

## Inhibitory Effects of Dihydraxidine on Catecholamine Release from the Rat Adrenal Medulla

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**Abstract** – The purpose of the present study was to examine the effect of dihydraxidine, a full D<sub>1</sub> receptor agonist, on the secretion of catecholamines (CA) from the perfused model of the rat adrenal gland, and to establish its mechanism of action. Dihydraxidine (10-100 μM), perfused into an adrenal vein for 60 min, relatively produced dose- and time-dependent inhibition in the CA secretory responses evoked by ACh (5.32 mM), high K<sup>+</sup> (56 mM), DMPP (100 μM) and McN-A-343 (100 μM). Dihydraxidine itself did fail to affect basal CA output. Also, in adrenal glands loaded with dihydraxidine (30 μM), the CA secretory responses evoked by Bay-K-8644 (10 μM), an activator of L-type Ca<sup>2+</sup> channels, cyclopiazonic acid (10 μM), an inhibitor of cytoplasmic Ca<sup>2+</sup>-ATPase, and veratridine, an activator of voltage-dependent Na<sup>+</sup> channels (10 μM), were also markedly inhibited, respectively. However, in the simultaneous presence of dihydraxidine (30 μM) and R (+)-SCH23390 (a selective antagonist of D<sub>1</sub> receptor, 3 μM), the CA secretory responses evoked by ACh, high K<sup>+</sup>, DMPP, McN-A-343, Bay-K-8644, cyclopiazonic acid and veratridine were considerably recovered to the extent of the corresponding control secretion compared with the inhibitory responses by dihydraxidine-treatment alone. In conclusion, these experimental results suggest that dihydraxidine significantly inhibits the CA secretion evoked by cholinergic stimulation (both nicotinic and muscarinic receptors) and membrane depolarization from the rat adrenal medulla. It seems that this inhibitory effect of dihydraxidine may be mediated by inhibiting influx of both Ca<sup>2+</sup> and Na<sup>+</sup> into the cytoplasm as well as by suppression of Ca<sup>2+</sup> release from cytoplasmic calcium store through activation of dopaminergic D<sub>1</sub> receptors located on the rat adrenomedullary chromaffin cells.

**Keywords:** Dihydraxidine, Dopamine D<sub>1</sub> receptors, Adrenal medulla, Catecholamine secretion

### INTRODUCTION

Dihydraxidine, [(+/-)-trans-10,11-dihydroxy-5,6,6a,7,8,12b-hexahydrobenzo[a]phenanthridine hydrochloride], a full D<sub>1</sub> receptor agonist, has been available for over 15 years (Lovenberg *et al.*, 1989; Brewster *et al.*, 1990); however, little information is known about the discriminative stimulus effects of this compound despite its clinical development for the treatment of working memory and cognitive deficits

in schizophrenia. A previous report demonstrated the ability of dihydraxidine to function as a discriminative stimulus in rats (Schechter, 1995). Gleason and Witkin (2004) found that the D<sub>1</sub> receptor partial agonist, SKF 38393, fully substituted and the D<sub>1</sub> receptor antagonist SCH 23390 attenuated the discriminative stimulus effects of dihydraxidine. Furthermore, in rats trained to discriminate SKF 38393 from saline, dihydraxidine produced full SKF 38393-like responding. Recently, it has been known that the full dopaminergic D<sub>1</sub> receptor agonist dihydraxidine produces prominent dopamine D<sub>1</sub> receptor agonist effects in vivo and is likely to produce subjective effects in humans similar to other D<sub>1</sub> receptor agonists (Gleason and Witkin, 2006). A D<sub>1</sub>-like receptor (the D<sub>1</sub> receptor class is composed of the D<sub>1</sub> and D<sub>5</sub> dopaminergic receptors) has also been identified on bovine chromaffin cells by fluorescence micro-

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scopy (Artalejo *et al.*, 1990). They have also found that stimulation of the D<sub>1</sub> receptors activates the facilitation of Ca<sup>2+</sup> current in the absence of pre-depolarization or repetitive activity, and that activation by D<sub>1</sub> agonists is mediated by cyclic AMP and protein kinase A. The recruitment of facilitation of Ca<sup>2+</sup> channels by dopamine in bovine chromaffin cells may form the basis of a positive feedback loop mechanism for secretion of catecholamines [CA] (Artalejo *et al.*, 1990). More recently, the D<sub>1</sub> agonist, SKF-38393, enhanced the number of exocytotic events as did prior exposure of the cell to epinephrine from bovine adrenal chromaffin cells (Villanueva and Wightman, 2007). In contrast to these findings, Dahmer and Senogles (1996a) have observed that the D<sub>1</sub>-selective agonists CI-APB and SKF-38393 inhibit DMPP-stimulated CA secretion in a concentration-dependent manner. Moreover, in bovine adrenal chromaffin cells, D<sub>1</sub>-selective agonists are found to inhibit secretagogue-stimulated Na<sup>+</sup> uptake in a cyclic AMP-independent manner (Dahmer and Senogles, 1996a).

It has also been demonstrated that apomorphine dose-dependently inhibits CA secretion by cholinergic receptor stimulation and also by membrane depolarization from the isolated perfused rat adrenal gland (Lim *et al.*, 1994).

Thus, it is clear that there are still several controversial reports about the role of dopaminergic D<sub>1</sub>-receptors in the CA release from the adrenal medulla. The purpose of the present study is to examine whether dihydraxidine, a full dopaminergic D<sub>1</sub> receptor agonist, can modify the CA release from the isolated perfused model of the rat adrenal gland, and to clarify its mechanism of action. This is the first report about the influence of dihydraxidine on the CA secretion from the rat adrenal medulla.

## MATERIALS AND METHODS

### Experimental procedure

Male Sprague-Dawley rats, weighing 200 to 300 grams, were anesthetized with thiopental sodium (50 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 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 gauze 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 peristaltic pump (ISCO<sup>®</sup> pump, WIZ Co. U.S.A.) 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<sub>2</sub>, 2.5; MgCl<sub>2</sub>, 1.18; NaHCO<sub>3</sub>, 25; KH<sub>2</sub>PO<sub>4</sub>, 1.2; glucose, 11.7. The solution was constantly bubbled with 95 % O<sub>2</sub> + 5% CO<sub>2</sub> 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

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

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

### Collection of perfusate

As a rule, prior to stimulation with various secretagogues, the perfusate was collected for 4 min to determine the spontaneous secretion of CA (background sample). Immediately after the collection of the background sample, collection of the perfusates was continued in another tube as soon as the perfusion medium containing the stimulatory agent reached the adrenal gland. Stimulated 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 dihydrexidine or R(+)-SCH23390 on the spontaneous and evoked secretion, the adrenal gland was perfused with Krebs solution containing dihydrexidine or R(+)-SCH23390 for 60 min, and then the perfusate was collected for a certain period (background sample). Then the medium was changed to the one containing the stimulating agent or along with dihydrexidine or R(+)-SCH23390, and the perfusates were collected for the same period as that for the background sample. The adrenal gland's perfusate was collected in chilled tubes.

### Measurement of catecholamines

CA content of perfusate was measured directly by the fluorometric method of Anton and Sayre (1962) without the intermediate purification alumina for the reasons described earlier (Wakade, 1981) using fluorospectrophotometer (Kontron Co., 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 (SEM). 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: 6-chloro-7,8-dihydroxy-1-phenyl-2,3,4,5-tetrahydro-1H-3-benzazepine (dihydrexidine), (*R*)-(+)-8-chloro-2,3,4,5-tetrahydro-3-methyl-5-phenyl-1H-benzazepine-7-ol [R(+)-SCH23390], cyclopiazonic acid, acetylcholine chloride, 1.1-dimethyl-4-phenyl piperazinium iodide (DMPP), norepinephrine bitartrate, methyl-1,4-dihydro-2,6-dimethyl-3-nitro-4-(2-trifluoro-methyl-phenyl)-pyridine-5-carboxylate (BAY-K8644), veratridine (Sigma Chemical Co., U.S.A.), and (3-(*m*-chloro-phenyl)-

carbamoyl-oxy)-2-butynyltrimethyl ammonium chloride [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 with Krebs-bicarbonate solution (final concentration of alcohol was less than 0.1%). Concentrations of all drugs are expressed in terms of molar base.

## RESULTS

### Effects of dihydrexidine on CA secretion evoked by ACh, high K<sup>+</sup>, DMPP and McN-A-343 from the perfused rat adrenal glands

After the perfusion with oxygenated Krebs-bicarbonate solution for 1 hr, basal CA release from the isolated perfused rat adrenal glands amounted to  $21 \pm 2$  ng for 2 min ( $n=9$ ). Since in bovine adrenal chromaffin cells, D<sub>1</sub>-selective agonists are found to inhibit secretagogue-stimulated Na<sup>+</sup> uptake in a cyclic AMP-independent manner (Dahmer and Senogles, 1996a), it was attempted initially to examine the effects of dihydrexidine itself on CA secretion from the perfused model of the rat adrenal glands. However, in the present study, dihydrexidine ( $10^{-5}$ - $10^{-4}$  M) itself did not produce any effect on basal CA output from perfused rat adrenal glands (data not shown). Therefore, it was decided to investigate the effects of dihydrexidine on cholinergic receptor stimulation- as well as membrane depolarization-mediated CA secretion. Secretagogues were given at 15 min-intervals. Dihydrexidine was present for 60 minutes after the establishment of the control release.

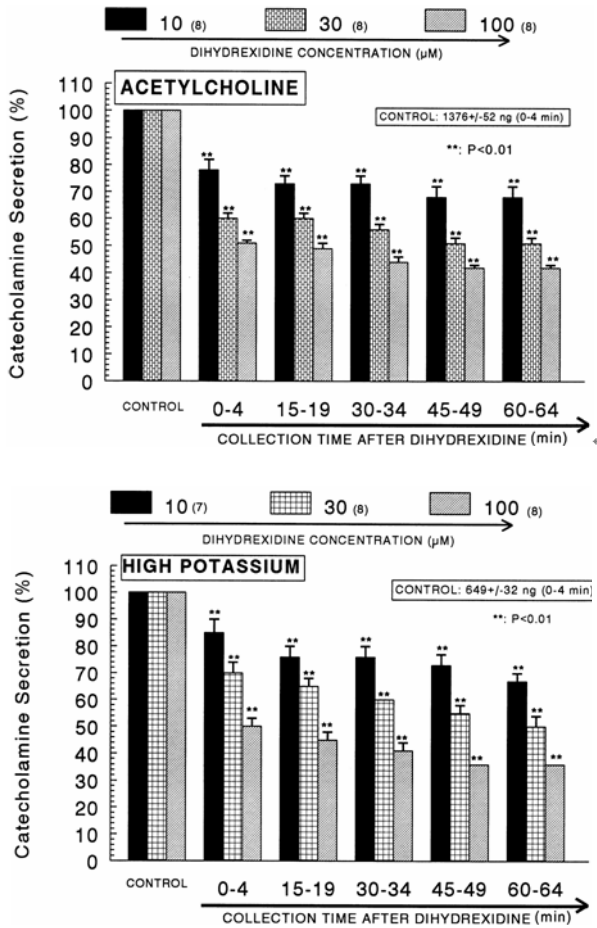
When ACh ( $5.32 \times 10^{-3}$  M) in a volume of 0.05 ml was injected into the perfusion stream, the amount of CA secreted was  $1,376 \pm 52$  ng for 4 min. However, in the presence of dihydrexidine in the range of  $10^{-5}$ - $10^{-4}$  M for 60 min, ACh-stimulated CA secretion was inhibited in concentration- and time-dependent fashion. As shown in Fig. 1 (Upper), in the presence of dihydrexidine, CA releasing responses were inhibited by 42% of the corresponding control release. Also, the depolarizing agent, high potassium markedly stimulated the CA secretion ( $649 \pm 32$  ng for 0-4 min). However, following the pretreatment with dihydrexidine ( $10^{-5}$  M- $10^{-4}$  M), high K<sup>+</sup> ( $5.6 \times 10^{-2}$  M)-stimulated CA secretion was significantly inhibited by 36% of the control at last period (60-64 min) as shown in Fig. 1 (Lower). DMPP ( $10^{-4}$  M), which is a selective nicotinic (N<sub>N</sub>) receptor agonist in autonomic sympathetic ganglia, evoked a sharp and rapid increase in CA secretion ( $1,278 \pm 41$  ng for 0-8 min). However, as shown in Fig. 2 (Upper), DMPP-evoked CA secretion after pretreatment with dihydrexidine was

greatly reduced to 42% of the control release (100%). McN-A-343 ( $10^{-4}$  M), which is a selective muscarinic  $M_1$ -receptor agonist (Hammer and Giachetti, 1982), perfused into an adrenal gland for 4 min also caused an increased CA secretion ( $514 \pm 27$  ng for 0-4 min). However, in the presence of dihydraxidine, McN-A-343-evoked CA

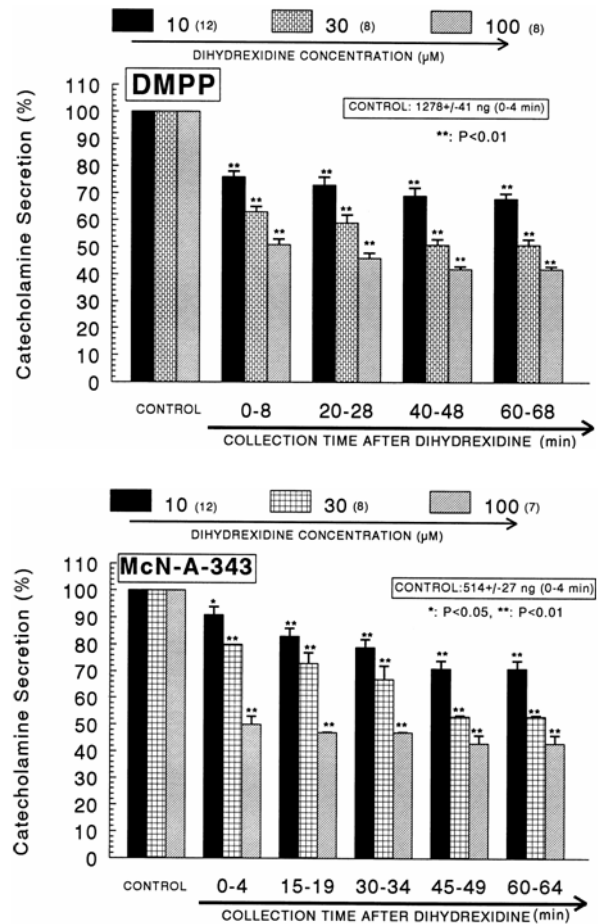
secretion was markedly depressed to 43% of the corresponding control secretion (100%) as depicted in Fig. 2 (Lower).

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

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



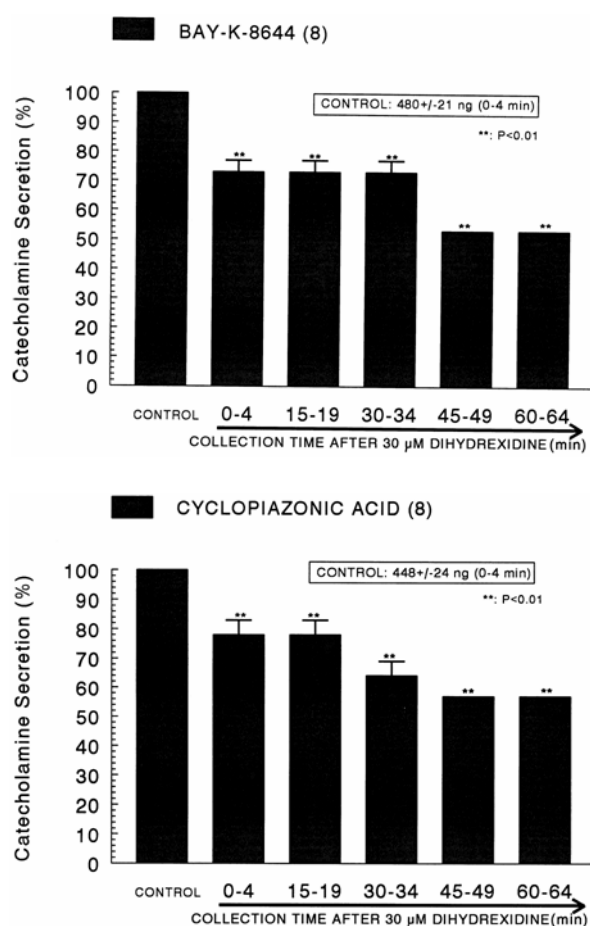
**Fig. 1.** Dose-dependent effect of dihydraxidine on the secretory responses of catecholamines (CA) evoked by acetylcholine (upper) and high potassium (lower) from the isolated perfused rat adrenal glands. The CA secretion by a single injection of ACh ( $5.32 \times 10^{-3}$  M) and  $K^+$  ( $5.6 \times 10^{-2}$  M) in a volume of 0.05 ml was evoked at 15 min intervals after preloading with 10, 30 and 100  $\mu$ M of dihydraxidine for 90 min as indicated at an arrow mark, respectively. Numbers in the parenthesis indicate number of rat adrenal glands. Vertical bars on the columns represent the standard error of the mean (S.E.M.). Ordinate: the amounts of CA secreted from the adrenal gland (% of control). Abscissa: collection time of perfusate (min). Statistical difference was obtained by comparing the corresponding control (CONTROL) with each concentration-pretreated group of dihydraxidine. ACh- and high  $K^+$ -induced perfusates were collected for 4 minutes, respectively. \*\* $p < 0.01$ .



**Fig. 2.** Dose-dependent effect of dihydraxidine on the CA secretory responses evoked by DMPP (upper) and McN-A-343 (lower) from the isolated perfused rat adrenal glands. The CA secretion by perfusion of DPPP ( $10^{-4}$  M) and McN-A-343 ( $10^{-4}$  M) for 2 min was induced at 15 and 20 min intervals after preloading with 10, 30 and 100  $\mu$ M of dihydraxidine for 90 min, respectively. Statistical difference was obtained by comparing the corresponding control (CONTROL) with each concentration-pretreated group of dihydraxidine. DMPP- and McN-A-343-induced perfusates were collected for 8 and 4 minutes, respectively. Other legends are the same as in Fig. 1. \* $p < 0.05$ , \*\* $p < 0.01$ . ns: Statistically not significant.

to determine the effect of dihydrexidine on Bay-K-8644-evoked CA secretion from the isolated perfused rat adrenal glands. Bay-K-8644 ( $10^{-5}$  M)-evoked CA secretion in the presence of dihydrexidine ( $30 \mu\text{M}$ ) was greatly blocked to 53% of the control at 45-64 min period as compared to the corresponding control release ( $480 \pm 21$  ng for 0-4 min) from 8 adrenal glands as shown in Fig. 3 (Upper).

Cyclopiazonic acid, a mycotoxin from *Aspergillus* and *Penicillium*, has been described as a highly selective inhibitor of  $\text{Ca}^{2+}$ -ATPase in skeletal muscle sarcoplasmic reticulum (Goeger and Riley, 1989; Seidler *et al.*, 1989). The inhibitory action of dihydrexidine on cyclopiazonic acid-evoked CA secretory response was observed as shown in Fig. 3 (Lower). In the presence of dihydrexidine



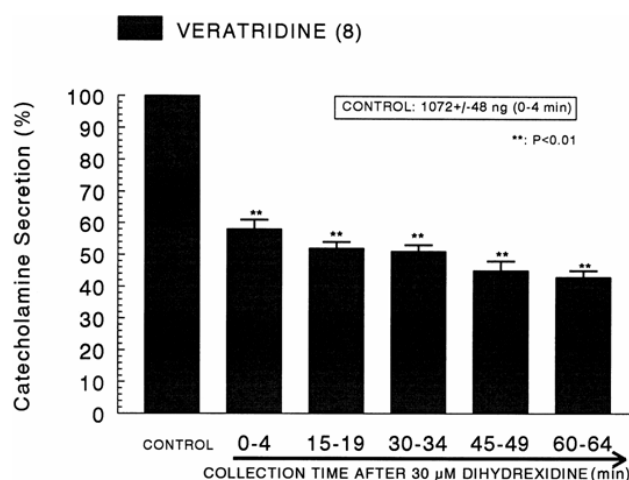
**Fig. 3.** Time-course effects of dihydrexidine on the CA release evoked by Bay-K-8644 (upper) and cyclopiazonic acid (lower) from the rat adrenal glands. Bay-K-8644 ( $10^{-5}$  M) and cyclopiazonic acid ( $10^{-5}$  M) were perfused into an adrenal vein for 4 min at 15 min intervals after preloading with dihydrexidine ( $30 \mu\text{M}$ ) for 90 min, respectively. Other legends are the same as in Fig. 1.  $**p < 0.01$ .

from 8 adrenal glands, cyclopiazonic acid ( $10^{-5}$  M)-evoked CA secretion was also inhibited to 57% of the control response ( $448 \pm 24$  ng for 0-4 min).

The voltage-dependent  $\text{Na}^+$  channels consist of the principal  $\alpha$ -subunit, which is associated with a noncovalently attached  $\beta_1$ -subunits, and a disulfide-linked  $\beta_2$ -subunit (Catterall, 2000). It has also been known that veratridine-induced  $\text{Na}^+$  influx mediated through  $\text{Na}^+$  channels increased  $\text{Ca}^{2+}$  influx via activation of voltage-dependent  $\text{Ca}^{2+}$  channels and produced the exocytotic secretion of CA in cultured bovine adrenal medullary cells (Wada *et al.*, 1985). To characterize the pharmacological action of dihydrexidine on voltage-dependent  $\text{Na}^+$  channels, the effect of dihydrexidine on the CA secretion induced by veratridine was examined here. As shown in Fig. 4, veratridine greatly produced CA secretion ( $1,072 \pm 48$  ng for 0-4 min). However, in the presence of dihydrexidine ( $30 \mu\text{M}$ ), veratridine ( $100 \mu\text{M}$ )-evoked CA secretion was greatly inhibited to 43% of the corresponding control release.

#### Effects of dihydrexidine plus R(+)-SCH23390 on CA release evoked by ACh, high $\text{K}^+$ , DMPP and McN-A-343 from the perfused rat adrenal glands

As shown in Fig. 1-3, it has also been shown that dihydrexidine inhibits the CA secretory response evoked by cholinergic stimulation in the perfused rat adrenal gland. Therefore, to study the relationship between dopaminergic  $\text{D}_1$  receptors and CA release, the effects of R(+)-SCH23390 on dihydrexidine-induced inhibitory responses

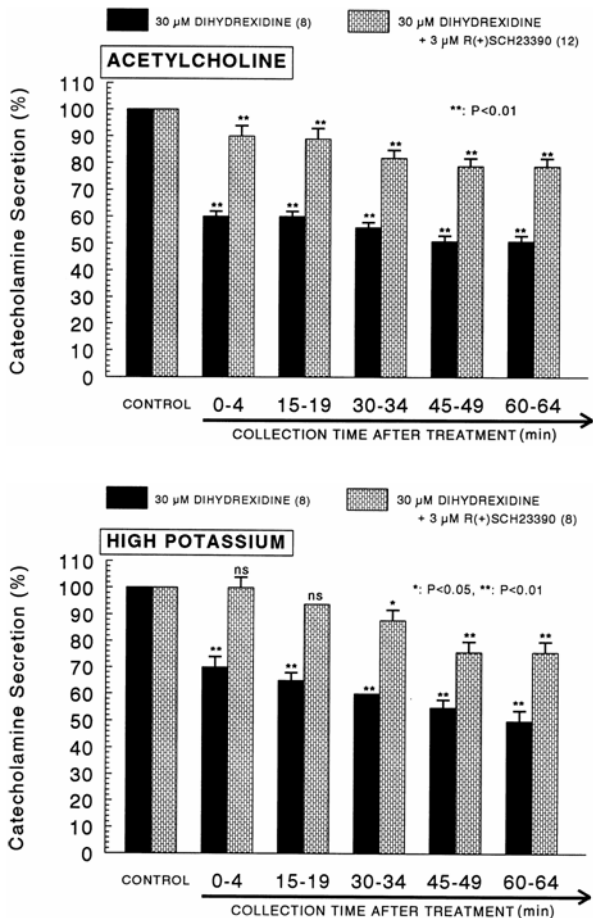


**Fig. 4.** Time-course effects of dihydrexidine on the CA release evoked by veratridine from the rat adrenal glands. Veratridine ( $10^{-4}$  M) was perfused into an adrenal vein for 4 min at 15 min intervals after preloading with dihydrexidine ( $30 \mu\text{M}$ ) for 90 min. Other legends are the same as in Fig. 1.  $**p < 0.01$ .

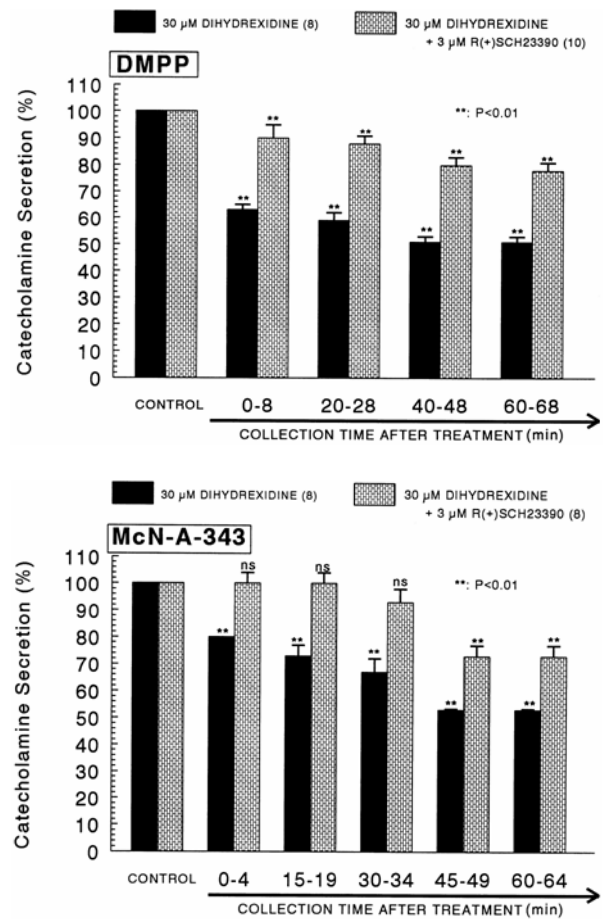
of CA secretion evoked by cholinergic receptor-stimulation as well as membrane depolarization were examined. In the simultaneous presence of dihydraxidine (30  $\mu$ M) and R (+)-SCH23390 (3  $\mu$ M) for 60 min, ACh-evoked CA release was recovered by 79-90% of the corresponding control release compared to results after loading of dihydraxidine alone as illustrated in Fig. 5 (Upper). High K<sup>+</sup> (56 mM)-evoked CA release in the simultaneous presence of dihydraxidine (30  $\mu$ M) and R (+)-SCH23390 (3  $\mu$ M) for 60 min was also recovered by 76-100% of the corresponding

control release during all periods in comparison to data of treatment with dihydraxidine alone (Fig. 5-Lower).

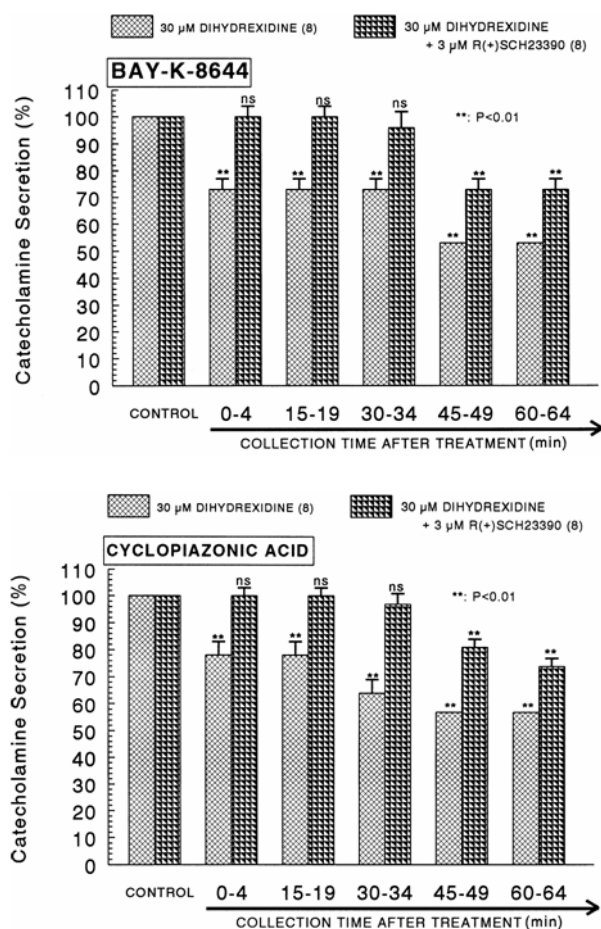
As shown in Fig. 6 (Upper), the simultaneous perfusion of dihydraxidine and R(+)-SCH23390 for 60 min got over the DMPP-evoked CA release to 78-90% of the corresponding control response in comparison to that of the dihydraxidine-treatment alone. Moreover, in the presence of dihydraxidine (30  $\mu$ M) and R(+)-SCH23390 (3  $\mu$ M), the CA secretory response evoked by McN-A-343 (10<sup>-4</sup> M for 4 min) was recovered to 73-100% of the corresponding control release compared to results of the dihydraxidine-treatment alone, as shown in Fig. 6 (Lower).



**Fig. 5.** Effects of dihydraxidine plus R(+)-SCH23390 on the CA secretory responses evoked by acetylcholine (upper) and high potassium (lower) from the perfused rat adrenal glands. The CA secretion by a single injection of ACh ( $5.32 \times 10^{-3}$  M) and K<sup>+</sup> ( $5.6 \times 10^{-2}$  M) in a volume of 0.05 ml was evoked at 15 min intervals after preloading with dihydraxidine (30  $\mu$ M) plus R(+)-SCH23390 (3  $\mu$ M) for 90 min, respectively. Statistical difference was obtained by comparing the corresponding control (CONTROL) with dihydraxidine-treated group or group treated with dihydraxidine plus R(+)-SCH23390. Other legends are the same as in Fig. 1. \* $p < 0.05$ , \*\* $p < 0.01$ . ns: Statistically not significant.



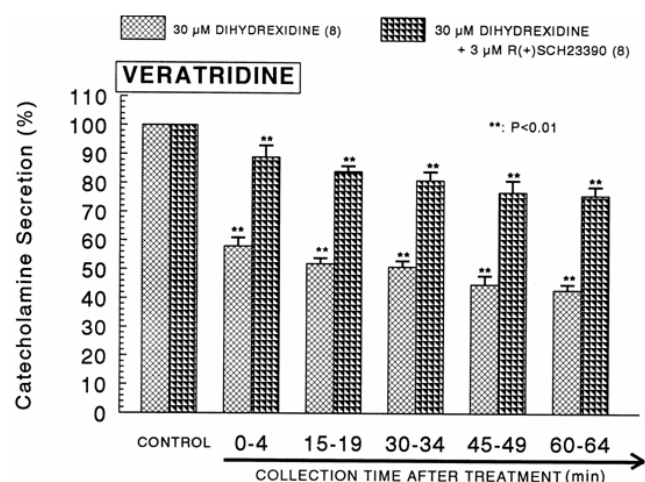
**Fig. 6.** Effects of dihydraxidine plus R(+)-SCH23390 on the CA secretory responses evoked by DMPP (upper) and McN-A-343 (lower) from the perfused rat adrenal glands. The CA secretion by perfusion of DPPP (10<sup>-4</sup> M) and McN-A-343 (10<sup>-4</sup> M) for 2 min was induced at 15 and 20 min intervals after preloading with dihydraxidine (30  $\mu$ M) plus R(+)-SCH23390 (3  $\mu$ M) for 90 min, respectively. Other legends are the same as in Fig. 1 and 5. \*\* $p < 0.01$ . ns: Statistically not significant.



**Fig. 7.** Effects of dihydrexidine plus R(+)-SCH23390 on the CA secretory responses evoked by Bay-K-8644 (upper) and cyclopiazonic acid (lower) from the rat adrenal glands. Bay-K-8644 ( $10^{-5}$  M) and cyclopiazonic acid ( $10^{-5}$  M) were perfused into an adrenal vein for 4 min at 15 min intervals after preloading with dihydrexidine (30  $\mu$ M) plus R(+)-SCH23390 (3  $\mu$ M) for 90 min. Other legends are the same as in Fig. 1 and 5. \*\* $p < 0.01$ . ns: Statistically not significant.

#### Effects of dihydrexidine plus R(+)-SCH23390 on CA release evoked by BAY-K-8644, cyclopiazonic acid and veratridine from the perfused rat adrenal glands

As shown in Fig. 7 (Upper), the simultaneous perfusion of dihydrexidine (30  $\mu$ M) and R(+)-SCH23390 (3  $\mu$ M) for 60 min made the CA release evoked by Bay-K-8644 to 73-100% of the corresponding control response compared to the results of dihydrexidine-treatment alone. After the simultaneous perfusion with dihydrexidine and R(+)-SCH-23390, cyclopiazonic acid-evoked CA release was also recovered by 74-100% of the control release in comparison to the results following the treatment with dihydrexidine alone (Fig. 7-Lower). As depicted in Fig. 8, in the simultaneous presence of dihydrexidine and R(+)-SCH23390,



**Fig. 8.** Effects of dihydrexidine plus R(+)-SCH23390 on the CA secretory responses evoked by veratridine from the rat adrenal glands. Veratridine ( $10^{-4}$  M) was perfused into an adrenal vein for 4 min at 15 min intervals after preloading with dihydrexidine (30  $\mu$ M) plus R(+)-SCH23390 (3  $\mu$ M) for 90 min. Other legends are the same as in Fig. 1 and 5. \*\* $p < 0.01$ .

veratridine-evoked CA release was also recovered by 76-89% of the corresponding control release compared to the results of dihydrexidine-treatment alone.

## DISCUSSION

The present study shows that dihydrexidine inhibits the CA secretion evoked by cholinergic stimulation (both nicotinic and muscarinic receptors) and membrane depolarization from the rat adrenal medulla. This inhibitory effect of dihydrexidine seems to be mediated by blocking the influx of  $\text{Na}^+$  and  $\text{Ca}^{2+}$  ions through their channels as well as by inhibiting the release of  $\text{Ca}^{2+}$  from cytoplasmic store through activation of dopaminergic  $\text{D}_1$  receptors located on the rat adrenomedullary chromaffin cells, which are relevant to adrenal nicotinic receptor blockade.

In support of the present results, previously Artalejo and his co-workers (1990) found specific binding of the rhodamine conjugate of the  $\text{D}_1$  antagonist SCH-23390 to almost all of the cells in chromaffin cell cultured. Because SCH-23390 will bind  $\text{D}_5$  receptors as well as  $\text{D}_1$  receptors, it is possible, given the results of RNA analysis by Dahmer and Senogles (1996b), that  $\text{D}_5$ -receptors were labeled on the cells. These observations suggested that  $\text{D}_5$  receptors on the cells are responsible for inhibition of secretion by  $\text{D}_1$ -selective agonists. Dahmer and Senogles (1996a) have also shown that dopaminergic  $\text{D}_1$ -selective agonists inhibit secretagogue-stimulated  $\text{Na}^+$  uptake into bovine adrenal chromaffin cells in a cyclic AMP-independent manner.

Albillos and his colleagues (1992) have reached two conclusions: First, the cat adrenal medulla chromaffin cell possesses a dopamine D<sub>1</sub>-receptor that seems to be coupled to an adenylyl cyclase. Second, this receptor regulates the muscarinic-mediated CA release through a negative feedback loop which uses cyclic AMP as a second messenger. In addition, D<sub>1</sub>-like receptors have been reported to inhibit secretion (Schoors *et al.*, 1991); however, such a function for members of the D<sub>1</sub> family of dopamine receptors is controversial.

These previous results are consistent with those obtained from the present study. In the simultaneous presence of R(+)-SCH23390 and dihydraxidine, the CA secretory responses evoked by ACh, high K<sup>+</sup>, and DMPP were recovered to the similar level of their corresponding control responses compared to the inhibitory results of dihydraxidine-treatment alone. This finding confirms that dihydraxidine inhibits the CA secretory responses evoked by cholinergic stimulation as well as membrane depolarization through activation of inhibitory dopaminergic D<sub>1</sub>-receptors on rat adrenal medullary chromaffin cells. Furthermore, electrophysiological studies in awake, behaving monkeys have also shown that iontophoresis of low concentrations of D<sub>1</sub> antagonists enhances memory-related neuronal firing (Williams and Goldman-Rakic, 1995). Infusions of the selective D<sub>1</sub> receptor antagonists R(+)-SCH23390 or SCH39166 into the prefrontal cortex of monkeys (Sawaguchi and Goldman-Rakic, 1991) or rats (Seamans *et al.*, 1995) impaired spatial working memory performance, without altering performance of a control task with identical motor and motivational demands but little mnemonic component (Sawaguchi and Goldman-Rakic, 1991). In terms of these findings, it is plausible that dopaminergic D<sub>1</sub> receptors exist on the rat adrenomedullary chromaffin cells. It has also been reported that, in sinoaortic denervated dogs (i.e. animals deprived from baroreflex pathways), the fenoldopam-induced decrease in arterial blood pressure was more important than in normal dogs (Damase-Michel *et al.*, 1995). Heart rate was unchanged. In these animals, D<sub>1</sub> stimulation induced a decrease in sympathetic tone, as shown by the significant fall in plasma noradrenaline levels. These "*in vivo*" data clearly demonstrate the inhibitory role of ganglionic D<sub>1</sub> receptors. Furthermore, recently, it has also been shown that the full dopaminergic D<sub>1</sub> receptor agonist dihydraxidine produces prominent dopamine D<sub>1</sub> receptor agonist effects *in vivo* and is likely to produce subjective effects in humans similar to other D<sub>1</sub> receptor agonists (Gleason and Witkin, 2006).

The nicotinic receptor is a neurotransmitter-gated cation-conducting ion channel that is opened by binding of ag-

onists such as ACh and DMPP (McGehee and Role, 1995). The opening of this channel triggers Ca<sup>2+</sup> uptake and secretion of CA from chromaffin cells (Wada *et al.*, 1985). To determine if the inhibition of DMPP-stimulated secretion by dopaminergic D<sub>1</sub> agonist was due to an effect on the activity of the nicotinic receptor, the effect of dihydraxidine, a full D<sub>1</sub>-selective agonist, on DMPP-stimulated CA secretion was examined. As shown in Fig. 2 (Upper), treatment with dihydraxidine greatly inhibited DMPP-evoked CA secretion, reducing by 42% of the control release. The present data are very similar to the result that C1-APB, a D<sub>1</sub>-selective agonist, inhibited DMPP-stimulated Na<sup>+</sup> uptake in bovine chromaffin cells (Dahmer and Senogles, 1996a). Previous studies have demonstrated that in bovine adrenal chromaffin cells both D<sub>1</sub>- and D<sub>2</sub>-selective dopamine receptor agonists inhibit CA secretion and Ca<sup>2+</sup> uptake stimulated by the nicotinic ACh receptor agonist DMPP (Dahmer and Senogles, 1996a). Both D<sub>1</sub>- and D<sub>2</sub>-selective agonists are found to inhibit CA release stimulated by veratridine, an agent that opens voltage-sensitive Na<sup>+</sup> channels (Dahmer and Senogles, 1996a). It is likely plausible that dihydraxidine can activate a signal transduction pathway that is altering the activity of both nicotinic receptors and voltage-sensitive Na<sup>+</sup> channels. There have been reports that D<sub>1</sub>-like dopamine receptors are linked to phosphoinositide metabolism (Felder *et al.*, 1989; Andersen *et al.*, 1990; Undie and Friedman, 1990). Activation of such a pathway could result in elevated levels of Ca<sup>2+</sup>, diacylglycerol, and inositol triphosphate in the cells. Consequently, Ca<sup>2+</sup>-dependent and protein kinase C-dependent pathways may be activated. Protein kinase C has been reported to attenuate the activity of both nicotinic receptors (Swope *et al.*, 1992) and voltage-sensitive Na<sup>+</sup> channels (Catterall, 1992). It has been proposed that activation of D<sub>1</sub> receptors on chromaffin cells causes activation of facilitation Ca<sup>2+</sup> channels on the cells in a cAMP-dependent manner (Artalejo *et al.*, 1990). However, Dahmer and Senogles (1996b) could find no evidence of message for D<sub>1</sub> dopamine receptors in chromaffin cells by either PCR analysis or northern blot analysis of RNA. In the present study, dihydraxidine, a D<sub>1</sub>-selective agonist inhibited the CA secretory responses by high potassium as well as by Bay-K-8644, an activator of L-type Ca<sup>2+</sup> channels, which facilitates the influx of Ca<sup>2+</sup> into the cells. The observation that D<sub>1</sub>-selective agonists inhibited the CA secretion evoked by Bay-K-8644 was surprising, as Artalejo and his colleagues (1990) have reported that D<sub>1</sub>-selective agonists recruit a facilitation Ca<sup>2+</sup> current in bovine chromaffin cells. Although Ca<sup>2+</sup> uptake measurements are clearly not the same as measuring Ca<sup>2+</sup>



channel activity, it is difficult to reconcile data indicating that D<sub>1</sub>-selective agonists can inhibit Ca<sup>2+</sup> uptake and facilitate Ca<sup>2+</sup> channel activity. Again, one possible explanation is that only a subpopulation of chromaffin cells responds to dopamine agonists by recruiting the facilitation channels.

It is unclear how activation of dopamine receptors results in the inhibition of secretion seen in these cells. The simplest interpretation is that the decrease in Ca<sup>2+</sup> uptake by D<sub>1</sub>-selective agonist is responsible for the observed inhibition of the CA secretion. However, such an interpretation is complicated by the complexity of the relationship between the CA secretion and intracellular free Ca<sup>2+</sup> levels. Both the intracellular location of the Ca<sup>2+</sup> level increase (Cheek, 1989; Ghosh and Greenberg, 1995) and the magnitude of the Ca<sup>2+</sup> level increase (Holz *et al.*, 1982) can affect the relationship between intracellular free Ca<sup>2+</sup> levels and secretion. Holz and his colleagues (1982) have reported that when Ca<sup>2+</sup> uptake is large, changes in Ca<sup>2+</sup> uptake resulted in less than proportional changes in CA secretion. Consequently, although the decrease in Ca<sup>2+</sup> uptake (influx) into the adrenal chromaffin cells may explain the decrease by dihydrexidine in CA secretion, it is still unclear whether this is only or even most important factor contributing to the inhibition of CA secretion by dopaminergic D<sub>1</sub> agonists. However, in view of the results so far obtained from the present study, it is felt that the voltage-sensitive Ca<sup>2+</sup> channels located on chromaffin cell membrane of the rat adrenal medulla could be the target site for dihydrexidine-mediated inhibition of CA secretion.

In the present study, dihydrexidine also inhibited the CA secretory responses evoked by cyclopiazonic acid, which is known to be a highly selective inhibitor of Ca<sup>2+</sup>-ATPase in skeletal muscle sarcoplasmic reticulum (Goeger and Riley, 1989; Seidler *et al.*, 1989). Therefore, it is felt that the inhibitory effect of dihydrexidine on CA secretion evoked by cholinergic stimulation as well as by membrane-depolarization may be associated with the mobilization of intracellular Ca<sup>2+</sup> in the chromaffin cells. This indicates that the activation of dopaminergic D<sub>1</sub>-receptors has an inhibitory effect on the release of Ca<sup>2+</sup> from the intracellular pools induced by stimulation of muscarinic ACh receptors, which is weakly responsible for the secretion of CA. In the present work, dihydrexidine time- and concentration-dependently produced the inhibition of CA secretion evoked by McN-A-343, a selective muscarinic M<sub>1</sub>-agonist. This fact suggests new other concept that dihydrexidine can modulate the CA secretory process induced by activation of muscarinic M<sub>1</sub>-receptors as well as neuronal nicotinic receptors in the rat adrenal medulla. In sup-

porting this finding, it has been shown that cyclopiazonic acid easily penetrates into the cytoplasm through the plasma membrane and reduces Ca<sup>2+</sup>-ATPase activity in sarcoplasmic/endoplasmic reticulum, resulting in increase in the subsequent Ca<sup>2+</sup> release from those storage sites and thereby increases of Ca<sup>2+</sup>-dependent K<sup>+</sup>-current (Suzuki *et al.*, 1992). Moreover, in bovine adrenal chromaffin cells, stimulation of muscarinic ACh receptors is also proposed to cause activation of phosphoinositide metabolism, resulting in the formation of inositol 1,4,5-trisphosphate, which induces the mobilization of Ca<sup>2+</sup> from the intracellular pools (Cheek *et al.*, 1989; Challiss *et al.*, 1991). However, in the present study, it is uncertain whether the inhibitory effect of the dihydrexidine on Ca<sup>2+</sup> movement from intracellular pools is due to their direct effect on the PI response or the indirect effect as a result of D<sub>1</sub>-receptor activation by dihydrexidine. Based on these previous results, this finding of the present work suggests that the inhibitory dopaminergic D<sub>1</sub>-receptors may be involved in regulating CA secretion evoked by muscarinic M<sub>1</sub>-receptor stimulation in the rat adrenal medullary chromaffin cells.

In contrast with the present experimental results, it has also been shown that stimulation of dopaminergic D<sub>1</sub>-receptors activates the facilitation of Ca<sup>2+</sup> currents in the absence of pre-depolarizations or repetitive activity from bovine chromaffin cells, and that activation by D<sub>1</sub> agonists is mediated by cAMP and protein kinase A (Artalejo *et al.*, 1990). This recruitment of facilitation of Ca<sup>2+</sup> channels by dopamine may form the basis of a positive feedback loop mechanism that augments CA secretion.

Taken together, these present results suggest that dihydrexidine inhibits the CA secretion evoked by cholinergic (both nicotinic and muscarinic) receptor stimulation and membrane depolarization from the rat adrenal medulla. It seems that this inhibitory of dihydrexidine is due to activation of dopaminergic D<sub>1</sub> receptors located on the rat adrenomedullary chromaffin cells, which are relevant to the blockade of influx of both Na<sup>+</sup> and Ca<sup>2+</sup> ions into chromaffin cells as well as to the inhibition of Ca<sup>2+</sup> release from the cytoplasmic store.

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