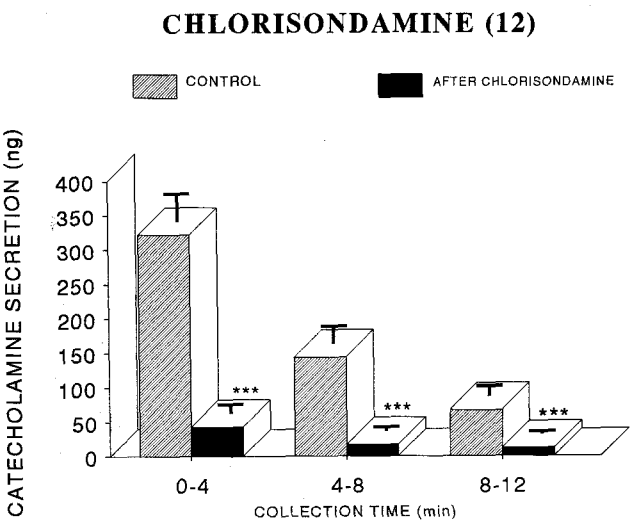

Fig. 3. The effect of chlorisondamine on the secretion of CA evoked by epibatidine in the perfused rat adrenal glands. Secretion of CA evoked by epibatidine (3×10^{-8} M) was evoked 20 min after perfusion of adrenal gland with Krebs solution containing 2×10^{-6} M-atropine. "CONTROL" and "AFTER" indicate amounts of CA released by epibatidine before (CONTROL) and after the preloading with chlorisondamine (10^{-6} M) Statistical differences were compared between amounts of CAs evoked by epibatidine before (CONTROL) and after pretreatment. The statistical difference was obtained by comparing the secretory effect of CA evoked by epibatidine after preloading with atropine with its corresponding control. Other legends are the same as in Fig. 1. ***: $P<0.01$.

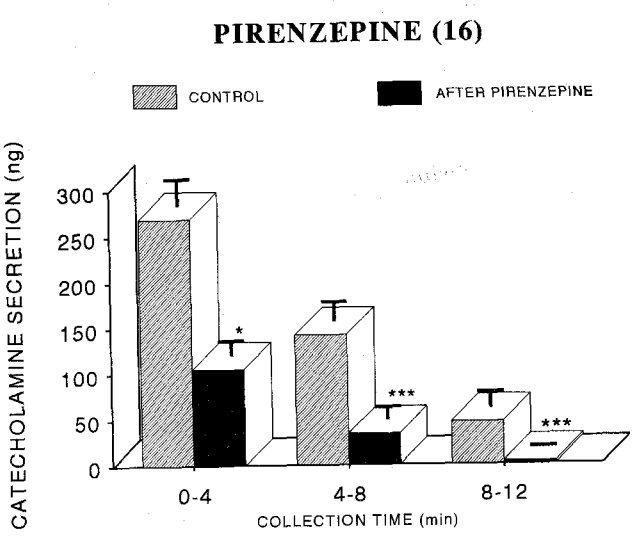

Fig. 4. The effect of pirenzepine on the secretion of CA evoked by epibatidine in the perfused rat adrenal glands. Secretion of CA evoked by epibatidine (3×10^{-8} M) was evoked 12 min after perfusion of adrenal gland with Krebs solution containing 2×10^{-6} M-pirenzepine. Other legends are the same as in Fig. 3 & 5. *: $P<0.05$, ***: $P<0.01$.

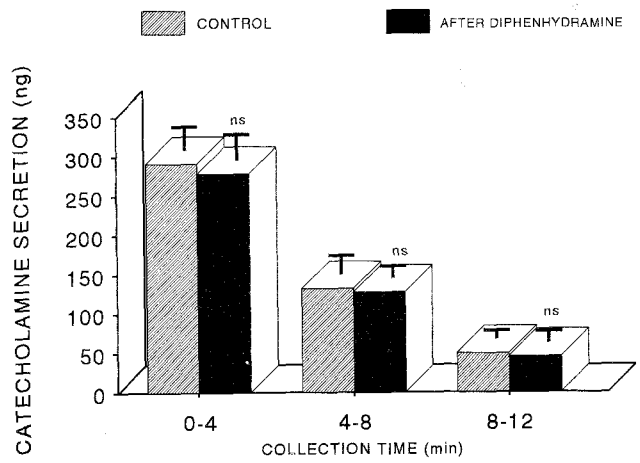

Fig. 5. The effect of diphenhydramine on the secretion of CA evoked by epibatidine in the perfused rat adrenal glands. Secretion of CA evoked by epibatidine (3×10^{-8} M) was produced 20 min after perfusion with Krebs solution containing diphenhydramine (10^{-5} M). Other legends are the same as in Fig. 3 & 5. ns: Statistically nonsignificant.

were greatly depressed to 12~18% of the control release, respectively as compared with their corresponding control from 12 experiments as shown in Fig. 3.

Hammer & 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 pirenzepine on CA release evoked by epibatidine. In the present work, the CA output induced by epibatidine was depressed in the rat adrenal gland preloaded with 2×10^{-6} M pirenzepine for 20 min. In 16 experiments, 3×10^{-8} M epibatidine-evoked CA releasing responses after pretreatment with pirenzepine were significantly reduced to 7~39% of the control release, respectively as compared with their control secretions as shown in Fig. 4.

Diphenhydramine (10^{-5} M), a selective H_1-histaminergic receptor antagonist, was preloaded into the rat adrenal gland for 20 min before the introducing epibatidine (3×10^{-8} M). In the presence of diphen-

hydramine, epibatidine-induced CA secretion amounted to 92~96% of the control release, respectively as compared to the corresponding control response from 6 rat adrenal gland as shown in Fig. 5.

The effect of perfusion of Ca^{2+}-free Krebs, nicardipine and TMB-8 on epibatidine-evoked CA secretion

It has been found that the physiological release of CA and dopamine-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 epibatidine is also related to extracellular calcium ions. Thus, the adrenal gland was perfused with calcium-free Krebs solution containing 5×10^{-3} M EGTA for 20 min prior to introduction of epibatidine. In the absence of extracellular calcium, CA releases by epibatidine (3×10^{-8} M) were significantly inhibited to 16~27% of the control release, respectively as compared with their control secretions form 11 rat adrenal glands as shown in Fig. 6. In order to investigate the effect of nicardipine, a dihydropyridine derivative and L-type Ca^{2+} channel blocker (Hardman et al, 1995) on epibatidine-evoked CA secretion, nicardipine (10^{-6} M) was preloaded into the adrenal gland for 20 min. In the presence of nicardipine effect, CA releases induced by perfusion of epibatidine (3×10^{-8} M) for 1 min were greatly depressed to 0~30% of the control release, respectively from 10 rat glands as compared with their corresponding control responses. Fig. 7 illustrates that nicardipine inhibits CA secretory responses evoked by epibatidine.

It has been reported that muscarinic, but not

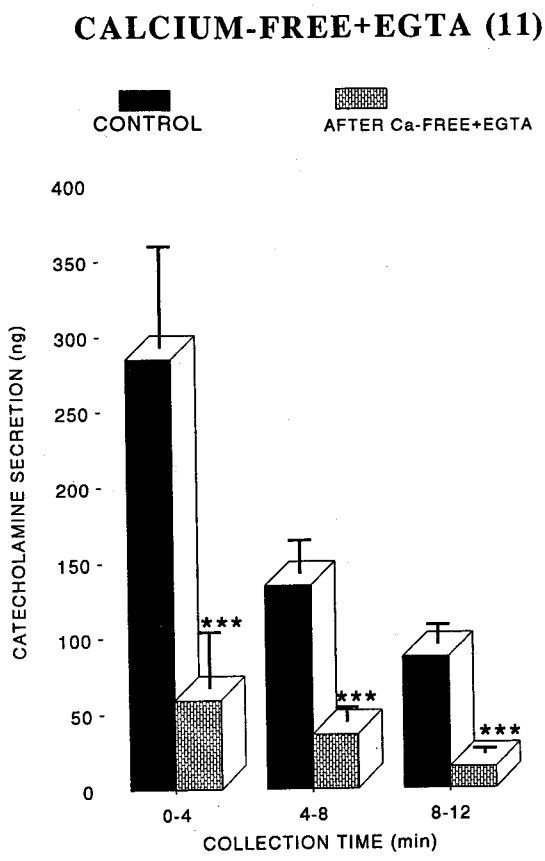

Fig. 6. The effect of Ca^{2+}-free Krebs-perfusion on the secretion of CA evoked by epibatidine in the perfused rat adrenal gland. Secretion of CA evoked by epibatidine (3×10^{-8} M) was induced 30 min following perfusion of adrenal gland with Ca^{2+}-free Krebs solution containing EGTA (5×10^{-3} M). Other legends are the same as in Fig. 3 & 5. *: $P<0.01$.

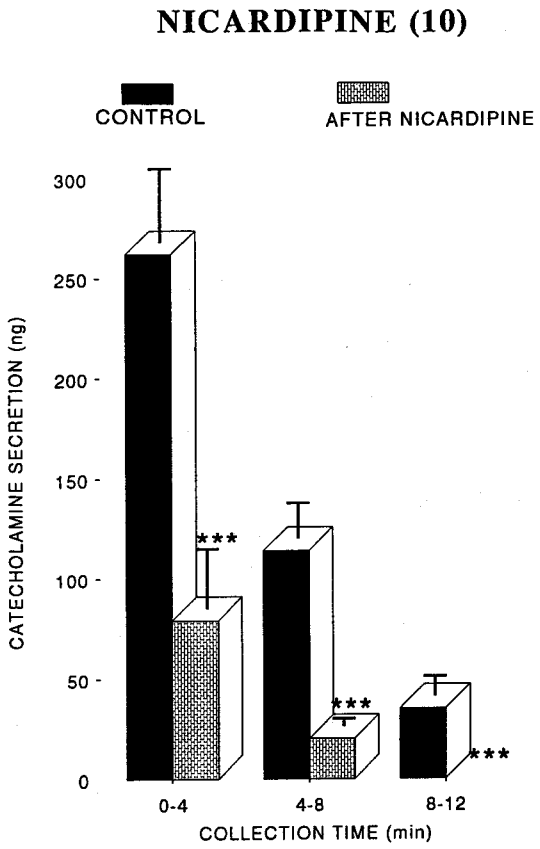

Fig. 7. The effect of nicardipine on epibatidine-evoked CA secretory responses in the perfused rat adrenal glands. Nicardipine (10^{-6} M) was perfused for 20 min before introducing epibatidine (3×10^{-8} M). Other legends are the same as in Fig. 3 & 5. ***: $P<0.01$

nicotinic activation causes CA secretion independent of extracellular calcium in the perfused cat adrenal glands (Nakazato et al, 1988). It suggests that the presence of an intracellular calcium pool is linked to a 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 & Hwang, 1991). It was attempted to test the TMB-8 on epibatidine-evoked CA secretion. In 10 rat adrenal glands, CA secretions evoked by 1 min-perfusion of epibatidine (3×10^{-8} M) after preloading with TMB-8 (10^{-5} M) for 20 min were perfectly blocked after the initial period (0~4 min) in comparison with their corresponding control responses as shown in Fig. 8.

The effect of epibatidine infusion on ACh- and high K^+-evoked CA releases

In terms of the facts that epibatidine-induced CA release was markedly inhibited by the pretreatment with atropine or chlorisondamine as well as pirenzepine as in Fig. 5, 6 & 7, it is of interest to examine the influence of epibatidine perfusion on ACh-and high K^+-induced CA secretory responses. The adrenal gland was preloaded with epibatidine (3×10^{-8} M) for 20 min before ACh and high K^+ were introduced. In the presence of epibatidine effect, ACh-induced CA output was greatly enhanced to 136% of the control release for first 4 min only, but the other CA

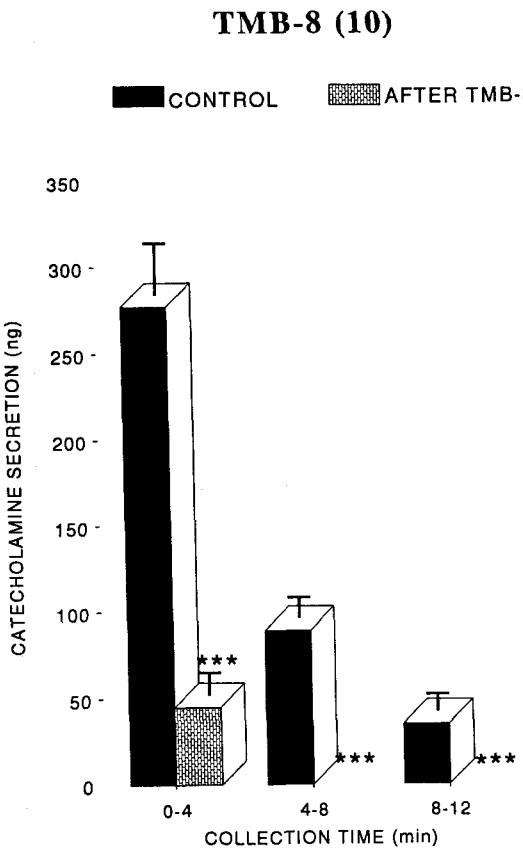

Fig. 8. The effect of TMB-8 on CA secretion evoked by epibatidine in the perfused rat adrenal glands. TMB-8 (10^{-5} M) was given into the perfusion stream for 20 min after obtaining the corresponding control responses of epibatidine (3×10^{-8} M). Other legends are the same as in Fig. 3 & 5. ***: $P<0.01$

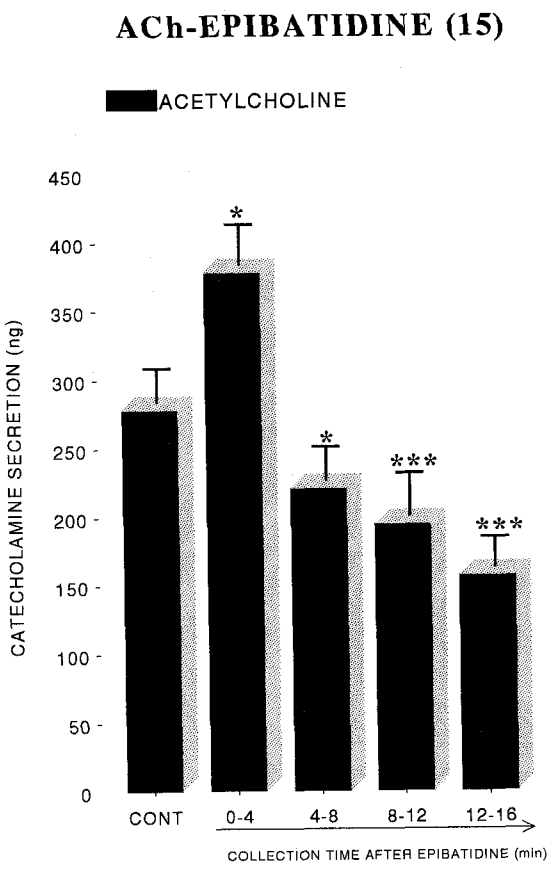

Fig. 9. The effect of epibatidine-infusion on ACh-evoked CA secretion in the perfused rat adrenal glands. ACh (5.32×10^{-3} M)-evoked CA secretory response was induced simultaneously along with epibatidine (1.5×10^{-6} M) after obtaining the control response of ACh. Statistical differences were obtained by comparing "CONT (control)" and "after" preloang with epibatidine. Other legends are the same as in Fig. 3 & 5. *: $P<0.05$, ***: $P<0.01$

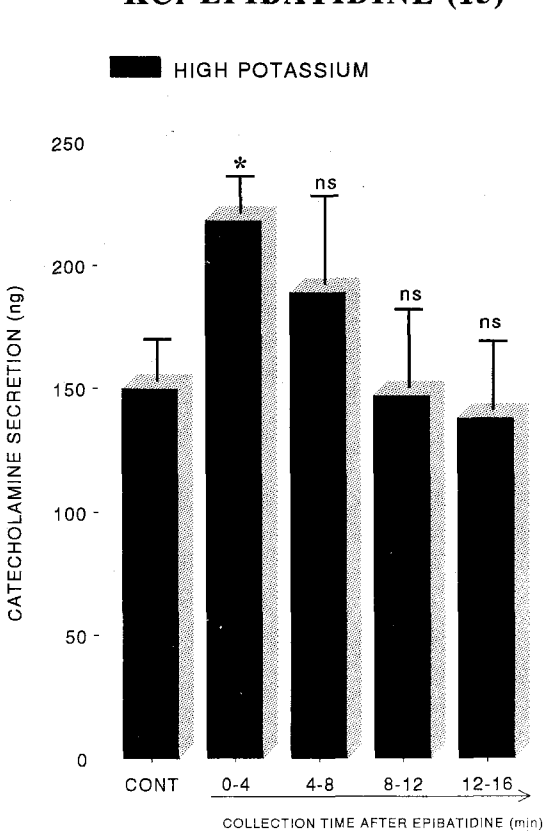

Fig. 10. The effect of epibatidine-infusion on high K^+-evoked CA secretion in the perfused rat adrenal glands. high K^+ (5.6×10^{-2} M) was injected into adrenal vein simultaneously along with epibatidine (1.5×10^{-8} M) perfusion after obtaining the control response of high K^+. Other legends are the same as in Fig. 1 & 3. *: $P<0.05$. ns: Statistically nonsignificant.

outputs after first 4 min were time- dependently rather inhibited to 56~79% of the control responses, respectively as compared to its corresponding CA secretion of 278±25 ng (0~4 min) as shown in Fig. 9.

Under the presence of epibatidine effect, high K^+ (5.6×10 M)-induced CA outputs were augmented to 145% of the control for first 4 min only, but the other CA outputs after first 4 min not affected to 106~92% of the control, respectively as compared to its corresponding CA secretion of 150±28 ng (0~4 min) as shown in Fig. 10.

DISCUSSION

These experimental data strongly suggest that epibatidine 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 support of this finding, it has been found in experiments using rat hippocampal neuron grown in culture that epibatidine can be used as a novel nicotinic agonist for the study of type II currents, and that the nicotinic ACh receptor related to type II currents may be one of the key target sites through which agonists such as epibatidine and nicotine elicit their behavioral action *in vivo* (Alkondon & Albuquerque, 1995). Moreover, as mentioned in introduction, epibatidine-induced analgesia is most likely mediated by ACh receptors because it is blocked by the selective nicotinic antagonist mecamylamine (Qian et al, 1993; Badio et al, 1994; Damaj et al, 1994). Epibatidine is also known to compete selectively with nicotine and cytisine in rat brain preparations (Qian et al, 1993; Dukat et al, 1994; Sullivan et al, 1994). Gerzanich & his coworkers (1995) have shown that epibatidine behaves as an extremely potent agonist for several subtypes of chicken and human neuronal nicotinic ACh receptors, whereas it has rather moderate affinity for muscle type ACh receptors. Particularly, in bovine chromaffin cells, epibatidine at 30 ηM evoked a maximal release of adrenaline and noradrenaline (Krause et al, 1997). Eibatidine is a nicotinic agonist with a very high potency (nanomolar activity). The present experimental results confirm that epibatidine at a relatively low concentration (30 ηM) induces a potent and rapid release of CAs from the perfused rat adrenal gland through activation of neuronal nicotinic ACh receptors. In the perfused rat adrenal gland, upon the repeated administration of epibatidine (3×10^{-8} M) at 15min intervals, the CA secretory response was rapidly decreased after second administration of epibatidine. Tachyphylaxis to releasing effects of CA evoked by epibatidine was observed by the repeated administration. In support of this finding, Collect & Story (1984) have found that the CA release evoked by DMPP declined abruptly between the first and second periods of DMPP exposure in the rabbit isolated adrenal gland and guinea pig atria. This reduction may be shown to be due to agonist densensitization of the nicotinic receptors. In the present study, the repeated perfusion of epibatidine four times at 15 min intervals also produced densensitization-like effect (tachyphylaxis)

between 1st and 2nd~4th periods. However, Lim & Hwang (1991) have found that the repetitive perfusion of DMPP in the isolated perfused rat adrenal gland does not produce any densensitization-like effect (tachyphylaxis) between 1st and 2nd~3rd periods. In terms of these results, it could not be excluded that there exists species difference in CA secretion evoked by epibatidine. Although the repeated perfusion of epibatidine at 15 min-intervals produced tachyphylaxis, there was no statistical difference between 1st and 2nd administration groups in CA releasing responses evoked by epibatidine at 120 min-intervals. Therefore, epibatidine was not given more than twice through all experiments of this study.

The epibatidine-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 in the present work. Chlorisondamine is known to be a selective antagonist of neuronal nicotinic cholinergic receptors (Hardman et al, 1995).

Also, in this study, the epibatidine-evoked CA secretory response was inhibited by pretreatment with pirenzepine in addition to the inhibition by pretreatment with chlorisondamine. This finding indicates that epibatidine-evoked CA release is exerted at least partly by stimulation of muscarinic ACh receptors.

In general, subtypes of muscarinic receptors have been recognized in many tissues (Eglen & Whiting, 1986). Receptor binding studies have supported the classification of muscarinic receptors into M_1 and M_2 on the basis of the selectivity profile of pirenzepine; receptors with a high affinity for pirenzepine are designated as M_1 and those with low affinity as M_2 receptors (Hammer et al, 1980; Hammer & Giachetti, 1982). Doods & his colleagues (1987) have classified muscarinic receptors into M_1 (pirenzepine sensitive, neuronal), M_2 (cardiac) and M_3 (smooth muscle and glandular). In view of above studies, the finding of this study that epibatidine-evoked CA release was inhibited by pretreatment with pirenzepine strongly demonstrates that epibatidine-evoked CA secretion is mediated partly through activation of muscarinic M_1-receptor in the perfused rat adrenal gland.

In the present work, epibatidine-evoked CA releasing effect was not influenced in presence of diphenhydramine. This demonstrates that epibatidine-evoked CA secretion is not relevant to activation of histaminergic receptors in the perfused rat adrenal medulla.

The indispensable role of calcium in the neurosecretory process has been well established. According to the assumption of Baker & Knight (1978; 1980), the relationship between the concentration of intracellular calcium and the transmitter release has not been determined in the nerve terminals. Furthermore, it has been found that nicotinic (but not muscarinic) stimulation also releases soluble ACh from the chromaffin cells by a calcium-dependent mechanism (Mizobe & Livett, 1983). The activation of nicotinic receptors stimulates CA secretion by increasing Ca^{2+} entry through receptor-linked and/or voltage-dependent Ca^{2+} channels in the perfused rat adrenal glands (Wakade & Wakade, 1983) and isolated bovine adrenal chromaffin cells (Kilpatrick et al, 1981; 1982; Knight & Kesteven, 1983).

In the present study, removal of extracellular Ca^{2+} depressed markedly CA secretion evoked by epibatidine. Furthermore, the pretreatment of nicardipine, a dihydropyridine derivative and L-type Ca^{2+} channel blocker, also blocked the epibatidine-evoked CA release. Based on these findings, the secretory effect of epibatidine seems to be apparently mediated by increasing Ca^{2+} entry through nicotinic receptor-linked Ca^{2+} channels in the perfused rat adrenal glands.

However, in this experiment, the reason for considerable response to epibatidine in Ca^{2+}-free Krebs plus EGTA solution or in the presence of nicardipine is not clear. In the presence of TMB-8, an inhibitor of the intracelluar calcium release from the store, epibatidine-evoked CA secretion was greatly inhibited in this perfused adrenal gland. TMB-8 is also known to inhibit caffeine-induced $^{45}Ca^{2+}$ release from, but not the uptake of $^{45}Ca^{2+}$ by, a sarcoplasmic reticulum preparation of skeletal muscle (Chiou & Malagodi, 1975) and in isolated bovine adrenomedullary cells (Sasakawa et al, 1984; Misbahuddin et al, 1985). Moreover, it has been shown that caffeine-evoked CA secretion is also inhibited from the perfused cat adrenal gland in the absence of extracellualr calcium (Yamada et al, 1988). Thus, in the present experiments, the inhibition of epibatidine-evoked CA secretion suggests that chromaffin cells of the rat adrenal gland contain the intracellular store of calcium that participates in the secretion of CA as shown in the bovine gland (Baker & Knight, 1978). Such a store may not be easily depleted by mere removal of extracellualr calcium. In support of this idea, some

investigators have reported that intracelluar Ca^{2+} stores may play some roles in contraction of smooth muscle produced by noradrenaline or ACh in Ca^{2+}-free medium (Boxler, 1967; Malagodi & Chiou, 1974; Ohashi et al, 1974; Casteels & Raeymaeker, 1979; Takahara et al, 1990). Moreover, it has been shown that the activation of muscarinic receptor causes an increase in adrenal CA secretion independent of extracellular Ca^{2+} in various species (Wakade et al, 1986; Harish et al, 1987; Nakazato et al, 1988) and also in cytosolic free Ca^{2+} in the isolated bovine adrenal chromaffin cells without associated CA secretion (Cheek & Burgoyne, 1985; Kao & Schneider, 1985; 1986; Misbahuddin et al, 1985). In term of these findings, the result that epibatidine-evoked CA release was inhibited by pretreatment with pirenzepine strongly demonstrate that epibatidine-evoked CA secretion is mediated at least partly through activation of M_1-muscarinic receptor in the perfused rat adrenal gland.

Interestingly, in this study, the reason that CA secretory responses evoked by ACh were time-dependently depressed in the presence of epibatidine (continuous infusion) except for first period (0~4 min) immedately after the initiation of epibatidine perfusion is not clear. However, high K^+-evoked CA release was not affected except for first period (0~4 min) immedately after the initiation of epibatidine perfusion. In supports of this idea, it has been found that a maximal release of adrenaline and noradrenaline was evoked by 30 ηM epibatidine, but their releases by higher concentrations were less active (Krause et al, 1997). This implies that the lower CA release after repeated or strong stimulation of the cells is not caused by exhaustion of the releasable CA pool. The present results showing a time-dependent inhibition of ACh-evoked CA release by continuous perfusion of epibatidine provide additional evidence that cells desensitized by pretreatment with epibatidine respond less when stimulated with nicotinic receptor agonist ACh, not with high K^+. Furthermore, Papke & Heinemann (1994) have reported that cytisine is a true partial agonist for β_2-containing ACh receptors and can inhibit the response of these receptors to ACh through a competitive mechanism. Although cytisine was relatively ineffective in stimulating current, the coapplication of cytisine and ACh reduced the responses to ACh (Papke & Heinemann, 1994). Therefore, in terms of these facts, it could not be excluded that epibatidine has a partial agonist activity.

Taken together, these experimental data suggest that epibatidine causes secretion of catecholamines in a calcium-dependent fashion from the perfused rat adrenal gland through the activation of neuronal nicotinic ACh receptors as well as muscarinic ACh receptors located in adrenomedullary chromaffin cells. It also seems that epibatidine-evoked catecholamine release is not relevant to stimulation of histaminergic receptors.

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