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Cotinine Inhibits Catecholamine Release Evoked by Cholinergic Stimulation from the Rat Adrenal Medulla

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The aim of the present study was to clarify whether cotinine affects the release of catecholamines (CA) from the isolated perfused rat adrenal gland, and to establish the mechanism of its action, in comparison with the response of nicotine. Cotinine (0.3~3 mM), when perfused into an adrenal vein for 60 min, inhibited CA secretory responses evoked by ACh (5.32 mM), DMPP (a selective neuronal nicotinic agonist, 100 μM for 2 min) and McN-A-343 (a selective muscarinic M_1-agonist, 100 μM for 2 min) in dose- and time-dependent manners. However, cotinine did not affect CA secretion by high K^+ (56 mM). Cotinine itself also failed to affect basal CA output. Furthermore, in the presence of cotinine (1 mM), CA secretory responses evoked by Bay-K-8644 (an activator of L-type Ca^{2+} channels, 10 μM) and cyclopiazonic acid (an inhibitor of cytoplasmic Ca^{2+}-ATPase, 10 μM) were relative time-dependently attenuated. However, nicotine (30 μM), given into the adrenal gland for 60 min, initially rather enhanced CA secretory responses evoked by ACh and high K^+, followed by the inhibition later, while it time-dependently depressed the CA release evoked by McN-A-343 and DMPP. Taken together, these results suggest that cotinine inhibits greatly CA secretion evoked by stimulation of cholinergic (both nicotinic and muscarinic) receptors, but does fail to affect that by the direct membrane-depolarization. It seems that this inhibitory effect of cotinine may be exerted by the cholinergic blockade, which is associated with blocking both the calcium influx into the rat adrenal medullary chromaffin cells and Ca^{2+} release from the cytoplasmic calcium store. It also seems that there is a big difference in the mode of action between cotinine and nicotine in the rat adrenomedullary CA secretion.

Key words: Cotinine, Nicotine, Catecholamine release, Adrenal medulla, Cholinergic blockade

INTRODUCTION

No matter how nicotine is used, it is metabolized in the body. Cotinine is found to be the major peripheral oxidative metabolite of (*S*)-(-)-nicotine in several animal species, including humans, and is able to pass the blood-brain barrier from the periphery (Gorrod and Wahren, 1993; Benowitz *et al.*, 1994; Crooks *et al.*, 1997). Because of its long terminal elimination half-life, serum cotinine concentrations in cigarette smokers are substantially higher than those of nicotine (Benowitz *et al.*, 1983; Hurt *et al.*, 1993; Sastry *et al.*, 1995; Benowitz and Jacob, 1999). It has been shown that plasma cotinine levels are approximately 2 folds higher than nicotine levels in rats following intravenous nicotine self-administration (Shoaib and Stolerman, 1999). In some study, chronic infusion of nicotine delivered via osmotic mini-pumps in rats have produced cotinine levels that are at least 5 times that of nicotine blood levels (Winders *et al.*, 1998). Animal studies have found that high doses of cotinine can produce behavioral and physiological effects that are similar and effects that are opposite to those of nicotine (Hatsukami *et al.*, 1998). In human studies, cotinine was observed to have effects that were antagonistic to that of nicotine (Hatsukami *et al.*, 1998). Moreover, it has also been reported that pretreatment with a high dose of cotinine in rabbits blocked the hypertension action of a high dose nicotine (Chahine *et al.*, 1996). In rat aorta, cotinine antagonized nicotines inhibition of prostacyclin biosynthesis (Chahine *et al.*, 1990).

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Cotinine has also been observed to block the contraction response to nicotine in rabbit duodenum and ileum (Kim *et al.*, 1968). Recently, it has been also found that cotinine inhibits activation of protein kinase C and norepinephrine release induced by nicotine agonists in primary cultures of bovine adrenal chromaffin cells (Vainio *et al.*, 1998).

In contrast, several studies have shown that cotinines physiological effects are similar to those of nicotine (Goldberg *et al.*, 1989; Takada *et al.*, 1989; Yeh *et al.*, 1989; Erenmemisoglu and Tekol, 1994). Moreover, the most likely explanation for the finding of positive relation of systolic blood pressure with greater cotinine concentration is that the higher blood pressure is due to greater nicotine exposure (Gravey *et al.*, 1995). The larger increase in blood pressure seen with greater nicotine exposure is probably caused by more pronounced nicotine-induced catecholamine release (Cryer *et al.*, 1976). Gravey and his colleagues (1995) have also concluded that cotinine concentration is more strongly related to the systolic pressure level than is the number of cigarettes smoked per day. On the other hand, human studies have shown that cotinine at high dose or doses that are similar to those observed in daily cigarette smokers produces either no effect (Hatsukaml *et al.*, 1997; 1998) or some minimal effects (Benowitz 1983; Keenan *et al.*, 1994) among abstinent smokers. There is a big controversy in the pharmacological actions of cotinine so far. Therefore, the present study was designed to clarify whether cotinine affects the release of catecholamines (CA) evoked by cholinergic stimulation and the membranedepolarization from the perfused rat adrenal gland and to elucidate the mechanism of action, in comparison with the response of nicotine.

MATERIALS AND METHODS

Male Sprague-Dawley rats, weighing 180 to 250 g, were anesthetized with thiopental sodium (40 mg/kg) intraperitoneally. 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. 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±1°C.

Perfusion of adrenal gland

The adrenal glands were perfused by means of a peristaltic pump (WIZ Co., U.S.A.) at a rate of 0.3 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 catecholamines.

Drug administration

The perfusions of DMPP (100 μM), McN-A-343 (100 μM) for 2 minutes, Bay-K-8644 (10 μM) and cyclopiazonic acid (10 μM) for 4minutes were made into perfusion stream, respectively. A single injection of ACh (5.32 mM) and KCl (56 mM) in a volume of 0.05 mL was given 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 pre-injection 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, 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 effects of cotinine on the spontaneous and evoked secretion, the adrenal gland was perfused with Krebs solution containing cotinine for 60 min immediately after the perfusate was collected for a certain minute (background sample). And then the medium was changed to the one containing the stimulating agent and the perfusates were collected for the same period as that for the background sample. Generally, 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 (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 secretogagues 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 significance between groups was determined by utilizing the Student's *t*-test. 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: cotinine, acetylcholine chloride, 1,1-dimethyl-4-phenylpiperazinium iodide (DMPP), norepinephrine bitartrate, nicotine tartrate, methyl-1,4-dihydro-2,6-dimethyl-3-nitro-4-(2-trifluoromethylphenyl)-pyridine-5-carboxylate (BAY-K8644), nicotine tartrate (Sigma Chemical Co., U.S.A.), cyclopiazonic acid and (3-(*m*-chlorophenyl-carbamoyl-oxy)-2-butynyltrimethylammonium chloride [McN-A-343] (RBI, U.S.A.). Drugs were dissolved in distilled water (stock) and added to the normal Krebs solution as required except Bay-K-8644, which was dissolved in 99.5% ethanol and diluted appropriately (final concentration of alcohol was less than 0.1%). Concentrations of all drugs used are expressed in terms of molar base.

RESULTS

Effect of cotinine on CA secretion evoked by ACh, high K^+, DMPP and McN-A-343 from the perfused rat adrenal glands

After the initial perfusion with oxygenated Krebs-bicarbonate solution for 1 h, the basal CA release from the isolated perfused rat adrenal glands amounted to 21±3 ng/2 min (n=10). Several studies have demonstrated that cotinine's physiological effects are similar to nicotines' effects (Goldberg *et al.*, 1989; Takada *et al.*, 1989; Yeh *et al.*, 1989; Erenmemisoglu and Tekol, 1994; Gravey *et al.*, 1995). Therefore, it was decided initially to examine the effects of cotnine on cholinergic receptor stimulation- as well as membrane depolarization-mediated CA secretion from perfused rat adrenal glands. Secretagagues were given at 15 or 20 min-intervals. Cotinine was present for 60 min, including stimulation with each secretagogue. In the present study, the cotinine itself was found not to produce any effect on the spontaneous CA secretion (data not shown).

When injected in a volume of 0.05 mL into the perfusion stream, the ACh (5.32×10^{-3} M) evoked the CA secretion of 227±24 ng (0-4 min). However, in the presence of cotinine (0.3~3.0 mM), the ACh-stimulated CA secretion was decreased dose- and time-dependently by ~40% of that of the control release (100%), as shown in Fig. 1. It has been found that direct membrane-depolarizing agent, like high potassium, sharply stimulates CA secretion. In this work, high K^+ (5.6×10^{-2} M)-stimulated CA secretion, following the pretreatment with cotinine, was not affected. In the presence of cotinine (0.3~3.0 mM), high K^+-evoked CA output was 107~92% of its corresponding control secretion (241±19 ng, 0-4 min) as depicted in Fig. 2.

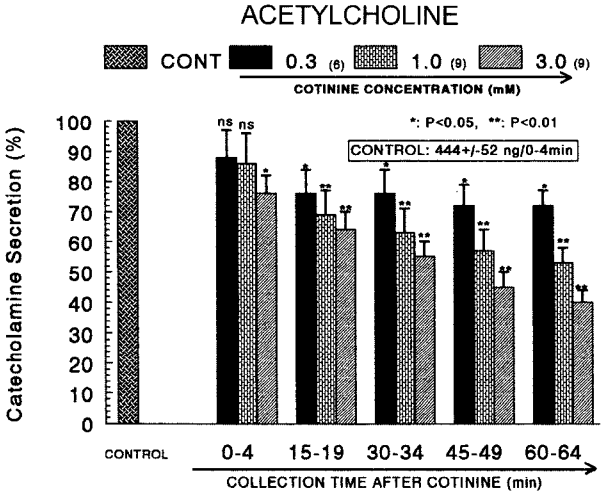

Fig. 1. The effect of cotinine on the secretory responses of catecholamines (CA) evoked by acetylcholine (ACh) from the isolated perfused rat adrenal glands. CA secretion by a single injection of ACh (5.32×10^{-3} M) in a volume of 0.05 mL was evoked at 15 min intervals after preloading with 0.3, 1.0, and 3.0 mM of cotinine for 60 min as indicated at an arrow mark, respectively. Numbers in the parenthesis indicate number of rat adrenal glands. Vertical bars on the columns represent the standard error of the mean (S.E.M.). Ordinate: the amounts of CA secreted from the adrenal gland (% of control, 444±52 ng for 4 min). Abscissa: collection time of perfusate (min). Statistical difference was obtained by comparing the corresponding control (CONT) with each concentration-pretreated group of cotinine. ACh-induced perfusate was collected for 4 minutes. ns: Statistically not significant.

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. 3, the DMPP-stimulated CA secretion, prior to the loading with cotinine, was 779±75 ng (0-8 min), while in the presence of cotinine (0.3~3.0 mM), they were time- and dose-dependently reduced by 91~0% from that of the control (100%), respectively. As illustrated in Fig. 4, the McN-A-343 (10^{-4} M), which is a selective muscarinic M_1-receptor agonist (Hammer and Giachetti, 1982), perfused into an adrenal vein for 2 min caused an increased in the CA secretion to 170±19 ng (0-4 min). However, the McN-A-343-evoked CA secretion in the presence of cotinine (0.3~3.0 mM) was gradually diminished by 89~0% from that of control (100%), respectively.

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

Bay-K-8644 has been shown to be a 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 cotinine on the Bay-K-8644-stimulated CA secretion from the isolated perfused rat adrenal glands.

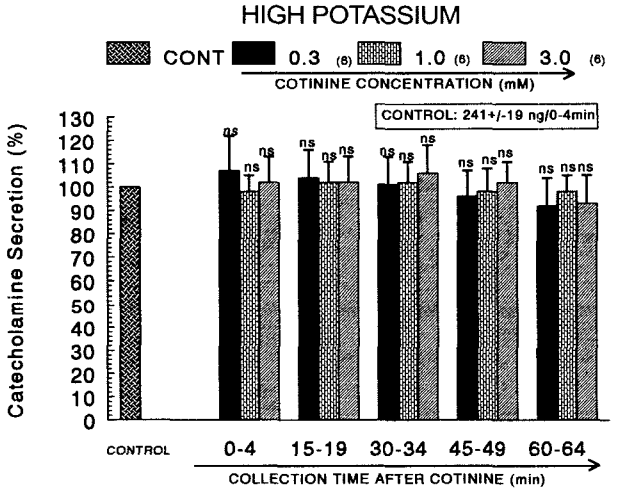

Fig. 2. The effect of cotinine on the secretory responses of catecholamines (CA) evoked by high K^+ from the isolated perfused rat adrenal glands. CA secretion by a single injection of K^+ (56 mM) was injected in a volume of 0.05 mL at 15 min intervals after preloading with 0.3, 1.0, and 3.0 mM of cotinine for 60 min, respectively. Statistical difference was obtained by comparing the corresponding control (CONT, 241±19 ng for 4 min) with each concentration-pretreated group of cotinine. K^+-induced perfusate was collected for 4 minutes. Other legends are the same as in Fig. 1. ns: Statistically not significant.

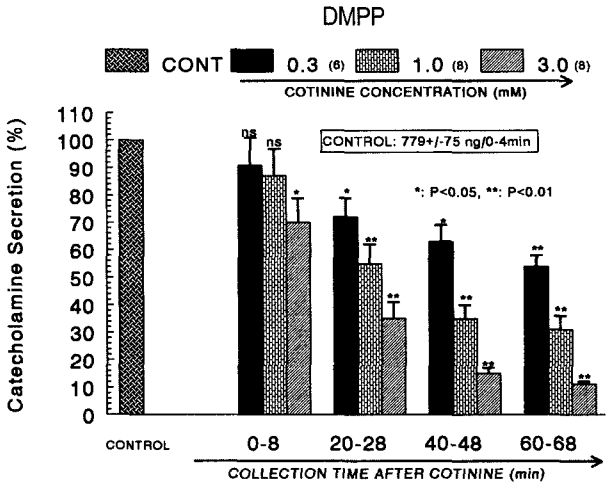

Fig. 3. The effect of cotinine on the secretory responses of catecholamines (CA) evoked by DMPP from the isolated perfused rat adrenal glands. CA secretion by a single injection of DPPP (10^{-4} M) was infused for 2 min at 20 min intervals after preloading with 0.3, 1.0, and 3.0 mM of cotinine for 60 min, respectively. Statistical difference was obtained by comparing the corresponding control (CONT, 779±75 ng for 8 min) with each concentration-pretreated group of cotinine. DMPP-induced perfusate was collected for 8 minutes. Other legends are the same as in Fig. 1. ns: Statistically not significant.

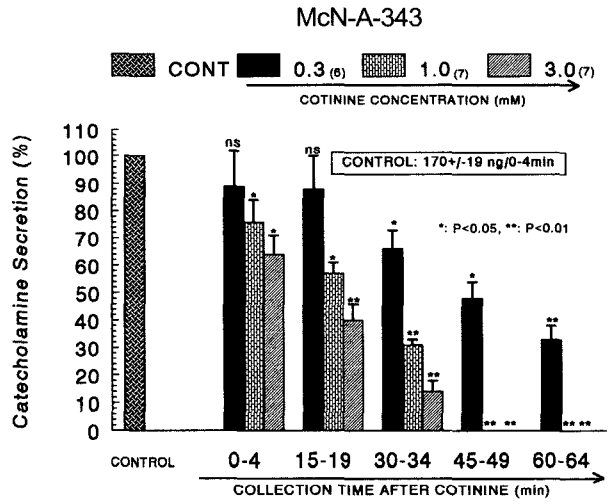

Fig. 4. The effect of cotinine on the secretory responses of catecholamines (CA) evoked by McN-A-343 from the isolated perfused rat adrenal glands. CA secretion by a single injection of McN-A-343 (10^{-4} M) was infused for 4 min at 15 min intervals after preloading with 0.3, 1.0, and 3.0 mM of cotinine for 60 min, respectively. Statistical difference was obtained by comparing the corresponding control (CONT, 170±19 ng for 4 min) with each concentration-pretreated group of cotinine. McN-A-343-induced perfusate was collected for 4 minutes. Other legends are the same as in Fig. 1. ns: Statistically not significant.

Fig. 5 (upper) shows the inhibitory effect of cotinine (1.0 mM) on the Bay-K-8644-evoked CA secretory re sponses. In absence of cotinine, Bay-K-8644 (10^{-5} M) introduced into the perfusion stream evoked the CA secretion of

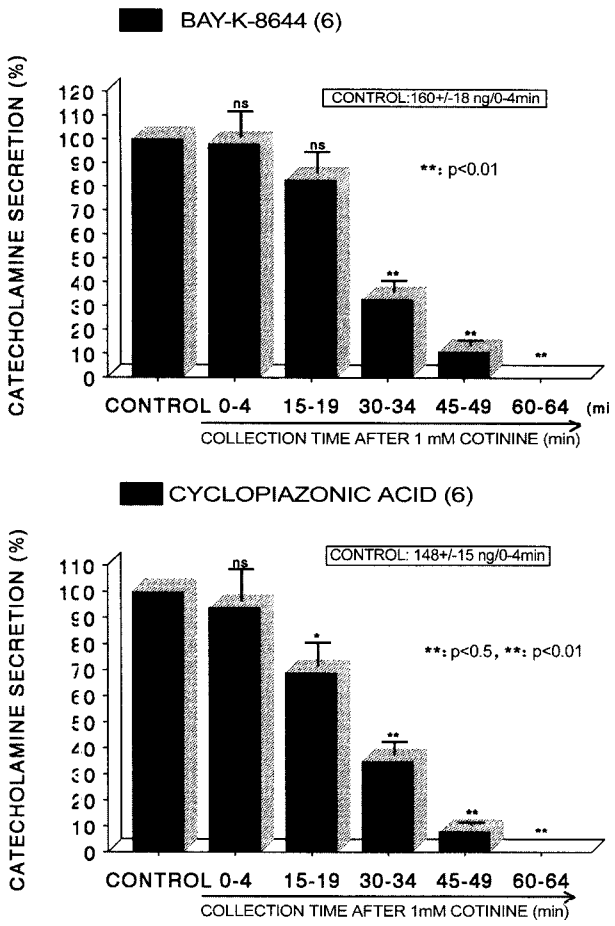

Fig. 5. Effects of cotinine on CA release evoked by Bay-K-8644 (upper) and cyclopiazonic acid (lower) from the rat adrenal glands. Bay-K-8644 (10^{-5} M) and cyclopiazonic acid (10^{-5} M) were perfused into an adrenal vein for 4 min at 15 min intervals after preloading with cotinine (1.0 mM) for 60 min, respectively. Other legends are the same as in Fig. 1. ns: Statistically not significant.

160±18 ng (0-4 min) in 6 rat adrenal glands. However, in the presence of cotinine (1.0 mM), the Bay-K-8644-stimulated CA secretion was time-dependently inhibited by 98~0% from that of the corresponding control release (100%).

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 (Georger and Riley, 1989; Seidler *et al.*, 1989). It may be an extremely valuable pharmacological tool for investigating intracellular Ca^{2+} mobilization and ionic current regulated by intracellular calcium (Suzuki *et al.*, 1992). As shown in Fig. 5 (lower), in the presence of cotinine (1.0 mM), the cyclopiazonic acid (10^{-5} M)-evoked CA secretion was largely attenuated by 94~0% from that of the control response (100%) from 6 rat adrenal glands.

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

From the experimental results, as shown in Fig. 1~5, cotinine showed time- and dose-dependent inhibition of the CA secretory responses. It has already been reported that (*S*)-(-)-cotinine, a constituent of tobacco products and the major metabolite of nicotine, stimulates nicotinic receptors to evoke the release of dopamine (DA) in a calcium-dependent manner from [^{3}H]DA-preloaded superfused rat striatal slices (Dwoskin *et al.*, 1999). Therefore, it was likely of interest to examine effects of nicotine on CA secretion evoked by ACh, high K^+, DMPP and McN-A-343 from the isolated perfused rat adrenal glands.

As shown in Fig. 6 (upper), in the present study, ACh (5.32 mM)-stimulated CA secretion, prior to the preloading with nicotine was 316±29 ng (0-4 min) from 6

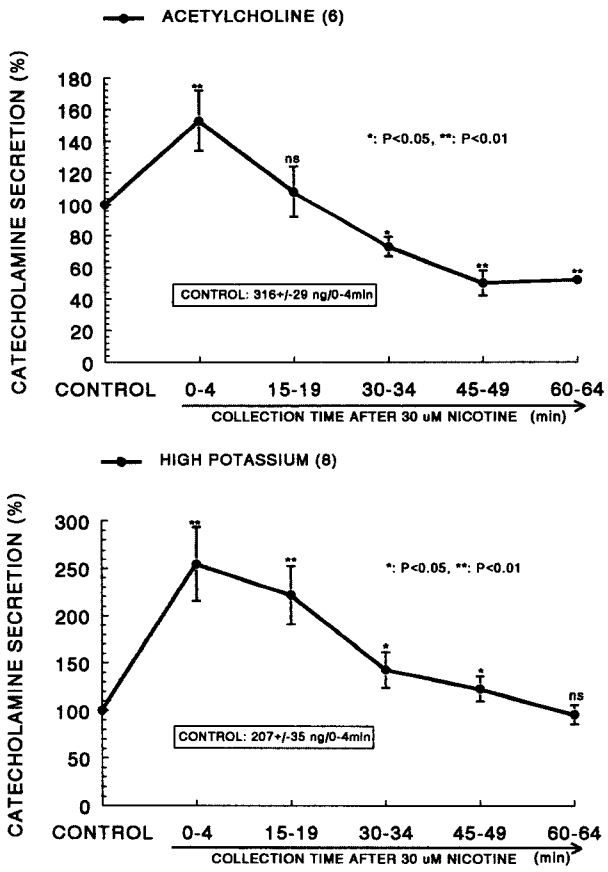

Fig. 6. Effects of nicotine on the secretory responses of catecholamines (CA) evoked by acetylcholine (upper) and high potassium (lower) from the isolated perfused rat adrenal glands. CA secretion by a single injection of ACh (5.32 mM) and high K^+ (56 mM) were induced before (CONTROL) and after preloading with 30 μM nicotine for 60 min, respectively. ACh- and high K^+-perfusates were collected for 4 minutes at 15 min-intervals, respectively. Other legends are the same as in Fig. 1. ns: Statistically not significant.

glands. However, in the presence of nicotine (30 μM) perfused for 60 min, it was markedly enhanced by 153% of the control secretion (100%), followed later by inhibition to ~52% of the control. Interestingly, high K^+ (56 mM)-stimulated CA secretion in the presence of nicotine (30 mM) was largely increased by 255% from the of the control release (100%) from 8 glands, as shown in Fig. 6 (lower).

When perfused into the adrenal gland, DMPP (100 μM), a neuronal nicotinic receptor agonist, evoked the CA secretion of 688±85 ng (0-8 min). However, following perfusion with nicotine (30 μM), it was time-dependently decreased by ~14% from that of the control release (100%) in 9 adrenal glands (Fig. 7-upper). In 8 adrenal glands, McN-A-343 (100 μM)-stimulated CA secretion before the administration of nicotine was 169±20 ng (0-4 min), but in the presence of nicotine (30 μM), it was also time-dependently reduced by ~15% from that of the control secretion (100%), as shown in Fig. 7 (lower).

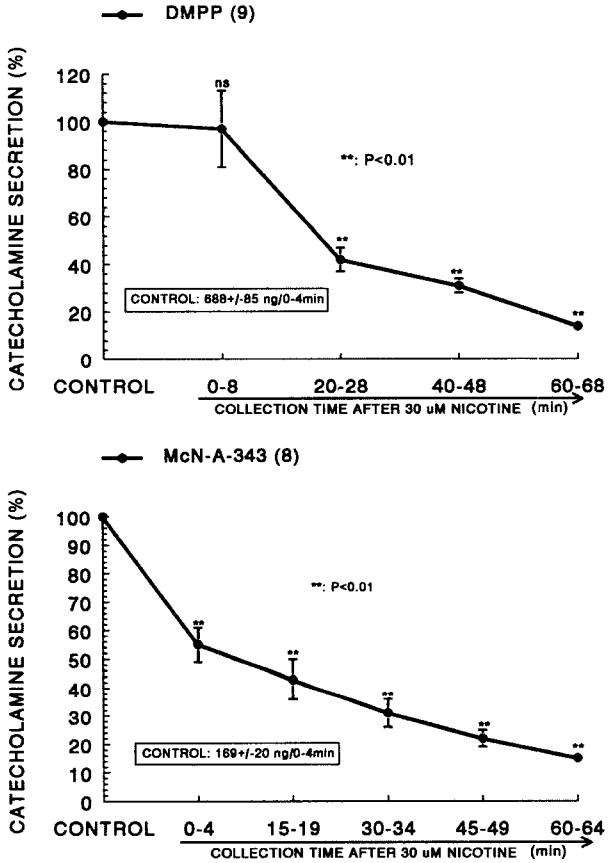

Fig. 7. Effects of nicotine on the secretory responses of catecholamines (CA) evoked by DMPP (upper) and McN-A343 (lower) from the isolated perfused rat adrenal glands. CA secretion by perfusion of DMPP (10^{-4} M) and McN-A343 (10^{-4} M) for 2 min was induced before (CONTROL) and after preloading with 30 μM nicotine for 60 min, respectively. DMPP- and McN-A343-induced perfusates were collected for 8 and 4 minutes at 20 and 15 min intervals, respectively. Other legends are the same as in Fig. 1. ns: Statistically not significant.

DISCUSSION

These experimental results demonstrate that cotinine inhibits greatly CA secretion evoked by stimulation of cholinergic (both nicotinic and muscarinic) receptors, but does fail to affect the secretion by direct membrane-depolarization. This inhibitory effect of cotinine is thought to be mediated by blocking both the calcium influx into the rat adrenal medullary chromaffin cells and Ca^{2+} release from the cytoplasmic calcium store. There also seems to be a big difference in the mode of action between cotinine and nicotine in rat adrenomedullary CA secretion.

In support of this idea, many evidences have been accumulated. Cotinine was found to be hypotensive and to abolish the nicotine-induced vasopressor response in dogs (Borzelleca *et al.*, 1962). It has also been reported that pretreatment with a high dose of cotinine in rabbits blocked the hypertensive action of a high dose nicotine (Chahine *et al.*, 1996). In rat aorta, cotinine antagonized nicotines inhibition of prostacyclin biosynthesis (Chahine *et al.*, 1990). Cotinine has also been observed to block the contraction response to nicotine in rabbit duodenum and ileum (Kim *et al.*, 1968). It has been also found that cotinine inhibits activation of protein kinase C and norepinephrine release induced by nicotine agonists in primary cultures of bovine adrenal chromaffin cells (Vainio *et al.*, 1998). Based on these previous facts, the present findings that continine relatively inhibited dose- and time-dependently CA secretory responses evoked by DMPP and McN-A-343 as well as by ACh from the perfused rat adrenal medulla suggest that this inhibitory effect of cotinine may be mediated by the blockade of cholinergic receptors located on the rat adrenomedullary chromaffin cells.

In contrast to the present results, in mice, cotinine has been reported to mimic nicotine as an antinociceptive compound (Erenmemisoglu and Tekol, 1994) and in counteracting ethanol-induced incoordination (Dar *et al.*, 1994). Recently, cotinine-evoked dopamine release from rat striatal slices has been reported (Dwoskin *et al.*, 1999). In humans, cotinine slightly worsens nicotine withdrawal symptoms (Keenan *et al.*, 1994), and it eliminates the beneficial effects of a nicotine patch (Hatsukami *et al.*, 1998). Outside the nervous system, cotinine has been reported to affect eicosanoid release both in a nicotine-like manner (Saareks *et al.*, 1993) and differently from nicotine (Chahine *et al.*, 1990). Moreover, in steroid metabolism both nicotine-like (Patterson *et al.*, 1990) and nicotine-unlike effects (Andersson *et al.*, 1993) have been detected.

In bovine adrenal medulla, increased concentration of extracellular potassium causes membrane depolarization and opening of voltage-gated calcium channels leading to

CA secretion (Douglas and Rubin 1961; Douglas *et al.* 1967) that can be inhibited by L-type calcium channel blockers (Pinto and Trifaro 1976; Kilpatrick *et al.*, 1981). In the present work, to exclude the possibility that cotinine could block calcium channels, cotinine was pretreated prior to exposure to 56 mM K^+. Since no inhibition of the potassium-induced exocytosis was detected, cotinine likely interacts with nicotine in a manner not involving voltage-gated calcium channels. This finding is also in agreement with the previous results that cotinine failed to affect high potassium-evoked CA secretion from the cultured bovine chromaffin cells (Vainio *et al.*, 1998).

In the present investigation, the results that cotinine as well as nicotine inhibits CA secretion evoked by stimulation of muscarinic receptors with McN-A-343, a selective muscarinic M_1-receptor agonist, suggest strongly that muscarinic M_1-receptors are involved in the regulation of the secretory responses in the rat adrenal medulla. In support of this hypothesis, it has been shown that muscarinic stimulation generates a depolarizing signal which triggers the firing of action potentials, resulting in the increased CA release in the rat chromaffin cells (Akaike *et al.*, 1990; Lim and Hwang, 1991). These observations are in line with a previous report showing that Bay-K-8644 almost crippled the peak secretory response to muscarine in perfused cat adrenal glands (Ladona *et al.*, 1987; Uceda *et al.*, 1992). In the present experiment, cotinine also depressed greatly CA secretion induced by Bay-K-8644, which is found to potentiate the release of CA by increasing Ca^{2+} influx through L-type Ca^{2+} channels in chromaffin cells (Garcia *et al.*, 1984). These findings that cotinine inhibited CA secretion evoked by Bay-K-8644, but did not that by high K^+ suggest that inhibits directly the voltage-dependent Ca^{2+} channels. In the bovine chromaffin cells stimulation of nicotinic, but not muscarinic ACh receptors is known to cause CA secretion by increasing Ca^{2+} influx largely through voltage-dependent Ca^{2+} channels (Burgoyne, 1984; Oka *et al.*, 1979). Therefore, it seems that cotinine inhibits DMPP-evoked CA secretion by inhibiting Ca^{2+} influx through voltage-dependent Ca^{2+} channels activated by nicotinic ACh receptors.

The present study has also shown that cotinine inhibits the CA secretion evoked by cyclopiazonic acid. Cyclopiazonic acid is known to be a highly selective inhibitor of Ca^{2+}-ATPase in skeletal muscle sarcoplasmic reticulum (Geoger and Riley, 1989; Siedler *et al.*, 1989) and a valuable pharmacological tool for investigating intracellular Ca^{2+} mobilization and ionic currents regulated by intracellular Ca^{2+} (Suzuki *et al.*, 1992). Therefore, it is felt that the inhibitory effect of cotinine on CA secretion evoked by cholinergic stimulation may be associated with the mobilization of intracellular Ca^{2+} from the cytoplasmic calcium store. This indicates that the cotinine has an inhibitory effect on the release of Ca^{2+} from the intracellular pools induced by stimulation of muscarinic ACh receptors, which is weakly responsible for the secretion of CA. It has been shown that Ca^{2+}-uptake into intracellular storage sites susceptible to caffeine (Ilno, 1989) is almost completely abolished by treatment with cyclopiazonic acid during the proceding Ca^{2+} load (Suzuki *et al.*, 1992). This is consistent with the findings obtained in skinned smooth muscle fibers of the logitudinal layer of the guinea-pig ileum, where Ca^{2+}-uptake was also inhibited by cylopiazonic acid (Uyama *et al.*, 1992). Suzuki and his coworkers (1992) have shown that cyclopiazonic acid easily penetrates into the cytoplasm through the plasma membrane and reduces Ca^{2+}-ATPase activity in sarcoplasmic/endoplasmic reticulum, resulting in increase in the subsequent Ca^{2+} release from those storage sites. Moreover, in bovine adrenal chromaffin cells, stimulation of muscarinic ACh receptors is also proposed to cause activation of phosphoinositide metabolism, resulting in the formation of inositol 1,4,5-trisphosphate, which induces the mobilization of Ca^{2+} from the intracellular pools (Cheek *et al.*, 1989; Challis *et al.*, 1991). However, in the present study, it is uncertain whether the inhibitory effect of cotinine on Ca^{2+} movement from intracellular pools is due to its direct effect on the PI response or the indirect effects.

The inhibitory effect of cotinine on nicotinic responses may have clinical relevance at least in the peripheral nervous system. Cotinine may e.g. sustain the addictive changes or relieve the abstinence symptoms at hours when nicotine is not present, which has been suggested by an uncontrolled study in nicotine addicts (Benowitz *et al.* 1983). By blocking the maximal response of nicotinic agonists, cotinine could also enhance the abstinence symptoms as suggested by Keenan *et al.* (1994). On the other hand, cotinine may antagonize nicotinic effects upon nicotine administration and thus inhibit the rewarding effect of self-administered nicotine, and be helpful in quitting smoking. However, one should be cautious in making firm conclusions of *in vivo* phenomena from *in vitro* data. Actually, the 10 μM concentration of nicotine which is frequently used in cell culture experiments, exceeds the peak nicotine concentrations of heavy smokers. Also, the lowest effective cotinine concentration in the present study, 300 μM, is some 75 times higher than that detected in the heavy smoker.

In conclusion, these results suggest that cotinine inhibits CA secretion evoked by stimulation of cholinergic (both nicotinic and muscarinic) receptors, but does fail to affect that by the direct membrane-depolarization. This inhibitory effect of cotinine is thought to be mediated by blocking both the calcium influx into the rat adrenal medullary chromaffin cells and Ca^{2+} release from the cytoplasmic calcium store. There also seems to be a big difference in

the mode of action between cotinine and nicotine in rat adrenomedullary CA secretion.

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