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Influence of 5'-(N'-Ethylcarboxamido) Adenosine on Catecholamine Secretion Evoked by Cholinergic Stimulation and Membrane Depolarization in the Rat Adrenal Gland

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Abstract – The present study was attempted to determine the effect of 5'-(N'-ethylcarboxamido) adenosine (NECA), which is an potent A_2-adenosine receptor agonist, on catecholamine (CA) secretion evoked by cholinergic stimulation, membrane depolarization and calcium mobilization from the isolated perfused rat adrenal gland. NECA (20 nM) perfused into the adrenal vein for 60 min produced a time-related inhibition in CA secretion evoked by ACh (5.32×10^{-3} M), high K^+ (5.6×10^{-2} M), DMPP (10^{-4} M for 2 min), McN-A-343 (10^{-4} M for 2 min), cyclopiazonic acid (10^{-5} M M for 4 min) and Bay-K-8644 (10^{-5} M for 4 min). Also, in the presence of β,γ-methylene adenosine-5'-triphosphate (MATP), which is also known to be a selective P_{2x}-purinergic receptor agonist, showed a similar inhibition of CA release evoked by ACh, high potassium, DMPP, McN-A-343, Bay-K-8644 and cyclopiazonic acid. However, in adrenal glands preloaded with 20 μM NECA for 20 min under the presence of 20 μM 3-isobutyl-1-methyl-xanthine (IBMX), an adenosine receptors antagonist, CA secretory responses evoked by ACh, high potassium, DMPP, McN-A-343, Bay-K-8644 and cyclopiazonic acid were much recovered in comparison to the case of NECA-treatment only. Taken together, these results indicate that NECA causes the marked inhibition of CA secretion evoked by stimulation of cholinergic (both nicotinic and muscarinic) receptors as well as by membrane depolarization. This inhibitory effect may be mediated by inhibiting influx of extracellular calcium and release in intracellular calcium in the rat adrenomedullary chromaffin cells through the adenosine receptor stimulation. Therefore, it is suggested that the inhibitory mechanism of adenosine receptor stimulation may play a modulatory role in regulating CA secretion.

Key words ☐ NECA, Adrenal Gland, Catecholamine Secretion, Adenosine Receptor, Cholinergic Stimulation, Membrane-Depolarization

By means of indirect studies, it has been demonstrated that 5'-(N-ethylcarboxamido) adenosine (NECA), A_1 and A_2 agonist is able to activate adenosine transport. This activation appears as a consequence of an increase in the number of transporters, as demonstrated by the increase in number of nitrobenzyl-thioinosine binding sites (Delicado *et al*, 1990). Adenosine receptors present in a given cell membrane are classified by their biochemical characterization, including the order of potency of agonists and antagonists and the calculation of the equilibrium binding parameters of the more specific ligands. For A_1 receptors, specific ligands are R-pheny lisopropyladenosine (R-PIA) as agonist and 8-cyclopentyl-1,3-dipropylxanthine (DPCPX) as antagonist (Bruns *et al*, 1986; Lohse *et al*, 1987; Stone *et al*, 1988). For the A_2 subclass, the agonist that has been widely used is NECA (Bruns *et al*, 1986; Friedman *et al*, 1989). However, NECA also binds to A_1 receptors (Bruns *et al*, 1986; Reddington *et al*, 1986). On the other hand, in some A_2 systems two components are identified in the binding of agonists: one of high affinity (A_{2a}) and the other of low affinity (A_{2b}) (Bruns *et al*, 1986; Jarvis *et al*, 1989). Furthermore, Casado and his coworkers (1992) have demonstrated that the receptors present on the plasma membrane of bovine chromaffin cells are exclusively of the A_{2b} subtype, which is low-affinity form of the A_2-receptor. Recently, prolonged exposure to NECA is found not to affect the adenosine A_{2a}-mediated vasodilatation in porcine coronary arteries (Conti et al, 1997). It has been also suggested that dephosphorylation of a protein involved in 1.1-dimethyl-4-phenyl piperazinium iodide (DMPP)-evoked Ca^{2+} influx pathway could be the mechanism of the inhibitory action of adenosine receptor stimulation on CA secretion from bovine chromaffin cells (Mateo *et al*, 1995). The effects

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of both adenosine and adenosine agonists on CA secretion (Chern *et al*, 1988; 1992) and adenosine transport (Delicado *et al*, 1990) are not associated with a modification of cAMP levels in chromaffin cells. However, NECA elevates cellular cAMP content in the presence of forskolin without having any positive effect on secretion. This finding suggests that the rise in cAMP level may not be the sole cause of the increase in secretion by adenosine (Chern *et al*, 1988). Gurden and co-workers (1993) have found that receptors present in dog coronary artery, human platelet and neutrophils, probably correspond to the A_{2a}, while those present in the guinea-pig aorta may be of the A_{2b} subtype. In addition, it has been suggested that NECA causes vasorelaxation in the rabbit and guinea-pig aorta by interfering with the Ca^{2+} influx via receptor-operated channels induced by norepinephrine (Ford and Broadly, 1996). Thus, there is considerable controversy in the physiological effects of adenosine, especially on adrenomedullary chromaffin cells. The purpose of the present study is an attempt to investigate whether NECA, a potent agonist at A_2-type adenosine receptors, affect CA secretory response evoked by stimulation of muscarinic and nicotinic receptors from the perfused rat adrenal gland and to establish the mechanism of its action.

MATERIALS AND METHODS

Experimental procedure

Male Sprague-Dawley rats, weighing 180 to 300 grams, 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 mid-line 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 (ISCO Co., Lincoln, NB, USA) at a rate of 0.31 ml/min. The perfusion was carried out with Krebs-bicarbonate solution of following composition (mM) : NaCl, 118.4; KCl, 4.7; $CaCl_2$, 2.5; $MgCl_2$, 1.18; $NaHCO_3$, 25; KH_2PO_4, 1.2; glucose, 11.7. The solution was constantly bubbled with 95% O_2 +5% CO_2 and the final pH of the solution was maintained at 7.4 0.05. The solution contained disodium EDTA (10 μg/ml) and ascorbic acid (100 μg/ml) to prevent oxidation of catecholamine.

Drug administration

The perfusions of DMPP (100 μM) and McN-A-343 (100 μM) for 2 minutes, and a single injection of ACh (5.32 mM) and KCl (56 mM) in a volume of 0.05 ml were made into perfusion stream via a three way stopcock, respectively. 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, 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 perfusate 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 secreted in the background sample have been subtracted from those secreted from the stimulated sample to obtain the net secretion value of CA, which is shown in all of the figures. In order to study the effect of NECA on the spontaneous and evoked secretion, the adrenal gland was perfused with Krebs solution containing NECA for 20 min. Then the perfusate was collected for the 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 content of CA in the perfusate was expressed in terms of norepinephrine (base) equivalents.

Statistical analysis

The statistical significance between groups was determined by the Student's t-test. A P-value of less than 0.05 was considered to be significant 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 purchased from Sigma Chemical Co., U.S.A. : 5'-(N'-ethylcarboxamido) adenosine), acetylcholine chloride, 1,1-dimethyl-4-phenyl piperazinium iodide (DMPP), β,γ-methylene adenosine-5'-triphosphate (MATP), 3-isobutyl-1-methylxanthine (IBMX), norepinephrine bitartrate, and methyl-1,4-dihydro-2, 6-dimethyl-3-nitro-4-(2-trifluoromethylphenyl)-pyridine-5-carboxylate (BAY-K-8644). cyclopiazonic acid, (3-(m-chloro-phenyl-carbamoyl-oxy)-2-butynyl-trimethyl ammonium chloride [McN-A-343] were purchased from 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 5'-(N'-ethylcarboxamido) adenosine on CA secretion evoked by ACh, high K^+, DMPP and McN-A-343

After the initial perfusion with oxygenated Krebs-bicarbonate solution for 1 hr, basal CA release from the isolated perfused rat adrenal glands amounted to 16.3 ± 1.8 ng/2 min (n=6). Since it has shown that in cultured bovine adrenal chromaffin cells, the NECA reduces CA secretory response evoked by DMPP (Mateo *et al*, 1995), it was attempted initially to examine the effects of NECA itself on CA secretion from perfused rat adrenal glands. However, in the present study, NECA itself did not produce any effect on basal CA output from perfused rat adrenal glands (data not shown). Therefore, it was decided to investigate the effects of NECA on cholinergic receptor stimulation- as well as membrane depolarizationmediated CA secretion. Secretagogues were given at 15 to 20 minintervals. NECA was present 15~20 min before initiation of stimulation.

When ACh (5.32×10^{-3} M) in a volume of 0.05 ml was injected into the perfusion stream, the amounts of CA secreted was 486±54 ng for 4 min. However, the pretreatment with 20 μM NECA for 60 min inhibited significantly ACh-stimulated CA secretion in a time-dependent manner from 6 adrenal glands, as shown in Fig. 1. Also, it has been found that depolarizing agent like KCl stimulates sharply CA secretion. In the present work, excess K^+ (5.6×10^{-2} M)-stimulated CA secretion after the pretreatment with NECA was significantly inhibited as compared with its corresponding control secretion (171

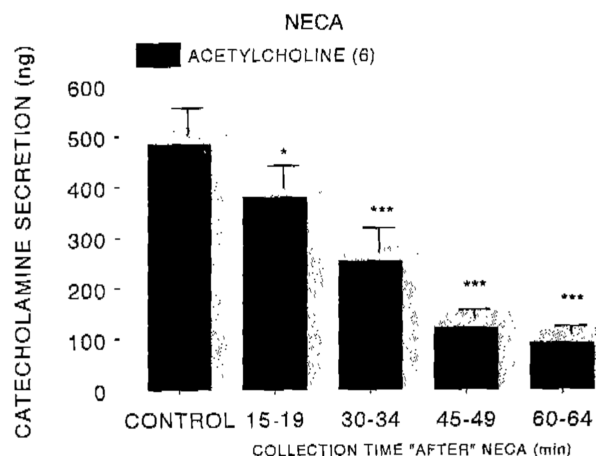

Fig. 1. Influence of 5'-(N'-ethylcarboxamido) adenosine (NECA) on ACh-stimulated catecholamine (CA) secretion from the isolated perfused rat adrenal glands. CA secretion was induced by a single injection of ACh (5.32×10^{-3} M) after perfusion with normal Krebs solution for one hour prior to initiation of the experimental protocol. "CONTROL" and "AFTER" denote CA secretion evoked by ACh before and after loading with 20 μM NECA, respectively. Number in the parenthesis indicates number of experiments. Vertical bars represent the standard error of the mean (S.E.M.). Ordinate: the amounts of CA secreted from the adrenal gland in ng. Abscissa: Collecting time of the perfusate (min). Statistical difference was obtained by comparing the control with the NECA-treated group. Each perfusate was collected for 4 minutes. *P<0.05, ***P<0.01.

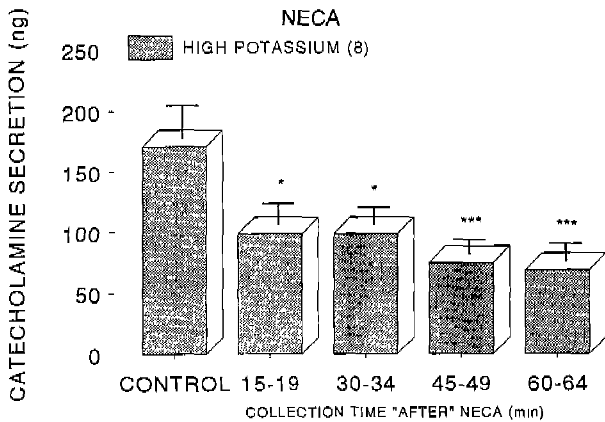

Fig. 2. Influence of NECA on high K^+-stimulated CA secretion from the isolated perfused rat adrenal glands. Excess K^+ (5.6×10^{-2} M) was given into an adrenal vein before and after the perfusion with 20 μM NECA, respectively. Other legends are the same as in Fig. 1. The perfusate was collected for 4 minutes. *P <0.05, ***P<0.01.

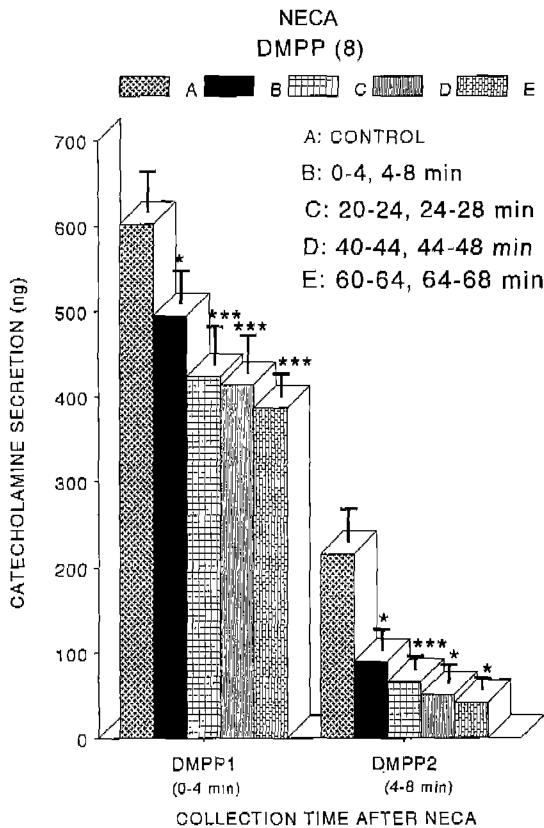

Fig. 3. Influence of NECA on DMPP-stimulated CA secretion from the isolated perfused rat adrenal glands. DMPP (10^{-4} M) was given into an adrenal vein before and after the perfusion with 20 μM NECA, respectively. The perfusates for DMPP were collected twice at 4 min interval for 8 min. Other legends are the same as in Fig. 1. DMPP-induced perfusates were collected twice at 4 min-interval for 8 min: DMPP1 (0~4 min) and DMPP2 (4~8 min). *P<0.05, ***P<0.01. DMPP: dimethyl phenyl piperazinium.

±27 ng for 0~4 min) from 8 glands (Fig. 2).

When perfused through the rat adrenal gland, DMPP (10^{-4} M for 2 min), which is a selective nicotinic receptor agonist in autonomic sympathetic ganglia, evoked a sharp and rapid increase in CA secretion. However, as shown in Fig. 3, DMPP-stimulated CA secretion after pretreatment with 20 μM NECA was relatively time-dependently reduced as compared with its corresponding control secretion (603±48 ng for 0~4 min; 216±39 ng for 4~8 min) in 6 rat adrenal glands. McN-A-343 (10^{-4} M), which is a selective muscarinic M_1-agonist (Hammer and Giachetti, 1982), perfused into an adrenal gland for 2 min caused an increased CA secretion (108± 15 ng for 0~4 min) from 10 glands. However, McN-A-343-stimulated CA secretion in the presence of 20 μM NECA was almost perfectly blocked as compared to the corresponding control secretion as depicted in Fig. 4.

Since Bay-K-8644 is known to be a calcium channel activator which enhances basal Ca^{2+} uptake (Garcia *et al.*, 1984) and CA release (Lim *et al.*, 1992), it was of interest to determine the effects of NECA on Bay-K-8644-stimulated CA secretion from the isolated perfused rat adrenal glands. Bay-K-8644 (10^{-5} M)-stimulated CA secretion under the presence of 20 μM NECA was strikingly depressed to 30~10% of the corresponding control release (189±39 ng for 0~4 min) from 6 glands as shown in Fig. 5.

Cyclopiazonic acid, a mycotoxin from *Aspergillus* and *Penicillium*, has been described as a highly selective inhibitor

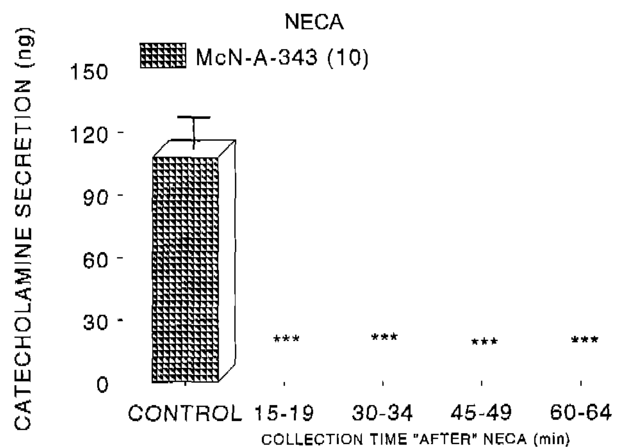

Fig. 4. Influence of NECA on McN-A-343-stimulated CA secretion from the isolated perfused rat adrenal glands. McN-A-343 (10^{-4} M) was given into an adrenal vein before and after the perfusion with 20 μM NECA, respectively. Other legends are the same as in Fig. 1. The perfusate was collected for 4 minutes. ***P<0.01. McN-A-343: (3-(m-chloro-phenyl-carbamoyl-oxy)-2-butynyltrimethyl ammonium chloride.

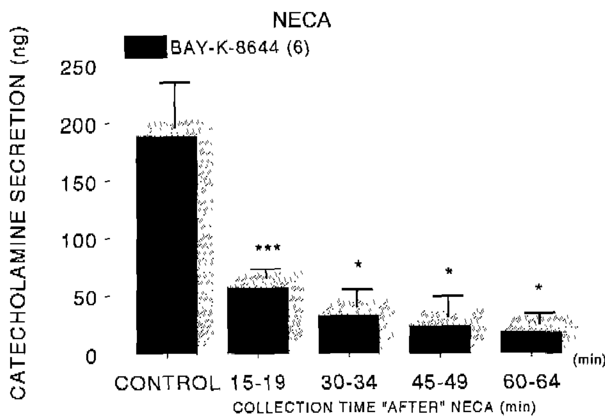

Fig. 5. Influence of NECA on Bay-K-8644 stimulated CA secretion from the isolated perfused rat adrenal glands. Bay-K-8644 (10^{-5} M) was given into an adrenal vein before and after the perfusion with 20 μM NECA, respectively. Other legends are the same as in Fig. 1. The perfusate was collected for 4 minutes. *P<0.05, ***P<0.01

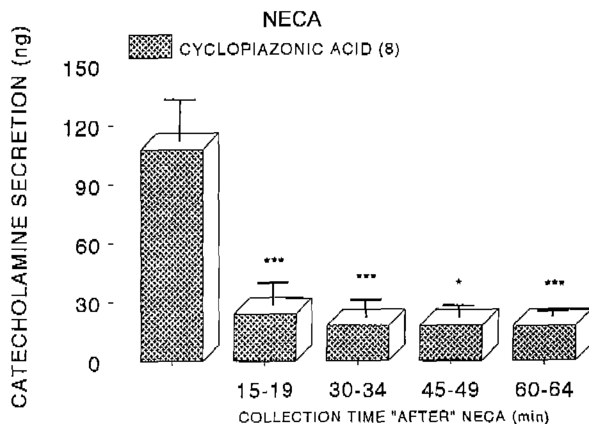

Fig. 6. Influence of NECA on CPA-stimulated CA secretion from the isolated perfused rat adrenal glands. CPA (10^{-4} M) was given into an adrenal vein before and after the perfusion with 20 μM NECA, respectively. Other legends are the same as in Fig. 1. The perfusate was collected for 4 minutes. *P<0.05, ***: P<0.01. CPA: Cyclopiazonic acid.

of Ca^{2+}-ATPase in skeletal muscle sarcoplasmic reticulum (Goeger and Riley, 1989; Seidler *et al.*, 1989). The inhibitory action of NECA on cyclopiazonic acid-evoked CA secretory response was observed as shown in Fig. 6. Under the effect of 20 μM NECA in 6 rat adrenal glands, cyclopiazonic acid (10^{-5} M)-evoked CA secretion was greatly reduced by 22~17% of the control response (108 ± 21 ng for 0~4 min).

The effects of NECA plus 3-isobutyl-1-methylxanthine (IBMX) on CA release evoked by ACh, excess K^+, DMPP, McN-A-343, Bay-K-8644 and cyclopiazonic acid

Since IBMX is a potent phosphodiesterase inhibitor and

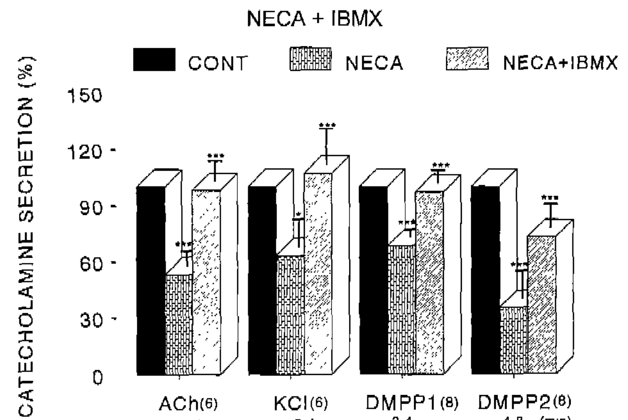

Fig. 7. Effect of NECA in the presence of 3-isobutyl-1-methylxanthine (IBMX) on CA release evoked by ACh, high K^+ and DMPP. ACh (5.32 mM), excess K^+ (56 mM) and DMPP (100 μM) were given before and after preloading with 20 μM NECA only or 20 μM NECA plus 20 μM IBMX for 20 min, respectively. "CONT", "NECA" and "NECA+IBMX" denote CA release induced by each secretagogue before (CONT) and after pretreatment with 20 μM NECA or along with 20 μM IBMX (NECA+IBMX) for 20 min, respectively. Statistical differences were compared between groups of CONT and NECA, and between groups of NECA and NECA+IBMX, respectively. Other legends are the same as in Fig. 1. Cont: Control. P<0.05, ***P<0.01.

more active than theophylline at adenosine receptors (Bruns *et al*, 1986), it was tried to determine the effect of NECA in the presence of IBMX on CA secretion evoked by various secretagogues from the isolated rat adrenal glands. NECA plus IBMX treatment did not alter the basal CA secretory response (data not shown).

ACh (5.32×10^{-3} M)- and excess K^+ (5.6×10^{-2} M)-evoked CA releases in the presence of 20 μM NECA along with 20 μM IBMX were recovered to 98 ± 11% (P<0.01, n=6) and 107 ± 19% (P<0.01, n=6) of their control secretion (100%), respectively as compared to their secretory responses of 53 ± 8% and 63 ± 15% of their controls in the presence of 20 μM NECA only (Fig. 7). On the other hand, 20 μM NECA along with 20 μM IBMX treatment did fail to affect the basal CA secretory response (data not shown). As depicted in Fig. 7 and 8, CA secretions under the presence of 20 μM NECA along with 20 μM IBMX were also greatly recovered to 99 ± 7% (0~4 min, P<0.01, n=8) and 73 ± 13% (4~8 min, P<0.01, n=8) in response to DMPP and 92 ± 20% (0~4 min, P<0.01, n=8) in response to McN-A-343 of their corresponding control responses, respectively as compared to the secretory responses of 68 ± 4% (0~4 min) and 35 ± 15% (4~8 min) for DMPP, and 0 ± 0 % (0~4 min) for McN-A-343 of the control

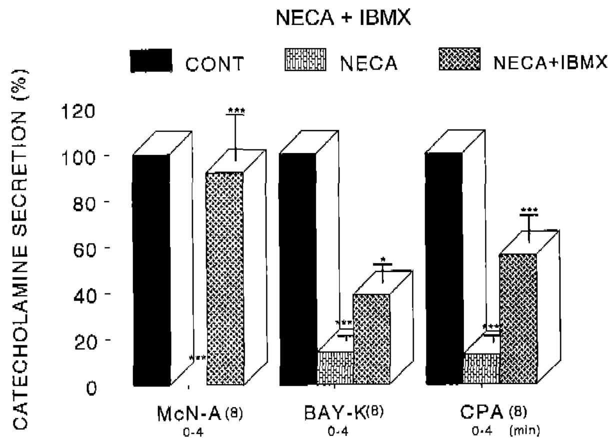

Fig. 8. Effect of NECA plus IBMX on CA release evoked by McN-A-343, Bay-K-8644 and cyclopiazonic acid. Secretagogues were administered before and after pretreatment with Krebs solution containing 20 μM NECA plus 20 μM IBMX for 20 min, respectively. Other legends are the same as in Fig. 1 and 7. McN-A: McN-A-343, BAY-K: Bay-K-8644, CPA: Cyclopiazonic acid, *P<0.05, ***P<0.01.

in the presence of 20 μM NECA only.

CPA (10^{-5} M)- and Bay-K-8644 (10^{-5} M)-induced CA secretory responses after preloading with Krebs solution containing 20 μM NECA along with 20 μM IBMX were reduced to 39 ± 8 % (P <0.05, n=6) and 56 ± 12% (P< 0.01, n=6) of each corresponding control (100%), respectively as compared to the secretory responses of 14 ± 2 % and 13 ± 3% of the controls in the presence of 20 μM NECA only as shown in Fig. 8.

Effect of β,γ-methylene adenosine-5'-triphosphate (MATP) on CA secretion evoked by ACh, excess K^+, DMPP, McN-A-343, Bay-K-8644 and cyclopiazonic acid

In the previous experimental results as shown in Fig. 1~6, it was found that NECA showed a time-dependent inhibition in CA secretory responses evoked by cholinergic stimulation and membrane depolarization. MATP is known to be an ATP receptor agonist in guinea-pig (Hourani *et al*, 1986). It has been also shown that MATP induces Ca^{2+} entry mainly through L-type Ca^{2+} channels by a pertussis toxin-insensitive mechanism, consistent with activation of P_{2x} receptors at presynaptic glial cells of the frog neuromuscular junction (Robitaille, 1995). Therefore, it is likely of very interest to examine the effect of MATP on CA secretion evoked by various secretagogues.

As shown in Fig. 9, CA release evoked by ACh (5.32×10^{-3} M) after preloading with 20 μM MATP for 20 min amounted to 86% of the corresponding control secretion (0~4 min, 435 ± 51 ng) from 8 rats. Excess K^+ (5.6 ± 10^{-2} M)-evoked CA release after preloading with 20 μM MATP was significantly inhibited to 72% of the corresponding control secretion (0~4 min, 293 ± 35 ng) in 8 glands as shown in Fig. 9. In 6 adrenal glands, the neuronal nicotinic agonist, DMPP (10^{-4} M)-evoked CA secretion following the pretreatment with MATP was also markedly depressed to 61% (0~4 min) and 36% (4~8 min) of the control secretion (0~4 min, 354 ± 51 ng; 4~8 min, 141 ± 24 ng), respectively as shown in Fig. 9.

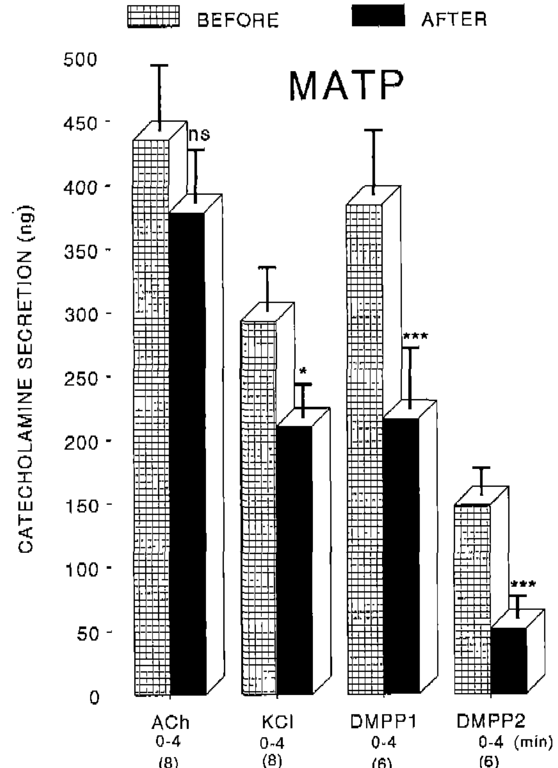

Fig. 9. Effect of β,γ-methyl adenosine-5'-triphosphate (MATP) on CA release evoked by ACh, high K^+ and DMPP. Secretagogues were administered before and after pretreatment with Krebs solution containing 10 μM MATP for 20 min, respectively. DMPP-induced perfusates were collected twice at 4 min-interval for 8 min: DMPP1 (0-4 min) and DMPP2 (4-8min). Other legends are the same as in Fig. 1 and 7. Ns: statistically nonsignificant. *P<0.05, ***P<0.01.

The muscarinic M_1-recptor agonist, McN-A-343 (10^{-4} M)-stimulated CA releases after preloading with MATP were also significantly reduced (Fig. 10). In the presence of 20 μM MATP, the CA secretory response by cyclopiazonic acid (10^{-5} M) given into the adrenal gland was greatly reduced to 32% of the corresponding control response (0~4 min, 95 ± 12 ng) from 8 experiments as shown in Fig. 10,. In 8 glands, Bay-K-8644-evoked CA secretion under the presence of 20 μM MATP was strikingly depressed as compared to the corre-

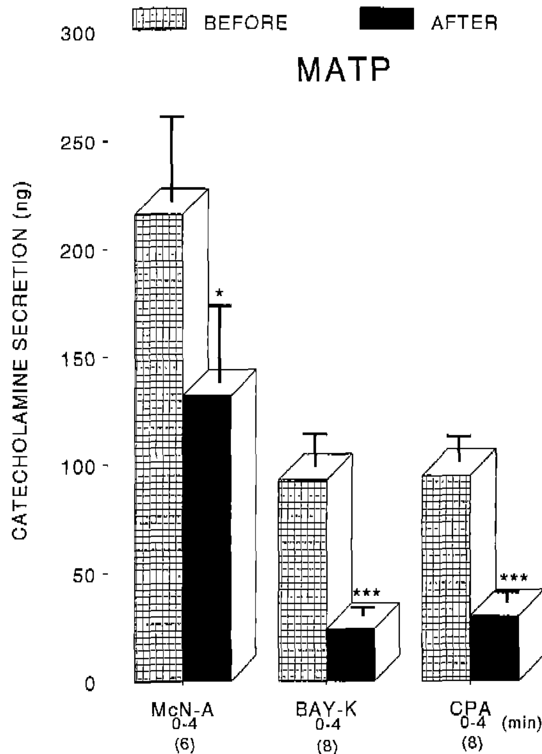

Fig. 10. Effect of β,γ-methyl adenosine-5'-triphosphate (MATP) on CA release evoked by McN-A-343, Bay-K-8644 and cyclopiazonic acid. Secretagogues were administered before and after pretreatment with Krebs solution containing 10 μM MATP for 20 min, respectively. Other legends are the same as Fig. 1 and 7. *P<0.05, ***P<0.01.

sponding control release; thus, the release was reduced to 26% of the control secretion (0~4 min, 93 ± 15 ng) as shown in Fig. 10.

DISCUSSION

The present experimental results demonstrate that NECA causes a great inhibition of CA secretory responses evoked by stimulation of cholinergic (both nicotinic and muscarinic) receptors as well as by membrane depolarization. This effect may be mediated by inhibiting influx of extracellular calcium and release in intracellular calcium in the rat adrenomedullary chromaffin cells, which appeared to be due to the inhibitory mechanism of adenosine receptor activation.

The present experiment results are in agreement with previous reports (Chern *et al*, 1987; Sasakawa *et al*, 1989; Mateo *et al*, 1995), while not in agreement with the results obtained by some investigators (Wakade and Wakade, 1978; Wakade, 1981; Lim and Choi; 1986). In the present study in the isolated perfused rat adrenal gland, NECA time-dependently caused decrease in responses on cholinergic stimulation- and membrane depolarization-induced CA secretion. The reason for differences between results of the present and previous studies in the culture adrenal chromaffin cells or the perfused adrenal glands is uncertain. But it may be related to species differences or differences in experimental design (Wakade and Wakade, 1978; Wakade, 1981; Lim and Choi, 1986; Diverse-Pierlussi, 1991; Mateo *et al*, 1995; Reichsman *et al*, 1995).

NECA is known to be used widely as an agonist of A_2-subclass (Bruns *et al*, 1986; Friedman *et al*, 1989). It has been also found that adrenal chromaffin cells express selectively the A_{2b}- subtype of the adenosine receptors (Casado *et al*, 1992). The present experimental findings that NECA inhibited both cholinergic stimulation- and depolarization-induced CA secretory responses from the perfused rat adrenal gland suggest strongly that this NECA-induced inhibitory action may be exerted via activation of adenosine A_{2b}-receptors located on the rat adrenomedullary chromaffin cells. In support of this finding, recently, it has been found that prolonged exposure to NECA does not affect the adenosine A_{2a}-mediated vasodilatation in porcine coronary arteries (Conti et al, 1997).

In the present investigation, NECA-induced inhibitory effects on CA secretory responses evoked by cholinergic stimulation and membrane depolarization were recovered nearly to the control level of each secretagogue when NECA and IBMX were perfused simultaneously into the adrenal gland. In view of these facts, this finding suggests that NECA can cause the inhibitory effect on CA secretion through the inhibitory mechanism of adenosine A_2-receptor activation. IBMX is found to be a potent phosphodiesterase inhibitor and to be more active than theophylline at adenosine receptors (Bruns *et al*, 1986). In support of this idea, it has been shown that vasopressin-secreting neurons can be brought to maturity in culture by growing them in the presence of IBMX, and forskolin, an activator of adenylate cyclase for 3 weeks. At this time, the cultures contain numerous large immunoreactive vasopressin-neurophysin neurons and secrete vasopressin into the culture medium. However, if the IBMX and forskolin are withdrawn at this point, vasopressin expression declines and 1 week later the cultures contain only occasional immunoreactive neurons and vasopressin is no longer detectable in the medium. Re-exposures of the cultures to IBMX and forskolin results in re-expression of vasopressin synthesis and secretion (Sladek and Gallagher, 1993; Mathiasen *et al*, 1996). More-

over, it has been found that excitatory behavior on CA secretion with NECA in the cultured bovine chromaffin cells did not occur (Mateo *et al*, 1995) as was seen with adenosine when assayed together with forskolin or an inhibitor of cyclic AMP phosphodiesterase (Chern *et al*, 1988). The present experimental result that NECA inhibits DMPP-, McN-A-343- and excess K^+-induced CA secretory responses cannot rule out completely the possibility in relation to the cAMP. cAMP analogs have variously been reported to enhance the nicotinic response of bovine adrenal chromaffin cells (Higgins and Berg, 1988; Peach, 1972), to have no specific effect on the nicotinic response (Adams and Boarder, 1987; Morita *et al*, 1987; Dubin *et al*, 1992) or to decrease it (Cheek and Burgoyne, 1987; Marriott *et al*, 1988). Nevertheless, the role of cyclic AMP is only modulatory because it is not required for exocytosis itself (Knight and Baker, 1982; Bittner *et al*, 1986). The cyclic AMP-mediated excitatory action of adenosine on evoked CA secretion from chromaffin cells has been shown to be blocked by inhibitors of adenosine transport and it has been proposed that intracellular adenosine could inhibit cAMP phosphodiesterase and thus potentiate cAMP effect (Turner and Njus, 1993). In fact, NECA, which is not transported into the cell (Balwierzak *et al*, 1989), lacks this excitatory effect.

Thus, selective stimulation of A_2 receptors by NECA appears more specific than treatment of chromaffin cells with high concentration of adenosine, which could activate an extended set of targets. In view of the fact that NECA inhibits CA secretory responses evoked by DMPP (a nicotinic receptor agonist), McN-A-343 (a muscarinic M_1-receptors agonist) and high potassium (a direct membrane-depolarizer) in the isolated perfused rat adrenal gland, it suggests that this inhibitory effect of NECA is not mediated by the increased cAMP. Both the effects of adenosine and adenosine agonists on CA secretion (Chern *et al*, 1988; 1992) and adenosine transport (Delicado *et al*, 1990) are not associated with a modification of cAMP levels in chromaffin cells. However, NECA elevates cellular cAMP content in the presence of forskolin without having any positive effect on secretion. This finding suggests that the rise in cAMP level may not be the sole cause of the increase in secretion by adenosine (Chern *et al*, 1988).

Furthermore, in the present investigation, the findings that MATP, a potent ATP receptor agonist, inhibited time-dependently CA secretory responses evoked by cholinergic stimulation as well as membrane depolarization can support surely A_2-receptor located on the rat adrenomedullary chromaffin cells. MATP is known to be an ATP receptor agonist in guinea-pig (Hourani *et al*, 1986). It has been also shown that MATP induces Ca^{2+} entry mainly through L-type Ca^{2+} channels by a pertussis toxin-insensitive mechanism, consistent with activation of P_{2x} receptors at presynaptic glial cells of the frog neuromuscular junction (Robitaille, 1995).

In the present investigation, the results that NECA as well as MATP inhibits CA secretion evoked by stimulation of nicotinic and muscarinic receptors with DMPP and McN-A-343 suggest strongly that adenosine receptors are involved in the regulation of the overall secretory responses evoked by nicotinic and muscarinic stimulation. 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 & Hwang, 1991). These observations are in line with a previous report (Ladona *et al*, 1987; Uceda *et al*, 1992) showing that Bay-K-8644 almost tripled the peak secretory response to muscarine in perfused cat adrenal glands. In the present experiment, NECA 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 NECA inhibited CA secretion evoked by high K^+ and also by Bay-K-8644 suggest that this NECA inhibits directly the voltage-dependent Ca^{2+} channels through adenosine receptors, just like Ca^{2+} channel blockers (Cena *et al.*, 1983), which have direct action on voltage-dependent Ca^{2+} channels. Therefore, it seems that the NECA inhibits CA secretion evoked by DMPP by inhibiting Ca^{2+} influx through voltage-dependent Ca^{2+} channels activated by nicotinic ACh receptors with DMPP. Similarly as in the present study, the time-dependent inhibition of CA secretion evoked by NECA has been found to correlate quite well with the inhibition of DMPP-evoked $[Ca^{2+}]_i$ transients after pre-incubation with NECA (Mateo *et al*, 1995). The $[Ca^{2+}]_i$ rise induced by the nicotinic agonist DMPP is considered to be caused almost exclusively by extracellular Ca^{2+} influx through voltage-sensitive Ca^{2+} channels activated by depolarization evoked by nicotinic receptor stimulation (Kilpatrick *et al*, 1982; Kim and Westhead, 1989; O'Sullivan *et al*, 1989). Thus, the inhibitory effect of NECA in the present work should be attributed to the inhibition of Ca^{2+} influx.

In the present study, it has been also shown that NECA inhibits the increase in 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 & 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 NECA on CA secretion evoked by cholinergic stimulation as well as by membrane-depolarization may be associated with the mobilization of intracellular Ca^{2+} in the chromaffin cells. This indicates that the NECA 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 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 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 (PI) metabolism, resulting in the formation of inositol 1, 4, 5-trisphosphate (IP_3), 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 the NECA on Ca^{2+} movement from intracellular pools is due to their direct effect on the PI response or an indirect effect as a result of the membrane hyperpolarization induced by adenosine receptors. In the present study, the fact that McN-A-343-evoked CA secretion was depressed by pretreatment with NECA appears to be consistent with these previous results.

In conclusion, the present experimental results demonstrate that the NECA inhibits CA secretory responses evoked by cholinergic (both nicotinic and muscarinic) stimulation as well as by membrane depolarization from the isolated perfused rat adrenal glands. And it leads to inhibit Ca^{2+} influx through the L-type voltage-dependent calcium channels and also Ca^{2+} mobilization from intracellular pools. These findings suggest that this inhibitory mechanism of NECA-sensitive adenosine receptor stimulation may play a modulatory role in CA secretion from the rat adrenomedullary chromaffin cells.

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