Full-length article

Arecoline inhibits catecholamine release from perfused rat adrenal gland[1]

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Abstract

Aim: To study the effect of arecoline, an alkaloid isolated from *Areca catechu*, on the secretion of catecholamines (CA) evoked by cholinergic agonists and the membrane depolarizer from isolated perfused rat adrenal gland. **Methods:** Adrenal glands were isolated from male Sprague-Dawley rats. The adrenal glands were perfused with Krebs bicarbonate solution by means of a peristaltic pump. The CA content of the perfusate was measured directly using the fluorometric method. **Results:** Arecoline (0.1–1.0 mmol/L) perfused into an adrenal vein for 60 min produced dose- and time-dependent inhibition in CA secretory responses evoked by acetylcholine (ACh) (5.32 mmol/L), 1.1-dimethyl-4-phenyl piperazinium iodide (DMPP) (100 µmol/L for 2 min) and 3-(m-choloro-phenyl-carbamoyl-oxy)-2-butynyl trimethyl ammonium chloride (McN-A-343) (100 µmol/L for 2 min). However, lower doses of arecoline did not affect CA secretion of high K^+ (56 mmol/L); higher doses greatly reduced CA secretion of high K^+. Arecoline also failed to affect basal catecholamine output. Furthermore, in adrenal glands loaded with arecoline (0.3 mmol/L), CA secretory response evoked by Bay-K-8644 (10 µmol/L), an activator of L-type Ca^{2+} channels, was markedly inhibited, whereas CA secretion by cyclopiazonic acid (10 µmol/L), an inhibitor of cytoplasmic Ca^{2+}-ATPase, was not affected. Nicotine (30 µmol/L), which was perfused into the adrenal gland for 60 min, however, initially enhanced ACh-evoked CA secretory responses. As time elapsed, these responses became more inhibited, whereas the initially enhanced high K^+-evoked CA release diminished. CA secretion evoked by DMPP and McN-A-343 was significantly depressed in the presence of nicotine. **Conclusion:** Arecoline dose-dependently inhibits CA secretion from isolated perfused rat adrenal gland evoked by activation of cholinergic receptors. At lower doses arecoline does not inhibit CA secretion through membrane depolarization, but at larger doses it does. This inhibitory effect of arecoline may be mediated by blocking the calcium influx into the rat adrenal medullary chromaffin cells without the inhibition of Ca^{2+} release from the cytoplasmic calcium store. There seems to be a difference in the mode of action of nicotine and arecoline in rat adrenomedullary CA secretion.

Introduction

The areca nut, popularly known as the betel nut, is almost symbolic of oriental culture, and is one of the oldest known masticatories in Asia. The composition of the betel quid varies regionally. In general, betel quid is composed of betel leaf, areca nut, catechu and lime, to which tobacco is sometimes added[1]. Betel nut is the endosperm of the fruit of the *Areca catechu* tree. It contains several alkaloids, of which arecoline is the most abundant[2,3]. The pharmacological effects of betel nut include addiction, euphoria, excessive salivation and tremors, which are attributable to the cholinergic effects of arecoline, the major alkaloid of the betel nut[4].

Molinengo and coworkers found that lower doses of arecoline caused neither modification of acetylcholine (ACh)

levels nor mouse motility, but higher doses of arecoline caused a reduction in motility and an increase in the ACh levels in the subcortical structures of the mouse central nervous system[5]. It has also been shown that arecoline causes a reduction in levels of ACh in the cortex and subcortex of mice at the limit of statistical significance, but a statistically significant reduction in levels of norepinephrine[5]. The major constituent of betel nut, arecoline, may aggravate bronchial asthma[6] and change urine volume and urinary electrolyte excretion[7]. Arecoline has been used to treat patients with Alzheimer's presenile dementia[8]. In such patients, it has been shown to improve verbal memory and performance in picture memory tasks[9]. In experimental models, arecoline prevented scopolamine-induced impairment in task acquisition[10], and restored passive avoidance performance[11], indicating that certain kinds of memory depend, at least in part, on cholinergic activity.

Arecoline has also been considered an M_1/M_3 partial agonist, with a peak effect of approximately half the maximum obtained with pilocarpine in rat brain slices[12,13]. However, arecoline appears to be a full agonist in membranes prepared from rat cortical slices, regardless of guanosine 5'-O-(3-thiotriphosphate) concentrations[14]. Recently, it has been suggested that the underlying mechanism by which phosphoinositide turnover is inhibited in rat cortical slices is arecoline-induced receptor sequestration[15]. Yang and colleagues reported that arecoline exerted its excitatory action by binding M_2-muscarinic receptors on the cell membrane of neurons of the locus coeruleus[16].

It has been concluded that the pressor effects brought about by quaternary compounds can be attributed to the stimulation of nicotinic and muscarinic receptors in the sympathetic ganglia of both pithed and anesthetized rats[17]. Moreover, Polinsky and coworkers found that the increased plasma epinephrine levels following arecoline treatment in normal subjects and patients with multiple system atrophy might result from nicotinic adrenal stimulation[18]. It has also been reported that betel chewing increases plasma concentrations of norepinephrine and epinephrine[19]. However, the mechanism underlying the pressor effect and increased plasma catecholamine (CA) concentration remains poorly understood. Therefore, the present study was designed to investigate the effects of arecoline on catecholamine secretion in an isolated perfused model of the rat adrenal gland, and to clarify its mechanism of action.

Materials and methods

Experimental procedure Male Sprague-Dawley rats,

weighing 200–300 g, were anesthetized with thiopental sodium (50 mg/kg) intraperitoneally. Adrenal glands were isolated by modification of the methods described previously[20]. 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 a portion of the liver were not removed, but pushed over to the right side and covered with saline-soaked gauge pads while urine in the bladder was removed, in order to obtain enough working space for tying blood vessels and carrying out the cannulations. A cannula, which was used for perfusion of the adrenal gland, was inserted into the distal end of the renal vein after all branches of the adrenal vein (if any), vena cava and aorta were ligated. Heparin (400 IU/mL) was injected into the vena cava to prevent blood coagulation before ligating the vessels and carrying out the cannulations. A small slit was cut into the adrenal cortex just opposite the entrance of the adrenal vein. Perfusion of the gland was started, making sure that no leakage occurred, and the perfusion fluid escaped only from the slit made in the adrenal cortex. Then the adrenal gland, along with the ligated blood vessels and the cannula, was carefully removed from the animal and placed on a platform in a leucite chamber. Water heated to 37±1 °C was continuously circulated in the chamber.

Drugs and their sources The following drugs were used: arecoline hydrobromide, acetylcholine chloride, 1.1-dimethyl-4-phenyl piperazinium iodide (DMPP), (-) nicotine, norepinephrine bitartrate, methyl-1,4-dihydro-2,6-dimethyl-3-nitro-4-(2-trifluoromethylphenyl)-pyridine-5-carboxylate (Bay-K-8644) (Sigma, USA), cyclopiazonic acid, 3-(m-choloro-phenyl-carbamoyl-oxy)-2-butynyl trimethyl ammonium chloride (McN-A-343) (RBI, USA). 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.

Perfusion of adrenal gland The adrenal glands were perfused by means of an ISCO pump (WIZ Co) at a rate of 0.33 mL/min. The perfusion was carried out with Krebs bicarbonate solution of the following composition (mmol/L): 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 in the range of 7.4–7.5. The solution contained disodium ethylenediamine tetraacetic acid (EDTA) ($2.97×10^{-5}$ mol/L) and ascorbic acid ($5.68×10^{-4}$ mol/L) to prevent oxidation of CA.

Drug administration The perfusions of DMPP ($1×10^{-4}$

mol/L) and McN-A-343 (1×10^{-4} mol/L) for 2 min, and a single injection of ACh (5.32 mmol/L) and KCl (5.6 mmol/L) in a volume of 0.05 mL were made into the perfusion stream via a three-way stopcock, respectively. Bay-K-8644 (1×10^{-5} mol/L) and cyclopiazonic acid (1×10^{-5} mol/L) were also perfused for 4 min, respectively.

In the preliminary experiments, it was found that after administration of these drugs, secretory responses to ACh, KCl, McN-A-343, Bay-K-8644 and cyclopiazonic acid returned to pre-injection levels in approximately 4 min, but the responses to DMPP took 8 min.

Collection of perfusate Prior to stimulation with various secretagogues, the perfusate was collected for 4 min to determine the spontaneous secretion of CA (background sample). Immediately after these samples were taken, collection of the perfusates was continued in another tube as soon as the perfusion medium containing the stimulatory agent reached the adrenal gland. These samples were collected for 4–8 min. The amounts secreted in the background sample were subtracted from those secreted in the stimulated sample to obtain the net secretion value of CA.

To study the effect of arecoline on spontaneous and evoked secretion, adrenal glands were perfused with Krebs solution containing arecoline for 60 min. Then the perfusate was collected for a certain period as a background sample. The medium was changed to the one containing the stimulating agent along with arecoline, and the perfusates were collected for the same period as the background sample. The adrenal gland perfusates were collected in chilled tubes.

Measurement of catecholamines CA content of perfusate was measured directly by using the fluorometric method of Anton and Sayre[21], without the intermediate purification alumina for the reasons described earlier[20], using a fluorospectrophotometer (Kontron, Milan, Italy). A volume of 0.2 mL of the perfusate was used for the reaction. The CA content in the perfusate of the stimulated glands achieved by using secretagogues used in the present work was high enough to obtain readings several times greater than the reading of control samples (unstimulated). The sample blanks were also lowest for perfusates of stimulated and non-stimulated samples. The content of CA in the perfusate was expressed in terms of norepinephrine (base) equivalents.

Statistical analysis The statistical difference between the control and pretreated groups was determined by using Student's *t*-test and ANOVA. 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 the means and the standard errors of the mean (SEM). The statistical analysis of the experimental results was done by the computer program described by Tallarida and Murray[22].

Results

Effect of arecoline on CA secretion evoked by ACh, excess K+, DMPP and McN-A-343 from the perfused rat adrenal glands After perfusion with oxygenated Krebs bicarbonate solution for 1 h, basal CA release from the isolated perfused rat adrenal glands amounted to 23.1±2.2 ng per 2 min (*n*=6). Because the addition of arecoline in rat brain cortical slices inhibited the carbachol-stimulated phosphoinositide breakdown[15], we initially attempted to examine the effects of arecoline itself on CA secretion from the perfused model of the rat adrenal glands. However, in the present study, arecoline (1×10^{-4}–1×10^{-3} mol/L) by itself did not produce any effect on basal CA output of the perfused rat adrenal glands (data not shown). Therefore, it was decided to investigate the effects of arecoline on cholinergic receptor stimulation, as well as membrane depolarization-mediated CA secretion. Secretagogues were given at 15 min intervals. Arecoline was present 15 min before initiation of stimulation.

When ACh (5.32×10^{-3} mol/L) in a volume of 0.05 mL was injected into the perfusion stream, the amount of CA secreted was 283±36 ng for 4 min. Pretreatment with arecoline for 60 min at concentrations ranging from 1×10^{-4} to 1×10^{-3} mol/L inhibited ACh-stimulated CA secretion in 6 adrenal glands in a time-dependent manner. As shown in Figure 1 (upper panel), in the presence of arecoline, CA-releasing responses were inhibited to 88%–30% of the corresponding control release. It has also been found that depolarizing agents such as KCl markedly stimulate CA secretion (128±16 ng for 0–4 min). Excess K+ (5.6×10^{-2} mol/L)-stimulated CA secretion, after pretreatment with a lower concentration of arecoline (1×10^{-4} mol/L), was not affected as compared with its corresponding control secretion (100%) (Figure 1, lower panel). However, following pretreatment with higher concentrations of arecoline (3×10^{-4} mol/L and 1×10^{-3} mol/L), excess K+ (5.6×10^{-2} mol/L)-stimulated CA secretion was significantly inhibited. When perfused through the rat adrenal gland, DMPP (1×10^{-4} mol/L), which is a selective nicotinic receptor agonist in autonomic sympathetic ganglia, evoked a sharp and rapid increase in CA secretion (512±24 ng for 0–8 min). However, as shown in Figure 2 (upper panel), DMPP-stimulated CA secretion after pretreatment with arecoline was significantly reduced to a maximum of 16% of the control release (100%) in 6 rat adrenal glands. McN-A-343 (1×10^{-4} mol/L), which is a selective muscarinic M_1-agonist[23], when
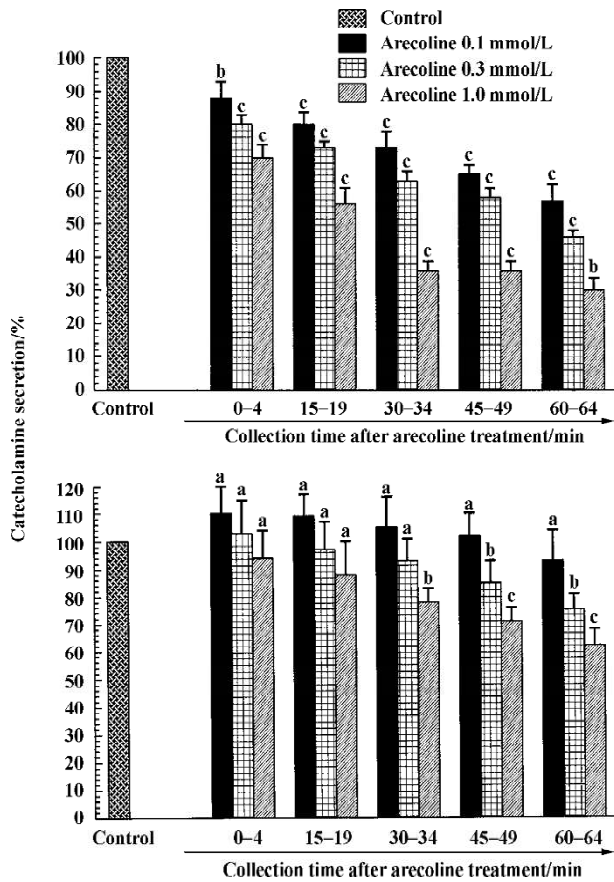
Figure 1. Dose-dependent effect of arecoline on the secretory responses of catecholamines (CA) from isolated perfused rat adrenal glands evoked by acetylcholine (upper) and high K^{+} (lower). CA secretion by a single injection of ACh (5.32×10^{-3} mol/L) and K^{+} (5.6×10^{-2} mol/L) in a volume of 0.05 mL was evoked at 15 min intervals after preloading with 0.1, 0.3, 1.0 mmol/L of arecoline for 60 min as indicated by the arrow. Numbers in parentheses indicate the number of rat adrenal glands. Vertical bars on the columns represent the standard error of the mean (SEM). Ordinate: amounts of CA secreted from the adrenal gland (% of control). Abscissa: collection time of perfusate (min). Statistical difference was assessed by comparing the corresponding control (CONT) with each group pretreated with a particular concentration of arecoline. ACh- and high K^{+}-induced perfusates were collected for 4 min, respectively. $n=6$. Mean±SEM. [a]$P>0.05$, [b]$P<0.05$, [c]$P<0.01$ *vs* control.

perfused into an adrenal gland for 4 min, caused increased CA secretion (79±8 ng for 0–4 min) from 6 glands. However, McN-A-343-stimulated CA secretion in the presence of arecoline was markedly depressed to 75%–0% of the corresponding control secretion (100%) as depicted in Figure 2 (lower panel).

Effect of arecoline on CA secretion evoked by Bay-K-8644 and cyclopiazonic acid from perfused rat adrenal glands Because Bay-K-8644 is known to be a calcium chan-
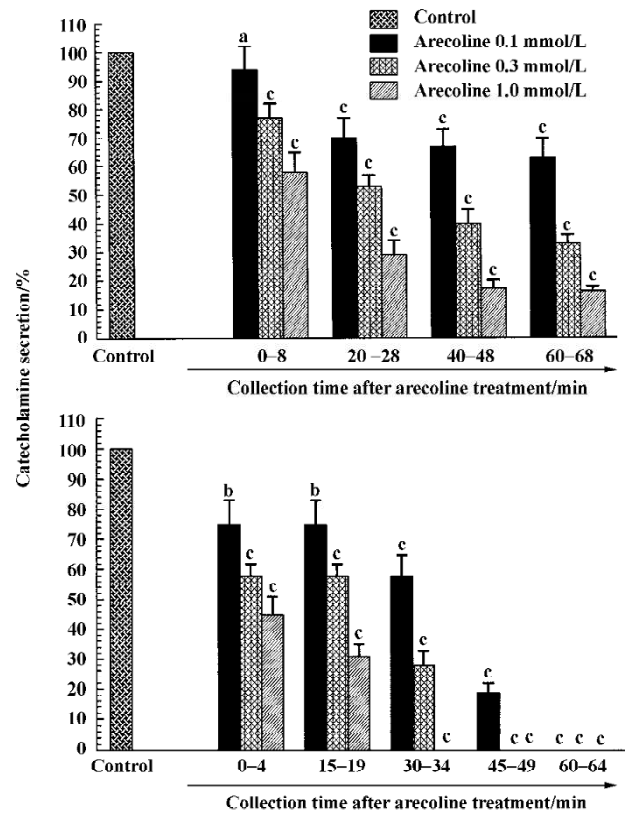

Figure 2. Dose-dependent effect of arecoline on the secretory responses of catecholamines (CA) from isolated perfused rat adrenal glands evoked by DMPP (upper) and McN-A-343 (lower). CA secretion by a single injection of DMPP (1×10^{-4} mol/L) and McN-A-343 (1×10^{-4} mol/L) was infused for 2 min at 20 min intervals after preloading with 0.1, 0.3, 1.0 mmol/L of arecoline for 60 min, respectively. Statistical difference was assessed by comparing the corresponding control (CONT) with each group pretreated with a particular concentration of arecoline. DMPP- and McN-A-343-induced perfusates were collected for 8 and 4 min, respectively. $n=6$. Mean±SEM. [a]$P>0.05$, [b]$P<0.05$, [c]$P<0.01$ *vs* control.

nel activator that enhances basal Ca^{2+} uptake[24] and CA release[25], it was of interest to determine the effects of arecoline on Bay-K-8644-stimulated CA secretion from isolated perfused rat adrenal glands. As shown in Figure 3 (upper panel), Bay-K-8644 (1×10^{-5} mol/L)-stimulated CA secretion in the presence of arecoline was significantly blocked to 85%–33% as compared with the control release (92±7 ng for 0–4 min). The only exception to this decrease was the first 4 min, during which an increase occurred.

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[26,27]. The inhibitory action of arecoline on cyclopiazonic acid-evoked CA secretory response was observed as shown
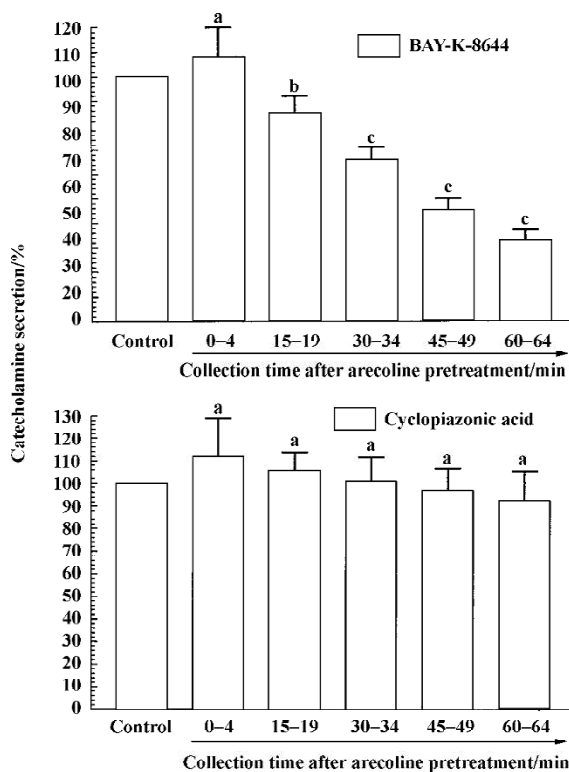
Figure 3. Effects of arecoline on catecholamine (CA) release from rat adrenal glands evoked by Bay-K-8644 (upper) and cyclopiazonic acid (lower). Bay-K-8644 (1×10^{-5} mol/L) and cyclopiazonic acid (1×10^{-5} mol/L) were perfused into an adrenal vein for 4 min at 15 min intervals after preloading with arecoline (0.3 mmol/L) for 60 min, respectively. Other legends are the same as in Figure 1. *n*=6. Mean±SEM. [a]$P>0.05$, [b]$P<0.05$, [c]$P<0.01$ *vs* control.
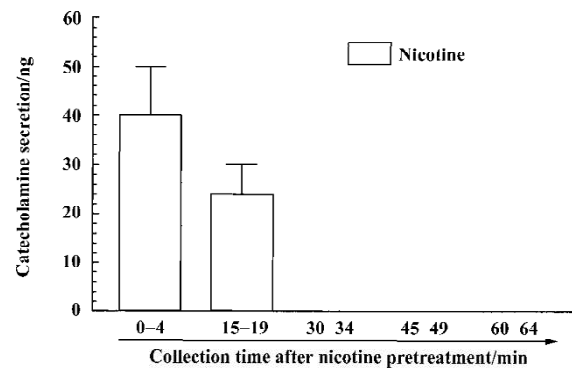

Figure 4. Time-course effects of continuous infusion of nicotine on the secretion of catecholamines (CA) from perfused rat adrenal glands. Nicotine (30 μmol/L) was infused continuously for 60 min into an adrenal vein. Nicotine-evoked perfusates were collected for 4 min at 15 min intervals, respectively. Other legends are the same as in Figure 1. *n*=8. Mean±SEM.

in Figure 3 (lower panel). However, in the presence of arecoline in 6 rat adrenal glands, cyclopiazonic acid (1×10^{-5} mol/L)-evoked CA secretion was not altered in comparison with the control response (52±4 ng for 0–4 min).

Effect of nicotine on CA secretion evoked by ACh, excess K$^+$, DMPP, and McN-A-343 from the perfused rat adrenal glands It has been reported that arecoline increases plasma epinephrine levels in normal subjects and patients with multiple system atrophy, which may result from nicotinic adrenal stimulation[18]. Therefore, in order to compare the effects of nicotine with those of arecoline, it was of interest to examine the effect of nicotine on CA secretion from isolated perfused rat adrenal glands evoked by ACh, high K$^+$, and DMPP. In order to test the effect of nicotine on cholinergic receptor-stimulated CA secretion as well as membrane depolarization-mediated secretion, when nicotine (3×10^{-5} mol/L) was loaded into the adrenal medulla, the released CA amounted to 40±10 ng (0–4 min) and 24±6 ng (15–19 min) without any more release after 30 min (Figure 4). In

the present experiment, ACh (5.32×10^{-3} mol/L)-evoked CA release, before perfusion with nicotine, was 358±39 ng (0–4 min). In the presence of nicotine (3×10^{-5} mol/L) for 60 min, ACh-evoked CA release initially increased to 149% of the control in the first 4 min period. As time elapsed, this CA release gradually reduced to 46% of the control (Figure 5, upper panel). High K$^+$ (5.6×10^{-2} mol/L)-evoked CA release, in the presence of nicotine, was significantly enhanced to 260% in the first 4 min, and then gradually changed to 131% of the control secretion (120±14 ng, 0–4 min). Only in the last period (98%±9%, 60–64 min) did the secretions return to the control level, as shown in Figure 5 (lower panel). In 8 rat adrenal glands, DMPP (1×10^{-4} mol/L) perfused into the adrenal gland evoked a marked CA secretion of 537±59 ng (0–8 min) before loading with nicotine. Following perfusion with the nicotine, the secretion was considerably diminished to a maximum of 19% of the corresponding control release (Figure 6, upper panel). Moreover, in the presence of nicotine, McN-A-343 (10^{-4} mol/L)-evoked CA secretory responses were also time-dependently inhibited to 23% of the control secretion (81±9 ng, 60–64 min) from 6 glands, as shown in Figure 6 (lower panel).

Discussion

The present study shows that arecoline dose- and time-dependently inhibits CA secretory responses from the perfused rat adrenal gland evoked by ACh, DMPP, and McN-A-343. Arecoline in lower doses did not affect CA secretion induced by high K$^+$; however, higher doses greatly reduced CA secretion of high K$^+$. Furthermore, in adrenal glands loaded with arecoline, CA secretory responses evoked by
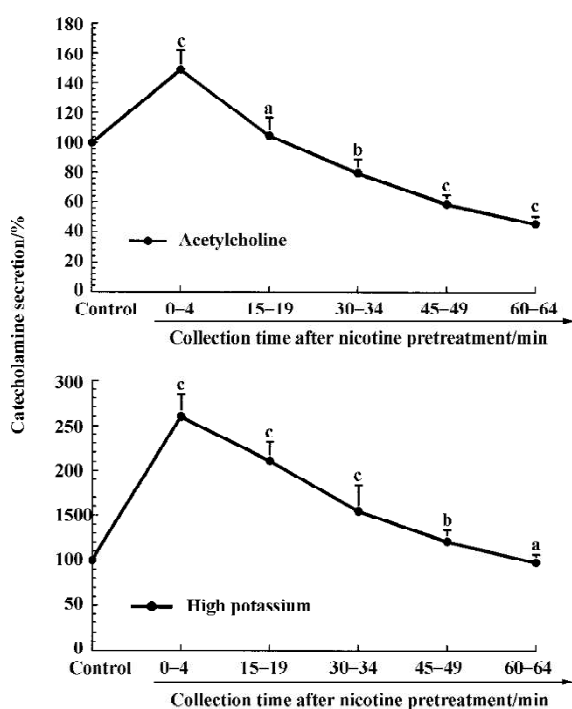
Figure 5. Effects of nicotine on the secretory responses of catecholamines (CA) evoked from isolated perfused rat adrenal glands by acetylcholine (upper) and high K^+ (lower). CA secretion by a single injection of ACh (5.32×10^{-3} mol/L) and high K^+ (5.6×10^{-2} mol/L) were induced before and after preloading with 30 μmol/L nicotine for 60 min, respectively. ACh- and high K^+-evoked perfusates were collected for 4 min at 15 min intervals, respectively. Other legends are the same as in Figure 1. $n=8$. Mean±SEM. [a]$P>0.05$, [b]$P<0.05$, [c]$P<0.01$ *vs* control.
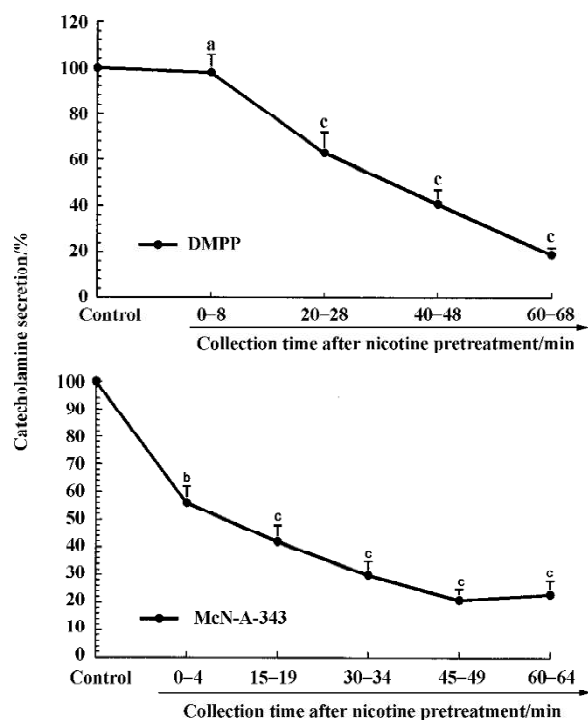

Figure 6. Effects of nicotine on the secretory responses of catecholamines (CA) from isolated perfused rat adrenal glands evoked by DMPP (upper) and McN-A343 (lower). CA secretion by perfusion of DMPP (1×10^{-4} mol/L) and McN-A343 (10^{-4} mol/L) for 2 min was induced before (Control) and after preloading with 30 μmol/L nicotine for 60 min, respectively. DMPP- and McN-A-343-evoked perfusates were collected for 8 and 4 min at 20 and 15 min intervals, respectively. Other legends are the same as in Figure 1. $n=8$. Mean±SEM. [a]$P>0.05$, [b]$P<0.05$, [c]$P<0.01$ *vs* control.

Bay-K-8644, an activator of L-type Ca^{2+} channels, was markedly inhibited, whereas CA secretion evoked by cyclopiazonic acid, an inhibitor of cytoplasmic Ca^{2+}-ATPase, was not affected. In contrast to arecoline exposure, exposure of the adrenal gland to nicotine (30 μmol/L) for 60 min initially enhanced the ACh-evoked CA secretory responses. These responses were inhibited as time elapsed, whereas the initially enhanced high K^+-evoked CA release was time-dependently diminished. CA secretion evoked by DMPP and McN-A-343 was significantly depressed in the presence of nicotine. Taken together, these results suggest that arecoline greatly inhibits CA secretion evoked by the activation of cholinergic (both nicotinic and muscarinic) receptors. At lower doses arecoline does not inhibit CA secretion by membrane depolarization, but at larger doses it does. We suggest that this inhibitory effect of arecoline may be mediated by blocking the calcium influx into the rat adrenal medullary chromaffin cells without the inhibition of Ca^{2+} release from the cytoplasmic calcium store. It seems that there is a differ-

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

In support of this idea, it has been found that arecoline (10 mg/kg) injected subcutaneously caused a reduction in levels of ACh in the mouse cortex and subcortex, and a great reduction in levels of norepinephrine[28]. However, higher doses (28.5 and 60 mg/kg per day) were found to cause a reduction in mouse motility and an increase of the ACh levels in the subcortical structure of the CNS of the mouse[5]. However, Polinsky and colleagues have shown that increased plasma epinephrine levels, following arecoline administration in normal subjects and patients with multiple system atrophy, may result from nicotinic adrenal stimulation[18]. Recently, it has been found that chewing betel, which contains arecoline, and Piper betel flower or leaf, which contain aromatic phenolic compounds that have been found to stimulate the release of catecholamine in *in vitro* studies, also increase plasma concentrations of norepinephrine and epi-

nephrine in normal subjects[19]. However, in the present study, our finding that arecoline greatly inhibited the CA secretory responses evoked by ACh and DMPP, selective neuronal nicotinic receptor agonists, clearly suggests that arecoline can act as an antagonist of the nicotinic receptors in rat adrenal medulla. Furthermore, at 1 mmol/L (the highest concentration tested) arecoline completely blocked the CA secretory responses that would have been evoked by cholinergic stimulation. Therefore, the results of the present experiments are not in agreement with those of previous studies[18,19], in which plasma CA levels were elevated following arecoline treatment or betel chewing. All concentrations (0.1–1.0 mmol/L) of arecoline used in these experiments failed to evoke any secretions of CA. In terms of these findings, it seems that arecoline at the concentrations used in the present study does not act as a nicotinic receptor agonist in perfused rat adrenal medulla.

Generally, arecoline has been considered as a M_1/M_3 partial agonist with a peak effect of approximately half that of the maximum obtained with pilocarpine in rat brain slices[12,13]. However, arecoline appears to be a full agonist in membranes prepared from rat cortical slices regardless of guanosine 5'-O-(3-thiotriphosphate) concentrations[14]. To assess the influence of arecoline treatment on muscarinic receptor-associated phosphoinositide signaling pathways, Lee and colleagues[15] investigated the effects of acute and chronic administration of arecoline on muscarinic cholinergic receptor-stimulated phosphoinositide turnover in rat brain cortical slices. They found that administration of arecoline inhibited carbachol-stimulated phosphoinositide turnover. In terms of this finding, our finding that arecoline completely abolishes the CA secretion evoked by McN-A-343, a selective muscarinic M_1-receptor agonist, suggests that arecoline has an anti-muscarinic activity. In the present experiment, more than 90% of the inhibitory response could also be reversed by perfusion of the adrenal medulla with an arecoline-free medium for 1.5 h after arecoline exposure. It would also seem that this inhibition did not result from cytotoxicity.

In the present investigation, arecoline at higher concentrations (0.3–1.0 mmol/L) greatly attenuated CA secretions evoked by high K^+, a direct membrane-depolarizing agent. This result suggests that arecoline can block voltage-sensitive Ca^{2+} channels. In support of this finding, our finding that arecoline greatly attenuates the CA secretion evoked by Bay-K-8644, an activator of L-type Ca^{2+} channels, indicates that arecoline may act as a Ca^{2+} channel antagonist in the rat adrenal medulla. Bay-K-8644 has been found to enhance the release of CA by increasing Ca^{2+} influx through L-type Ca^{2+} channels in cultured bovine chromaffin cells[24].

Moreover, previous studies on primary cultures of bovine chromaffin cells have shown that dihydropyridines can partially inhibit CA secretion induced by depolarization with ACh, nicotine or K^+. The degree of inhibition varies between studies depending on the dihydropyridine used, its concentration, and the concentration of agonist[29–31]. However, at high concentrations (≥ 1 µmol/L), dihydropyridines block the nicotinic receptor-related ion channels, and at these concentrations they also inhibit calcium uptake and CA secretion induced by nicotinic agonists without comparable effects on K^+-evoked responses[32]. Nitrendipine at a concentration of 1 µmol/L was found to be sufficient to reduce the contraction of pig coronary artery rings induced by 30 mmol/L K^+[33].

The most plausible explanation of this finding is that arecoline also has a direct blocking effect on Ca^{2+} channels. Therefore, the present experimental results imply that arecoline itself blocks Ca^{2+} entry into the adrenomedullary chromaffin cells by inhibiting voltage-dependent Ca^{2+} channels and, as consequence, it inhibits the Ca^{2+}-dependent release of CA evoked by cholinergic stimulation as well as membrane-depolarization.

However, in the present work, arecoline did not inhibit the increase in CA secretion evoked by cyclopiazonic acid. Cyclopiazonic acid is a highly selective inhibitor of Ca^{2+}-ATPase in skeletal muscle sarcoplasmic reticulum[26,27], and a valuable pharmacological tool for investigating intracellular Ca^{2+} mobilization and ionic currents regulated by intracellular Ca^{2+}[34]. In light of these facts, therefore, we think that the inhibitory effect of arecoline on CA secretion evoked by cholinergic stimulation as well as by membrane-depolarization is not associated with the mobilization of intracellular Ca^{2+} in chromaffin cells.

In the present study, nicotine (30 µmol/L) initially enhanced the CA secretion evoked by ACh and high K^+, but later inhibited the secretion in a time-dependent manner. In light of this finding, it appears that the mechanism by which arecoline exerts its influence on the CA-releasing effects evoked by cholinergic stimulation, as well as by membrane depolarization, in the perfused rat adrenal medulla is quite different from that of nicotine. In support of this idea, it has been found that the arecoline-induced excitatory effects in rat brain slices were not antagonized by hexamethonium[16]. In contrast, the effect of nicotine (endogenous ACh, splanchnic nerve stimulation) on CA secretion from the rat adrenal gland was greatly reduced (75%) by hexamethonium alone[35]. Based on these results, it seems that there is clearly a large difference in the modes of action of arecoline and nicotine, at least with respect to rat adrenomedullary CA

secretion. There do, however, exist controversial findings that arecoline appears as a partial agonist in whole cells and a full agonist in membranes prepared from rat cortical slices[14]. The data obtained here, that arecoline inhibits the CA secretory responses evoked by cholinergic stimulation (ACh and DMPP), imply that it has the properties of an antagonist at the nicotinic ACh receptors, which mediate nicotinic effects in adrenomedullary chromaffin cells.

In conclusion, the results of the present study using isolated perfused rat adrenal glands demonstrate that arecoline greatly inhibits the CA secretion evoked by stimulation of cholinergic (both nicotinic and muscarinic) receptors in a dose- and time-dependent fashion. However, at lower doses, arecoline does not inhibit CA secretion by membrane depolarization, but at larger dose it does. It seems that this inhibitory effect of arecoline may be mediated by blocking calcium influx into the rat adrenal medullary chromaffin cells without the inhibition of Ca^{2+} release from the cytoplasmic calcium store. These data indicate that nicotine and arecoline have different modes of action with respect to rat adrenomedullary CA secretion.

Acknowledgement

References

1 International Agency for Research on Cancer. Betel-quid and areca-nut chewing. IARC Monographs on the Evaluation of the Carcinogenic Risk of Chemicals to Humans. Volume 37. Lyon: IARC; 1985. p 141–202.

2 Mujumder AM, Kapadi AH, Pendse GS. Chemistry and pharmacology of betel nut *Areca catechu* Linn. J Plant Crops 1979; 7: 69–92.

3 Holdsworth DK, Jones RA, Self R. Volatile alkaloids from *Areca catechu*. Phytochemistry 1998; 48: 581–2.

4 Bhonsle RB, Murti PR, Gupta PC. Tobacco habits in India. In: Gupta PC, Hamner JE, Murti PR, editors. Control of tobacco-related cancers and other diseases. Bombay: Oxford University Press; 1992. p 25–6.

5 Molinengo L, Fundaro AM, Cassone MC. Action of a chronic arecoline administration on mouse motility and on acetylcholine concentrations in CNS. J Pharm Pharmacol 1988; 40: 821–2.

6 Taylor RF, Al-Jarad N, John LM, Conroy DM, Barnes NC. Betel-nut chewing and asthma. Lancet 1992; 339: 1134–6.

7 Trimarchi GR, Germano A, Campo GM, De Luca R, Caputi AP. Changes in urine volume and urinary electrolyte excretion after intracerebroventricular injection of arecoline and hemicholinium-3. Life Sci 1991; 48: 2097–107.

8 Mondadore C, Hengerer B, Ducret T, Borkowski J. Delayed emergence of effects of memory-enhancing drugs: implication for dynamics of long-term potentiation. Proc Natl Acad Sci USA 1994; 91: 2041–5.

9 Raffaele KC, Berardi A, Asthana S, Morris P, Haxby JV, Soncrant TT. Effects of long-term continuous infusion of the muscarinic cholinergic agonist arecoline on verbal memory in dementia of the Alzheimer type. Psychopharmacol Bull 1991; 27: 315–9.

10 Carey GJ, Costall B, Domeney AM, Gerald PA, Jones DN, Naylor RJ, *et al*. Ondansetron and arecoline prevent scopolamine-induced cognitive deficits in the marmoset. Pharmacol Biochem Behav 1992; 42: 75–83.

11 Riekkinen P Jr, Ridkkinen M, Sirvil J. Cholinergic drugs regulate passive avoidance performance via the amygdala. J Pharmacol Exp Ther 1993; 267: 1484–92.

12 Fisher SK, Bartus RT. Regional differences in the coupling of muscarinic receptors to inositol phospholipid hydrolysis in guinea pig brain. J Neurochem 1985; 45: 1085-95.

13 Freedman SB, Harley EA, Iversen LL. Biochemical measurement of muscarinic receptor efficacy and its role in receptor regulation. Trends Pharmacol Sci 1988; 3: 54–60.

14 Wallace MA, Claro E. Comparison of serotonergic to muscarinic cholinergic stimulation of phosphoinositide specific phospholipase C in rat brain cortical membranes. J Pharmacol Exp Ther 1990; 255: 1296–300.

15 Lee HM, Tsai KJ, Lin CH, Huang CL, Tung CS. Arecoline desensitizes carbachol-stimulated phosphatidylinositol breakdown in rat brain cortices. J Neurochem 1998; 70: 1189–98.

16 Yang YR, Chang KC, Chen CL, Chiu TH. Arecoline excites rat *locus coeruleus* neurons by activating the M_2-muscarinic receptor. Chin J Physiol 2000; 43: 23–8.

17 Porsius AJ, Coilffert B, Lambsecht G, Moser Cl, Mutschler E. Muscarinic effects of various isoarecaidine esters in sympathetic ganglia. J Auton Pharmacol 1981; 1: 119–26.

18 Polinsky RT, Brown RT, Curras MT, Baser SM, Baucom CE, Hooper DR, *et al*. Central and peripheral effects of arecoline in patients with autonomic failure. J Neurol Neurosurg Psychiatry 1991; 54: 807–12.

19 Chu NS. Effects of betel chewing on the central and autonomic nervous systems. J Biomed Sci 2001; 8: 229–36.

20 Wakade AR. Studies on secretion of catecholamines evoked by acetylcholine or transmural stimulation of the rat adrenal gland. J Physiol 1981; 313: 463–80.

21 Anton AH, Sayre DF. A study of the factors affecting the aluminum oxidetrihydroxy indole procedure for the analysis of catecholamines. J Pharmacol Exp Ther 1962; 138: 360–75.

22 Tallarida RJ, Murray RB. Manual of pharmacologic calculation with computer programs. 2nd ed. New York: Springer-Verlag; 1987.

23 Hammer R, Giachetti A. Muscarinic receptor subtypes: M_1 and M_2 biochemical and functional characterization. Life Sci 1982; 31: 2992–8.

24 Garcia AG, Sala F, Reig JA, Viniegra S, Frias J, Fonteriz R, *et al*. Dihydropyridine Bay-K-8644 activates chromaffin cell calcium channels. Nature 1984; 309: 69–71.

25 Lim DY, Kim CD, Ahn KW. Influence of TMB-8 on secretion of catecholamines from the perfused rat adrenal glands. Arch Pharm Res 1992; 15: 115–25.

26 Goeger DE, Riley RT. Interaction of cyclopiazonic acid with rat skeletal muscle sarcoplasmic reticulum vesicles. Effect on Ca^{2+} binding and Ca^{2+} permeability. Biochem Pharmacol 1989; 38:

3995–4003.

27 Seidler NW, Jona I, Vegh N, Martonosi A. Cyclopiazonic acid is a specific inhibitor of the Ca^{2+}-ATPase of sarcoplasmic reticulum. J Biol Chem 1989; 264: 17816–23.

28 Molinengo L, Orsetti M. Dose versus survival time curves in the elevation of "prompt" and "delayed" acute toxicities. J Pharm Pharmacol 1986; 38: 54–6.

29 Cena V, Nicolas GP, Sanchez-Garcia P, Kirpekar SM, Garcia AG. Pharmacological dissection of receptor-associated and voltage-sensitive ionic channels involved in catecholamine release. Neuroscience 1983; 10: 1455–62.

30 Boarder MR, Marriott D, Adams M. Stimulus secretion coupling in cultured chromaffin cells, dependency on external sodium and on dihydropyridine-sensitive calcium channels. Biochem Pharmacol 1987; 36: 163–7.

31 Owen PJ, Marriott DB, Boarder MR. Evidence for a dihydropyridine-sensitive and conotoxin-insensitive release of noradrenaline and uptake of calcium in adrenal chromaffin cells. Br J Pharmacol 1989; 97: 133–8.

32 Lopez MG, Fonteriz R, Gandia L, De la Fuente M, Villarroya M, Garcia-Sancho J, *et al*. The nicotinic acetylcholine receptor of the bovine chromaffin cell, a new target for dihydro-pyridines. Eur J Pharmacol Mol Biol 1993; 247: 199–207.

33 O'Farrell N, Zigas J, Marley PD. Effect of N- and L-type calcium channel antagonists and (t)-Bay-K-8644 on nerve-induced catecholamine secretion from bovine perfused adrenal glands. Br J Pharmacol 1997; 121: 381–8.

34 Suzuki M, Muraki K, Imaizumi Y, Watanabe M. Cyclopiazonic acid, an inhibitor of the sarcoplasmic reticulum Ca^{2+}-pump, reduces Ca^{2+}-dependent K^+ currents in guinea-pig smooth muscle cells. Br J Pharmacol 1992; 107: 134–40.

35 Wakade AR, Wakade TD. Contribution of nicotinic and muscarinic receptors in the secretion of catecholamines evoked by endogenous and exogenous acetylcholine. Neuroscience 1983; 10: 973–8.