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Studies on Secretion of Catecholamine Evoked by Caffeine from the Isolated Perfused Rat Adrenal Gland

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Abstract □ The influence of caffeine on secretion of catecholamines (CA) was examined in the isolated perfused rat adrenal gland. Caffeine (0.3 mM) perfused into an adrenal vein of the gland produced a marked increase in secretion of CA. This secretory effect of CA evoked by perfusion of caffeine for one minute was considerably prolonged, lasting for more than 90 minutes. The tachyphylaxis to releasing effect of CA induced by caffeine was observed by repeated perfusion of this drug. The caffeine-evoked CA secretion was markedly inhibited by pretreatment with ouabain, trifluoperazine, TMB-8 and perfusion with calcium-free Krebs solution containing 5 mM EGTA, but was not affected by perfusion of calcium-free Krebs solution without other addition. CA secretion evoked by caffeine was not reduced significantly by pretreatment with chlorisondamine but after the first collection of perfusate for 3 min was clearly inhibited. Interestingly, the caffeine-evoked CA secretion was considerably potentiated by pretreatment with atropine or pirenzepine, but after the first collection for 3 min it was markedly decreased. These experimental results suggest that caffeine causes a marked increase in secretion of CA from the isolated perfused rat adrenal gland by an extracellular calcium-independent exocytotic mechanism. The secretory effect of caffeine may be mainly due to mobilization of calcium from an intracellular calcium pool in the rat chromaffin cells and partly due to stimulation of both muscarinic and nicotinic receptors.

Keywords □ Caffeine, catecholamine-secretion, adrenal gland.

It is well-known that caffeine has stimulant effects on the central nervous system, resulting in increased alertness and wakefulness[1].

More recently, some reports confirm that coffee intake in human increases blood pressure and plasma-epinephrine levels and also enhances diuresis and natriuresis[2-4]. Immediately after caffeine absorption, both systolic and diastolic blood pressure were increased clearly and remained elevated for 3 hours. At this time, plasma epinephrine levels were increased by 15%, whereas plasma norepinephrine levels exhibited no significant changes[4]. Most investigators reported increases in plasma epinephrine content after caffeine intake[3,5]. The absence of a significant change in norepinephrine content may indicate a direct stimulation of the adrenal gland by caffeine[4,6].

It has been known that caffeine and theophylline cause increase in secretion of catecholamine (CA) from the perfused cat adrenal glands[6,7], ox glands[8] and cultured bovine adrenal chromaffin cells[9,10]. Some investigators of above authors claim that the secretory effect of CA evoked by caffeine is mediated by accumulation of cyclic AMP resulted from the inhibition of cyclic nucleotide phosphodiesterase, although no direct supporting evidence is available. Recently, Yamada *et al.*[7] (1989) report that caffeine produces an increase in CA secretion from the perfused cat adrenal gland by mobilization of Ca^{2+} from an intracellular storage site.

The stimulation evoked by caffeine is known to

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be as effective in increasing CA secretion in the absence of extracellular Ca^{2+} as in its presence, and the response to caffeine in Ca^{2+} -free medium is increased by the loading of Ca^{2+} stores in the perfused bovine adrenal glands[11]. Yamada *et al.* (1988) [12] showed that caffeine was much more effective in increasing CA secretion in the absence of extracellular Ca^{2+} and Mg^{2+} than in their presence, and that the responses to caffeine and muscarinic receptor activation were reversibly inhibited by an intracellular Ca^{2+} antagonist, TMB-8, in perfused cat adrenal glands. There is a clear controversy about the mechanism of CA secretory effect evoked by caffeine as previously described. The present investigation was carried out i) to study whether caffeine causes secretion of CA from the isolatied rat adrenal gland as in other animals, and ii) to elucidate the mechanism of CA secretion induced by caffeine.

EXPERIMENTAL METHODS

Experimental animals

Mature male Sprague Dawley rats, weighing 180-300 g, were anesthetized with ether. The adrenal gland was isolated by the method described previously[13]. The abdomen was opened by a midline incision, and the left adrenal gland and surrounding area were exposed by placing three hook retractors. The stomach, intestine and portions of the liver were not removed, but pushed over to the right side and covered by saline-soaked gauze pads and urine in bladder was removed in order to obtain enough working space for tying blood vessels and cannulations.

As shown in Fig. 1, a cannula, used for perfusion of the adrenal gland (A), was inserted into the distal end of the renal vein after all branches of adrenal vein, renal vein (if any), vena cava and aorta were ligated. Heparin (400 IU/m*l*) 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 the 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 in a leucite chamber. The chamber was continuously circulated with water heated at 37± 1℃(B).

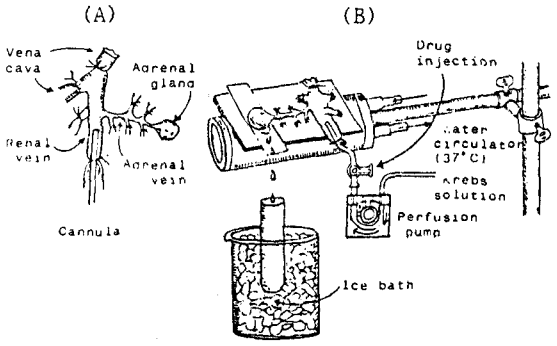

Fig. 1 .Schematic drawing of the preparation used to study secretion of catecholamine in the isoated perfused adrenal gland of the rat.

Perfusion of the adrenal gland

The adrenal glands were perfused by means of a ISCO pump (WIZ Co.) at a rate of 0.4 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; $NaHCO_3$, 2.5; 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± 0.5. The solution contained disodium EDTA (10 μg/m*l*) and ascorbic acid (100 μg/m*l*) to prevent oxidation of catecholamine.

Drug administration

The 1 minute perfusions of caffeine citrate (0.3 mM) or a single injection of Ach (50 μg) in a volume of 0.05 m*l* were made into the perfusion stream *via* a three way stopcock (Fig. 1).

In the preliminary experiments it was found that upon administration of the above of drugs, secretory response to Ach returned to preinjection level in about 4 min, but the responses to caffeine lasted more than 90 min. Therefore, each sample to Ach was collected for 4 min, and samples for caffeine response were collected for 30 min, at three minute intervals. Generally, the adrenal glands were perfused with normal Krebs solution for about one hour. The adrenal perfusate was collected in chilled tubes. Details of the collection of samples are given in results section.

Collection of perfusate

As a rule, prior to each stimulation with cholinergic agonists perfusate samples were collected (4 min)

to determine the spontaneous secretion of CA ("background sample"). Immediately after the collection of the "background sample", collection of the perfusates was continued in another tube as soon as the perfusion medium containing the stimulatory agent reached the adrenal gland. Each perfusate was collected for 4 to 30 min samples. The amounts secreted in the "background sampe" have been substracted from those secreted from the "stimulate sample" to obtain the net secretion value of CA, which is shown in all of the figures.

To study the effects of a blocking agent on the spontaneous and evoked secretion, the adrenal gland was perfused with Krebs solution containing the agent for about 20-30 min, then the perfusate was collected for a specific time period ("background sample"), and then the medium was changed to the one containing the blocking agent plus the stimulating agent and the perfusates were collected for the same period as that for the "background sample".

Measurement of catecholamines

CA content of perfusate was measured directly by the fluorometric method of Anton and Sayre[14] (1962) without the intermediate purification alumina, for the reasons described earlier[13], using fluorospectrophotometer (Shimazu Co.). A volume of 0.2 ml of the perfusate was used for the reaction. The CA content in the perfusate of stimulated glands by Ach or caffeine was high enough to obtain readings several-fold greater than the readings 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) equivalences. All data are presented as means with their standard errors, and the significances of differences were analyzed by using Student's t-test.

Drugs and their sources

The following drugs were used: caffeine citrate (Mallinckrodt Chemical Works, USA), acetylcholine chloride, 3,4,5-trimethoxy benzoic acid 8-(diethylamino) octyl ester (TMB-8), norepinephrine bitartrate, ouabain octahydrate, ethylene glycol bis (β-aminoethyl ether) N,N,N',N'-tetracetic acid (EGTA), trifluoperazine 2 HCl (Sigma Chemicl Co., USA.), pirenzepine 2 HCl (Shinpoong Pharmaceutical Manufac. Korea), chlorisondamine chloride (Ciba Co., USA.) and atropine sulfate (Merk Co., West Germany). Drugs were dissolved in distilled water (Stock) and added to the Krebs solution as required. All drug concentrations are expressed in terms of molar base except the case of Ach.

RESULTS

Secretion of catecholamines evoked by caffeine or Ach from the adrenal gland

The spontaneous (resting) secretion of CA from the isolated perfused rat adrenal glands reached a steady level about 60 min after the start of perfusion with Krebs solution. When Ach (50 μg) was injected into the perfusion stream *via* a three way stopcock after obtaining the stabilized resting release of CA, CA secretion was 473.9± 27.96 ng ($p<0.001$) for 4 min in 20 rat adrenal glands.

Caffeine (0.3 mM) perfused into the adrenal vein for 1 min period resulted in a rapid and great increase in secretion of CA that was maximal in that fraction of caffeine-perfusion and this secretory effect lasted for more than 90 min, as shown in Fig. 2 and Table I. The secretions evoked for 30 min after 1 min perfusion of caffeine from 20 rat adrenal glands were 2419.5± 185.70 ng (0-3 min), 760.0± 52.05 (3-6 min) ng, 443.0± 42.73 ng (6-9 min), 338.0± 44.35 ng (9-12 min), 295.5± 55.34 ng (12-15 min), 225.8± 56.38 ng (15-18 min), 170.0± 50.53 ng (18-21 min), 100.0 ± 45.51 ng (21-24 min), 145.5± 23.29 ng (24-27 min) and 122.5± 23.03 ng (27-30 min). Interestingly, even 90 min after perfusion of caffeine, CA secretion was still remained considerably as evidenced by the amount of CA released in 87-90 min sample (27.6± 4.70 ng). All above data showed statistically significant differences compared to control responses. Particularly, caffeine-induced CA secretion was clearly decreased after 1st collection period (0-3 min). From our unpublished experimental results, it was found that the concentration of 0.3 mM-caffeine caused the most significant releasing effect of CA among 0.1, 0.3, 1.0, 3.0 mM of caffeine used in the present investigation. Therefore, in all subsequent experiments the concentration of 0.3 mM caffeine was used along with50 μg-Ach in order to compare each other.

Fig. 3. represents time course and influence of repeated perfusion of caffeine. As shown in figure, when caffeine was perfused into the perfusion stream of the adrenal gland three times consecutively

Table I. Time course of catecholamine secretion evoked by caffeine and acetylcholine

Agents to evoke secretoin	Dosage of administration	Time of collection (min)	Secretion of catecholamines (ng)
Acetylcholine	50 μg	0- 4	473.9± 27.96
Caffeine	0.3 mM	0- 3	2419.5± 185.70
		3- 6	760.0± 52.05
		6- 9	443.0± 42.73
		9-12	338.0± 44.35
		12-15	295.5± 55.34
		15-18	225.8± 56.38
		18-21	170.5± 50.53
		21-24	150.0± 45.51
		24-27	145.5± 23.29
		27-30	122.5± 23.03
		.	
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		.	
		87-90	27.6± 4.70

Data obtained are expressed with mean± S. E. from 20 rat adrenal glands. About 60 min after perfusion with normal Krebs solutin, the adrenal glands are stimulated with perfusion of caffeine (0.3 mM) for 1 min, or with single injection of acetylcholine. The perfusate to acetylcholine was collected for 4 min. but to caffeine for 30 min at 3 min intervals.

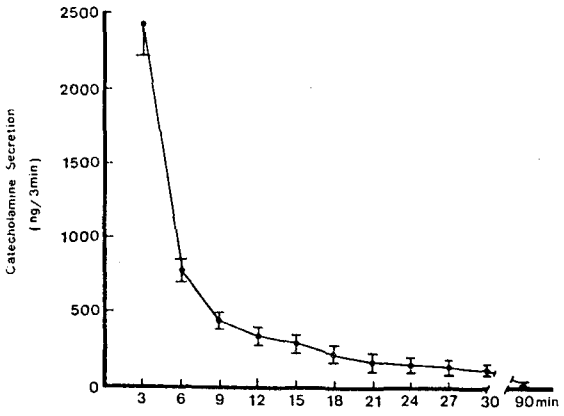

Fig. 2. The time course of catecholamine release evoked by perfusion of caffeine.
Secretion of catecholamines (CA) was induced by introducing perfusion of caffeine (0.3 mM) for 1 min into the perfusion stream 60 min after the begining of perfusion with Krebs solution. After the injection of drugs, the perfusate was collected for 30 min at 3 min intervals. Vertical bars denote S. E. of the mean. Ordinate: the amounts of CA secreted from the adrenal gland. Abscissa: the lapse time after the caffeine perfusion.

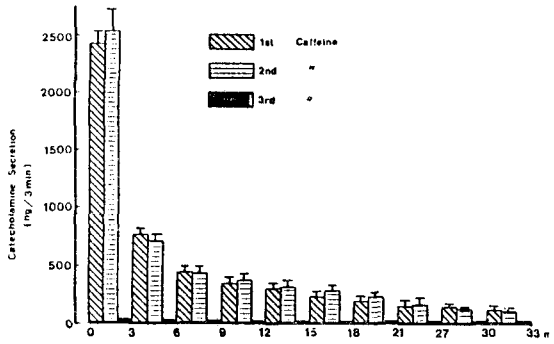

Fig. 3. Effects of repetitive perfusion of caffeine on secretion of catecholamines from the rat adrenal gland.
Caffeine (0.3 mM) was perfused repeatedly into the perfusion stream for 1 min at 2.5 hours intervals 60 min after begining of perfusion with normal Krebs solution. No significance between 1st and 2nd perfusion of caffeine on CA secretion was obtained from 20 rat adrenal glands, except 3rd perfusion. Other legends are the same as in Fig. 1.

with 150 min intervals: there was no difference between 1st and 2nd periods after perfusion of caffeine, but in 3rd period there was a typical tachyphylaxis

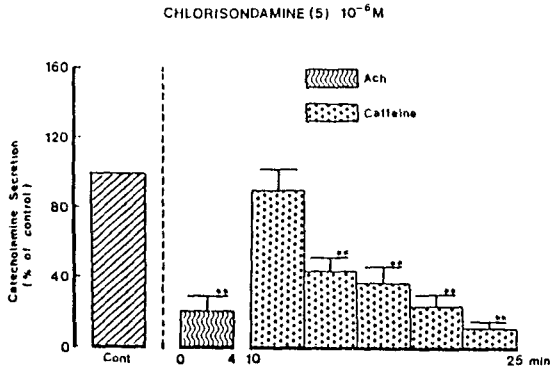

Fig. 4. The effect of chlorisondamine on secretion of catecholamines evoked by Ach or caffeine.
Chlorisondamine (10^{-6} M) was introduced 30 min before perfusion of caffeine. The histograms represent the mean value of caffeine-evoked secretion of catecholamines expressed as percentages of agent-induced secretion as the control (100%). Caffeine was perfused 6 min after Ach injection.
The number of experiments is shown in the upper bracket. The asterisks denote a significant reduction (or increase) from the corresponding control (cont): **p<0.01.

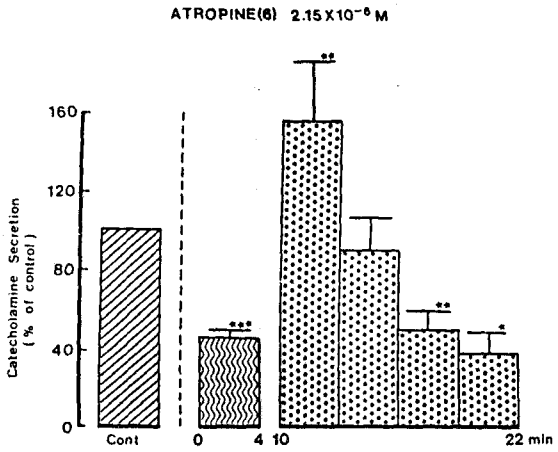

Fig. 5. The effect of atropine on caffeine-evoked secretion of catecholamines.
Atropine was present 30 min before perfusion of caffeine. Other legends and methods are the same as in Fig. 2 and Fig. 3 *p<0.05, **p<0.01, ***p<0.001.

to releasing effect of CA, as observed by the fact that there was no CA secretion at 3rd period sample. Therefore, through all experiment, caffeine was not perfused more than twice.

Influence of chlorisondamine on caffeine-evoked secretion of CA

To study the effect of chlorisondamine, a selective ganglionic nicotinic receptor antagonist[15], on secretion of CA induced by caffeine, the adrenal gland was perfused with chlorisondamine (10^{-6} M) for 20 min before the introduction of caffeine. In the presence of chlorisondamine the CA release evoked by 0.3 mM caffeine from 5 rats was considerably reduced by 87.94± 12.45% (0-3 min, NS), 45.50± 7.58 % (3-6 min, p<0.01), 37.62± 9. 63% (6-9 min, p< 0.01), 23.58± 7.67% (9-12 min, p<0.01) and 10.54± 3.04 % (12-15 min, p<0.01) of the corresponding control value, respectively. It should be noted that there was no significant difference in the 1st collection period (0-3 min) as shown in Fig. 4. At this time, Ach-evoked secrection was depressed by 19.12± 9.84% (p< 0.01, N=5) of the corresponding control value (Fig 4)

The influence of atropine and pirenzepine on caffeine-evoked secretion of CA

It is well known that both atropine and pirenzepine are muscarinic antagonists and pirenzepine is also more selective M_1-muscarinic antagonist[16,17]. Thus it appears to be interesting to examine the effect of atropine or pirenzepine on CA secretion induced by caffeine.

In the present experiment, the CA secretion of caffeine was induced from the perfused gland pretreated with 2.15×10^{-6} M atropine and 2×10^{-6} M pirenzepine for 30 min, respectively. From 6 rat adrenal glands, caffeine-induced secretion of CA in the presence of atropine was greatly enhanced to 155.9± 29.59% (p<0.01) of the control value during the first 3 min following caffeine introduction but after the first collection period (0-3 min), the secretion was rather depressed by 89.2± 16.5% (3-6 min, NS), 49.8± 10.25% (6-9 min, p<0.01) and 38.9± 11.58 % (9-12 min, p<0.05) of the corresponding control responses, respectively. Ach-evoked secretion after treatment of atropine was markedly diminished by 46.3± 2.30% (N=6, p<0.001) of the control value. Fig. 5 shows the influence of atropine on caffeine-induced secretion of CA.

Interestingly, after pirenzepine caffeine-evoked CA secretion was the same as the case of atropine-treat-

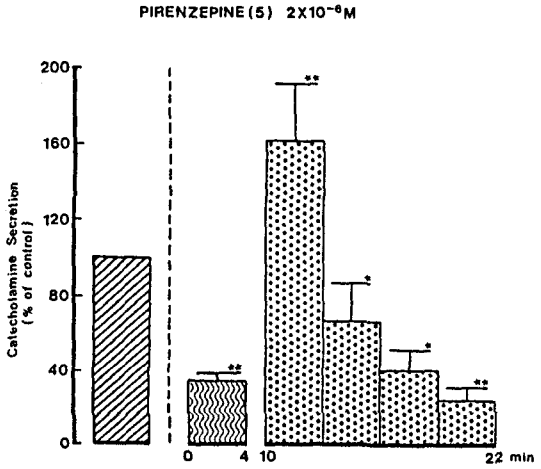

Fig. 6. The effect of pirenzepine on secretion of catecholamines evoked by caffeine.
Pirenzepine (2μM) was present 20 min before perfusion of caffeine. Other legends and methods are the same as in Fig. 2 and 3. *p<0.05, **p<0.01.

ment. In 5 rats after pretreatment with 2×10^{-6} M pirenzepine for 30 min caffeine-induced secretion of CA was markedly increased by 162.3± 33.58% of the control response only for the first collection period (0-3 min) but following the 1st collection secretion of CA was significantly reduced by 66.5± 13.67 (3-6 min, $p<0.05$), 40.9± 13.44 (6-9 min, $p<0.05$) and 24.2± 7.26% (9-12 min, $p<0.01$) of the corresponding control responses, respectively as shown in Fig. 6. Ach-induced secretion was also clearly depressed by 34.8± 3.48% of the control after pretreatment of pirenzepine (Fig. 6).

The influence of perfusion with Ca^{2+}-free medium Ca^{2+}-free medium plus EGTA on caffeine-evoked CA secretion

Since the physiological release of CA and dopamine-β-hydroxylase from the perfused cat adrenal gland is dependent on the extracellular calium concentration[18] and caffeine is much more effective in increasing CA secretion in the absence of extracellular Ca^{2+} and Mg^{2+} than in their presence[12], it is of particular interest to examine whether the secretory effects induced by caffeine (0.3 mM) in this preparations also related to extracellular Ca^{2+} -free Krebs solution for 30 min prior to perfusion of caffeine or Ach.

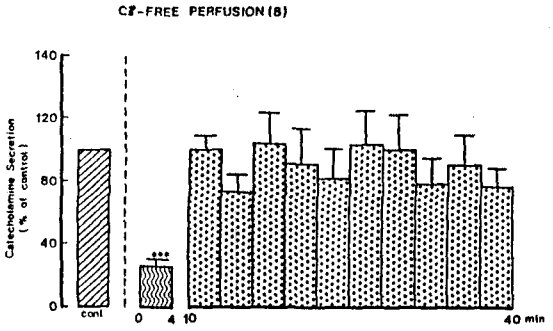

Fig. 7. The effect of perfusion of Ca^{2+} -free Krebs solution on secretion of catecholamines evoked by caffeine. Ca^{2+} -free Krebs solution was perfused 30 min before perfusion of caffeine.
Other legends and methods are the same as in Fig. 2 and 3. ***p<0.001.

After perfusion with Ca^{2+} -free Krebs solution, caffeine-evoked CA secretion was not affected as compared with the corresponding control values while Ach-induced secretion was greatly inhibited by 25.1± 4.84% (N=8, $p<0.01$) of the control response as shown in Fig. 7.

However, in 6 rats perfusion with Ca^{2+} -free Krebs solution plus EGTA led to considerable reduction in CA secretion evoked by caffeine to 65.1 ± 11.74% (0-3 min, $p<0.05$), 35.9± 6.51% (3-6 min, $p<0.01$), 32.9± 5.44% (6-9 min, $p<0.01$), 32.5± 5.15% (9-12 min, $p<0.01$), 25.5± 6.63% (12-15 min, $p<0.01$), 23.1± 7.22% (15-18 min, $p<0.01$), 21.8± 7.23% (18-21 min, $p<0.01$), 18.4± 5.51% (21-24 min, $p<0.01$), 7.83 ± 2.98% (24-27 min, $p<0.01$), and 9.4± 2.36% (27-30 min, $p<0.01$) of the corresponding control values, respectively. At the same time, Ach-evoked CA secretion was significantly diminished by 27.2± 7.98% ($p<0.01$) of the control responses. Fig. 8 shows the effect of perfusion with Ca^{2+} -free medium plus EGTA on caffeine-evoked secretion of CA.

The influence of ouabain on caffeine-evoked secretion of CA

It has been known that inhibition of Na^+-K^+ pump by cardiac glycosides increase spontaneous or evoked secretion of CA from perfused adrenal glands of various species[19-23], and from isolated adrenal chromaffin cells[24-27]. Therefore, it may be of particular interest to investigate the effect of ouabain on CA secretion evoked by caffeine.

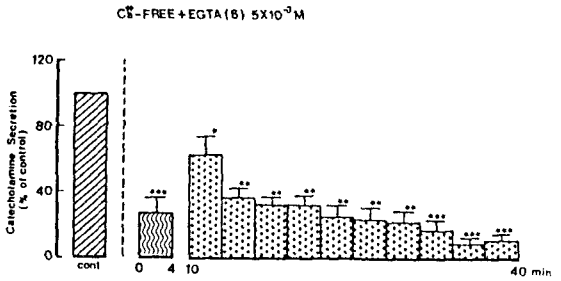

Fig. 8. The effect of perfusion of Ca^{2+} -free medium plus EGTA on catecholamine secretion evoked by caffeine.

Ca^{2+} -free Krebs solution containing 5 mM EGTA was perfused 30 min before perfusion of caffeine. Other legends and methods are the same as in Fig. 2 and 3. *p<0.05, **p<0.01, ***p<0.001.

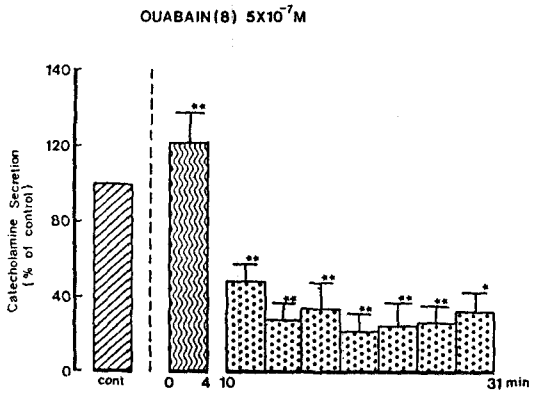

Fig. 9. The effect of ouabain on caffeine-evoked secretion of catecholamines.

Ouabain(0.5 μM) was introduced 30 min before perfusion of caffeine. Other legends and methods are the same as in Fig. 2 and 3. *p<0.05, **p<0.01.

As shown in Fig. 9, after perfusion of 5×10^{-7} M ouabain for 30 min CA secretion of Ach was clearly enhanced to 121.9± 16.22% (N=8, p<0.01) of the control response, while secretory effect of CA evoked by caffeine was markedly inhibited by 47.2 ± 9.80% (0-3 min, p<0.01), 27.6± 8.70% (3-6 min, p< 0.01), 34.6± 14.52% (6-9 min, p<0.01), 20.6± 8.74% (9-12 min, p<0.01) 24.3± 9.17% (12-15 min, p<0.01), 25.4 ± 11.68% (15-18 min, p<0.01) and 32.3± 10.14% (18-21 min, p<0.05) of the corresponding control values, respectively.

The influence of trifluoperazine on caffeine-evoked secretion of CA

Recently, trifluoperazine and other phenothiazine compounds have received considerable attention because these drugs inhibit calcium regulatory protein-calmodulin[28,29] and very effectively block Ca^{2+}-mediated physiological responses in different cells. Wakade and Wakade[30] (1984) have also reported that trifluoperazine reduces CA secretion evoked by Ach from the adrenal medulla. Therefore, it is very exciting to observe the effect of trifluoperazine on secretory effect of CA evoked by caffeine. In 6 rats, adrenal glands were perfused with trifluoperazine (10^{-4} M) for 30 min before the stimulation with caffeine. Ach-induced CA secretion after trifluoperazine treatment was greatly depressed to 26.2± 6.57 % (p<0.01) of the control value and CA secretion of caffeine was also significantly inhibited by 31.3± 7.81% (0-3 min, p<0.01), 3.92± 1.08% (3-6 min, p< 0.01) and 0.9± 0.09% (6-9 min, p<0.01) of the corresponding control responses, respectively, as shown in Fig. 10.

The influence of TMB-8 on caffeine-evoked secretion of CA

Since it has been found that muscarinic, but not nicotinic activation causes catecholamine secretion independent of extracellular Ca^{2+} in perfused adrenal glands of the cat[31] and guinea pig[32], suggesting the presence of an intracellular Ca^{2+} pool linked to a muscarinic receptor, attempt was made to test the effect of TMB-8 on caffeine-evoked secretion of CA. The Ach-induced CA secretion was almost completely blocked after perfusion of 10^{-4} M TMB-8 for 20 min and secretion evoked by caffeine was also greatly inhibited by 11.8± 2.77% (0-3 min, p< 0.01) and 5.22± 2.7% (3-6 min, p<0.01) of the corresponding control values, respectively, Fig. 11 shows that treatment of TMB-8 led to blockades of CA secretion evoked by caffeine or Ach.

DISCUSSION

The analysis of the data presented in this study shows that caffeine greatly enhances secretion of CA from the isolated perfused rat adrenal gland by exocytotic mechanism independent of extracellular calcium and that this secretory effect of caffeine

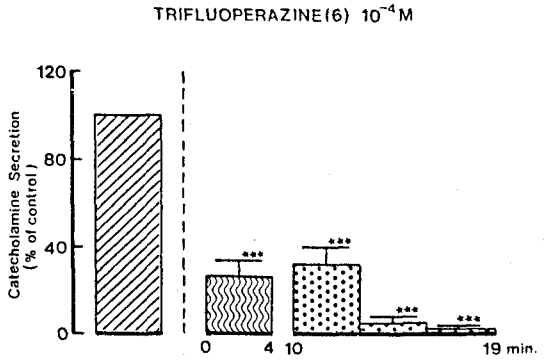

Fig. 10. The effect of trifluoperazine on caffeine-evoked secretion of catecholamines.
Trifluoperazine was present 20 min before perfusion of caffeine. Other legends and methods are the same as in Fig. 2 and 3. ***$p<0.001$.

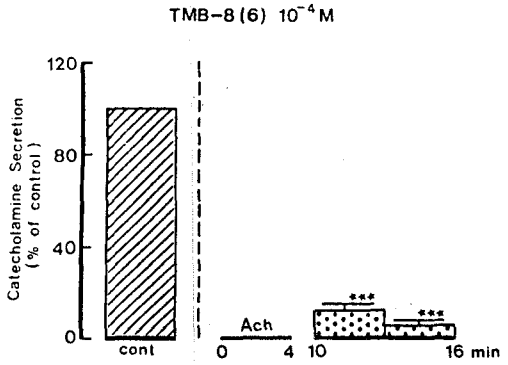

Fig. 11. The effect of TMB-8 on caffeine-evoked secretion of catecholamines.
TMB-8 (100 μM) was present 20 min before introduction of caffeine. Other legends and methods are the same as in Fig. 2 and 3. Ach-evoked CA secretion was almost blocked by TMB-8. ***$p<0.001$.

may be due to direct mobilization of calcium from an intracellular calcium pool in the rat adrenal chromaffin cells and partly due to stimulation of both muscarinic and nicotinic receptors.

It has been found in the present experiment that caffeine-evoked CA secretion disappeared abruptly following the third perfusion period of caffeine by repeated administration and the reason for this tachyphylaxis to releasing effect of CA evoked by caffeine is not clear.

In general, the adrenal medulla has been employed as the model system to study numerous cellular functions involving not only noradrenergic nerve cells but also neurons. One of such functions is neurosecretion. During the neurogenic stimulation of the adrenal medulla, Ach is released from the splanchnic nerve endings and activates cholinergic receptors on the chromaffin cell membrane[33]. This activation triggers a series of events known as stimulus-secretion coupling, culminating in the exocytotic secretion of CA and other components of the secretory vesicles in the extracellular space. Ach, the physiological neuro-transmitter at the adrenal medulla, releases CA and dopamine-beta hydroxylase by calcium-dependent secretory processes[34,35].

Since chlorisondamine, a well-known ganglionic blocking agent, did not significantly modify the secretory response evoked by caffeine, during the first 3 min collection period it is clear that caffeine effect is due to a direct action on the chromaffin cell as previously shown in the case of ouabain. However, the findings that in the present study pretreatment of chlorisondamine inhibited the secretory effect evoked by caffeine after the first 3 min collection period suggest that caffeine may partly produce CA release from the rat adrenal gland by weak activation of nicotinic receptors.

Surprisingly, the caffeine-evoked CA release was greatly enhanced by pretreatment with atropine or pirenzepine during the first 3 min collection period but was depressed considerably immediately after the first collection period. In the present experiments, the reasons why muscarinic antagonists potentiate CA release evoked by caffeine are not known. It appears that caffeine-induced secretory effect may be partly due to the stimulation of M_1-muscarinic receptors in the adrenal chromaffin cells because of the marked reduction after the 1st collection period.

More recently, subtypes of muscarinic receptors have been recognized in many tissues[36]. Receptor binding studies have supported the classification of muscarinic receptors into M_1 and M_2 on the basis of the selective profile of pirenzepine; receptors with a high affinity for pirenzepine are designated as M_1 and those with low affinity as M_2 receptors[37-38]. Doods and his colleagues[16] (1987) have classified muscarinic receptors into M_1 (pirenzepine sensitive, neuronal), M_2 (cardiac) and M_3 (smooth muscle and grandular). In the light of above investigations, the present work shows that caffeine may cause CA secretion partly through weak activation of M_1-mus-

carinic receptors in adrenal chomaffin cells.

Furthermore, enhancement of caffeine-evoked CA secretion by atropine or pirenzepine during the first 3 min collection period suggest that caffeine may have the another direct action on CA secretion.

Yamada *et al.*[12] (1988) have previously reported that caffeine is much more effective in releasing CA in the absence of extraecllular Ca^{2+} than in its presence, but in the present study caffeine was almost equieffective in CA secretion both in the absence and in the presence of extracellular calcium. In addition, perfusion of the adrenal gland with Ca^{2+} -free Krebs containing 5 mM EGTA for 30 min led to considerable inhibition of the caffeine-induced secretion of CA. Generally, the indispensible role of calcium in the neurosecretory process has been thoroughly established. Yet, according to the assumptions of Baker and Knight (1978; 1980)[39,40], the relationship between the concentration of intracellular calcium and the transmitter release has not been determined in nerve terminals. As aforementioned, calcium plays the crucial role in depolarization-neurotransmitter release coupling in many other types of secretory cells[41-43]. In the present investigation, removal of extracellular calcim did not affect CA secretion evoked by caffeine, but that induced by Ach was depressed greatly.

This secretory effect of caffeine seems to be unaffected by extracellular calcium while that of Ach is considerably dependent on the concentration of extracellular calcium.

In support of this result, it is known that caffeine-evoked stimulation is as effective in increasing catecholamine secretion in the absence of extracellular Ca^{2+} as in its presence, and the response to caffeine in Ca^{2+} -free medium is increased by loading of Ca^{2+} -stores in perfused bovine adrenal glands[11]. More recently, Yamada *et al.* (1988)[12] have found that caffeine is much more effective in increasing CA secretion in the absence of extracellular Ca^{2+} and Mg^{2+} than in their presence.

It seems that chromaffin cells of the rat adrenal gland contain an intracellular store of calcium which participates in the secretion of CA as shown in the bovine adrenal gland. Such a store may not be easily depleted by more removal of extracellular calcium. Some investigators[44-47] have reported that intracellular stores of calcium can play some role in contraction of smooth muscle produced by noradrenaline or Ach in calcium-free medium.

It has also been known that cardiac glycosides increase both spontaneous and evoked CA secretion from the perfused adrenal glands of various species[19-23] and isolated chromaffin cells of bovine adrenal medulla[24-27].

In the present work, in view of the fact that caffeine-evoked CA secretion was greatly inhibited in the adrenal gland pretreated with ouabain, it is felt that caffeine may cause CA release through the same mode of action with ouabain. However, Ach-induced secretion was markedly augmented by ouabain-treatment and this effect is known to be enhanced probably by increasing the rate of Ca^{2+} entry through the Ach receptor-linked Ca^{2+} channel as a result of increased rate of Na^{+} -Ca^{2+} exchange or by reducing Ca^{2+} efflux, both of which are resulting from the reduction of the Na^{+} electrochemical gradient[23].

In support of this evidence, it is shown that the activation of nicotinic receptors stimulates CA release by increasing Ca^{2+} influx through receptor-mediated and/or voltage-dependent Ca^{2+} channels in both perfused rat adrenal gland[48] and bovine isolated adrenal chromaffin cells[49-51].

The present experimental data suggest that caffeine may possess the secretory effect of CA partly even through weak activation of nicotinic receptors in the adrenal chromaffin cells.

Furthermore, it is found that muscarinic receptor activation causes an increase in adrenal CA secretion independent of Ca^{2+} in various species[31,32,52,53] and in cytosolic free Ca^{2+} in isolated bovine adrenal cells without associated CA secretion[54-57].

Previously, some researchers found that muscarinic, but not nicotinic, receptor activation caused CA secretion independent on extracellular Ca^{2+} in perfused adrenal glands of the cat[31] and guinea pig[32], suggesting the presence of an intracellular Ca^{2+} pool linked to muscarinic receptors. In the present study, it was shown that CA releases evoked by caffeine as well as Ach were almost blocked by the pretreatment with the intracellular calcium antagonist, TMB-8. These results agree with the view of Yamada *et al.* (1988)[12], in which Ach- and caffeine-evoked CA secretions in the absence of extracellular calcium in the perfused cat adrenal glands are exerted by inhibiting the mobilization of intracellular Ca^{2+}. TMB-8 is known to inhibit the Ach-

evoked increase in the intracellular Ca^{2+} concentration in isolated bovine adrenal medullary cells, and carbamylcholine-induced CA secretion and $^{45}Ca^{2+}$ uptake in cultured bovine adrenal chromaffin cells[58].

Yamada *et al.* (1989)[59] also suggested that ouabain enhances the response to muscarinic stimulation by intracellular Ca^{2+} entry, which in turn increases the capacity of the intracellular Ca^{2+} pool linked to muscarinic receptors. However, it was also found that TMB-8 blocked CA secretions evoked by nicotinic agent and excess potassium in the presence of extracellular Ca^{2+} in perfused cat adrenal glands[12].

In any case the present experimental results do not confirm the finding that caffeine causes CA secretion entirely by increasing intracellular Ca^{2+} pool linked to muscarinic receptors and by facilitating Ca^{2+} entry through nicotinic receptor-linked Ca^{2+} channels as shown in the case of ouabain[59].

The CA secretion evoked by caffeine in the isolated rat adrenal gland was not clearly blocked by pretreatment with chlorisondamine, pirenzepine or atropine and there was only weak inhibition by them.

However, it seems to be sure that only in the perfused rat adrenal gland caffeine increase CA secretion by the direct mobilization of calcium from the intracellular calcium pool of the adrenal chromaffin cells.

Moreover, in view of the fact that caffeine-evoked CA release was significantly depressed by pretreatment with a calmodulin inhibitor, trifluoperazine, it is thought that caffeine has the direct secretory effect of CA through release of intracellular calcium.

Trifluoperazine and other phenothiazine compounds have received considerable attention in recent years because these drugs inhibit calcium regulatory protein, calmodulin[28,29] and very effectively block Ca^{2+} -mediated physiological responses in different cells: glucose-induced secretion of beta-cells of the pancreas is blocked by trifluoperazine[29,60-64]. Wakade and Wakade (1984)[30] have shown that trifluoperazine and chlorpromazine reduced greatly CA secretion evoked by Ach, excess K^+ or transmural stimulation of splanchnic nerves.

In general, trifluoperazine and other antidepressant inhibit the activity of calmodulin, and therefore many investigators have made use of these agents as "many calmodulin inhibitors" to unravel the role of calmodulin in different systems. Essentially in all cases including the current investigation on the adrenal gland, these agents very effectively block Ca^{2+} -dependent responses. Interestingly, inhibition of a Ca^{2+} -dependent response is not accompanied with a reduction in Ca^{2+} uptake[60,63,65,66]. This dissociation between blockade of Ca^{2+} -dependent response and lack of effect on Ca^{2+} influx has strengthened the idea that not influx of Ca^{2+} but its intracellular utilization *via* calmodulin is affected by neuroleptics and antidepressants[30].

Anyway, the present experimental data do not fit with the view of some investigators that caffeine, as well as muscarinic agonists, possesses an intracellular calcium storage site from which these agonists release calcium into the cytosol to cause CA secretion[11,31,32]. However, the process of caffeine-evoked CA secretion seems to differ from that of muscarinic agonists, because muscarinic agonists are able to increase CA release both in the presence and in the absence of extracellular Ca^{2+} and Mg^{2+} [7,12] and in the present work the caffeine-evoked CA was not blocked clearly by pretreatment with atropine or pirenzepine, and Ca^{2+} store linked to caffeine and muscarinic agonists do not entirely overlap, because Ach is still effective in releasing CA after a substantial secretory response to caffeine during perfusion with Ca^{2+} -free Locke solution containing hexamethonium. Recently, it was found that calcium stores in smooth muscle consisted of two classes; one possessing both caffeine (Ca^{2+} -induced Ca^{2+} release) and inositol 1,4,5-triphosphate (IP3)-induced Ca^{2+} release mechansim, and the other only an IP3-induced Ca^{2+} release mechanism[67].

From the above discussion, it is felt that the present data in caffeine-evoked CA secretion are different from the previous report[7,11,31,32] and this may be due to different animals used in our experiment, and that caffeine absorption clearly increase the blood pressure by the direct stimulation of the adrenal gland, resulting in increased plasma epinephrine levels as shown in the investigation of Nussberger *et al.* (1990).

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