응용약물학회지, The Journal of Applied Pharmacology, 9, 201-208(2001)

Influence of Bromocriptine on Release of Norepinephrine and Epinephrine Evoked by Cholinergic Stimulation from the Rat Adrenal Medulla

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(Received June 29, 2001; accepted September 10, 2001)

Astract – The present study was conducted to examine the effects of cholinergic stimulation and membrane depolarization on secretion of epinephrine (EP) and norepinephrine (NE) in the perfused model of the rat adrenal gland and to investigate the effect of bromocriptine on secretion of EP and NE evoked by these secretagogues. Acetylcholine (ACh, 5.32 mM), high K^+ (56 mM), 1.1-dimethyl-4-phenyl piperazinium iodide (DMPP, 100 μM for 2 min), (3-(m-cholro-phenyl-carbamoyl-oxy)-2butynyl trimethyl ammonium chloride (McN-A-343, 100 μM for 2 min), cyclopiazonic acid (10 μM for 4 min) and methyl-1,4-dihydro-2,6-dimethyl-3-nitro-4-(2-trifluoromethylphenyl) -pyridine-5-carboxylate (Bay-K-8644, 10 μM for 4 min) evoked a 1.3~5.3-fold greater secretion of EP than NE in the perfused rat adrenal gland. The perfusion of bromocriptine (1~10 μM) into an adrenal vein for 20 min produced relatively dose-dependent inhibition in secretion of EP and NE evoked by ACh, high K^+, DMPP, and McN-A-343. Moreover, under the presence of bromocriptine (1 ~10 μM), releasing responses of EP and NE evoked by cyclopiazonic acid and Bay-K-8644 were also greatly reduced. Taken together, these results suggest that cholinergic stimulation and membrane depolarization enhance more release of EP than NE in the perfused rat adrenal medulla, and that bromocriptine inhibits the release of EP and NE evoked by stimulation of cholinergic receptors as well as by membrane depolarization. It seems that this inhibitory effect of bromocriptine is associated with inhibition of calcium channels through activation of dopaminergic D_2-receptors located in the rat adrenomedullary chromaffin cells.

Key words □ Bromocriptine, Adrenal Gland, Norepinephrine and Epinephrine, Dopaminergic D_2-Receptors.

It has been found that epineprine (EP) and norepinephrine (NE) are stored in and secreted from two distinct chromaffin cell types (Hillarp & Hokfelt, 1953; Coupland *et al.*, 1964; Douglas & Poisner, 1965) and there are several studies indicating functional differences between EP and NE-containing cells. Nicotinic agonists and depolarizing agents are shown to elicit the secretion of a greater percentage of NE stores than EP stores in bovine adrenal chromaffin cells (Marely & Livett, 1987; Owen *et al.*, 1989; Cahill & Perlman, 1992; Teraoka *et al.*, 1993), Whereas histamine causes a preferential secretion of EP in the same cells (Livett & Marley, 1986; Choi *et al.*, 1993). These results suggest that stimulus-secretion coupling or the exocytotic machinery may be different for EP- and NE-containing cells.

The biosynthetic pathways of the two catecholamines (CA), EP and NE secreted by the gland from which such cultures are derived are well-documented (Goodall & Kirshner, 1957; Molinoff & Axelrod, 1971; Sabban & Goldstein; 1984). Dopamine formed from 3, 4-dihydroxy phenylalanine is cornered to NE by dopamine β-hydroxylase (Levin *et al.*, 1960), and this is subsequently converted by the enzyme phenylethanolamine N-methyltransferase into EP (Axelrod, 1962). However, a remarkable diversity in the relative levels of both EP and NE and of the cell populations that synthesize these amines exists between different species. In the pig, for example, the EP/NE ratio measured in the intact gland is ~0.5 (Kong *et al.*, 1989), and that for the cat is ~1, whereas that for the intact bovine adrenal gland is ~1.7, when analyzed by HPLC techniques. A figure of ~6 had been reported much earlier for the intact bovine gland (West, 1955), which is similar to that reported more recently by Unsicker *et al.* (1980) for Percoll gradient-separated cells from bovine tissue, but this differs from that found by Livett *et al.* (1983), estimated with freshly isolated cells. More

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recently, it has been reported that ACh, DMPP, high K^+, calcimycin, histamine and angiotensin induced the secretion of a 1.3~2-fold greater percentage of NE stores then EP stores in the intact bovine chromaffin cells (Kuwashima *et al.*, 2000). It has been shown that dopaminergic inhibition of CA secretion from adrenal medulla is known to be mediated by D_2-like but not D_1-like dopaminergic receptors (Damase-Michel *et al.*, 1996). However, Dahmer and Senogles (1996) have shown that D_1- and D_2- selective agonists inhibit Ca^{2+} uptake and CA secretion by activating D_4 and D_5 dopaminergic receptors on bovine chromaffin cells. Therefore, the present study was performed to examine the effect of cholinergic stimulation and membrane depolarization on the levels of NE and EP secreted from the isolated perfused model of the rat adrenal gland. In addtion, it was also tried to investigate the effect of bromocriptine, a D_2-dopiminergic agonist, on the secretion of NE and EP evoked by these secretagogues.

MATERIALS AND METHODS

Isolation of adrenal gland

Male Sprague-Dawley rats, weighing 180 to 250 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 midline 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 to 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 (WIZ Co.) at a rate of 0.3 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 μg/ml) and ascorbic acid (100 μg/ml) to prevent oxidation of catecholamines.

Drug administration

DMPP (100 μM), McN-A-343 (100 μM) for 2 minutes, and Bay-K-8644 (10 μM) and cyclopiazonic acid (10 μM) for 4 minutes were given into perfusion stream, respectively. 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.

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 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, 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 was collected for 4 to 8 min. The amounts secreted in the background sample have been subtracted from that secrected from the stimulated sample to obtain the net secretion value of CA, which is shown in all of the figures.

To study the effects of bromocriptine on the spontaneous and evoked secretion, the adrenal gland was perfused with Krebs solution containing bromocriptine for 20 min. The perfusate was collected for a certain minute (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

The contents of NE and EP of perfusate were measured

directly by the modified method of Garcia *et al.* (1994). For simultaneous detection of NE and EP in the perfusate from the rat adrenal gland an HPLC procedure with electrochemical detection was employed. The adrenal perfusate was put into a sample tube along with 5% $HClO_4$ and 1.5 M sodium acetate and stirred up. This perfusate was immediately centrifuged for 10 min at 10,000 g at 40C and protein free supernatants were passed through a 0.22 um membrane filter. A sample volume of 20 μl was injected onto a Hitach isocratic HPLC Sysytem consisting of a Hitachi pump L-7110, Hitachi Autosampler L-7200 and an Esa Coulochem II electrochemical detector set at 70 mV and 50 nA of sensitivity.

Separation was carried out on a column (Waters Co., U.S.A., 4.6×150 mm I.D.). The signal from the electrochemical detector was recorded on a Hitachi Integrater L-7500. The composition of the mobile phase consists of 0.1 M potassium phosphate buffer (pH 3.1-3.3) containing 10% methanol, 0.4 mg/ml sodium heptanesulfonate and 100 μM disodium EDTA. The flow rate was 0.7 ml/min. The contents of NE and EP of perfusate were expressed in nanograms.

Statistical analysis

The statistical significance between groups was determined by utilizing the Student's *t*-test. 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: bromocriptine mesylate, ACh chloride, DMPP, NE bitartrate, EP bitartrate, BAY-K-8644, cyclopiazonic acid (Sigma Chemical Co., U.S.A.), McN-A-343 (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 bromocriptine on secretion of norepinephrine and epinephrine evoked by ACh, high K^+, DMPP and McN-A-343 from the perfused rat adrenal glands

After the initial perfusion with oxygenated Krebs-bicarbonate solution for one hour, basal CA release from the isolated perfused rat adrenal glands amounted to 21±2 ng/2 min (n=7). And then CA release evoked by various secretagogues from the isolated perfused rat adrenal gland was initiated. And then the effects of bromocriptine on cholinergic receptor stimulation- as well as membrane depolarization-mediated CA secretion from perfused rat adrenal glands were examined, and secretagogues were given before and after the treatment with bromocriptine. In the present study, bromocriptine itself at concentrations used in the present experiments did not produce any effect on basal CA output (data not shown).

When ACh (5.32 mM) in a volume of 0.05 ml was injected into the perfusion stream, the amount of NE and EP secreted was 135±15 ng (0-4 min) and 258±28 ng (0-4 min), respectively. The ratios of EP/NE secretion in responses to various secretagogues were calculated. The ratio value obtained with ACh was average 1.91, which EP release was greater than NE release in the present perfused model. However, after the preperfusion with bromocriptine (1~10 μM) for 20 min, ACh-evoked secretory responses of NE and EP were dose-depen-

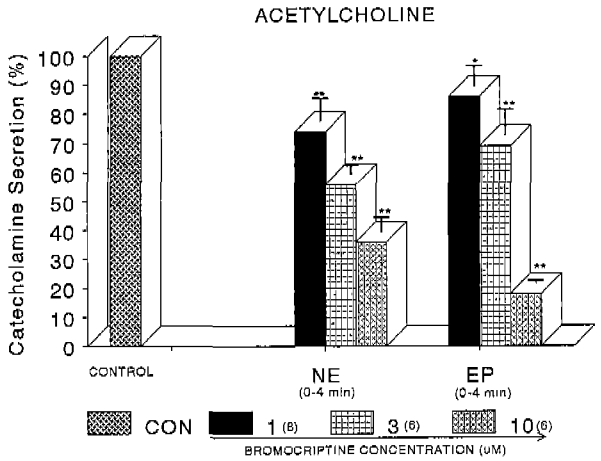

Fig. 1. Influence of bromocriptine on acetylcholine (ACh)-evoked secretion of norepinephrine (NE) and epinephrine (EP) from the isolated perfused rat adrenal glands. A single injection of ACh (5.32 mM) after perfusion with normal Krebs solution for one hour prior to initiation of the experimental protocol was evoked. Analyses of NE and EP evoked by ACh before (CONTROL) and after preloading with 1, 3 and 10 μM of bromocriptine for 20 min, respectively were performed. A numeral in the parenthesis indicates number of experimental rat adrenal glands. Vertical bars represent the standard error of the mean (S.E.M.). Ordinate the amounts of NE and EP secreted from the adrenal gland for 4 min (%). Abscissa: NE and EP. Statistical difference was obtained by comparing the control (100%) with bromocriptine-pretreated group. Each perfusate was collected for 4 minutes.
*P< 0.05, **P<0.01.

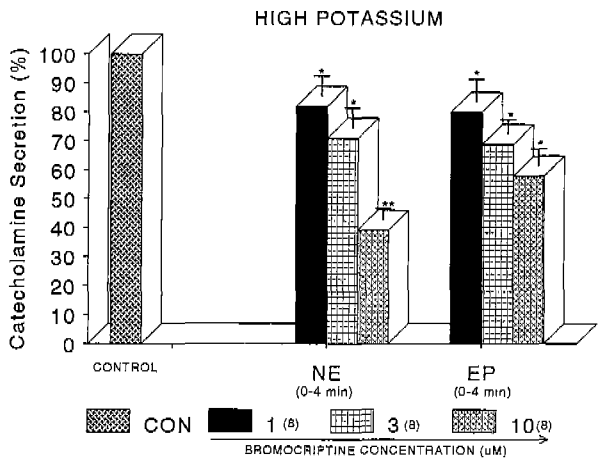

Fig. 2. Influence of bromocriptine on high potassium-evoked secretion of norepinephrine (NE) and epinephrine (EP) from the isolated perfused rat adrenal glands. A single injection of high potassium (56 mM) was made before and after the perfusion with 1, 3 and 10 μM of bromocriptine for 20 min, respectively. *: P< 0.05, **: P< 0.01.

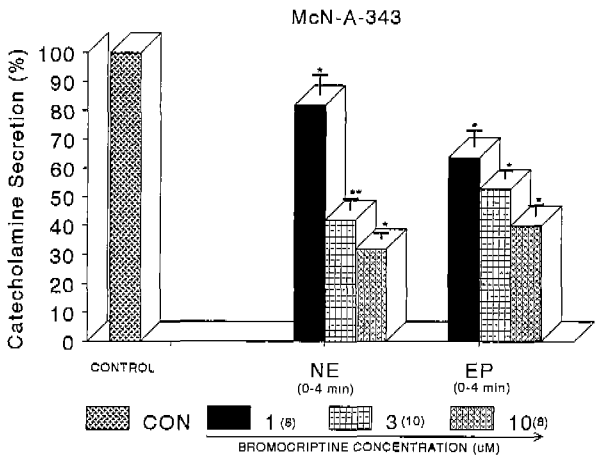

Fig. 4. Influence of bromocriptine on secretion of norepinephrine (NE) and epinephrine (EP) evoked by McN-A-343 from the isolated perfused rat adrenal glands. McN-A-343 (100 μM) was given into an adrenal vein before and after the perfusion with 1, 3 and 10 μM of bromocriptine for 20 min, respectively. *P< 0.05, **P< 0.01.

dently inhibited to 36~74% and 18~86% of their corresponding control responses, respectively, as shown in Fig. 1. High potassium (56 mM)-evoked secretion of NE and EP was 80±15 ng (0-4 min) and 148~26 ng (0-4 min), respectively. The EP release was also greater than NA. Its ratio of EP/NE was 1.85. In the presence of bromocriptine (1~10 μM), high potassium-evoked secretory responses of NE and EP were dose-dependently attenuated to 38~82% and 58~80% of their corresponding control responses, respectively (Fig. 2).

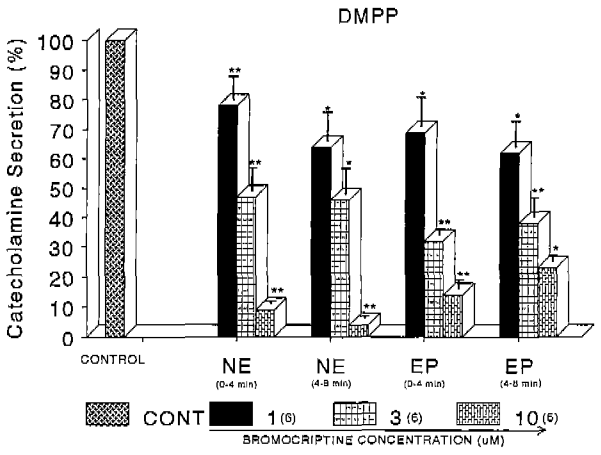

Fig. 3. Influence of bromocriptine on DMPP-evoked secretion of norepinephrine (NE) and epinephrine (EP) from the isolated perfused rat adrenal glands. DMPP (100 μM) was given into an adrenal vein before and after the perfusion with 1, 3 and 10 μM of bromocriptine for 20 min, respectively. Perfusate for DMPP was collected for 8 minutes at 4 min-interval.
*P< 0.05, **P< 0.01.

When perfused through the rat adrenal gland, DMPP (100 μM for 2 min), which is a selective nicotinic receptor agonist in autonomic sympathetic ganglia, evoked a sharp and rapid increase in secretion of NE and EP. As shown in Fig. 3, DMPP-evoked release of NE and EP before pre-loading with bromocriptine was 106±13 ng (0-4 min) and 270±16 ng (0-4 min) for 1st period, and 24±3 ng (4-8 min) and 38±4 ng (4-8 min) for 2nd period, respectively. The ratios of EP/NE were 2.55 for 1st period and 1.58 for 2nd period, respectively. However, after pretreatment with bromocriptine (1~10 μM) for 20 min, they were dose-dependently reduced to 9~78% and 4~64% for 1st period, and 14~69% and 23~62% for 2nd period, respectively as compared to their corresponding control responses. As illustrated in Fig. 4, McN-A-343 (100 μM), which is a selective muscarinic M_1- agonist (Hammer and Giachetti, 1982), perfused into an adrenal gland for 2 min released the NE and EP of 32±4 ng (0-4 min) and 106±15 ng (0-4 min), respectively. Its ratio of EP/NE was 3.19. However, in the presence of bromocriptine (1~10 μM), McN-A-343-evoked release of NE and EP was dose-dependently inhibited to 32~82% and 4~64% of their control secretions, respectively.

Effect of bromocriptine on secretion of norepinephrine and epinephrine evoked by Bay-K-8644 and cyclopiazonic acid from the perfused rat adrenal glands

It has been found that Bay-K-8644 is a calcium channel activator that causes positive inotropy and vasoconstriction in

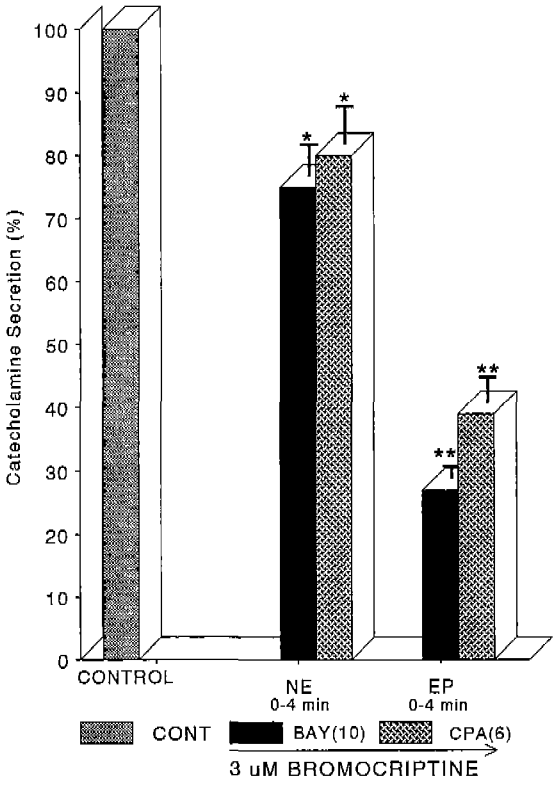

Fig. 5. Influence of bromocriptine on secretory responses of norepinephrine (NE) and epinephrine (EP) evoked by Bay-K-8644 and cyclopiazonic acid from the isolated perfused rat adrenal glands. Bay-K-8644 (100 μM) and cyclopiazonic acid (100 μM) were injected into an adrenal vein before and after the perfusion with 3 μM bromocriptine for 20 min, respectively. CONT: Control, BAY-K: Bay-K-8644, CPA: Cyclopiazonic Acid. *P< 0.05, **P< 0.01.

isolated tissues and intact animals (Schramm *et al.*, 1982; Wada *et al.*, 1985) and also enhances basal Ca^{2+} uptake (Garcia *et al.*, 1984) and release of catecholamines (Lim *et al.*, 1992). It was of interest to determine the effects of 3 μM bromocriptine on Bay-K-8644-evoked secretion of NE and EP from the isolated perfused rat adrenal glands. Fig. 5 illustrates the inhibitory effect of 3 μM bromocriptine on Bay-K-8644-evoked CA secretion. Bay-K-8644 (10 μM)-evoked contents of NE and EP were 4±1 ng (0-4 min) and 45±6 ng (0-4 min), respectively, from 10 rat adrenal glands. The ratio of EP/NE secretion in responses to ACh was 11.25, which EP release was much greater than NE release in the present model. However, after the pre-loading with 3 μM bromocriptine for 20 min, Bay-K-8644-evoked secretory responses of NE and ED were significantly inhibited to 75% and 27% of the control responses, respectively (Fig. 5).

Cyclopiazonic acid, a mycotoxin from *Aspergillus* and *Penicillium*, has been described as a highly selective inhibitor of Ca^{2+}-ATPase in skeletal muscle sarcoplasmic reticulum (Georger & Riley, 1989; Seidler *et al.*, 1989). It may be extremely valuable pharmacological tool for investigating intracellular Ca^{2+} mobilization and ionic current regulated by intracellular calcium (Suzuki *et al.*, 1992). Cyclopiazonic acid (10 μM)-evoked secretion of NE and EP was 14±2 ng (0-4 min) and 57±8 ng (0-4 min), respectively, which EP release was also greater than NE. Its ratio of EP/NE was 4.07. Under the presence of 3 μM bromocriptine, cyclopiazonic acid-evoked secretory responses of NE and EP were markedly attenuated to 80% and 39% of their control secretions, respectively (Fig. 5).

DISCUSSION

These experimental results obtained from the present study demonstrate that cholinergic stimulation and membrane depolarization may enhance the preferential release of EP than NE in the perfused rat adrenal medulla. It has been found that bromocriptine inhibits the release of both EP and NE evoked by stimulation of cholinergic receptors as well as by membrane depolarization. It seems that this inhibitory effect of bromocriptine is associated with inhibition of calcium mobilization through activation of dopaminergic D_2-receptors located in the rat adrenomedullary chromaffin cells.

In the present work, ACh, high potassium (a direct membrane-depolarizing agent), DMPP (a selective agonist of neuronal nicotinic receptor), McN-A-343 (a selective agonist of muscarinic M_1-receptor), cyclopiazonic acid (an inhibitor of cytoplasmic Ca^{2+}-ATPase) and Bay-K-8644 (an activator of dihydropyridine Ca^{2+} channel) evoked a 1.3~5.3-fold greater secretory responses of EP than NE in the isolated perfused rat adrenal gland, respectively. These findings are in agreement with the results that histamine causes the preferential release of EP from the adrenal medulla because it is a more effective stimulus for catecholamine secretion from EP-containing cells than from NE-containing cells (Choi *et al.*, 1993; Livett & Marley, 1986). The present results can be also supported by the report that the adrenergic cell-enriched fraction consists of >90% adrenergic cells, whereas the noradrenergic cell-enriched fraction contains >60% noradrenergic cells (Moro *et al.*, 1991). They also demonstrated that these cells may be cultured with their secretory machinery intact: analysis of secreted catecholamines from nicotine- or high potassium concentration-evoked cells cultured from each fraction confirms that EP is the major catecholamine secreted by one fraction, whereas NE is mainly secreted by the other. Moreover, as described in

introduction, a remarkable diversity in the ratio of EP/NE exists between different species, such as in the pig (Kong *et al.*, 1989), the cat and the intact bovine adrenal gland (Michelena *et al.*, 1991). Therefore, base on these findings, it is felt that cholinergic stimulation and membrane-depolarization cause the preferential release of EP from the perfused model of the isolated rat adrenal gland. These secretagogues appear to be more preferentially effective stimuli for catecholamine secretion from EP-containing cells than from NE-containing cells of the rat adrenal medullary chromaffin cells.

However, .the present results is not consistent with the finding in bovine chromaffin cells reported by some previous studies (Owen *et al.*, 1989; Livett *et al.*, 1983), estimated with freshly isolated cells. More recently, it has been reported that ACh, DMPP, high K^+, calcimycin, histamine and angiotensin induced the secretion of a 1.3~2-fold greater percentage of NE stores then EP stores in the intact bovine chromaffin cells (Kuwashima *et al.*, 2000). They have insisted that the different results seem to be due to differences in the incubation period of histamine. The incubation period was 20~120 min in their experiments (Livett & Marley, 1986; Choi *et al.*, 1993) and 3 min in another experiments (Livett *et al.*, 1983; Owen *et al.*, 1989; Kuwashima *et al.*, 2000). The uptake of catecholamines into cells may reduce the estimated catecholamine secretion (% of total content) during such long incubation periods. The uptake of NE into chromaffin cells has been reported to be greater than that of EP. Therefore, the reduction may be larger in NE secretion than EP secretion. This may explain the discrepancies in the preferential secretion between EP- and NE-containing cells.

The present results obtained from ACh, DMPP, McN-A-343 and high potassium are out of accordance with previous findings that the preferential secretion of NE was elicited by nicotinic receptor stimulation or membrane depolarization in bovine adrenal chromaffin cells (Marley & Livett, 1987; Owen *et al.*, 1989; Cahill & Perlman, 1992; Teraoka *et al.*, 1993). Nicotinic receptor activation promotes Na^+ and Ca^{2+} influx through receptor-linked ion channels and the resulting depolarization produces Ca^{2+} influx through voltage-operated Ca^{2+} channels (VOCC); the subsequent elevation of intracellular Ca^{2+} triggers catecholamine secretion. The different density and properties of nicotinic receptors or VOCC have been suggested as mechanisms underlying the differential responses of EP- and NE-containing cells. The results of the present study suggest a possible contribution of effectiveness of Ca^{2+} to the preferential secretion of EP in response to ACh, DMPP, McN-A-343 and high K^+. This postulate may be supported indirectly by the finding that the secretion of a greater percentage of EP stores was also elicited by the Ca^{2+} channel activator Bay-K-8644, which increases Ca^{2+} entry without activation of receptors or VOCC.

It is well established that elevation of intracellular Ca^{2+} is an essential role in adrenal catecholamine secretion. Studies in digitonin-permeabilized chromaffin cells have suggested that exocytosis involves at least two distinct steps, each having different Ca^{2+} and ATP dependence (Holz *et al.*, 1989; Bittner & Holz, 1992; 1992). Ca^{2+}-induced catecholamine secretion from permeabilized chromaffin cells exhibits a biphasic response characterized by early ATP-independent component and slow ATP-dependent release (Bittner & Holz, 1992; 1992; Hay & Martin, 1992; Lawrence *et al.*, 1994; Parson *et al.*, 1995). The ATP-independent phase presumably represents the Ca^{2+}-mediated steps that ultimately lead to fusion, while the ATP-dependent phase may be associated with vesicle priming events. The primed vesicles are readily releasable in response to transient Ca^{2+} elevation that triggers the fusion event. Therefore, it seems possible that differences in the effectiveness of Ca^{2+} for secretion at the priming or fusion steps between EP- and NE-containing cells contribute to the differential secretion of EP and NE in response to various secretagogues. Only a little information is available suggesting that preferential secretion of NE in response to acetylcholine (ACh), high K^+ and bradykinin may be due to preferentially greater effectiveness of Ca^{2+} in NA-containing cells (Teraoka *et al.*, 1993). In accordance with results from a previous study in digitonin-permeabilized bovine adrenal chromaffin cells (Teraoka *et al.*, 1993). It has been also suggested that, in the presence of MgATP, the effectiveness of Ca^{2+} for NE secretion was higher (1.4-fold) than that for EP secretion, but the value was almost identical to that (1.5-fold) in the absence of MgATP. Thus, the greater effectiveness of Ca^{2+} on NE-containing cells in the presence of MgATP may also be explained by the size of the primed granule pool (Kuwashima *et al.*, 2000).

Collectively, these results suggest that cholinergic stimulation and membrane depolarization enhance more release of EP than NE in the perfused rat adrenal medulla, and that bromocriptine inhibits the release of EP and NE evoked by stimulation of cholinergic receptors as well as by membrane depolarization. It is felt that this inhibitory effect of bromocriptine is associated with inhibition of calcium channels through activation of dopaminergic D_2-receptors located in

the rat adrenomedullary chromaffin cells.

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