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Influence of FCCP on Catecholamine Release in the Rat Adrenal Medulla

Dong-Yoon LIM[1], Seong-Ho JO[1], Young-Woo KEE[1], Ji-Yeon LIM[1], Deok-Ho CHOI[1], Young-Joo BAEK[1], and Soon-Pyo HONG[2]

[1]*Department of Pharmacology, College of Medicine, Chosun University, Kwang Ju 501-759, KOREA*
[2]*Department of Internal Medicine (Cardiology), Chosun University, Kwang Ju 501-759, KOREA*

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Abstract – The aim of the present study was to investigate the effect of FCCP (carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone), which is a potent mitochondrial uncoupler, on secretion of catecholamines (CA) from the perfused model of the rat adrenal gland and to establish the mechanism of its action. The perfusion of FCCP (3×10^{-5} M) into an adrenal vein of for 90 min resulted in great increases in CA secretions. Tachyphylaxis to CA-releasing effect of FCCP was not observed by repeated perfusion of it. The CA-releasing effects of FCCP were depressed by pre-treatment with pirenzepine, chlorisondamine, nicardipine, TMB-8, and the perfusion of EGTA plus Ca^{2+}-free medium. In the presence of FCCP (3×10^{-5} M), the CA secretory responses induced by Ach (5.32×10^{-3} M), and DMPP (10^{-4} M) were significantly enhanced. Furthermore, the perfusion of CCCP (3×10^{-5} M), a similar mitochondrial uncoupler, into an adrenal vein for 90 min also caused an increased response in CA secretion. Taken together, these experimental results indicate that FCCP causes the CA secretion from the perfused rat adrenal medulla in a calcium-dependent fashion. It is suggested that this facilitatory effects of FCCP may be mediated by cholinergic receptor stimulation, which is relevant to both stimulation of the Ca^{2+} influx and Ca^{2+} release from cytoplasmic Ca^{2+} stores.

Keywords □ FCCP (carbonylcyanide *p*-trifluoromethoxyphenylhydrazone), catecholamine secretion, cholinergic receptor stimulation, adrenal Medulla

INTRODUCTION

FCCP (carbonylcyanide *p*-trifluoromethoxyphenylhydrazone) is a potent mitochondrial uncoupler (Heytler, 1979) that inhibits oxidative phosphorylation by dissipating the proton gradient across the inner mitochondrial membrane, and as a result blocks mitochondrial ATP synthesis (Gunter *et al.*, 1994; Yuan *et al.*, 1996). Various mitochondrial uncouplers, including FCCP, have been widely used as a tool to produce cellular models of ischemia or hypoxia (Duchen *et al.*, 1990; Rounds and McMurtry, 1981; Suh *et al.*, 2000).

However, it has been assumed that FCCP acts mainly on the mitochondrial membrane, and the effects on the plasma membrane have been relatively ignored. FCCP evoked a rapid depolarization in rat carotid body type I cells, which was found to be due to the inhibition of a background K^+ conductance and the generation of an unidentified small inward current (Buckler and Vaughan-Jones, 1998). However, FCCP-induced depolarization was also found in rat astrocytes, where the opening of Cl^- channels was suggested to be the mechanism of depolarization (Juthberg and Brismar, 1997). Smith and his coworkers (1999) found that both tolbutamide and the mitochondrial uncoupler FCCP mobilized intracellular Ca^{2+} and prolonged Ca^{2+} transients elicited by cholinergic mobilization of intracellular Ca^{2+} stores in pancreatic α-cells. Mitochondrial inhibitors and uncouplers also excite the carotid body and stimulate CA release from glomus cells (Gonzalez *et al.*, 1994), but the mechanisms by which these agents exert their effects are controversial: previous studies have shown that such agents raise $[Ca^{2+}]_i$ in glomus cells (and so presumably trigger CA release), yet hyperpolarize these cells by causing release of Ca^{2+} from intracellular stores, including mitochondria (Biscoe and Duchen, 1989; Duchen and Biscoe, 1992). Others have suggested that mitochondrial uncouplers act as acidic stimuli and cause the plasma membrane Na^+-Ca^{2+} exchanger to operate in reverse mode, due to Na^+ loading of cells via high Na^+-H^+ exchanger activity (Rocher *et al.*, 1991). Buckler and Vaughan-Jones (1998) have provided evidence that mitochondrial uncouplers,

*Corresponding author
Tel: +82-62-230-6335, Fax: +82-62-227-4693
E-mail: dylim@chosun.ac.kr

like hypoxia, depolarize glomus cells of the rat carotid body via inhibition of K^+ channels, causing Ca^{2+} entry via voltage-gated Ca^{2+} channels. Moreover, it has been shown that FCCP and carbonyl cyanide m-chlorophenylhydrazone (CCCP) stimulated the synthesis of ^{14}C-CA from [^{14}C]tyrosine in cultured bovine chromaffin cells (Yokota *et al.*, 1988). Recently, Montero and his coworkers (2001) found that blocking mitochodrial Ca^{2+} uptake with protonophores or mitochondrial inhibitors also enhanced CA secretion induced by histamine in chromaffin cells.

In contrast, several investigators reported that mitochondrial uncouplers, including FCCP, affect the membrane potential and ionic currents across the plasma membrane. In rat dissociated hippocampal neurons, FCCP hyperpolarized the majority of the cells (Hyllienmark and Brismar, 1996), mimicking the responses to hypoxia in those cells (Fujiwara *et al.*, 1987). This hyperpolarization was clearly driven by an increased K^+ conductance (Murai *et al.*, 1997), but the proposed nature of the K^+ conductance mediating this response differs between studies (Hyllienmark and Brismar, 1996; Krause *et al.*, 1995; Nowicky and Duchen, 1998).

However, there seems to be big controversy in the effects of FCCP on cytosolic Ca^{2+} mobilization. Therefore, the present study was attempted to investigate the effects of FCCP on CA secretion in the isolated perfused model of the rat adrenal gland, and to clarify the mechanism of action.

MATERIALS AND METHODS

Experimental procedure

Male Sprague-Dawley rats, weighing 200 to 350 grams, were intraperitoneally anesthetized with thiopental sodium (40 mg/kg). 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 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 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 contained disodium EDTA (10 mg/ml) and ascorbic acid (100 mg/ml) to prevent oxidation of CAs.

Drug administration

FCCP (3×10^{-5} M) and CCCP (3×10^{-5} M) were perfused into an adrenal vein for 90 min. The perfusions of DMPP (10^{-4} M) for 2 minutes and/or a single injection of ACh (5.32×10^{-3} M) in a volume of 0.05 ml were made into perfusion stream via a three-way stopcock, respectively. In the preliminary experiments, it was found that upon administration of the above drugs, secretory responses to ACh returned to pre-injection level in about 4 min, but the responses to DMPP in 8 min.

Collection of perfusate

As a rule, prior to stimulation with various secretagogues, 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 sample's perfusate was collected for 4 to 8 min. The amounts secreted in the background sample have been subtracted from that secreted from the stimulated sample to obtain the net secretion value of CA, which is shown in all of the figures.

To study the effect of FCCP on the spontaneous and evoked secretion, the adrenal gland was perfused with Krebs solution containing FCCP for 90 min. Then, the perfusate was collected for a certain period (background sample). Then the medium was changed to the one containing the blocking agent or along with FCCP, 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 CAs

CA content of perfusate was measured directly by the fluorometric method of Anton and Sayre (Anton and Sayre, 1962) without the intermediate purification alumina for the reasons described earlier (Wakade, 1981) using fluorospectrophotometer (Kontron Co., Milano, Italy). A volume of 0.2 ml of the perfusate was used for the reaction. The CA content in the perfusate of stimulated glands by secretagogues used in the present work was high enough to obtain readings several folds greater than the reading of control samples (unstimulated). The sample blanks were also lowest for perfusates of stimulated and non-stimulated samples. The content of CA in the perfusate was expressed in terms of norepinephrine (base) equivalents.

Statistical analysis

The statistical difference between the control and pretreated groups was determined by the Student's *t*- and *ANOVA*- tests. A P-value of less than 0.05 was considered to represent statistically significant changes unless specifically noted in the text. Values given in the text refer to means and the standard errors of the mean (S.E.M.). The statistical analysis of the experimental results was made by computer program described by Tallarida and Murray (1987).

Drugs and their sources

The following drugs were used: FCCP, CCCP, acetylcholine chloride, 1.1-dimethyl-4-phenyl piperazinium iodide (DMPP), norepinephrine bitartrate, nicardipine hydrochloride and 3.4.5-trimethoxy benzoic acid 8-(diethylamino) octylester (TMB-8), and pirenzepine hydrochloride were purchased from Sigma Chemical Co., U.S.A., and chlorisondamine chloride from Ciba Co., U.S.A., cyclopiazonic acid, from RBI, U.S.A. Drugs were dissolved in distilled water (stock) and added to the normal Krebs solution as required except nicardipine, FCCP and CCCP. Nicardipine and FCCP were dissolved in 99.5% ethanol and CCCP in DMSO. They were diluted appropriately (final concentration of alcohol or DMSO was less than 0.1%). Concentrations of all drugs used are expressed in terms of molar base.

RESULTS

The secretory effect of CA evoked by FCCP

When the adrenal gland was perfused with oxygenated Krebs-bicarbonate solution for 60 min before experimental protocol is initiated, the spontaneous CA secretion reached steady state. The basal CA release from the perfused rat adrenal medulla amounted to 22±2 ng for 2 min from 8 experiments. The releasing effects to the initial perfusion of FCCP (3×10^{-5} M) for 90 min are shown in Fig. 1-upper. Time-course effect of FCCP (3×10^{-5} M) infusion into the perfusion stream for 90 min at 120 min-interval exerted significant CA secretion over the background release. This result seems to be similar to the findings that both FCCP and CCCP stimulated the synthesis of ^{14}C-CA from [^{14}C]tyrosine in cultured bovine chromaffin cells (Yokota *et al.*, 1988), and that both protonophores applied at 2 μM in the standard bath solution without histamine in bovine chromaffin cells showed an intracellular Ca^{2+} rise (Bödding, 2001). In 8 rat adrenal glands, this FCCP-evoked CA secretory responses were 16~168 ng (0~90 min) for the 1st period, and 18~170 ng (0~90 min) for the 2nd period, respectively. There was no statistically significant difference between 1st and 2nd period groups. The tachyphylaxis to CA-releasing effects of FCCP was not observed. However, in all subsequent experiments, FCCP was not administered more than twice at 120 min-intervals.

Effect of pirenezepine and chlorisondamine on FCCP-evoked CA secretion

In order to examine the effect of chlorisondamine, a selective nicotinic receptor antagonist, on FCCP-induced CA release, the rat adrenal gland was preloaded with 10^{-6} M chlorisondamine for 20min before FCCP was introduced. In the presence of chlorisondamine effect, the CA outputs evoked by perfusion with FCCP (3×10^{-5} M) for 90 min amounted to 90~ 0% of their corresponding control from 8 experiments (Fig. 1-lower).

Hammer and Giachetti (1982) demonstrated that two types of muscarinic receptors (M_1 and M_2) characterized by high or low affinity for the muscarinic antagonist pirenzepine were present in sympathetic ganglia. Therefore, it would be interesting to examine the effect of pirenzepine on CA release evoked by FCCP. In the present work, the CA output induced by FCCP was greatly reduced in the rat adrenal gland preloaded with 2×10^{-6} M pirenzepine. In 8 rat adrenal glands, 3×10^{-5} M FCCP-evoked CA releasing responses after pretreatment with pirenzepine were depressed by 90~0% of their control secretions as shown in Fig. 2-upper.

The effect of perfusion of Ca^{2+}-free Krebs, nicardipine and TMB-8 on FCCP-evoked CA secretion

It has been found that the physiological release of CA and

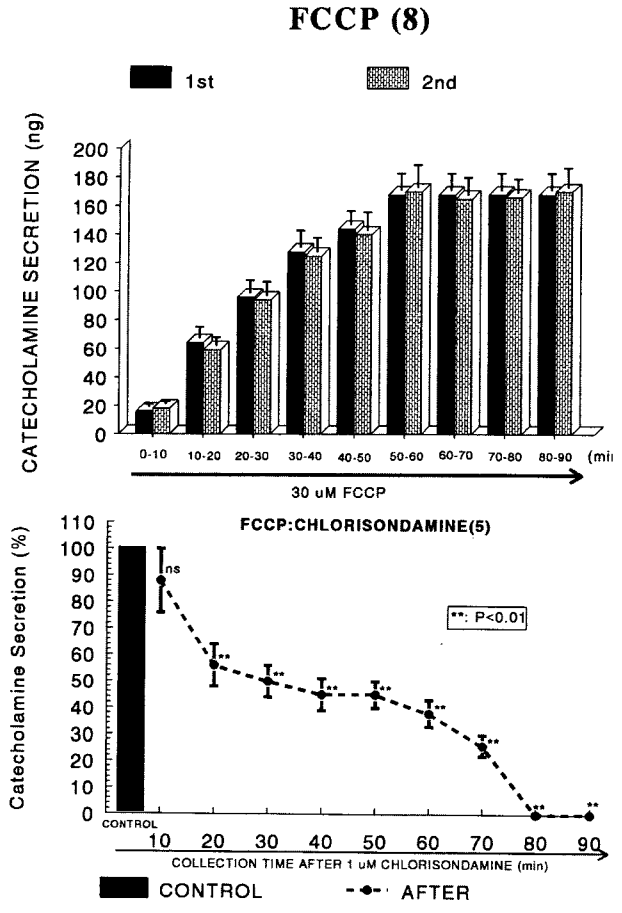

Fig. 1. Upper: Time-course effect of FCCP on secretion of catecholamines (CA) from the perfused rat adrenal glands. FCCCP (3×10^{-5} M) was perfused into an adrenal vein twice for 90 min at 120 min interval. Perfusion of FCCP was made after perfusion with normal Krebs-bicarbonate solution for one hour before the experimental protocols were initiated. The data are expressed with mean±S.E. from 8 rat adrenal glands. The perfusate was collected for 90 min at 10 min-intervals. The statistical significance was compared between the 1st group and 2nd group. Abscissa: Time of collection (min). Ordinate: secretion of CA in ng for 10 min. The vertical columns and bars denote means and the standard errors of the corresponding means, respectively. Number in the parenthesis indicates the number of animals used in the experiments. There was no significant difference between two groups.

Lower: The effect of chlorisondamine on the secretion of CA evoked by FCCP in the perfused rat adrenal glands. Secretion of CA evoked by FCCP (3×10^{-5} M) was evoked for 90 min after perfusion of adrenal gland with Krebs solution containing 10^{-6} M chlorisondamine. "CONTROL" and "AFTER" indicate amounts of CA released by FCCP before (CONTROL) and after the preloading with chlorisondamine (10^{-6} M). Statistical differences were compared between amounts of CAs evoked by FCCP before (CONTROL) and after the pretreatment. **: $P < 0.01$. ns: Statistically not significant.

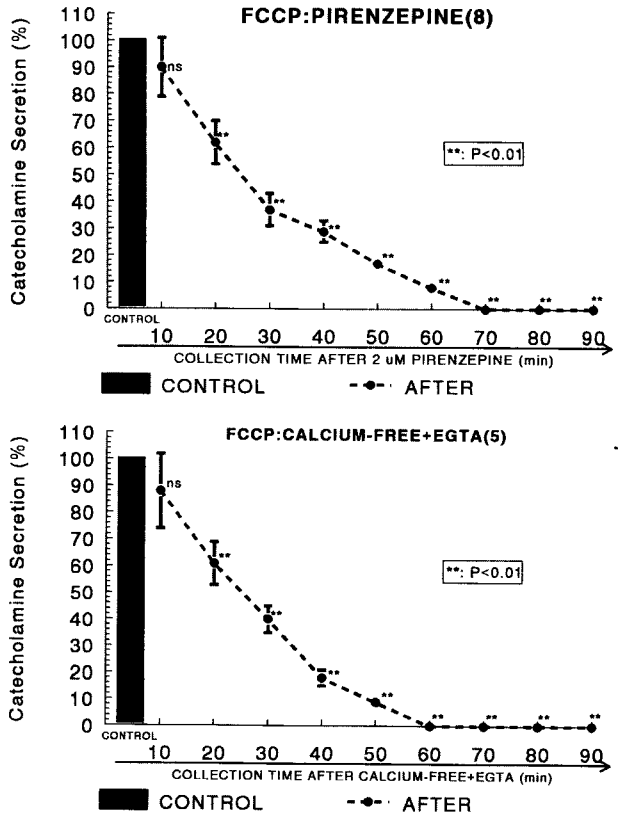

Fig. 2. Upper: The effect of pirenzepine on the secretion of CA evoked by FCCP in the perfused rat adrenal glands. Secretion of CA evoked by FCCP (3×10^{-5} M) was evoked for 90 min after perfusion of adrenal gland with Krebs solution containing 2×10^{-6} M-pirenzepine.

Lower: The effect of Ca^{2+}-free Krebs-perfusion on the secretion of CA evoked by FCCP in the perfused rat adrenal gland. Secretion of CA evoked by FCCP (3×10^{-5} M) was induced for 90 min following perfusion of adrenal gland with Ca^{2+}-free Krebs solution containing EGTA (5×10^{-3} M). Other legends are the same as in Fig. 1. **: $P < 0.01$. ns: Statistically not significant.

dopamine-â-hydroxylase from the perfused cat adrenal gland is dependent on the extracellular calcium concentration (Dixon *et al.*, 1975). It was of particular interest to test whether the secretory effect induced by FCCP is also related to extracellular calcium ions. Thus, the adrenal gland was pre-perfused with calcium-free Krebs solution containing 5×10^{-3} M EGTA for 20 min prior to introduction of FCCP. In the absence of extracellular calcium, CA releases by FCCP (3×10^{-5} M) were significantly inhibited to 88~0% from 5 rat glands as compared with their corresponding control responses as shown in Fig. 2-lower.

In order to investigate the effect of nicardipine, a dihydropyridine derivative and L-type Ca^{2+} channel blocker (Hardman *et al.*, 2001), on FCCP-evoked CA secretion, nicardipine (10^{-6} M)

was preloaded into the adrenal gland for 20min. In the presence of nicardipine effect, CA releases induced by perfusion of FCCP (3×10^{-5} M) for 90 min were greatly depressed to 29~0% of their corresponding control responses from 8 rat glands as shown in Fig. 3-upper.

It has been reported that muscarinic, but not nicotinic activation causes CA secretion independent of extracellular calcium in the perfused cat adrenal glands (Nakazato *et al.*, 1988). It suggests that the presence of an intracellular calcium pool is linked to muscarinic receptors, and that TMB-8, an intracellular calcium antagonist, inhibits both nicotinic and muscarinic stimulation-induced CA release in the rat adrenal glands (Lim and Hwang, 1991). Therefore, it was attempted to test the effect of TMB-8 on FCCP-evoked CA secretion. In 8 rat adrenal glands, CA secretions evoked by perfusion of FCCP (3×10^{-5} M) after preloading with TMB-8 (10^{-5}M) for 20min were inhibited to 88

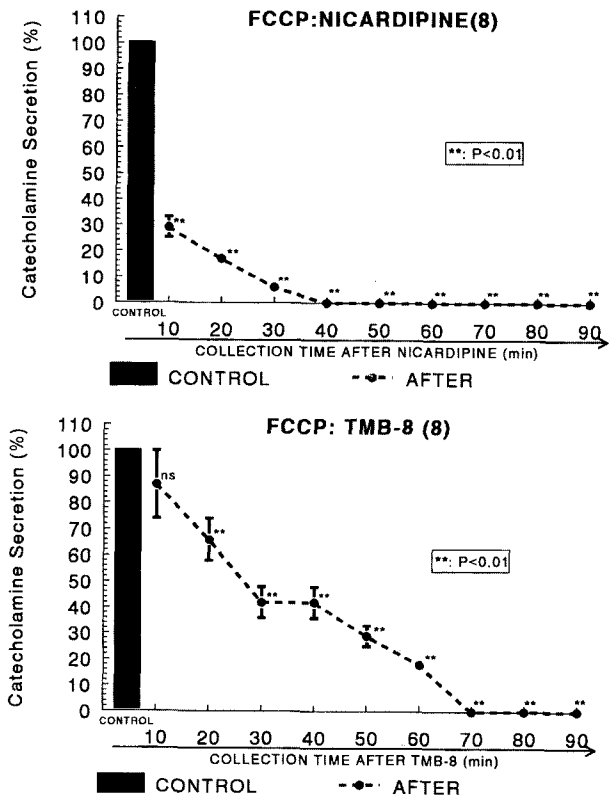

Fig. 3. Upper: The effect of nicardipine on FCCP-evoked CA secretory responses in the perfused rat adrenal glands. Nicardipine (10^{-6} M) was perfused for 90 min before intro-ducing FCCP (3×10^{-5} M). **Lower:** The effect of TMB-8 on CA secretion evoked by FCCP in the perfused rat adrenal glands. TMB-8 (10^{-5} M) was given into the perfusion stream for 90 min after obtaining the corresponding control responses of FCCP (3×10^{-5} M). Other legends are the same as in Fig. 1. **: $P < 0.01$. ns: Statistically not significant.

~0% compared with their corresponding control response (100 %) as shown in Fig. 3-lower.

Effect of FCCP on CA secretion evoked by ACh and DMPP from the perfused rat adrenal gland

It has been reported that, in the endoplasmic reticular Ca^{2+}-depleted bovine chromaffin cells, the protonophore FCCP (20 μM given during the 5 s preceding each pulse) augmented the secretory responses to ACh responses fourfold for all pulse durations applied (1-5 s) whereas responses to K^+ were potentiated twofold with 1 to 2 s pulses but were not affected with longer pulse durations (Cuchillo-Ibáñez *et al.*, 2002). Therefore, it would be interesting to examine effect of FCCP on CA secretion evoked by ACh and DMPP from the isolated perfused rat adrenal glands. In order to test the effect of FCCP on cholinergic receptor-stimulated CA secretion, the concentration of 3×10^{-5} M FCCP was loaded into the adrenal medulla. In the present experiment, ACh (5.32 mM)-evoked CA release before perfusion with FCCP was 358±20 ng (0-4 min) from 10 rat adrenal glands. However, in the presence of FCCP (3×10^{-5} M) for 90 min, it was gradually enhanced to 129~189% of the control release (100%) as illustrated in Fig. 4-upper. When DMPP (10^{-4} M for 2 min), a selective nicotinic receptor agonist in autonomic sympathetic ganglia, was perfused through the rat adrenal gland, a sharp and rapid increase in CA secretion was evoked. As shown in Fig. 4-lower, DMPP-evoked CA release prior to the perfusion with FCCP was 355±18 ng (0-8 min), while in the presence of FCCP (3×10^{-5} M), it was potentiated by 120~140% of the control (100%).

The secretory effect of CA evoked by CCCP

In the present work, it was found that FCCP increases CA secretion in the perfused rat adrenal gland as shown in Fig. 1~4. Therefore, in order to establish whether mitochodrial uncoupler affects CA release in the adrenal gland, it would be interesting to determine the effect of CCCP, another mitochondrial inhibitor like FCCP, on CA secretion in this perfused model of the rat adrenal gland.

As illustrated in Fig. 5, time-course effect of CCCP (3×10^{-5} M) infusion into the perfusion stream for 90 min exerted significant responses of CA secretion over the background release, leading to the peak release at 50~70 min period. In 9 rat adrenal glands, this CCCP (3×10^{-5} M)-evoked CA secretory responses were 22~106 ng (0~90 min). However, it seems likely that CCCP is less potent in CA release that FCCP. The tachyphylaxis to releasing effects of CA evoked by CCCP was not

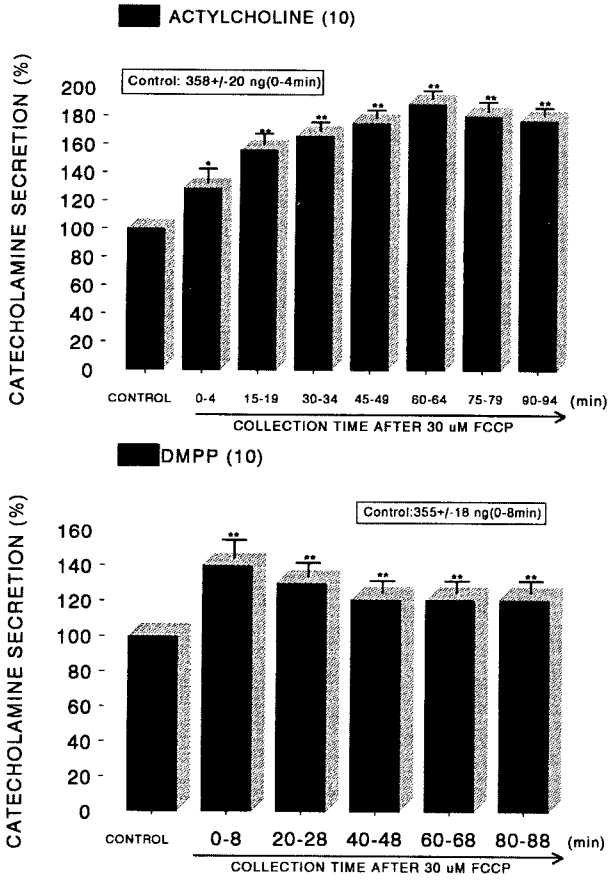

Fig. 4. Upper: The effect of FCCP-infusion on ACh-evoked CA secretion in the perfused rat adrenal glands. ACh (5.32×10^{-3} M)-evoked CA secretory response was induced simultaneously along with FCCP (3×10^{-5} M) after obtaining the control response of ACh. Statistical differences were obtained by comparing "control" and "after" preloading with FCCP. ACh-evoked perfusate was collected for 4 min at 15 min interval during the perfusion of FCCP.

Lower: The effect of FCCP-infusion on DMPP-evoked CA secretion in the perfused rat adrenal glands. DMPP (10^{-4} M) was perfused into adrenal vein for 2 min simultaneously along with FCCP (3×10^{-5} M) perfusion after obtaining the control response of DMPP. DMPP-evoked perfusate was collected for 8 min at 20 min interval during the perfusion of FCCP. Other legends are the same as in Fig. 1. *: $P < 0.05$, **: $P < 0.01$. ns: Statistically not significant.

observed (data not shown). However, in all subsequent experiments, CCCP was not administered more than twice at 120 min-intervals.

DISCUSSION

These experimental results indicate that FCCP causes the rat adrenomedullary CA secretion in a calcium-dependent fashion.

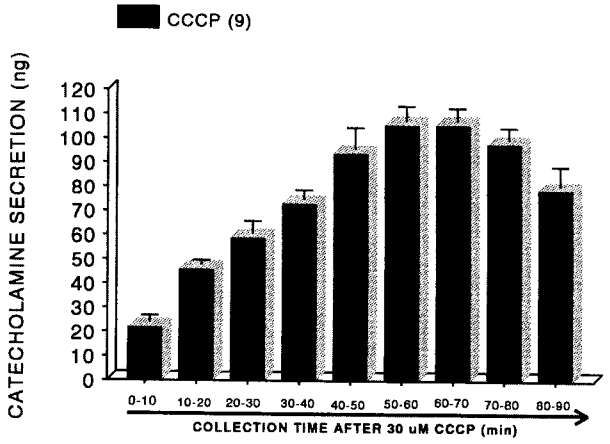

Fig. 5. Time-course effect of CCCP on secretion of catecholamines (CA) from the perfused rat adrenal glands. CCCP (3×10^{-5} M) was perfused into an adrenal vein for 90 min. Perfusion of CCCP was made after perfusion with normal Krebs-bicarbonate solution for one hour before the experimental protocols were initiated. Other legends are the same as in Fig. 1.

It is suggested that this facilitatory effects of FCCP may be mediated by cholinergic receptor stimulation, which is relevant to both stimulation of the Ca^{2+} influx and Ca^{2+} release from cytoplasmic Ca^{2+} stores (both endoplasmic reticulum and mitochondria).

In support of this finding, since the FCCP-induced release of CA was inhibited greatly in the presence of chlorisondamine in the present work, this secretory effect of CA was due presumably to exocytosis of CA storage vesicles subsequent to activation of nicotinic ACh receptors in the rat adrenomedullary chromaffin cells. Chlorisondamine is known to be a selective antagonist of neuronal nicotinic cholinergic receptors (Hardman *et al.*, 2001).

Also, in the present study, the FCCP-evoked CA secretory response was also inhibited by pretreatment with pirenzepine. This finding indicates that FCCP-evoked CA release is exerted at least partly by stimulation of muscarinic ACh receptors. In general, subtypes of muscarinic receptors have been recognized in many tissues (Eglen & Whiting, 1986). Receptor binding studies have supported the classification of muscarinic receptors into M_1 and M_2 on the basis of the selectivity profile of pirenzepine; receptors with a high affinity for pirenzepine are designated as M_1 and those with low affinity as M_2 receptors (Hammer *et al.*, 1980; Hammer and Giachetti, 1982). Doods and his colleagues (1987) have classified muscarinic receptors into M_1 (pirenzepine sensitive, neuronal), M_2 (cardiac) and M_3 (smooth muscle and glandular). In view of above studies, the finding of this study that FCCP-evoked CA release was inhib-

ited by pretreatment with pirenzepine suggests that FCCP-evoked CA secretion is mediated partly through activation of muscarinic M_1-receptor in the perfused rat adrenal gland.

The indispensable role of calcium in the neurosecretory process has been well established. Calcium is well found to play the crucial role in process of depolarization-neurotransmitter release coupling in many types of secretory cells (Douglas, 1968; Schulz and Stolze, 1980; Williams, 1980). Furthermore, it has been found that nicotinic (but not muscarinic) stimulation also releases soluble ACh from the chromaffin cells by a calcium-dependent mechanism (Mizobe and Livett, 1983). The activation of nicotinic receptors stimulates CA secretion by increasing Ca^{2+} entry through receptor-linked and/or voltage-dependent Ca^{2+} channels in the perfused rat adrenal glands (Wakade and Wakade, 1983) and isolated bovine adrenal chromaffin cells (Kilpatrick *et al.*, 1981; 1982; Knight and Kesteven, 1983).

An FCCP-induced $[Ca^{2+}]_i$ increase has been observed in various tissues (Nowicky and Duchen, 1998; Sato, 1997). There are three possible mechanisms for these effects: (1) increased Ca^{2+} influx, (2) impaired Ca^{2+} extrusion, such as the failure of Ca^{2+}-ATPases in the endoplasmic reticulum, and (3) release from mitochondria. Until now, several studies of other tissues have demonstrated that the $[Ca^{2+}]_i$ increase elicited by FCCP application is reduced by either extracellular Ca^{2+}-free solution or inorganic voltage-sensitive Ca^{2+} channel (VSCC) blockers (Buckler and Vaughan-Jones, 1998; Sato, 1997). Based on these findings, in the present study, the secretory effect of FCCP seems to be apparently mediated by increasing Ca^{2+} entry through nicotinic receptor-linked Ca^{2+} channels in the perfused rat adrenal glands. As illustrated in Fig. 2 and 3, in the present work, ongoing secretion of CA from the perfused rat adrenal gland continuously exposed to FCCP could be completely abolished when extracellular Ca^{2+} was removed along with deletion of Ca^{2+} with EGTA, indicating an absolute dependency on extracellular Ca^{2+}. Furthermore, the CA secretory responses to the same concentration of FCCP could be fully abolished when nicardipine, a dihydropyridine derivative L-type Ca^{2+} channel blocker, was applied to the rat adrenal gland in the continued presence of Ca^{2+}. These findings indicate that FCCP, like hypoxia (Taylor and Peers, 1998), evokes CA secretion from the perfused rat adrenal gland by stimulating Ca^{2+} influx through voltage-gated Ca^{2+} channels. Previously, it has been shown that hypoxia evokes secretion from PC12 cells primarily by promoting Ca^{2+} influx through ω-conotoxin-sensitive N-type Ca^{2+} channels (Taylor and Peers 1998). To investigate which Ca^{2+} channel subtype mediated Ca^{2+} influx coupled to secretion in response to FCCP, the ability of selective Ca^{2+} channel blockers to interfere with FCCP-evoked secretion was tested. The CA secretory responses to FCCP from the perfused rat adrenal gland in the absence of extracellular Ca^{2+}, or in the presence of TMB-8 to block the intracelluar calcium release from the store, and also in the presence of nicardipine to block L-type Ca^{2+} channels, were greatly inhibited. Thus, like hypoxia (Taylor and Peers, 1998), it is felt that FCCP promotes Ca^{2+} entry to trigger exocytosis primarily though L- (and N-) type Ca^{2+} channels in the rat adrenal gland. Moreover, In the presence of TMB-8, an inhibitor of the intracelluar calcium release from the store, FCCP-evoked CA secretion was greatly inhibited in the perfused rat adrenal gland. TMB-8 is also known to inhibit caffeine-induced $^{45}Ca^{2+}$ release from, but not the uptake of $^{45}Ca^{2+}$ by, a sarcoplasmic reticulum preparation of skeletal muscle (Chiou and Malagodi, 1975) and in isolated bovine adrenomedullary cells (Misbahuddin *et al.*, 1985; Sasakawa *et al.*, 1984). It has also been shown that caffeine-evoked CA secretion is inhibited from the perfused cat adrenal gland in the absence of extracellular calcium (Yamada *et al.*, 1988). Thus, in the present experiments, the inhibition of FCCP-evoked CA secretion by TMB-8 suggests that chromaffin cells of the rat adrenal gland really contain the intracellular store of calcium that participates in the secretion of CA as shown in the bovine gland (Baker and Knight, 1978).

In order to investigate whether FCCP exerted its effect to promote the CA secretion from the perfused rat adrenal gland via its ability to inhibit mitochondrial function (rather than through any other, unknown action), the effects of another mitochondrial inhibitor, CCCP was investigated. As illustrated in Fig. 5, this CCCP was capable of promoting exocytosis. These findings indicate that mitochondrial uncouplers stimulate CA release that is dependent on Ca^{2+} entry via voltage-gated Ca^{2+} channels. This study indicates that these two distinct agents all of which interfere with mitochondrial function, are capable of stimulating quantal CA release from the perfused rat adrenal gland. Mitochondrial uncouplers are, like hypoxia and acidosis, potent stimuli of the carotid body and cause CA release from glomus cells (Gonzalez *et al.*, 1994). Hypoxia and acidosis similarly evoke CA release from PC12 cells (Taylor and Peers, 1998; Taylor *et al.*, 1999a). As shown in the present study, the CA secretory responses to mitochondrial uncouplers are apparent. In support of this idea, using rabbit glomus cells, Biscoe and Duchen (1989) showed that cyanide hyperpolarized cells, due to activation of a Ca^{2+}-dependent K^+ current via

release of Ca^{2+} from intracellular stores, including mitochondria (Duchen and Biscoe, 1992). Indeed, cyanide (and also rotenone and FCCP) was found to depolarize the mitochondrial membrane potential, which is required for these organelles to sequester Ca^{2+} (Biscoe and Duchen, I989; Duchen and Biscoe, 1992).

Rocher *et al.* (1991), also employing rabbit carotid bodies, found that the uncoupler DNP evoked catecholamine release by acting as an acidic stimulus (DNP is a protonophore). Thus, DNP caused intracelluIar acidosis, which stimulated plasma membrane Na^+-H^+ exchange, which in turn caused a sufficiently large rise of $[Na^+]_i$ to reverse the Na^+-Ca^{2+} exchanger, so permitting Ca^{2+} entry via a transporter. Buckler and Vaughan-Jones (I998) studied the effects of two uncouplers, DNP and FCCP, on rat carotid body cells. These agents only caused slight intracellular acidification, but evoked substantial rises of $[Ca^{2+}]_i$ due to cell depolarization and Ca^{2+} influx via voltage-gated Ca^{2+} channels. The depolarizing response was primarily due to inhibition of a K^+ conductance, which appears similar to the one sensitive to hypoxia and hypercapnia (Buckler and Vaughan-Jones, 1994a; 1994b; 1998). Finally, cell depolarization was secondary to a depolarization of the mitochondriaI membrane potential, and a small inward current was also observed. Interestingly, Inoue *et al.*, (1999) recently reported in chromaffin cells that cyanide and anoxia aIso stimulated a non-selective inward current, providing further evidence that mitochondrial inhibition is an important element of O_2-sensing. However, it would appear from the present study that this influx pathway, if present in the perfused rat adrenal gland, does not itself contribute significant Ca^{2+} influx, since Ca^{2+} channel bIocker (nicardipine) was able to reduce FCCP-evoked exocytosis dramatically. The present results, using real-time measurements of CA secretion from the perfused rat adrenal gland, are in good agreement with the work of Buckler and Vaughan-Jones (I998). In the present study, both mitochondrial uncouplers (FCCP and CCCP) were found to evoke CA release via Ca^{2+} inflIux through voltage-gated Ca^{2+} channels (primarily L- type, even though N-type Ca^{2+} channels are not excluded). Thus, these agents are likely to cause membrane depolarization, presumably via inhibition of K^+ channels, as proposed for the effects of hypoxia and acidosis in bovine chromaffin cells (Zhu *et al.*, 1996; Taylor and Peers, l998; Taylor *et al.*, 1999a). More recently, Inoue and his co-workers (1999) have found that application of CCCP into guinea-pig adrenal chromaffin cells, which does not stimulated generation of reactive oxygen species, reversibly induced CA secretion. This CCCP-evoked secretion was abolished by removal of external Ca^{2+} ions.

However, whether both mitochondrial uncouplers (FCCP and CCCP) evoke CA release via Ca^{2+} influx entirely through voltage-gated N-type Ca^{2+} channels remains to be resolved in the future study.

Taken together, these experimental results indicate that FCCP causes the rat adrenomedullary CA secretion in a calcium-dependent fashion. It is suggested that this facilitatory effects of FCCP may be mediated by cholinergic receptor stimulation, which is relevant to both stimulation of the Ca^{2+} influx and Ca^{2+} release from cytoplasmic Ca^{2+} stores (both endoplasmic reticulum and mitochondria).

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