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Influence of Naloxone on Catecholamine Release Evoked by Nicotinic Receptor Stimulation in the Isolated Rat Adrenal Gland#

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The present study was designed to investigate the effect of naloxone, a well known opioid antagonist, on the secretion of catecholamines (CA) evoked by cholinergic stimulation and membrane-depolarization in the isolated perfused rat adrenal glands, and to establish its mechanism of action. Naloxone (10^{-6}~10^{-5} M), perfused into an adrenal vein for 60 min, produced dose- and time-dependent inhibition of CA secretory responses evoked by ACh (5.32×10^{-3} M), high K^+ (5.6×10^{-2} M), DMPP (10^{-4} M) and McN-A-343 (10^{-4} M). Naloxone itself also failed to affect the basal CA output. In adrenal glands loaded with naloxone (3×10^{-6} M), the CA secretory responses evoked by Bay-K-8644, an activator of L-type Ca^{2+} channels, and cyclopiazonic acid, an inhibitor of cytoplasmic Ca^{2+}-ATPase, were also inhibited. In the presence of met-enkephalin (5×10^{-6} M), a well known opioid agonist, the CA secretory responses evoked by ACh, high K^+, DMPP, McN-A-343, Bay-K-8644 and cyclopiazonic acid were also significantly inhibited. Taken together, these results suggest that naloxone greatly inhibits the CA secretion evoked by stimulation of cholinergic (both nicotinic and muscarinic) receptors as well as that by membrane depolarization. It seems that these inhibitory effects of naloxone does not involve opioid receptors, but might be mediated by blocking both the calcium influx into the rat adrenal medullary chromaffin cells and the uptake of Ca^{2+} into the cytoplasmic calcium store, which are at least partly relevant to the direct interaction with the nicotinic receptor itself.

Key words: Naloxone, Opioid receptors, Secretion of catecholamines, Adrenal gland, Nicotinic receptors

INTRODUCTION

Bovine chromaffin cells co-release opioid peptides together with catecholamines (CA), following stimulation of the nicotinic receptor (Livett *et al.*, 1981) and their membranes possess μ-, δ- and κ-opioid receptors (Lemaire *et al.*, 1984). The secreted opioid peptides might inhibit nicotine-induced CA secretion from chromaffin cells (Kumakura *et al.*, 1980), but this effect does not seem to be mediated by opioid receptors since opioid antagonists, like naloxone, have the same effect (Dean *et al.*, 1982). Moreover, it has been shown that activation of opioid κ-receptors inhibits nicotine-induced Ca^{2+} entry into chromaffin cells but this effect is not prevented by opioid antagonists, such as Mr2266 (Bunn and Dunkley, 1991). Marley and Livett (1987) found that adrenal opioid peptides from cultured bovine adrenal chromaffin cells probably do not act on adrenal opioid binding sites, as characterized from ligand binding studies for prevention of from desensitization of the nicotinic response. Therefore, they are unlikely to be involved in a mechanism that maintains the CA secretion during stress. Dermitzaki and his colleagues (2001) suggested that the suppressive effect of opioids on basal and nicotine-induced CA secretions in the PC12 (rat pheochromocytoma cell line) may result from an opioid-provoked stabilization of cortical actin. It has been suggested that the cultured porcine adrenal chromaffin cells possess μ-, β-, and κ-opioid receptors, and activation of opioid receptors mainly inhibits *N*-type voltage-dependent calcium channels (VDCCs) *via* pertussis toxin-sensitive G-proteins (Kitamura *et al.*, 2002).

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It has been shown that codeine is an autocrine regulator, which suppresses CA release *via* naloxone-insensitive receptors from perifused chromaffin cells of the eel, and stimulates CA release *via* opiate receptor(s). Co-released morphine may modulate the action of codeine (Epple *et al.*, 1994).

In contrast, morphine, [D-Ala^2-D-Leu^5] enkephalin and U50, 488H, relatively selective ligands for the μ-, δ- and κ-receptors, injected intracerebroventricularly into conscious rats, produced dose-dependent elevations in the levels of norepinephrine and epinephrine, the order of potency being enkephalin ≃ morphine > U50,488H (Conway *et al.*, 1984). These responses to morphine were blocked in the presence of naloxone but were unaltered by RX 781094, a selective α_2-adrenoceptor antagonist. Therefore, to elucidate the mechanism of action on the inhibition of nicotinic stimulation-induced CA secretion by opioid antagonists, the present study was carried out to investigate the effect of naloxone, a well known opioid antagonist, on nicotinic stimulation-induced CA secretion in the isolated perfused model of the rat adrenal gland, and compared to the responses to met-enkephalin, an opioid peptide.

MATERIALS AND METHODS

Experimental procedure

Male Sprague-Dawley rats, weighing 180 to 300 g, were anesthetized with thiopental sodium (40 mg/kg), administered 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 the placement of three-hook retractors. The stomach, intestine and portion of the liver were not removed, but pushed over to the right side and covered by saline-soaked gauge pads, and urine in bladder was removed in order to obtain enough working space for tying blood vessels and cannulations.

A cannula, used for perfusion of the adrenal gland, was inserted into the distal end of the renal vein after all branches of 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 prior to ligating the 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. The adrenal gland, along with the ligated blood vessels and the cannula, was then carefully removed from the animal and placed on a platform of a leucite chamber. The chamber was continuously circulated with water heated at 37 ±10°C.

Perfusion of adrenal gland

The adrenal glands were perfused by means of ISCO pump (WIZ Co.) at a rate of 0.33 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 also contained disodium EDTA (10 μg/mL) and ascorbic acid (100 μg/mL) to prevent oxidation of catecholamines.

Drug administration

The perfusions of DMPP (10^{-4} M) for 2 minutes and/or a single injection of ACh (5.32×10^{-3} M) and KCl (5.6×10^{-2} M) in a volume of 0.05 ml were made into perfusion stream *via* a three-way stopcock, respectively. McN-A-343 (10^{-4} M), Bay-K-8644 (10^{-5} M) and cyclopiazonic acid (10^{-5} M) were also perfused for 4 min, respectively.

In the preliminary experiments, it was found that upon administration of the above drugs, secretory responses to ACh, KCl, McN-A-343, Bay-K-8644 and cyclopiazonic acid returned to preinjection level in about 4 min, but the responses to DMPP in 8 min.

Collection of perfusate

As a rule, prior to stimulation with various secretagogues, the perfusate was collected for 4 min to determine the spontaneous secretion of CA (background sample). Immediately after the collection of the background sample, collection of the perfusates was continued, in another tube, as soon as the perfusion medium containing the stimulatory agent reached the adrenal gland. Stimulated samples were collected for 4 to 8 min. The amounts of CA secreted in the background sample was 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 naloxone on the spontaneous and evoked secretions, the adrenal gland was perfused with Krebs solution containing naloxone for 60 min, and the perfusate then was collected for a certain period (background sample). Then the medium was changed to the one containing the stimulating agent alone or along with naloxone, and the perfusates were collected for the same period as that for the background sample. The adrenal gland's perfusate was collected in chilled tubes.

Measurement of catecholamines

The CA content of the perfusate was measured directly by the fluorometric method of Anton and Sayre (Anton and Sayre, 1962) without the intermediate alumina purification for the reasons described earlier (Wakade, 1981) using a fluorospectrophotometer (Kontron Co., Milano, Italy).

A perfusate volume of 0.2 mL was used for the reaction. The CA content in the perfusate of the glands stimulated by the secretagogues used in the present work was high enough to obtain readings several folds greater than those of the 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 using the Student's *t* and ANOVA tests. A P-value of less than 0.05 was considered to represent a statistically significant change 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 performed using the computer program described by Tallarida and Murray (1987).

Drugs and their sources

The following drugs were used: naloxone, ACh chloride, 1,1-dimethyl-4-phenyl piperazinium iodide (DMPP), methionine-enkephalin, norepinephrine bitartrate, methyl-1, 4-dihydro-2, 6-dimethyl-3-nitro-4-(2-trifluoromethyl-phenyl)-pyridine-5-carboxylate (BAY-K8644) (Sigma Chemical Co., U.S.A.), naltrexone hydrochloride (Jeil Pharmaceutical Co., Korea), and cyclopiazonic acid, (3-(*m*-chloro-phenyl-carbamoyl-oxy)-2-butynyltrimethyl ammonium chloride [McN-A-343] (RBI, U.S.A.). The drugs were dissolved in distilled water (stock) and added to the normal Krebs solution as required, with the exception of Bay-K-8644, which was dissolved in 99.5% ethanol and diluted appropriately (final concentration of alcohol was less than 0.1%). The concentrations of all drugs used are expressed in molarity.

RESULTS

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

After the perfusion with oxygenated Krebs-bicarbonate solution for 1 h, the basal CA released from the isolated perfused rat adrenal glands was 22 ± 3 ng for 2 min (*n*=6). Since the nicotine-induced CA secretion and inward ionic currents were inhibited, in a dose-dependent manner, by the opioid antagonist naloxone in cultured bovine chromaffin cells (Tomé *et al.*, 2001), the effects of naloxone itself on CA secretion from the perfused model of the rat adrenal glands was initially examined. However, in the present study, naloxone (10^{-6}~10^{-5} M) itself produced no effect on the basal CA output from perfused rat adrenal glands (data not shown). Therefore, it was of interest to investigate the effects of naloxone on the CA secretion evoked by cholinergic receptor stimulation as well as by membrane depolarization. The secretagogues were given at 15 min-intervals. Naloxone was introduced immedately before the initiation of stimulation.

When ACh (5.32×10^{-2} M) in a volume of 0.05 mL was injected into the perfusion stream, the amount of CA secreted was 346 ± 28 ng for 4 min. However, the pretreatment with naloxone, in the range of 10^{-6}~10^{-5} M for 60 min, concentration- and time-dependently inhibited the ACh-stimulated CA secretion. As shown in Fig. 1 (upper),

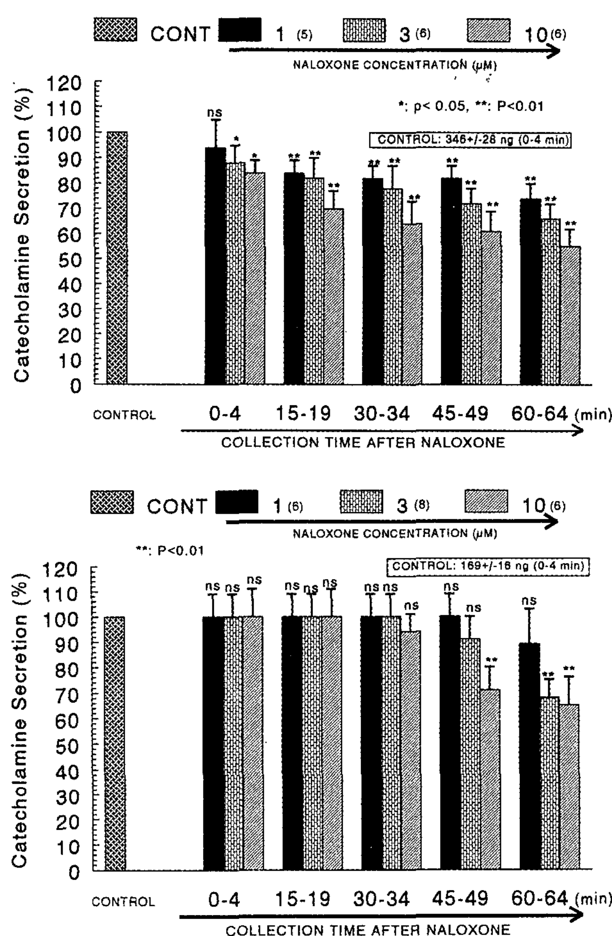

Fig. 1. The effects of naloxone on the secretory responses of catecholamines (CA) evoked by acetylcholine (ACh, **Upper**) and high K$^+$ (**Lower**) in isolated perfused rat adrenal glands. CA secretion by a single injection of ACh (5.32×10^{-3} M) or K$^+$ (56 mM) in a volume of 0.05 mL was evoked at 15 min intervals after preloading with 1, 3, and 10 μM of naloxone for 60 min as indicated by the arrow. The numbers in the parenthesis indicate the 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). Abscissa: collection time of perfusate (min). Statistical difference was obtained by comparing the corresponding control (CONT) with each naloxone concentration pretreated group. Perfusates induced by ACh and high K$^+$ were collected for 4 minutes. *: P < 0.05, **: P < 0.01. ns: Statistically not significant.

in the presence of naloxone, CA releasing responses were *inhibited by 55% of the corresponding control release.* Also, it has been found that the depolarizing agent, such as KCl, markedly stimulates the CA secretion (169 ± 16 ng for 0-4 min). Excess K^+ (5.6×10^{-2} M)-stimulated CA secretion, following the pretreatment with 10^{-6} M naloxone, was not affected as compared with its corresponding control secretion (100%) (Fig. 1-lower). However, following the pretreatment with higher concentrations of naloxone (3×10^{-6} M and 10^{-5} M), excess K^+ (5.6×10^{-2} M)-stimulated CA secretion was significantly inhibited by 65% of the control value after 45 min period, although initially it was *not affected by naloxone.* DMPP (10^{-4} M), *a* selective nicotinic receptor agonist in autonomic sympathetic ganglia, evoked a sharp and rapid increase in CA secretion (333 ± 25 ng for 0-8 min). However, as shown in Fig. 2 (upper), DMPP-stimulated CA secretion following the pretreatment with naloxone was greatly reduced to 59% of the control release (100%). McN-A-343 (10^{-4} M), a selective muscarinic M_1-agonist (Hammer and Giachetti, 1982), perfused into an adrenal gland for 4 min, caused an increase in CA secretion (170 ± 15 ng for 0-4 min). However, McN-A-343-stimulated CA secretion in the presence of naloxone was markedly depressed to 60% of the corresponding control secretion (100%), as depicted in Fig. 2 (lower).

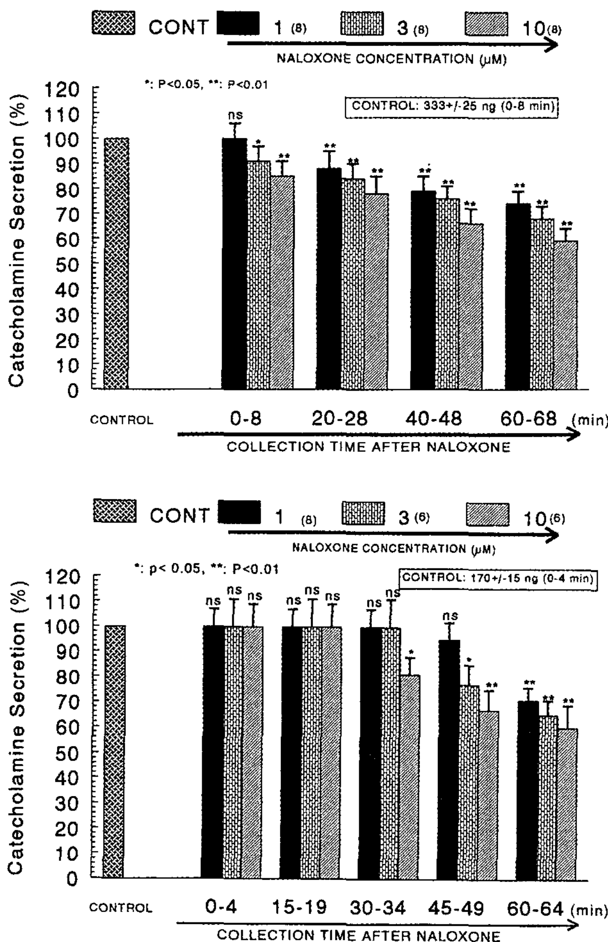

Fig. 2. The effects of naloxone on the secretory responses of the catecholamines (CA) evoked by DMPP (**Upper**) and McN-A-343 (**Lower**) in the isolated perfused rat adrenal glands. The CA secretory responses induced by the perfusion of DMPP (10^{-4} M) and McN-A-343 (10^{-4} M) for 2 min at 20 and 15 min intervals were induced after preloading with 1, 3, and 10 μM naloxone for 60 min. Perfusates induced by DMPP and McN-A-343 were collected for 8 and 4 minutes. Other legends are the same as in Fig. 1. *: $P < 0.05$, **: $P < 0.01$. ns: Statistically not significant.

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

Since Bay-K-8644 is known as a calcium channel activator, which enhances basal Ca^{2+} uptake (Garcia *et al.*, 1984) and CA release (Lim *et al.*, 1992), it was of

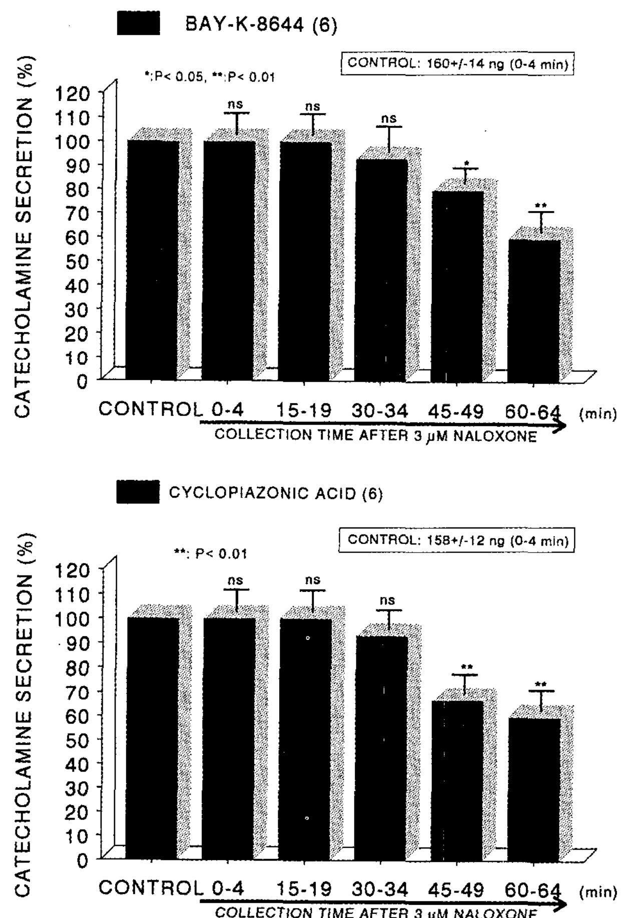

Fig. 3. The effects of naloxone on the CA release evoked by Bay-K-8644 (**Upper**) and cyclopiazonic acid (**Lower**) in the rat adrenal glands. Bay-K-8644 (10^{-5} M) and cyclopiazonic acid (10^{-5} M) were perfused into an adrenal vein for 4 min, at 15 min intervals, after preloading with naloxone (3 μM) for 60 min. Perfusates induced by Bay-K-8644 and cyclopiazonic acid were collected for 4 minutes. Other legends are the same as in Fig. 1. **: $P < 0.01$. ns: Statistically not significant.

interest to determine the effects of naloxone on the Bay-K-8644-stimulated CA secretion from the isolated perfused rat adrenal glands. Bay-K-8644 (10^{-5} M)-stimulated CA secretion in the presence of naloxone was appreciably blocked to 60% of the control, except for the first 30 min, compared to the corresponding control release (160 ± 14 ng for 0-4 min) from 6 rat adrenal glands as shown in Fig. 3 (upper).

Cyclopiazonic acid, a mycotoxin from *Aspergillus* and *Penicillium*, has been described as a highly selective inhibitor of Ca^{2+}-ATPase in skeletal muscle sarcoplasmic reticulum (Goeger and Riley, 1989; Seidler *et al.*, 1989). The inhibitory action of naloxone on the cyclopiazonic acid-evoked CA secretory response was observed as shown in Fig. 3 (lower). However, in the presence of naloxone in 6 rat adrenal glands, the cyclopiazonic acid (10^{-5} M)-evoked CA secretion was inhibited to 61% of the control response (158 ± 12 ng for 0-4 min).

Effect of met-enkephalin on CA secretion evoked by ACh, excess K^+, DMPP, McN-A-343, Bay-K-8644 and cyclopiazonic acid from the perfused rat adrenal glands

It has been found that opioids suppress CA secretion in bovine, human and rodent adrenal glands (Jarry *et al.*, 1989; Livett and Boksa, 1984; Mannelli *et al.*, 1986; Marley *et al.*, 1986; Twitchell and Rane, 1993). Therefore, to study the relationship between naloxone effect and the opioid receptors, the effect of met-enkephalin, an opioid peptide, was examined on CA secretory responses evoked by cholinergic receptor-stimulation as well as membrane depolarization. In the present study, the ACh (5.32 mM)-evoked CA release prior to met-enkephalin pretreatment was 393 ± 33 ng (0-4 min) from 7 rat adrenal glands. In the presence of met-enkephalin (5 μM) for 60 min, the ACh-evoked CA release was not affected for the first 0-4

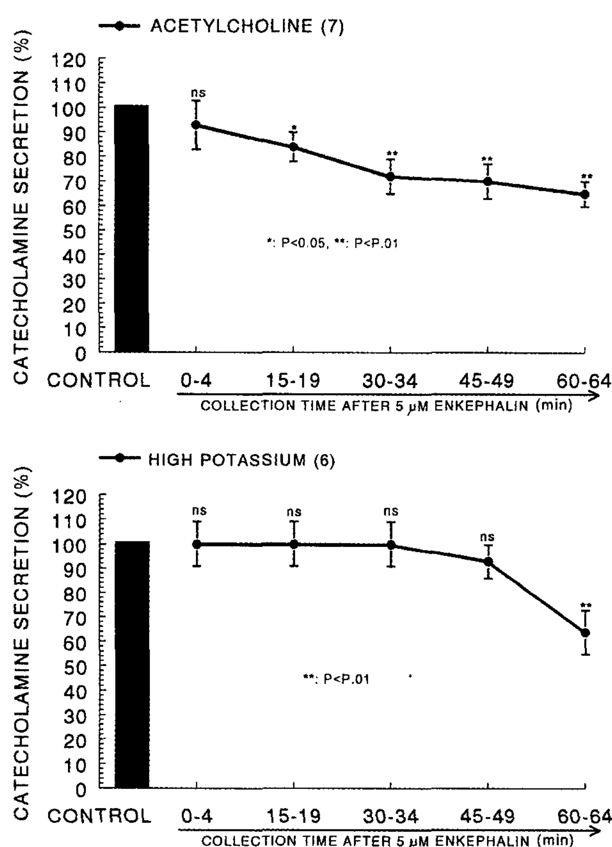

Fig. 4. Time course effects of met-enkephalin on the secretory responses of the catecholamines (CA) evoked by acetylcholine (**Upper**) and by high K^+ (**Lower**) in the isolated perfused rat adrenal glands. The CA secretory responses by a single injection of ACh (5.32×10^{-3} M) or K^+ (56 mM), in a volume of 0.05 mL, were induced before (CONTROL) and after preloading with 5 μM met-enkephalin for 60 min. Perfusate was collected for 4 minutes at 15 min-intervals. Other legends are the same as in Fig. 1. *: P < 0.05, **: P < 0.01. ns: Statistically not significant.

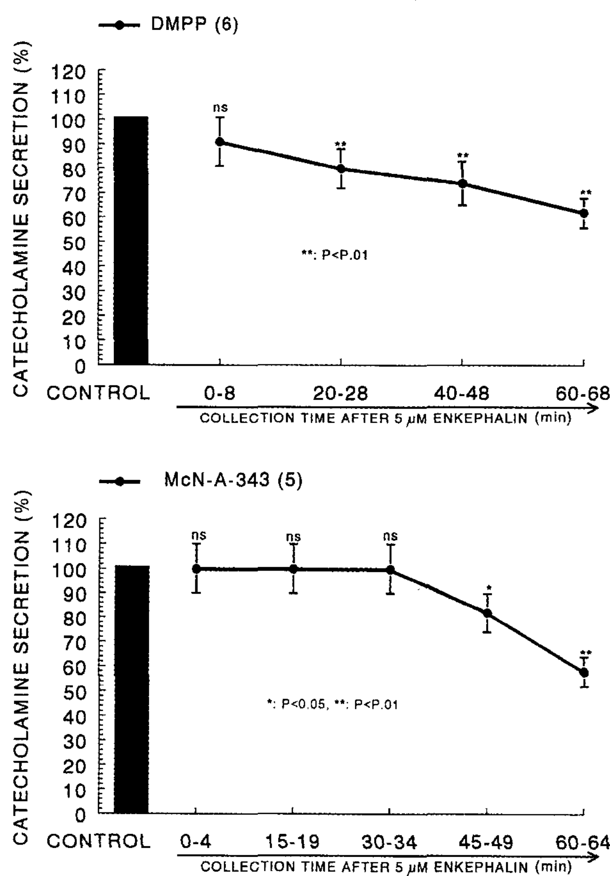

Fig. 5. Time course effects of met-enkephalin on the secretory responses of the catecholamines (CA) evoked by DMPP (**Upper**) and McN-A-343 (**Lower**) in the isolated perfused rat adrenal glands. The CA secretory responses evoked by the perfusion of DPPP (10^{-4} M) and McN-A-343 (10^{-4} M) for 2 min at 20 and 15 min intervals, respectively were induced before (CONTROL) and after preloading with 5 μM met-enkephalin for 60 min. Perfusates induced by DMPP and McN-A-343 were collected for 8 and 4 minutes, respectively. Other legends are the same as in Fig. 1. *: P < 0.05, **: P < 0.01. ns: Statistically not significant.

min, but was rather inhibited by 65% of the corresponding control release thereafter, as shown in Fig. 4 (upper). High K^+ (56 mM)-evoked CA release in the presence of met-enkephalin (5 μM) for 60 min was also unchanged at 0-45 min period compared to the control secretion (149 ± 13 ng, 0-4 min) from 6 glands (Fig. 4-lower), but was greatly inhibited by 64% of the control release, only for the last 60-64 min.

As shown in Fig. 5 (upper), the DMPP-evoked CA release prior to the perfusion with met-enkephalin was 373 ± 42 ng (0-8 min). In the presence of met-enkephalin (5 μM) for 60 min, the DMPP-evoked CA release was also depressed by 62% of the corresponding control from 6 experiments. Moreover, the McN-A-343 (10^{-4} M)-evoked CA release, in the presence of met-enkephalin (5 μM), was also reduced by 58% of the control release (154 ± 16 ng, 0-4 min) from 5 rat glands, as shown in Fig. 5 (lower).

As shown in Fig. 6, the perfusion of met-enkephalin (5 μM) for 60 min depressed the CA secretory responses evoked by Bay-K-8644 and cyclopiazonic acid at 45-60 min period by 57% and 61%, respectively, compared to their corresponding control responses (149 ± 13 ng/0-4 min and 139 ± 11 ng/0-4 min). However, they were unaffected for the initial 0-30 min.

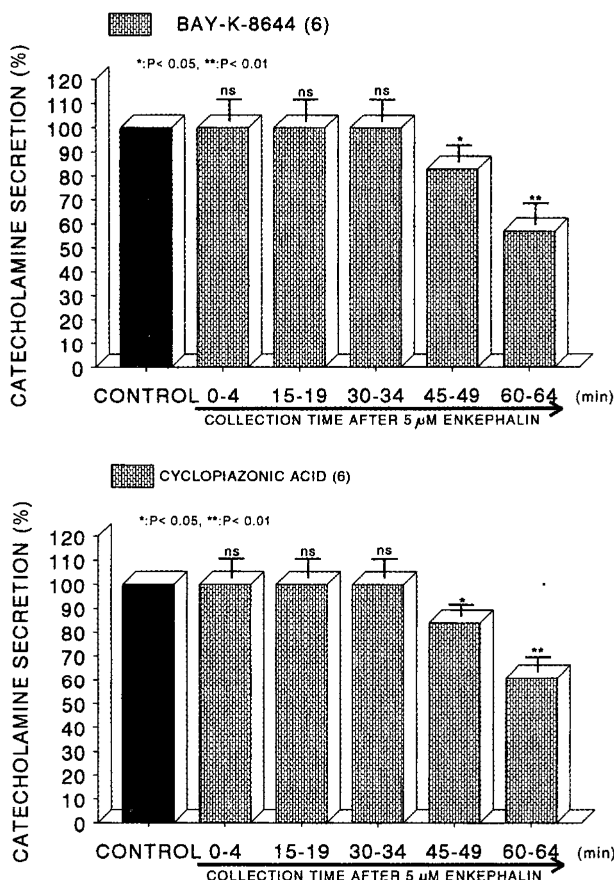

Fig. 6. Time course effects of met-enkephalin on the CA release evoked by Bay-K-8644 (**Upper**) and cyclopiazonic acid (**Lower**) in the rat adrenal glands. Bay-K-8644 (10^{-5} M) and cyclopiazonic acid (10^{-5} M) were perfused into an adrenal vein for 4 min, at 15 min intervals, after preloading with met-enkephalin (5 μM) for 60 min. Perfusates induced by Bay-K-8644 and cyclopiazonic acid were collected for 4 minutes. Other legends are the same as in Fig. 1. *: P < 0.05, **: P < 0.01. ns: Statistically not significant.

DISCUSSION

The experimental results presented here demonstrate that naloxone, a non-selective opioid antagonist, dose- and time-dependently blocks the CA secretory responses evoked by cholinergic (both nicotinic and muscarinic) receptor stimulation, as well as by membrane depolarization, from the isolated perfused rat adrenal gland. Furthermore, in adrenal glands loaded with naloxone, the CA secretory responses evoked by Bay-K-8644 as well as by cyclopiazonic acid were also inhibited. This inhibitory effect of naloxone might be explained by the reversible direct blockade of cholinergic receptors without the involvement of opioid receptors in rat adrenomedullary chromaffin cells, which is may be relevant to by blocking of both the calcium influx into the rat adrenal medullary chromaffin cells and the Ca^{2+} uptake into the cytoplasmic calcium store.

Generally, chromaffin cells synthesize, store and secrete CAs (dopamine, norepinephrine and epinephrine) in nerve stimulations. The cells have an excitable action in response to ACh or to electrical stimulation of the splanchnic nerve. Following activation of ACh nicotinic receptors, ACh causes the opening of the receptor-mediated ion channel, allowing the influx of Na^+, and to a lesser extent Ca^{2+} (Douglas *et al.*, 1967, 1967). This influx of Na^+ into the chromaffin cells results in a mild depolarization of the cell membrane, which is sufficient to activate voltage-dependent Na^+ channels (Cena *et al.*, 1983). The opening of Na^+ channels induces the activation of voltage-dependent Ca^{2+} channels (Garcia *et al.*, 1984). The opening of Na^+ and Ca^{2+} channels causes the firing of action potentials and the entry of Ca^{2+} from extracelluar spaces (Artalejo *et al.*, 1994). An increase in the $[Ca^{2+}]_i$ is the trigger for exocytosis of chromaffin granules (secretion of CA) and the stimulation of CA biosynthesis. Cholinergic nicotinic and muscarinic receptors are present in chromaffin cells, and these receptors stimulate the CA secretion in most species. In bovine adrenal chromaffin cells, however, only nicotinic receptor stimulation evokes the CA secretion. The CA secretion can also be evoked by high K^+, which directly activates the voltage-dependent channels. The depolarization induced by high K^+ directly opens the Ca^{2+} channels without the contribution of the Na^+ channel. Previous studies have shown that the CA release in PC12 cells, stimulated by carbamylcholine or

K^+-depolarization, is a transient phenomenon, which ceases after about 15 min (Ritchie, 1979; Dendorfer and Dominiak, 1995). Based on these findings, the present results, where naloxone was found to inhibit the CA secretory responses evoked by DMPP and McN-A-343, as well as high potassium, suggested that naloxone has the ability to directly block both nicotinic and muscarinic receptors located on the rat adrenomedullary chromaffin cells. Thus, naloxone seems to block the cholinergic stimulation that can openin the Na^+, and Ca^{2+} channels, causing the firing of action potentials and the entry of Ca^{2+} through L-type Ca^{2+} channels, and then increase the $[Ca^{2+}]_i$, triggering exocytosis from the rat adrenomedullary chromaffin granules (secretion of CA). In the present investigation, naloxone at higher concentrations (3~10 μM) greatly attenuated the CA secretion evoked by high potassium, a direct membrane-depolarizing agent. This result strongly suggests that naloxone can block the voltage-sensitive Ca^{2+} channels. In support of this finding, the result obtained from the present study, where naloxone greatly attenuated the CA secretion evoked by Bay-K-8644, an activator of L-type Ca^{2+} channels, indicates that naloxone may act as a Ca^{2+}channel antagonist in the rat adrenal medulla. Bay-K-8644 has been found to potentiate the release of CA by increasing Ca^{2+} influx through L-type Ca^{2+} channels, in cultured bovine chromaffin cells (Garcia *et al.*, 1984). Moreover, previous studies on primary cultures of bovine chromaffin cells have shown that dihydropyridines can partially inhibit the CA secretion induced by the depolarization with ACh, nicotine or K^+. The degree of inhibition varied between studies depending on the dihydropyridine used, its concentration, and the concentration of agonist (Cena *et al.*, 1983; Boarder *et al.*, 1987; Owen *et al.*, 1989). However, at high concentration (≥1 μM), dihydropyridines block the nicotinic receptor ion channels, and at these concentrations they inhibit calcium uptake and CA secretion induced by nicotinic agonists without comparable effects on the K^+-evoked responses (Lopez *et al.*, 1993). Nitrendipine at a concentration of 1 μM was found to be sufficient to reduce 30 mM potassium-induced contraction of pig coronary artery rings mounted in organ baths (O'Farrell *et al.*, 1997). The most plausible explanation of this finding is, that naloxone has a direct blocking effect on the Ca^{2+} channels. Therefore, the present experimental results imply that naloxone itself blocks the Ca^{2+} entry into the adrenomedullary chromaffin cells by inhibiting the voltage-dependent Ca^{2+} channels, which subsequently inhibits the Ca^{2+}-dependent release of CAs evoked by cholinergic stimulation as well as membrane-depolarization.

Moreover, the present study has also shown that naloxone 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 (Goeger & Riley, 1989; Seidler *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 naloxone on the CA secretion evoked by cholinergic stimulation as well as by membrane-depolarization may be associated with the mobilization of intracellular Ca^{2+} from the cytoplasmic calcium store. This indicates that the naloxone 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 that are susceptible to caffeine (Ilno, 1989) is almost completely abolished by treatment with cyclopiazonic acid during the proceeding Ca^{2+} load (Suzuki *et al.*, 1992). This is consistent with the findings obtained in skinned smooth muscle fibers of the longitudinal layer of the guinea-pig ileum, where Ca^{2+}-uptake was also inhibited by cyclopiazonic 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 an increase in the subsequent Ca^{2+} release from those storage sites. In bovine adrenal chromaffin cells, stimulation of muscarinic ACh receptors has also been 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 naloxone on movement of Ca^{2+} from intracellular pools is due to its direct effect on the PI response or the indirect effects.

In the present study, met-enkephalin also caused very similar inhibitory effects with naloxone on the CA release evoked by cholinergic stimulation and membrane depolarization, strongly suggesting that naloxone-induced inhibition of CA secretion is not relevant to the involvement of opioid receptors. In support of this idea, methionine-enkephalin and ATP in the secretory vesicles of adrenal chromaffin cells (Winkler and Westhead, 1980) are released on exocytosis together with CA (Livett *et al.*, 1981). It has been shown that these substances affect the activity of voltage-dependent calcium channels (VDCCs). The application of a soluble vesicular lysate of bovine chromaffin cells causes a rapid inhibition of the currents through VDCCs, which is recovered by antagonists of opioid receptors and purinoceptors (Albillos *et al.*, 1996). It has been reported that exogenously applied opioids suppress the VDCC activity in bovine chromaffin cells (Albillos *et al.*, 1996; Kleppisch *et al.*, 1992; Twitchell and Rane, 1993),

and inhibit the CA secretion evoked by nicotine (Kumakura *et al.*, 1980) and ACh (Saiani and Guidotti, 1982). Therefore, it is hypothesized that opioids co-released with CA regulate negative feedback control for the CA secretion through the suppression of the VDCC activity. Moreover, beta-endorphin and morphine have also been shown to reduce the CA secretion induced by nicotine to as much as fifty percent whereas [Met^5]-enkephalin decreased the CA release to seventy-five percent (Kumakura *et al.*, 1980). Barron and Hexum (1986) found that opiates modulate the secretion of CA and met-enkephalin-immunoreactive materials from the perfused bovine adrenal gland. It has also been shown that the splanchnic nerve stimulation-induced CA output was markedly reduced by opiate agonists (opioid peptides or morphine), but was enhanced by an opiate antagonist (naloxone) in the dog adrenal gland *in vivo*, and these effects are clearly associated with the opiate receptors located in the adrenal gland (Kimura *et al.*, 1988). In support of these hypotheses, Lim and his coworkers (1992) have demonstrated that both met-enkephalin and morphine greatly decreased the CA secretion evoked by DMPP and ACh in the perfused rat adrenal gland. In terms of these results, it seems that naloxone-induced inhibition of the CA release is not mediated through the opioid receptors, suggesting that the effect of naloxone might be due to the direct blockade of the cholinergic receptor itself.

Taken together, these results suggest that naloxone greatly inhibits the CA secretion evoked by stimulation of cholinergic (both nicotinic and muscarinic) receptors as well as that by membrane depolarization. It is thought that this inhibitory effect of naloxone does not involve opioid receptors, but may be mediated by blocking of both the calcium influx into the rat adrenal medullary chromaffin cells and Ca^{2+} uptake into the cytoplasmic calcium stores, which are at least partly relevant to the direct interaction with the nicotinic receptor itself.

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