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Influence of Cilnidipine on Catecholamine Release in the Perfused Rat Adrenal Medulla

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The present study was attempted to investigate the effect of cilnidipine (FRC-8635), which is a newly synthesized novel dihydropyridine (DHP) type of organic Ca^{2+} channel blockers, on secretion of catecholamines (CA) evoked by acetylcholine (ACh), high K^+, DMPP and McN-A-343 from the isolated perfused rat adrenal gland. Cilnidipine (1~10 μM) perfused into an adrenal vein for 60 min produced relatively dose- and time-dependent inhibition in CA secretory responses evoked by ACh (5.32×10^{-3} M), DMPP (10^{-4} M for 2 min) and McN-A-343 (10^{-4} M for 2 min). However, lower dose of cilnidipine did not affect CA secretion by high K^+ (5.6×10^{-2} M), higher dose of it reduced greatly CA secretion of high K^+. Cilnidipine itself did fail to affect basal catecholamine output. In the presence of cilnidipine (10 μM), the CA secretory responses evoked by Bay-K-8644 (10 μM), an activator of L-type Ca^{2+} channels and cyclopiazonic acid (10 μM), an inhibitor of cytoplasmic Ca^{2+}-ATPase were also inhibited. Moreover, ω-conotoxin GVIA (1 μM), a selective blocker of the N-type Ca^{2+} channels, given into the adrenal gland for 60 min, also inhibited time-dependently CA secretory responses evoked by Ach, high K^+, DMPP, McN-A-343, Bay-K-8644 and cyclopiazonic acid. Taken together, these results demostrate that cilnidipine inhibits CA secretion evoked by stimulation of cholinergic (both nicotinic and muscarinic) receptors from the isolated perfused rat adrenal gland without affecting the basal release. However, at lower dose, cilnidipine did not affect CA release by membrane depolarization while at larger dose inhibited that. It seems likely that this inhibitory effect of cilnidipine is exerted by blocking both L- and N-type voltage-dependent Ca^{2+} channels (VDCCs) on the rat adrenomedullary chromaffin cells, which is relevant to inhibition of both the Ca^{2+} influx into the adrenal chromaffin cells and intracellular Ca^{2+} release from the cytoplasmic store. It is thought that N-type VDCCs may play an important role in regulation of CA release from the rat adrenal medulla.

Key Words: Cilnidipine, L- & N-Type VDCCs, Catecholamines (CA), Adrenal Medulla

INTRODUCTION

Cilnidipine (FRC-8635) is a newly synthesized novel dihyropyridine (DHP) type of organic Ca^{2+} channel blockers that have been developed so far in Japan (Yoshimoto et al, 1991; Hosono et al, 1992). It also has a blocking action on L-type voltage-dependent Ca^{2+} channels (VDCCs) in the rabbit basilar artery (Oike et al, 1990) and a slow-onset and long-lasting hypotensive action in clinical and experimental studies (Ikeda et al, 1992; Tominaga et al, 1997). Unlike other DHP Ca^{2+} channel blockers. cilnidipine inhibits both pressor response and increase of plasma norepinephrine (NE) concentration induced by acute cold stress in the spontaneously hypertensive rats (Hosono et al, 1995). Cilnidipine also has been shown to inhibit the release of [^{3}H] NE from the rat mesenteric vasculature (Hosono et al, 1995) but failed to induce tachycardia evoked by hypotensive baroreflexes in humans (Saihara et al, 1993). These findings suggest that cilnidipine may have an inhibitory action on sympathetic neurotransmission. Furthermore, previously it has been demonstrated that the N-type VDCC blocker ω-conotoxin GVIA inhibits the secretion of CA induced by splanchnic nerve stimulation (SNS) and exogenous ACh in anesthetized dogs and suggested that N-type VDCCs may contribute to the CA secretion (Kimura et al, 1994). In spontaneously hypertensive rats, vasoconstriction induced by electrical sympathetic nerve stimulation was also blocked by cilnidipine (Hosono et al, 1995). In *in vitro* experiments, cilnidipine also inhibited [^{3}H] NE release evoked by electrical stimul-

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ABBREVIATIONS: CA, catecholamine; DMPP, 1.1-dimethyl-4-phenyl piperazinium iodide; SNS, splanchnic nerve stimulation; VDCCs, voltage-dependent Ca^{2+} channels; BAY-K-8644,ethyl-1,4-dihydro-2,6-dimethyl-3nitro-4-(2-trifluoromethylphenyl)-pyridine-5-carboxylate; DHP, dihydropyridine; McN-A-343, 4-(N-[3-Chlorophenyl]carbamoyloxy)-2-butynyltrimethyl ammonium chloride

ation in the rabbit mesenteric artery (Nakashima et al, 1991). Also, in addition to its well-known L-type Ca^{2+} channel blockade (Oike et al, 1990), it was clarified that cilnidipine had a potent inhibitory effect for CA secretion from nerve growth factor-differentiated PC12 cells via the blockade of extracellular Ca^{2+} influx through the N-type Ca^{2+} channel (Uneyama et al, 1998). Therefore, the present study was conducted to clarify whether cilnidipine affects the release of CA evoked by cholinergic stimulation and membrane depolarization from the isolated perfused model of the rat adrenal gland and to establish the mechanism of action.

METHODS

Experimental procedure

Male Sprague-Dawley rats, weighing 180 to 250 grams, were anesthetized intraperitoneally 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 placing three-hook retractor exposed the left adrenal gland and surrounding area. 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. Before ligating vessels and cannulations, heparin (400 IU/ml) was injected into vena cava to prevent blood coagulation. 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. The adrenal gland, along with ligated blood vessels and the cannula, was then carefully removed from the animal and placed on a platform of a leucite chamber. The chamber was continuously circulated with water heated at $37 \pm 1°C$.

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 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 CAs.

Drug administration

The perfusions of DMPP (100 μM) and McN-A-343 (100 μM) for 2 minutes, and Bay-K-8644 (10 μM) and cyclopiazonic acid (10 μM) for 4 minutes were made into perfusion stream, respectively. A single injection of ACh (5.32 mM) in a volume of 0.05 ml and KCl (56 mM) in a volume of 0.1 ml was injected into perfusion stream via a three-way stopcock.

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

Prior to stimulation with various secretagogues, perfusate was routinely collected for 4 min to determine spontaneous secretion of CA (background sample). Immediately after the collection of the background sample, the perfusates were continuously collected in another tube as soon as the perfusion medium containing the stimulatory agent reached the adrenal gland. Stimulated samples were collected for 4 to 8 min. The amounts secreted in the background sample have been subtracted from 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 effects of cilnidipine on the spontaneous and evoked secretion, the adrenal gland was perfused with Krebs solution containing cilnidipine for 60 min immediately after the perfusate was collected for a certain minute (background sample). And the medium was then changed to the one containing the stimulating agent, and the perfusates were collected for the same period as that for the background sample. Generally, the adrenal gland's perfusate was collected in chilled tubes.

Measurement of catecholamines

CA content of perfusate was fluorospectrophotometrically (Kontron Co. Italy) measured directly by the fluorometric method of Anton and Sayre (1962) without intermediate purification on alumina for the reasons described earlier (Wakade, 1981).

A volume of 0.2 ml perfusate was used for the reaction. The CA content in the glands perfusate stimulated by secretogagues in the present work was high enough to obtain several folds greater readings than that of control samples (unstimulated). The sample blanks were also the 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 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 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: cilnidipine (gift from UCV Japan Co., Japan and Boryung Pharmaceutical Manuf. Co., Korea), acetylcholine chloride, 1.1-dimethyl-4-phenyl piperazinium iodide (DMPP), norepinephrine bitartrate, methyl-1,4-dihydro-2,6-dimethyl-3-nitro-4-(2-trifluoromethylphenyl)-pyridine-5-carboxylate (BAY-K-8644), nicotine tartrate (Sigma Chemical Co., USA), cyclopiazonic acid and (3-(m-chloro-phenyl-carbamoyl-oxy)-2-butynyl trimethyl ammo-

nium chloride [McN-A-343] (RBI, USA), and ω-conotoxin GVIA (Bachem, Bubendorf, Switzerland). Drugs were dissolved in distilled water (stock) and diluted to the normal Krebs solution as required except Bay-K-8644 and clinidipine, which were dissolved in 99.5% ethanol and diluted appropriately (final concentration of alcohol was less than 0.1%). Bay-K-8644 and clinidipine are stored under dark light before use. Concentrations of all drugs used are expressed in terms of molar base.

RESULTS

Effect of cilnidipine on CA secretion 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 1 hr, basal CA release from the isolated perfused rat adrenal glands amounted to 22±3 ng/2 min (n=10). It has been shown that cilnidipine inhibits the secretion of adrenal NE and EP induced by splanchnic nerve stimulation as well as muscarine-induced secretion of adrenal NE in comparison with the pure L-type VDCC blocker nifedipine in anesthetized dogs (Nagayama et al, 1998). Therefore, it was decided initially to examine the effects of cilnidipine on cholinergic receptor stimulation- as well as membrane depolarization-mediated CA secretion from the isolated perfused rat adrenal glands. Secretagagues were given at 15 or 20 min-intervals. Cilnidipine was present for 60 min including stimulation with each secretagague.

In the present study, it was found that cilnidipine itself did not produce any effect on the spontaneous CA release (data not shown). When ACh (5.32×10^{-3} M) in a volume of 0.05 ml was injected into the perfusion stream, the amount of CA secreted was 330±30 ng for 4 min. However, after the simultaneous perfusion with cilnidipine (10^{-6} M), ACh-stimulated CA secretion was dose- and time-dependently inhibited to 37% of the control release (100%) as shown in Fig. 1 (upper). Also, it has been found that direct membrane-depolarizing agent like KCl stimulates sharply CA secretion (182±30 ng for 4 min). In the present work, high K^+ (5.6×10^{-2} M)-stimulated CA secretion after the pretreatment with lower dose of cilnidipine (10^{-6} M) was not affected. However, in presence of higher doses of cilnidipine (3×10^{-6} and 10^{-5} M), high K^+-evoked CA output was inhibited to 67% of the corresponding control secretion (100%) as shown in Fig. 1 (lower).

When perfused through the rat adrenal gland, DMPP (10^{-4} M), which is a selective nicotinic receptor agonist in autonomic sympathetic ganglia, evoked a sharp and rapid increase in CA secretion. As shown in Fig. 2 (upper), DMPP-stimulated CA secretion following the loading with cilnidipine (10^{-6}~10^{-5} M) was greatly inhibited to 32% of the control secretion (361±28 ng for 8 min).

As illustrated in Fig. 2 (lower), McN-A-343 (10^{-4} M), which is a selective muscarinic M_1-receptor agonist (Hammer and G iachetti, 1982), perfused into an adrenal vein for 4 min caused an increased CA secretion to 140±6 ng (0~4 min). However, in the presence of cilnidipine (10^{-6} ~10^{-5} M), McN-A-343-evoked CA secretion was also a dose-dependently diminished to 34% of the control release.

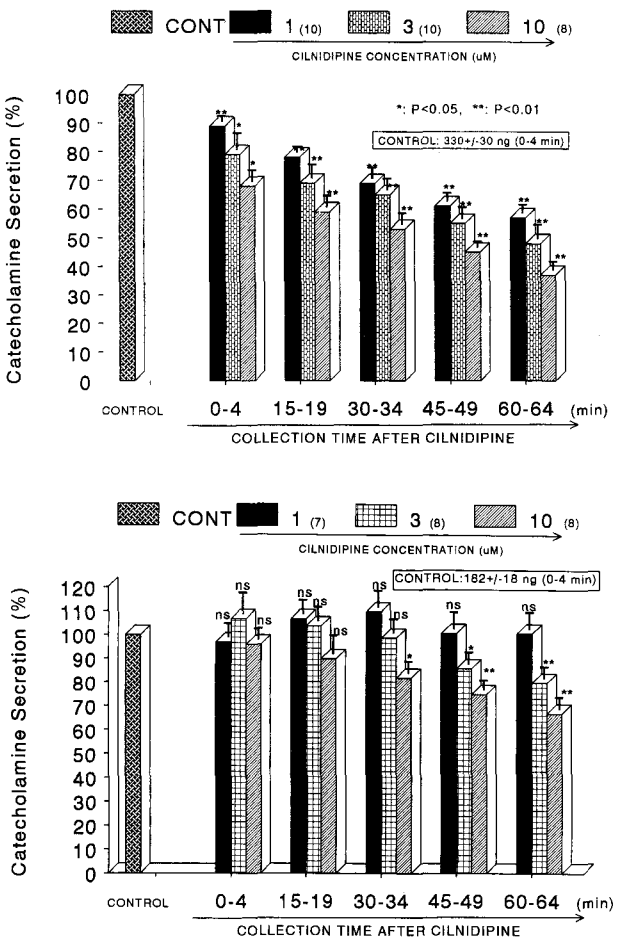


Fig. 1. Dose-dependent effects of cilnidipine on the secretory responses of catecholamines (CA) evoked by acetylcholine (ACh, Upper) and by high K^+ (Lower) from the isolated perfused rat adrenal glands. CA secretion by a single injection of ACh (5.32×10^{-3} M) or K^+ (56 mM) in a volume of 0.05 ml was evoked at 15 min intervals after preloading with 1, 3, 10 μM of cilnidipine for 60 min as indicated at an arrow mark. 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 (CONT) with each concentration-pretreated group of cilnidipine. Pefusates induced by ACh and high K^+ were collected for 4 minutes, respectively. *: $P<0.05$, **: $P<0.01$. ns: Statistically not significant.

K-8644 and cyclopiazonic acid from the perfused rat adrenal glands

It has been found that Bay-K-8644 is a selective L-type calcium channel activator, which causes positive inotropy and vasoconstriction in isolated tissues and intact animals (Schramm et al, 1982; Wada et al, 1985) and enhances basal Ca^{2+} uptake (Garcia et al, 1984) and CA release (Lim et al, 1992). Therefore, it was of interest to determine the effects of cilnidipine on Bay-K-8644-stimulated CA secretion from the isolated perfused rat adrenal glands. Fig. 3 (upper) illustrates the inhibitory effect of cilnidipine on

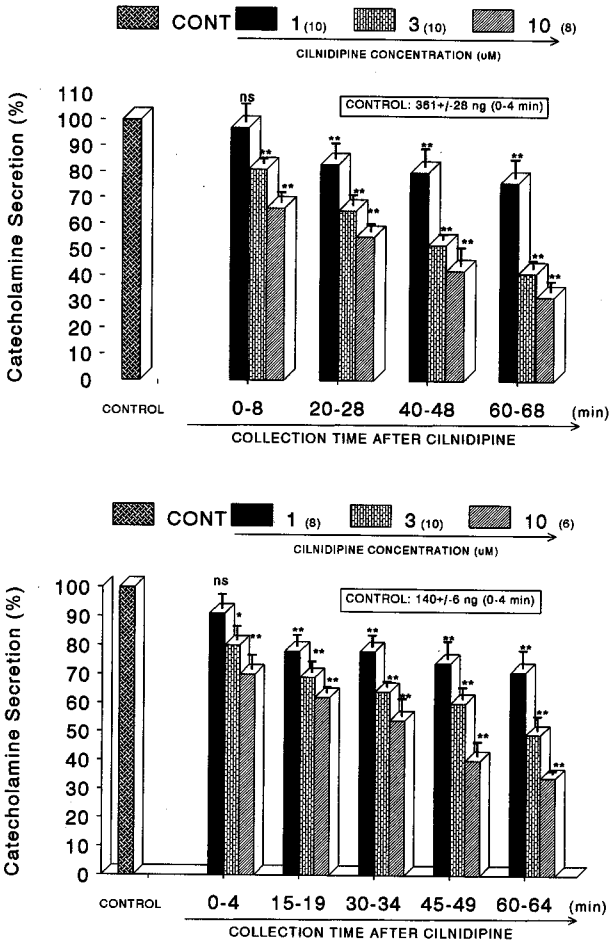


Fig. 2. Dose-dependent effects of cilnidipine on the secretory responses of catecholamines (CA) evoked by DMPP (Upper) and McN-A-343 (Lower) from the isolated perfused rat adrenal glands. The CA secretory responses by the perfusion of DPPP (10^{-4} M) and McN-A-343 (10^{-4} M) for 2 min at 20 and 15 min intervals were induced after preloading with 1, 3, 10 μM of cilnidipine for 60 min, respectively. Pefusates induced by DMPP and McN-A-343 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.

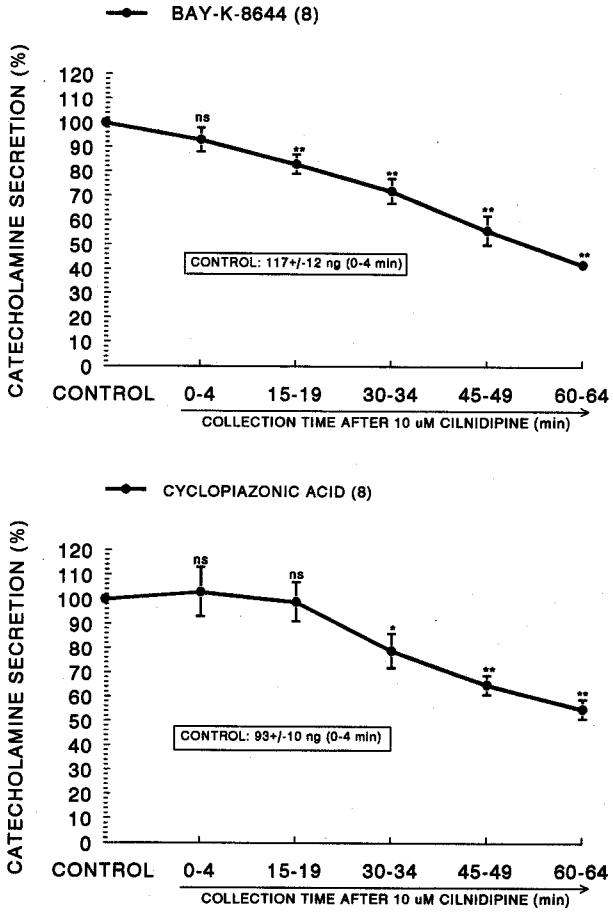


Fig. 3. Effects of cilnidipine on CA release evoked by Bay-K-8644 (Upper) and cyclopiazonic acid (Lower) from the rat adrenal glands. Bay-K-8644 (10^{-5} M) and cyclopiazonic acid (10^{-5} M) were perfused into an adrenal vein for 4 min at 15 min intervals after preloading with of cilnidipine (10 μM) for 60 min, respectively. Other legends are the same as in Fig. 1. *: $P<0.05$, **: $P<0.01$. ns: Statistically not significant.

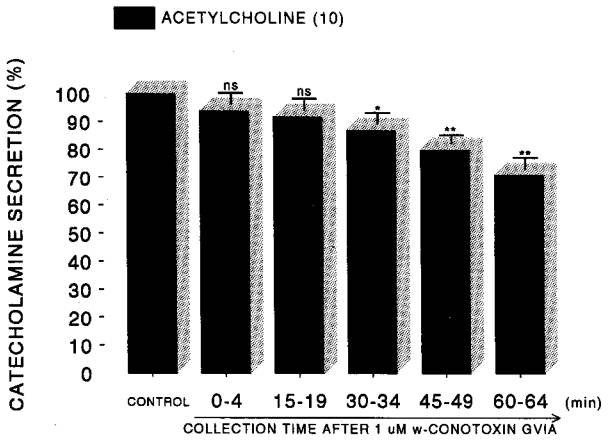
Bay-K-8644-evoked CA secretory responses. In the absence of cilnidipine, Bay-K-8644 (10^{-5} M) given into the perfusion stream produced CA secretion of 117 ± 12 ng (0~4 min) from 8 rat adrenal glands. However, in the presence of cilnidipine (10^{-6}~10^{-5} M), Bay-K-8644-stimulated CA secretion was greatly inhibited to 42% of the corresponding control release as time elapsed.

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). As shown in Fig. 3 (lower), in the presence of cilnidipine (10^{-6} ~10^{-5} M), cyclopiazonic acid (10^{-5} M)-evoked CA secretion was markedly inhibited to 55% of the control response ($93\pm$ 10 ng for 4 min) from 8 rat adrenal glands.

Effect of ω-conotoxin GVIA on CA secretion evoked by ACh, high K^+, DMPP, McN-A-343, Bay-K-8644 and cyclopiazonic acid from the perfused rat adrenal glands

It has been found that ω-conotoxins, ω-conotoxin MVIIA and ω-conotoxin GVIA potently block N-type Ca^{2+} channels (Kasai et al, 1987; McCleskey et al, 1987; Plummer et al, 1989; Regan et al, 1991; Miljanich and Ramachandran, 1995). Therefore, it was likely of interest to examine effects of ω-conotoxin GVIA on CA secretion evoked by ACh, high K^+, DMPP and McN-A-343 from the isolated perfused rat adrenal glands.

As shown in Fig. 4 (upper), in the present study, ACh (5.32×10^{-3} M)-stimulated CA secretion before pre-loading with ω-conotoxin GVIA was 336 ± 21 ng (0~4 min) from 10 glands, while in the presence of ω-conotoxin GVIA (10^{-6} M), it was gradually reduced to 71% the corresponding control secretory response. Excess K^+ (5.6×10^{-2} M)- stim-



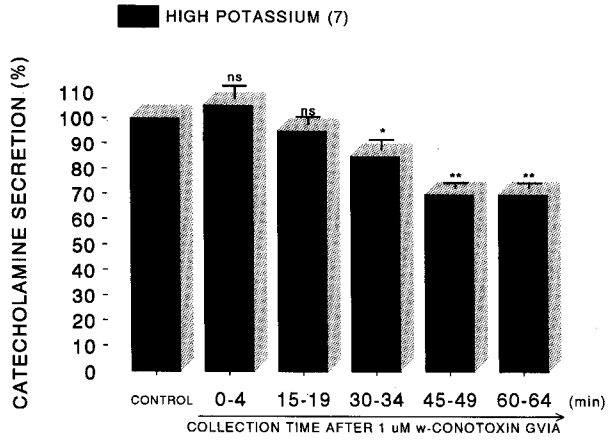
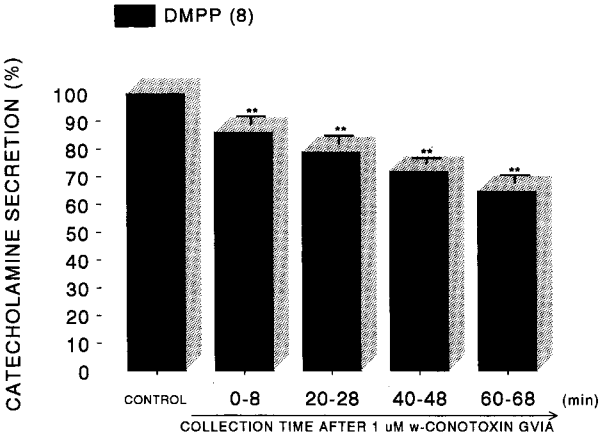


Fig. 4. Effects of ω-conotoxin GVIA on the secretory responses of catecholamines (CA) evoked by acetylcholine (Upper) and by high K^+ (Lower) from the isolated perfused rat adrenal glands. The CA secretory responses by a single injection of ACh (5.32×10^{-3} M) or K^+ (56 mM) in a volume of 0.05 ml were induced before (CONTROL) and after preloading with 1.0 μM ω-conotoxin GVIA for 60 min. Perfusate was collected for 4 minutes at 15 min-intervals. Other legends are the same as in Fig. 1. *: $P<0.05$, **: $P<0.01$. ns: Statistically not significant.



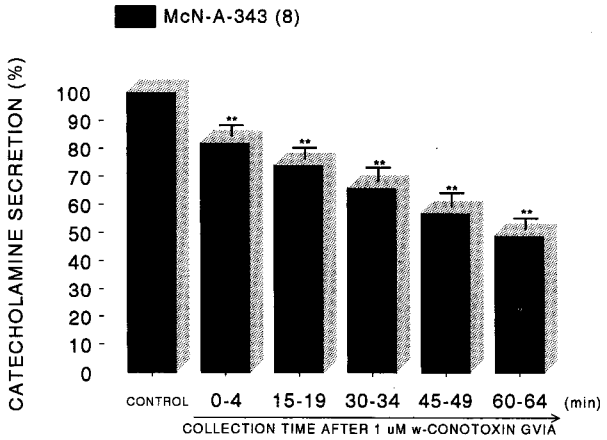


Fig. 5. Effects of ω-conotoxin GVIA on the secretory responses of catecholamines (CA) evoked by DMPP (Upper) and McN-A-343 (Lower) from the isolated perfused rat adrenal glands. The CA secretory responses by the perfusion of DPPP (10^{-4} M) and McN-A-343 (10^{-4} M) for 2 min at 20 and 15 min intervals were induced before (CONTROL) and after preloading with 1.0 μM ω-conotoxin GVIA for 60 min, respectively. Pefusates induced by DMPP and McN-A-343 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.

ulated CA secretion following the loading with ω- conotoxin GVIA (10^{-6} M) was significantly diminished to 70% of the corresponding control secretion (138±6 ng for 4 min) from 7 glands, as shown in Fig. 4 (lower). A neuronal nicotinic receptor agonist, DMPP (10^{-4} M) perfused into the adrenal gland evoked great CA secretion of 324±16 ng (0~8 min) from 8 rat adrenal glands. However, following perfusion with ω-conotoxin GVIA (10^{-6} M) it was significantly inhibited to 65% of the control secretion (Fig. 5-upper). In 8 adrenal glands, McN-A-343 (10^{-4} M)- stimulated CA secretion before the perfusion of ω-conotoxin GVIA was 120 ±6 ng (0~4 min), but in the presence of ω- conotoxin GVIA (10^{-6} M), it was significantly reduced to 49% of the control secretion as shown in Fig. 5 (lower).

In the absence of ω-conotoxin GVIA, Bay-K-8644 (10^{-5} M) produced the CA secretory response of 114±9 ng (0~4 min) from 8 rat adrenal glands. However, in the presence of ω- conotoxin GVIA (10^{-6} M), Bay-K-8644-stimulated CA secretion was greatly inhibited to 56% of the corresponding control release as time elapsed.

As shown in Fig. 6 (lower), in the presence of ω-conotoxin GVIA (10^{-6} M), CA secretion evoked by cyclopiazonic acid (10^{-5} M), a highly selective inhibitor of Ca^{2+}-ATPase, was significantly inhibited to 62% of the control response (95 ±8 ng for 4 min) from 8 rat adrenal glands.

DISCUSSION

These experimental results demonstrate that cilnidipine inhibits CA secretion evoked by stimulation of cholinergic (both nicotinic and muscarinic) receptors from the isolated perfused rat adrenal gland without affecting the basal release. However, at lower dose, cilnidipine did not affect that by membrane depolarization while at larger dose inhibited that. It seems that this inhibitory effect of cilnidipine is exerted by blocking both the Ca^{2+} influx into

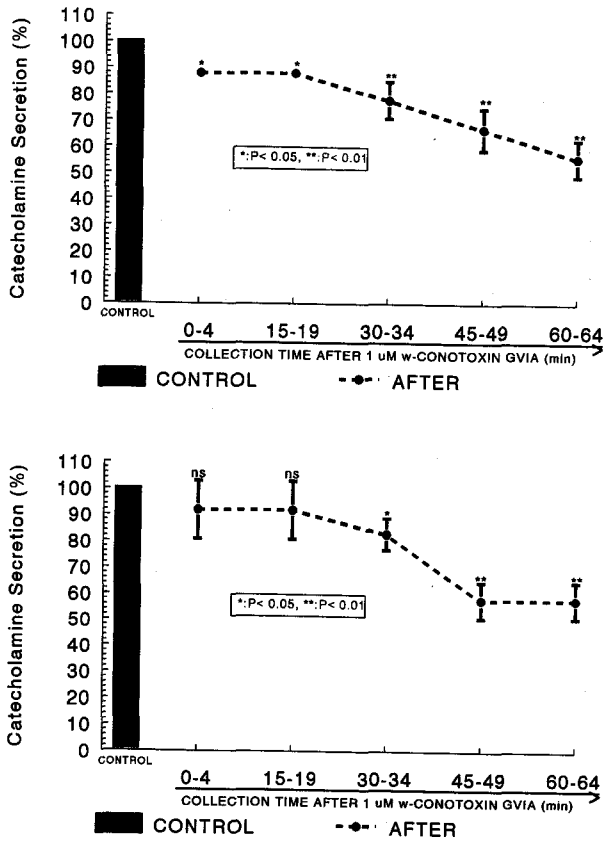


Fig. 6. Effects of ω-conotoxin GVIA on CA release evoked by Bay-K-8644 (Upper) and cyclopiazonic acid (Lower) from the rat adrenal glands. Bay-K-8644 (10^{-5} M) and cyclopiazonic acid (10^{-5} M) were perfused into an adrenal vein for 4 min at 15 min intervals after preloading with of ω-conotoxin GVIA (1 μM) for 60 min, respectively. Other legends are the same as in Fig. 1. *: $P<0.05$, **: $P<0.01$. ns: Statistically not significant.

the adrenal chromaffin cells and intracellular Ca^{2+} release from the cytoplasmic store.

Generally, it has been found that the adrenal medulla possesses characteristics of postganglionic sympathetic neurons, and both L- and N-type VDCCs have been identified in medullary chromaffin cells (Gandia et al, 1995). Adrenal CA secretion has been found to be mediated by muscarinic receptors as well as nicotinic receptors in various species (Harish et al, 1987; Nakazato et al, 1988), including the dog (Kimura et al, 1992).

In the present investigation, cilnidipine as well as ω-conotoxin GVIA infused into the rat adrenal gland significantly inhibited CA output evoked by DMPP as well as McN-A-343 and ACh without affecting the basal CA output. In support of this idea, it has been reported that cilnidipine has potent inhibitory actions on N-type as well as L-type VDCCs in rat dorsal root ganglion neurons (Fujii et al, 1997). Previously, by using experimental conditions similar to those in the anesthetized dog, it has been also demonstrated that the SNS-induced CA secretion is inhibited by ω-conotoxin GVIA, an N-type VDCC blocker, but not nifedipine and verapamil, L-type VDCC blockers (Kimura et al, 1994). Therefore, in terms of these findings, it is probable that cilnidipine inhibits the secretion of CA in response to DMPP as well as McN-A-343 and ACh through blockade of N-type VDCCs on the rat adrenomedullary chromaffin cells. In addition, the fact that ω-conotoxin GVIA also inhibited CA secretion by ACh as well as DMPP and McN-A343 indicates that it can depress the secretion of CA evoked by those agents through blockade of N-type VDCCs. Previously, it was also demonstrated that the exogenous ACh-induced CA secretion is inhibited by ω-conotoxin GVIA, nifedipine and verapamil in the anesthetized dog (Kimura et al, 1994). It has also been found that ω-conotoxins, ω-conotoxin MVIIA and ω-conotoxin GVIA potently block N-type Ca^{2+} channels (Kasai et al, 1987; McCleskey et al, 1987; Plummer et al, 1989; Regean et al, 1991; Miljanich and Ramachandran, 1995). From these results, it is suggested that cilnidipine inhibits the ACh-induced CA secretion through blockade of both L- and N-type VDCCs. Therefore, in the present study, it seems likely that the inhibition by cilnidipine of the ACh-induced CA secretion is attributed to its blocking action in N-type VDCCs.

Nagayama and his coworkers (1998) have reported that cilnidipine significantly inhibited both EP and NE out-put induced by DMPP, as well as SNS and ACh in the anesthetized dog. Previously, it was demonstrated, under the same experimental conditions, that the SNS-induced secretion of CA is mediated mainly by nicotinic receptors (Kimura et al, 1992; Shimamura et al, 1991). Based on these results, the inhibitory effect of cilnidipine on the DMPP-induced CA secretion indicates that cilnidipine inhibits the secretion by affecting the process mediated by nicotinic receptors. It has been also shown that ACh simulates the secretion of CA by activating both nicotinic and muscarinic receptors (Kimura et al, 1992). Therefore the inhibition by cilnidipine of the ACh-induced secretion of CA can be explained at least in part by its inhibitory action on nicotinic receptor-mediated pathway. Moreover, PC12 cells are derived from a rat pheochromocytoma cell line that is very popular for investigation neuronal differentiation. A typical L-type Ca^{2+} channel blocker such as nifedipine had a small effect on the Ca^{2+} channel- operated functions (Uneyama et al, 1998). This observation agreed with previous findings using rat superior ganglion neurons (Uneyama et al, 1997) and dorsal horn ganglion neurons (Fujii et al, 1997). In the present work, the results that cilnidipine greatly inhibited CA secretory responses evoked by cholinergic stimulation as well as by membrane depolarization suggest that the blockade of N-type Ca^{2+} channels by cilnidipine results in reduced Ca^{2+} influx through these channels and thereby reduces CA secretions closely linked with $[Ca^{2+}]_i$ elevation, which is evoked by various depolarizing stimulation. It is well characterized that Ca^{2+} influx through N-type Ca^{2+} channels is closely related to the CA secretion at the nerve endings of the sympathetic neurons as well (Hirning et al, 1988). Therefore, the blockade by cilnidipine of CA secretion from the isolated perfused model of adrenal gland might well explain why cilnidipine failed to induce elevation of plasma CA concentration by hypotension-evoked baroreflexes *in vivo* (Hosono et al, 1995) or why the drug blocked the NE release from isolated vessels (Nakashima et al, 1991). The present results are in agreement with the results in differentiated PC12 cells (Uneyama et al, 1998) and in anesthetized dog (Nagayama et al, 1998). In support of this idea, it has been well known that sources of plasma CA are mainly sympathetic nerve endings and adrenal chromaffin cells, at

which N-type Ca^{2+} channels are predominantly distributed. It has been also reported that the blockade of peripherally distributed N-type Ca^{2+} channels by ω-conotoxin GVIA led to reduction of plasma CA concentration by inhibition of its secretion from the vascular beds (Friedman and Duckles, 1994) and the anesthetized dog adrenal glands (Kimura et al, 1994). Especially, it is well known that CA secretion from sympathetic neurons is insensitive to L-type Ca^{2+} channel antagonist, but sensitive to ω-conotoxin GVIA (Hirning et al, 1988).

In the present investigation, the results that cilnidipine as well as ω-conotoxin GVIA inhibits CA secretion evoked by stimulation of muscarinic receptors with McN-A-343, a selective muscarinic M_1-receptor agonist, suggest strongly that muscarinic M_1-receptors are involved in the regulation of the secretory responses in the rat adrenal medulla. In support of this hypothesis, the muscarinic receptor- mediated secretion of adrenal CA is thought to be caused by Ca^{2+} mobilized from intracellular storage sites (Cheek and Burgoyne, 1985; Misbahuddin and Oka, 1988; Nakazato et al, 1988; Yamada et al, 1988). Furthermore, it has been shown that muscarinic stimulation generates a depolarizing signal, which triggers the firing of action potentials, resulting in the increased CA release in the rat chromaffin cells (Akaike et al, 1990), guinea pigs (Inoue and Kuriyama, 1991) and the perfused rat adrenal gland (Lim and Hwang, 1991). These observations are in line with a previous report showing that Bay-K-8644 almost tripled the peak secretory response to muscarine in perfused cat adrenal glands (Ladona et al, 1987; Uceda et al, 1992). In the present experiment, cilnidipine also depressed greatly CA secretion induced by Bay-K-8644, which is found to potentiate the release of CA by increasing Ca^{2+} influx through L-type Ca^{2+} channels in chromaffin cells (Garcia et al, 1984). Also, cilnidipine at higher concentrations ($3\sim10\,\mu M$) greatly attenuated CA secretions evoked by high potassium, a direct membrane-depolarizing agent. These findings that cilnidipine inhibited CA secretion evoked by Bay-K-8644 as well as by high K^+ suggest that cilnidipine inhibits directly the voltage-dependent Ca^{2+} channels.

The present study has also shown that cilnidipine inhibits the CA secretion evoked by cyclopiazonic acid. Cyclopiazonic acid is known to be a highly selective inhibitor of Ca^{2+}-ATPase in skeletal muscle sarcoplasmic reticulum (Geoger & Riley, 1989; Siedler et al, 1989) and a valuable pharmacological tool for investigating intracellular Ca^{2+} mobilization and ionic currents regulated by intracellular Ca^{2+} (Suzuki et al, 1992). Therefore, it is felt that the inhibitory effect of cilnidipine on CA secretion evoked by cholinergic stimulation as well as by membrane-depolarization may be associated with the mobilization of intracellular Ca^{2+} from the cytoplasmic calcium store. This indicates that the cilnidipine has an inhibitory effect on the release of Ca^{2+} from the intracellular pools induced by stimulation of muscarinic ACh receptors, which is weakly responsible for the secretion of CA. Suzuki and his coworkers (1992) have shown that cyclopiazonic acid easily penetrates into the cytoplasm through the plasma membrane and reduces Ca^{2+}-ATPase activity in sarcoplasmic/endoplasmic reticulum, resulting in increase in the subsequent Ca^{2+} release from those storage sites. Based on these results obtained from this study, it is felt that cilnidipine with N-type Ca^{2+} channel-blocking action may offer a new choice in treating hypertension, which is refractory to hypotensive Ca^{2+} channel antagonists or results from increased sympathetic nerve activity. In keeping with these therapeutic implications, some observations in rats and humans revealed that the oral administration of cilnidipine successfully reduced blood pressure elevated by stress stimulation (Saihara et al, 1993; Hosono et al, 1995). It might be also clinically used for controlling malignant hypertension resulting in hypersecretion of CA from human pheochromocytoma, because the PC12 cells are derived from the rat adrenal gland.

In conclusion, these results demonstrate that cilnidipine inhibits CA secretion evoked by stimulation of cholinergic (both nicotinic and muscarinic) receptors from the isolated perfused rat adrenal gland without affecting the basal release. However, at lower dose, cilnidipine did not affect CA release by membrane depolarization while at larger dose inhibited that. It seems likely that this inhibitory effect of cilnidipine is exerted by blocking both L- and N-type VDCCs on the rat adrenomedullary chromaffin cells, which is relevant to inhibition of both the Ca^{2+} influx into the adrenal chromaffin cells and intracellular Ca^{2+} release from the cytoplasmic store. It is thought that N-type VDCCs may play an important role in regulation of CA release from the rat adrenal medulla.

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REFERENCES

Akaike A, Mine Y, Sasa M, Takaori S. Voltage and current clamp studies of muscarinic and nicotinic excitation of the rat adrenal chromaffin cells. *J Pharmacol Expt Ther* 255: 333−339, 1990

Anton AH, Sayre DF. A study of the factors affecting the aluminum oxide trihydroxy indole procedure for the analysis of catecholamines. *J Pharmacol Exp Ther* 138: 360−375, 1962

Cheek TR, Burgoyne RD. Effects of activation of muscarinic receptors on intracellular free calcium and secretion in bovine chromaffin cells. *Biochim Biophys Acta* 846: 167−173, 1985

Friedman DJ, Duckles SP. Influence of age on control of norepinephrine release: Ca^{2+} channels and dopamine D2 receptors. *Eur J Pharmacol* 252:1−9, 1994

Fujii S, Kameyama K, Hosono M, Hayashi Y, Kitamura K. Effect of cilnidipine, a novel dihydropyridine Ca^{2+}-channel antagonist, on N-type Ca^{2+} channel in rat dorsal root ganglion neurons. *J Pharmacol Exp Ther* 280:1184-1191, 1997

Gandia L, Borges R, Albillos A, Garcia AG. Multiple calcium channel subtypes in isolated rat chromaffin cells. *Pflgers Arch* 430: 55−63, 1995

Garcia AG, Sala F, Reig JA, Viniegra S, Frias J, Fonteriz R, Gandia L. Dihydropyridine Bay-K-8644 activates chromaffin cell calcium channels. *Nature* 309: 69−71, 1984

Goeger DE, Riley RT. Interaction of cyclopiazonic acid with rat skeletal muscle sarcoplasmic reticulum vesicles. Effect on Ca^{2+} binding and Ca^{2+} permeability. *Biochem Pharmacol* 38: 3995−4003, 1989

Hammer R, Giachetti A. Muscarinic receptor subtypes: M_1 and M_2 biochemical and functional characterization. *Life Sci* 31: 2992−2998, 1982

Harish OE, Kao LS, Raffaniello R, Wakade AR, Shneider AS. Calcium dependence of muscarinic receptor-mediated catecholamine secretion from the perfused adrenal medulla. *J Neurochem* 48: 1730−1735, 1987

Hirning LD, Fox AP, McCleskey EW, Olivera BM, Thayer SA, Miller RJ, Tsien RW. Dominant role of N-type Ca^{2+} channels

in evoked release of norepinephrine from sympathetic neurons. *Science* 239:57−61, 1988

Hosono M, Iida H, Ikeda K, Hayashi Y, Dohmoto H, Hashiguchi Y, Yamamoto H, Watanabe N, Yoshimoto R. In vivo and ex vivo Ca-antagonistic effect of 2-methoxyethyl (E)-3-phenyl-2-propen-1-yl(+/−)-1,4-dihydro-2,6-dimethyl-4-(3-nitrophenyl) pyridine-3,5-dicarboxylate (FRC-8653), a new dihydropyridine derivative. *J Pharmacobiodyn* 15: 547−553, 1992

Hosono M, Fujii S, Himura T, Watanabe K, Hayashi Y, Ohnishi H, Takata Y, Kato H. Inhibitory effect of cilnidipine on vascular sympathetic neurotransmission and subsequent vasoconstriction in spontaneously hypertensive rats. *Jpn J Pharmacol* 69:127−134, 1995

Hosono M, Himura T, Watanabe K, Hayashi Y, Ohnishi H, Takata Y, Kato H. Inhibitory effect of cilnidipine on pressor response to acute cold stress in spontaneously hypertensive rats. *Jpn J Pharmacol* 69: 119−125, 1995

Ikeda K, Hosono M, Iida H, Ohnishi H. Antihypertensive and cardiovascular profiles of a newly synthesized dihydropyridine derivative 2-methoxyethyl (E)030phenyl-2-propen-1-yl(±)-1, 4-dihydro-2, 6-dimethyl-4-(3-nitrophenyl) pyridine-3, 5-dicarboxylate (FRC-8653). *Pharmacometrics* 44: 433−442, 1992

Inoue M, Kuriyama H. Muscarinic receptor is coupled with a cation channel through a GTP-binding protein in guinea-pig chromaffin cells. *J Physiol (Lond)* 436: 511−529, 1991

Kasai H, Aosaki H, Fukuda J. Presynaptic Ca^{2+}-antagonist omega-conotoxin irreversibly blocks N-type Ca^{2+} channels in chick sensory neurons. *Neurosci Res* 4: 228−235, 1987

Kimura T, Takeuchi A, Satoh S. Inhibition by omega-conotoxin GVIA of adrenal catecholamine release in response to endogenous and exogenous acetylcholine. *Eur J Pharmacol* 264: 169−175, 1994

Kimura T, Shimamura T, Satoh S. Effects of pirenzepine and hexamethonium on adrenal catecholamine release in responses to endogenous and exogenous acetylcholine in anesthetized dogs. *J Cardiovasc Pharmacol* 20: 870−874, 1992

Ladona MG, Aunis D, Gandia AG, Garcia AG. Dihydropyridine modulation of the chromaffin cell secretory response. *J Neurochemstry* 48: 483−490, 1987

Lim DY, Hwang DH. Studies on secretion of catecholamines evoked by DMPP and McN-A-343 in the rat adrenal gland. *Korean J Pharmacol* 27(1): 53−67, 1991

Lim DY, Kim CD, Ahn KW. Influence of TMB-8 on secretion of catecholamines from the perfused rat adrenal glands. *Arch Pharm Res* 15(2): 115−125, 1992

Mc Cleskey EW, Fox AP, Feldman D, Cruz LJ, Olivera BM, Tsien RW, Yoshikami D. ω-Conotoxin: direct and persistent block of specific types of Ca^{2+} channels in neurons but not muscle. *Proc Natl Acad Sci USA* 84: 4327−4331, 1987

Miljanich GP, Ramachandra J. Antagonists of neuronal calcium channels: structure, function and therapeutic implications. *Annu Rev Pharmacol Toxicol* 35: 707−734, 1995

Misbahuddin M, Oka M. Muscarinic stimulation of guinea pig adrenal chromaffin cells stimulates catecholamine secretion without significant increase in Ca^{2+} uptake. *Neurosci Lett* 87: 266−270, 1988

Nagayama T, Yoshida M, Suzuki-kusaba M, Hisa H, Kimura T, Satoh S. Effect of cilnidipine, a novel dihydropyridine Ca^{2+} channel blocker, on adrenal catecholamine secretion in anesthetized dogs. *J Cardiovasc Pharmacol* 32: 479−484, 1998

Nakazato Y, Oleshanskly M, Tomita U, Yamada Y. Voltage- independent catecholamine release mediated by the activation of muscarinic receptors in guinea-pig adrenal glands. *Br J Pharmacol* 93:101−109, 1988

Nakashima M, Kou M, Hashitani H, Chen G, Ono H, Kuriyama H. Suzuki H. Action of FRC-8653 on smooth muscle cells of the rabbit mesenteric artery. *Jpn J Pharmacol* 57: 51−61, 1991

Oike M, Inoue Y, Kitamura K, Kuriyama H. Dual action of FRC8653, a novel dihydropyridine derivative, on the Ca^{2+} current recorded from the rabbit basilar artery. *Circ Res* 67: 993−1006, 1990

Plummer MR, Logothetis DE, Hess P. Elementary properties and pharmacological sensitivities of calcium channels in mammalian peripheral neurons. *Neuron* 2: 1453−1463, 1989

Regan LJ, Sah DWY, Bean BP. Ca^{2+} channels in rat central and peripheral neurons: high threshold current resistant to dihydropyridine blockers and ω-conotoxin. *Neuron* 6: 269−280, 1991

Saihara S, Sumio M, Hosono N, Hayashi Y, Kitamura Y, Hashimoto S. Effect of FRC-8653 (cilnidipine) on blood pressure changes during the cold pressor test. *Jpn Pharmacol Ther* 21: 271−276, 1993

Schramm M, Thomas G, Towart R, Franckowiak G. Novel dihydropyridines with positive inotropic action through activation of Ca^{2+} channels. *Nature* 303: 535−537, 1982

Seidler NW, Jona I, Vegh N, Martonosi A. Cyclopiazonic acid is a specific inhibitor of the Ca^{2+}-ATPase of sarcoplasimc reticulum. *J Biol Chem* 264: 17816−17823, 1989

Shimamura T, Kimura T, Satoh S. Effects of pirenzepine, AF-DX 116 and gallamine on the release of catecholamines from the dog adrenal gland in response to splanchnic nerve stimulation: interaction of M_1 and M_2 receptors with nicotinic receptors. *J Pharmacol Exp Ther* 257: 369−373, 1991

Suzuki M, Muraki K, Imaizumi Y, Watanabe M. Cyclopiazonic acid, an inhibitor of the sarcoplasmic reticulum Ca^{2+}-pump, reduces Ca^{2+}-dependent K^+ currents in guinea-pig smooth muscle cells. *Br J Pharmacol* 107: 134−140, 1992

Tallarida RJ, Murray RB. Manual of pharmacologic calculation with computer programs. 2nd Ed. New York, Speringer-Verlag, p 132, 1987

Tominaga M, Ohya Y, Tsukashima A, et al. Ambulatory blood pressure monitoring in patients with essential hypertension treated with a new calcium antagonist, cilnidipine. *Cardiovasc Drugs Ther* 11: 43−48,1997

Uceda G, Artalejo AR, de la Fuente MT, Lopez MG, Albillos A, Michelena P, Garcia AG, Montiel C. Modulation by L-type Ca^{2+} channels and apamin-sensitive K^+ channels of muscarinic responses in cat chromaffin cells. *Am J Physiol* 266(5 Pt 1): C1432−1439, 1992

Uneyama H, Uchida H, Yoshimoto R, Ueno S, Inoue K, Akaike N. Effects of a novel antihypertensive drug, cilnidipine, on catecholamine secretion from differentiated PC12 cells. *Hypertension* 31: 1195−1199, 1998

Uneyama H, Takahara A, Dohmoto H, Yoshimoto R, Inoue K, Akaike N. Blockade of N-type Ca^{2+} current by cilnidipine (FRC-8653) in acutely dissociated rat sympathetic neurones. *Br J Pharmacol* 122: 37−42, 1997

Wada Y, Satoh K, Taira N. Cardiovascular profile of Bay-K-8644, a presumed calcium channel activator in the dog. *Naunyn-Schmiedebergs Arch Pharmacol* 328: 382−387, 1985

Wakade AR. Studies on secretion of catecholamines evoked by acetylcholine or transmural stimulation of the rat adrenal gland. *J Physiol* 313: 463−480, 1981

Yamada Y, Teraoka H, Nakazato Y, Ohga A. Intracellular Ca^{2+} antagonist TMB-8 blocks catecholamine secretion evoked by caffeine and acetylcholine from perfused cat adrenal glands in the absence of extracellular Ca^{2+}. *Neurosci Lett* 90: 338−342, 1988

Yoshimoto R, Dohmoto H, Yamada K, Goto A. Prolonged inhibition of vascular contraction and calcium influx by the novel 1,4-dihydropyridine calcium antagonist cinaldipine. *Jpn J Pharmacol* 56: 225−229, 1991