Effects of a Novel Antihypertensive Drug, Cilnidipine, on Catecholamine Secretion From Differentiated PC12 Cells

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Abstract—Effects of a novel dihydropyridine type of antihypertensive drug, cilnidipine, on the regulation of the catecholamine secretion closely linked to the intracellular Ca$^{2+}$ were examined using nerve growth factor (NGF)–differentiated rat pheochromocytoma PC12 cells. By measuring catecholamine secretion with high-performance liquid chromatography coupled with an electrochemical detector, we showed that high K$^+$ stimulation evoked dopamine release from PC12 cells both before and after NGF treatments. Cilnidipine depressed dopamine release both from NGF-treated and untreated PC12 cells in a concentration-dependent manner. In contrast, inhibition by nifedipine was markedly decreased in the differentiated PC12 cells. With intracellular Ca$^{2+}$ concentration ([Ca$^{2+}$]) measurements using fura 2, the elevation of high K$^+$–evoked [Ca$^{2+}$] was separated into nifedipine-sensitive and -resistant components. The nifedipine-resistant [Ca$^{2+}$] increase was also blocked by cilnidipine, as well as ω-conotoxin-GVIA. By the use of the conventional whole-cell patch-clamp technique, the compositions of the high-voltage–activated Ca$^{2+}$ channel currents in the NGF-treated PC12 cells were divided into types: L-type, N-type, and residual current components. It was also estimated that cilnidipine at 1 and 3 μmol/L strongly blocked the N-type current without affecting the residual current. These results suggest that cilnidipine inhibits catecholamine secretion from differentiated PC12 cells by blocking Ca$^{2+}$ influx through the N-type Ca$^{2+}$ channel, in addition to its well-known action on the L-type Ca$^{2+}$ channel. (Hypertension. 1998;31:1195-1199.)

Key Words: cilnidipine ■ calcium channels ■ calcium, cytoplasmic ■ dopamine

Cilnidipine (FRC-8653) is a newly synthesized DHP type of organic Ca$^{2+}$ channel blocker that has been developed as a slow-onset and long-lasting antihypertensive drug in Japan. Recent electrophysiological data indicate that cilnidipine might be a dual-channel antagonist for peripheral neuronal N-type and vascular L-type Ca$^{2+}$ channels. In humans and rodents, cilnidipine depressed the pressor response to acute cold stress but failed to induce tachycardia evoked by hypotensive baroreflexes. In spontaneously hypertensive rats, vasoconstriction induced by electrical sympathetic nerve stimulation was also blocked by cilnidipine. In vitro experiments, cilnidipine also inhibited [H]$^+$norepinephrine release evoked by electrical stimulation in the rabbit mesenteric artery.

PC12 cells are derived from a rat pheochromocytoma cell line that is very popular for investigating neuronal differentiation. In response to externally applied NGF, PC12 cells acquire sympathetic neuronal characteristics such as neurite extension, increased CA synthesis, and expression of neuronal types of voltage-dependent N-type Ca$^{2+}$ channels. Therefore, the differentiated PC12 cells are widely used as model cells for studying the intracellular mechanisms of the stimulus-secretion coupling, including the activation of the N-type Ca$^{2+}$ channel. With this model cell, it has been revealed that endogenous substances such as neuroptide Y and proadrenomedullin N-terminal 20 peptide inhibit CA release by inhibiting Ca$^{2+}$ influx through N-type Ca$^{2+}$ channels. In the present study, we examined the effects of cilnidipine on the cellular functions of the NGF-treated PC12 cells, including CA secretion and intracellular Ca$^{2+}$ mobilization triggered by membrane depolarizations. Consequently, in addition to its well-known L-type Ca$^{2+}$ channel blockade, it was clarified that cilnidipine had a potent inhibitory effect for CA secretion from differentiated PC12 cells via the blockade of extracellular Ca$^{2+}$ influx through the N-type Ca$^{2+}$ channel.

Methods

Cell Culture
PC12 cells were prepared as described previously. In brief, cells were cultured in Dulbecco’s modified Eagle’s medium containing 7% fetal bovine serum (Gibco), 7% heat-inactivated (56°C, 40 minutes) horse serum (Cell Culture Laboratories), 2 mmol/L L-glutamine, and 50 μg/mL gentamicin sulfate (Boehringer Mannheim GmbH) in a humidified atmosphere of 90% air and 10% CO$_2$ at 37°C. Cells were plated onto poly-L-lysine–coated glass coverslips (24×60×0.15 mm), placed in silicone rubber walls (Flexiperm, W.C. GmbH) at a density of 2.5×10$^3$ cells per well (8×11 mm), and cultured for an additional 2 days. For measurement of [Ca$^{2+}$], in
single cells, cells were plated onto poly-L-lysine–coated glass coverslips at a density of $2.5 \times 10^3$ cells per well ($8 \times 11$ mm).

**Dopamine Release**

The released DA was measured as described by Ohara-Imaizumi et al. All the procedures, including incubation, washing, and drug application, were performed at room temperature using 1 mL per dish of balanced salt solution (BSS) with the following composition (mmol/L): NaCl 150, KCl 5, CaCl$_2$ 1.2, MgCl$_2$ 1.2, d-glucose 10, and HEPES 25 (pH adjusted to 7.4 with NaOH). Cells were incubated with BSS for 1 hour, and then the BSS containing 80 mmol/L KCl was added to the dishes and incubated for 1 minute. At the end of the incubation period, solutions were transferred to tubes containing 0.25 mL of 1 mol/L HClO$_4$. Cells were then sonicated, and DA residing in cells was extracted with 0.2 mol/L HClO$_4$. After centrifugation (at 4°C for 5 minutes, 10 000g), supernatants of both incubation solutions and the sonicated cellular solutions were collected for the measurement of DA content. DA content was determined with high-performance liquid chromatography coupled with an electrochemical detector (HPLC/ECD). Each supernatant (20 μL) was applied to the HPLC/ECD system, which consisted of a reverse-phase HPLC column (ODS, 0.003 mm; 4.6 mm), and an ECD (LC-4B and TL-5A, Bioanalytical Systems) with the electrode potential set at +0.6 V versus the Ag/AgCl reference electrode. The mobile phase consisted of a monochloroacetate buffer (140 mmol/L, pH 3.05) containing 10% methanol, 30 mg/L sodium 1-octanesulfonate, and 1.8 mmol/L EDTA. Flow rate was 1.3 mL/min. The data were analyzed with a chromatographic processor (Shimazu C-R4A, Shimazu Corp). The percentage of DA release was calculated using the values obtained for the DA content in the incubation solution (A) and the DA content remaining in the cells (B) using the following equation: % of total DA contents = 100×A/(A+B).

**[Ca$^{2+}$]** Measurements

The increase in [Ca$^{2+}$], in single cells was measured by the fura 2 method as described by Grynkiewicz et al. with minor modifications. The cells were washed with BSS and incubated with 10 μmol/L fura 2-AM (Dojindo) at 37°C in BSS. Thirty minutes later, the cells were washed twice with 0.2 mL BSS. The coverslips were mounted on an Olympus IMT-2 inverted epifluorescence microscope equipped with a 75-W xenon lamp and band-pass filters of 340-nm wavelength for detection of Ca$^{2+}$-dependent signal (F$_{340}$) and 360-nm wavelength for detection of Ca$^{2+}$-independent signal (F$_{360}$). Measurements were obtained at room temperature. Image data, recorded by a high-sensitivity silicon intensified target camera (C-2741-08, Hamamatsu Photonics), were processed by a personal computer.

**Electrophysiological Measurements**

Electrophysiological measurements were performed in a whole-cell mode by using a conventional patch-clamp technique. The resistance between the recording electrode filled with internal solution and reference electrode in external solution was 2 to 5 MΩ. The current and voltage were measured with a patch-clamp amplifier (List Medical, EPC-7), monitored on a storage oscilloscope (Iwatsu DS-9121), and then stored on DAT tape with a PC processor (TEAC RD-120TE) after being filtered at 1 kHz (NF Instruments). Then, analog signals were reconverted into digital signals with an AD converter at a sampling frequency of 3 kHz; the signals were stored with an IBM-compatible computer using pClamp software.

**Data Analysis**

Experimental values are presented as mean±SEM. Statistical differences in values for DA release or [Ca$^{2+}$], increase were determined using ANOVA followed by Dunnett’s test for multiple comparison; a value of $P<.05$ was considered to be significant.

**Results**

**Effects of Cilnidipine on CA Secretion From Differentiated PC12 Cells**

Rat PC12 cells exhibited neurite outgrowth in response to externally applied NGF (100 ng/mL) for 8 days (Fig 1). Rat PC12 cells used in the present study were changed to contain norepinephrine in addition to DA. However, since the amount of norepinephrine content was less than that of DA (data not shown), we measured the rate of DA production as an indication of the CA release process in the following experiments. Depolarization with 80 mmol/L KCl evoked DA release from the PC12 cells both before and after the NGF treatments (8.53±0.65% release of a total DA content in undifferentiated PC12 cells and 7.21±0.49% in the differentiated PC12 cells; each $n=12$). This stimulation was dependent on extracellular Ca$^{2+}$ influx through voltage-dependent Ca$^{2+}$ channels because it was completely prevented by addition of 100 μmol/L Cd$^{2+}$, a nonselective inorganic Ca$^{2+}$ channel antagonist ($n=3$). The DHP types of Ca$^{2+}$ channel...
antagonists, cilnidipine and nifedipine, strongly blocked DA release from the undifferentiated PC12 cells (Fig 2). The IC₅₀ values for cilnidipine and nifedipine were 6×10⁻³ and 8×10⁻⁴ mol/L, respectively. In contrast, in the NGF-differentiated PC12 cells, inhibitory potency of nifedipine was markedly reduced (Fig 2B), whereas the inhibitory effect of cilnidipine was almost equal (Fig 2A) to that observed in the PC12 cells. The IC₅₀ values for cilnidipine and nifedipine were 7×10⁻⁸ and >10⁻⁷ mol/L, respectively. NGF differentiation also reduced the blocking potency of L-type Ca²⁺ channel antagonists other than nifedipine, such as verapamil (phenylalkylamine-type) and diltiazem (benzothiazepine-type). The inhibitory percentages of 10 μmol/L verapamil and 10 μmol/L diltiazem on 80 mmol/L KCl–evoked DA release were 82.48±6.6% and 37.96±5.53% (each, n=3) in the undifferentiated PC12 cells and 37.99±2.29% and 41.02±1.40% (each, n=3) in the NGF-differentiated PC12 cells, respectively. In contrast, an N-type Ca²⁺ channel antagonist peptide, ω-Cg-GVIA, had no effect on the high K⁺–evoked DA release from undifferentiated cells (0.84±6.29% inhibition, n=6) but inhibited the DA release from the differentiated PC12 cells (39±5.1% inhibition, n=6). This observation strongly indicated that the DA release mediated by the N-type Ca²⁺ channel activation was caused by the cell differentiation.

Effects of Cilnidipine on [Ca²⁺], Elevation in Differentiated Rat PC12 Cells

Because Ca²⁺ influx through Ca²⁺ channels is closely related to the CA secretion in rat PC12 cells, we measured [Ca²⁺], in differentiated PC12 cells. Eight days after NGF treatment, [Ca²⁺], elevated by 80 mmol/L KCl was blocked by 25±2% with nifedipine (n=18). As shown in Fig 4, 80 mmol/L KCl evoked a rapid [Ca²⁺], elevation even in the presence of a high concentration of nifedipine (10 μmol/L), and the nifedipine-resistant [Ca²⁺], elevation was successfully inhibited by the further administration of ω-Cg-GVIA (Fig 4A,a) or cilnidipine in a concentration-dependent manner (Fig 4A,b). Under this experimental condition, we confirmed that the initial high K⁺ response was reproducible at least for four stimulations (data not shown, n=3). The quantitative results are summarized in Fig 4B. The nifedipine-resistant [Ca²⁺], elevation was significantly blocked by cilnidipine as well as ω-Cg-GVIA. In addition, the nifedipine- and ω-Cg-GVIA-resistant [Ca²⁺], elevation was not influenced by the further application of 10 μmol/L cilnidipine (72±9% inhibition, n=10). These results suggest that cilnidipine blocks Ca²⁺ influx through nifedipine-resistant Ca²⁺ channels (possibly N-type).

Cilnidipine Blockade of HVA Ca²⁺ Current in Differentiated Rat PC12 Cells

Finally, the direct effect of cilnidipine on the Ca²⁺ channels in the differentiated rat PC12 cells was investigated by using the

As a next step, we estimated whether the potent blocking action of cilnidipine on the DA release from the differentiated cells might be derived from the blockade of channels other than the L-type Ca²⁺ channel. We examined the effect of cilnidipine on the residual releases after abolishing the L-type Ca²⁺ channel–dependent DA release by nifedipine. About 58% of the high K⁺–evoked DA releases from the NGF-differentiated cells remained, even in the presence of 10 μmol/L nifedipine (n=6). Under these conditions, it was clarified that cilnidipine (1 μmol/L) as well as ω-Cg-GVIA (1 μmol/L) inhibited the nifedipine-resistant DA release (Fig 3).
conventional whole-cell patch-clamp technique. To suppress the Ca\(^{2+}\)-dependent inactivation, we used 10 mmol/L Ba\(^{2+}\) instead of Ca\(^{2+}\) as a charge carrier through the HVA Ca\(^{2+}\) channels. The typical current traces are shown in Fig 5A.a. In this experiment, 1 mmol/L \(\omega\)-Cg-GVIA was first applied to the cell, then 10 mmol/L nifedipine was applied, and finally 100 mmol/L Cd\(^{2+}\) was applied. The composition of the HVA \(I_{\text{Ba}}\) was classified into current compounds that were \(\omega\)-Cg-GVIA-sensitive (N-type: 40\% \pm 6\%) and nifedipine-sensitive (L-type: 13\% \pm 6\%) (Fig 5A.a and 5B). Blocking potencies of 3 and 10 mmol/L nifedipine were 53 \% \pm 8\% and 75 \% \pm 10\%, respectively (n=4). Finally, the effect of cilnidipine on the residual non-L-type and N-type component was also examined (Fig 6). In this experiment, L- and N-type components were completely abolished by treatment with 10 mmol/L nifedipine and 1 mmol/L \(\omega\)-Cg-GVIA (Fig 6A). Consequently, cilnidipine failed to inhibit the \(\omega\)-Cg-GVIA–and nifedipine-resistant current up to 3 mmol/L. These results suggest that cilnidipine blocks \(\omega\)-Cg-GVIA–sensitive N-type current component in differentiated rat PC12 cells.

**Discussion**

In the present experiment, we investigated the effects of cilnidipine on DA release, [Ca\(^{2+}\)]i elevation, and Ca\(^{2+}\) channel currents in differentiated rat PC12 cells. The PC12 cells used in this study clearly showed neurite outgrowth, and \(\omega\)-Cg-GVIA–sensitive N-type Ca\(^{2+}\) currents. A typical L-type Ca\(^{2+}\) channel blocker such as nifedipine had a small effect on the Ca\(^{2+}\) channel–operated functions. These characteristics are consistent with those of differentiated PC12 cells, which have characteristics similar to those of peripheral sympathetic neurons, including the development of N-type Ca\(^{2+}\) channels.\(^{10\text{-}12,22}\)

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**Figure 4.** Effects of cilnidipine on nifedipine-resistant [Ca\(^{2+}\)]i elevation in differentiated rat PC12 cells. A, Typical traces obtained with two individual cells. Nifedipine (10 mmol/L) was continuously applied during experiments. \(\omega\)-Cg-GVIA (a) and cilnidipine (b) were applied for 2 minutes just before 80 mmol/L KCl stimulation. B, Effects of cilnidipine and \(\omega\)-Cg-GVIA. Each point and vertical bar represent the mean \pm SEM from three experiments. **P<.01 vs nifedipine-treated (control) group (Dunnett’s test for multiple comparison).

**Figure 5.** Cilnidipine blocks the nifedipine-resistant HVA Ca\(^{2+}\) channel currents. A, a: Composition of HVA \(I_{\text{Ba}}\). PC12 cells were held at -60 mV and HVA \(I_{\text{Ba}}\) was elicited by 200-ms depolarizing pulses to 0 mV. First, 1 mmol/L \(\omega\)-Cg-GVIA was applied, and subsequently 10 mmol/L nifedipine was applied. The residual current resistant to these antagonists was completely blocked by 100 mmol/L Cd\(^{2+}\). A, b: Effects of cilnidipine on the HVA \(I_{\text{Ba}}\). First, 10 mmol/L nifedipine was applied, and then 3 mmol/L cilnidipine was applied in the presence of nifedipine. \(\omega\)-Cg-GVIA (1 mmol/L) was applied last to the cell. All recordings were obtained from two individual cells. B, The concentration-dependent effects of cilnidipine in the presence of 10 mmol/L nifedipine. Each concentration of cilnidipine was applied to the neuron 2 minutes before the subsequent five depolarizing pulses. Each point plotted was typical of four reproducible observations.

**Figure 6.** Effects of cilnidipine on nifedipine- and \(\omega\)-Cg-GVIA-resistant HVA Ca\(^{2+}\) channel currents. A, Effects of 3 mmol/L cilnidipine. N-type and L-type current components were completely blocked by treatment with 1 mmol/L \(\omega\)-Cg-GVIA and 10 mmol/L nifedipine, respectively. Each current trace was obtained with the same PC12 cell. B, Concentration-dependent effects of cilnidipine on the \(\omega\)-Cg-GVIA- and nifedipine-resistant current. Each concentration of cilnidipine was applied to the neuron 2 minutes before the subsequent five depolarizing pulses were applied every 15 seconds. The amplitude of \(I_{\text{Ba}}\) was measured at the peak. The current recorded before application of cilnidipine was normalized as 1.0. Each point and vertical bar represent the mean \pm SEM from four experiments.
In the present study, we clearly showed that a DHP type of antihypertensive drug, cilnidipine, strongly inhibited the high K+-evoked CA secretion, [Ca2+]e, elevation, and HVA Ca2+ channel currents in differentiated rat PC12 cells. In contrast to cilnidipine, the selective L-type Ca2+ channel antagonist nifedipine showed a weak inhibition on all three parameters in the PC12 cells after differentiation by NGF (Fig 1B). Cilnidipine directly blocked the isolated N-type channel current (IC50 value, ~1 μmol/L) but had no effect on the R-type channel current in the differentiated rat PC12 cells (Figs 5 and 6). This observation agreed with previous findings using rat superior ganglion neurons1 and dorsal horn ganglion neurons.3 Blockade of N-type Ca2+ channels by cilnidipine results in reduced Ca2+ influx through these channels and thereby reduces CA secretions closely linked with [Ca2+]e, elevation, which is evoked by various depolarizing stimulations. It is well characterized that Ca2+ influx through N-type Ca2+ channels is closely related to the CA secretion at the nerve endings of the sympathetic neurons as well.23 The blockade of CA secretion from the differentiated PC12 cells might well explain why cilnidipine failed to induce elevation of plasma CA concentration by hypotension-evoked baroreflexes in vivo3 or why the drug blocked the norepinephrine release from isolated vessels.9 Sources of plasma CA were mainly sympathetic nerve endings and adrenal chromaffin cells, at which N-type Ca2+ channels were predominantly distributed. It was reported that the blockade of peripherally distributed N-type Ca2+ channels by α-Cg-GVIA led to reduction of plasma CA concentration by inhibition of its secretion from the vascular beds32 and adrenal glands.32 Especially, it is well known that CA secretion from sympathetic neurons is insensitive to L-type Ca2+ channel antagonist but sensitive to α-Cg-GVIA.23 Therefore, cilnidipine with N-type Ca2+ channel blocking action may offer a new choice in treating hypertension which is refractory to hypotensive Ca2+ channel antagonists or results from increased sympathetic nerve activity. In keeping with these therapeutic implications, recent observations in rats and humans revealed that the oral administration of cilnidipine successfully reduced blood pressure elevated by stress stimulations.6,8 It might also be clinically used for controlling malignant hypertension resulting in hypersecretion of CA from human pheochromocytoma, because the PC12 cells used here are derived from rat pheochromocytoma cell line. J Physiol (Lond). 1990;426:95–116. 9. Passafaro M, Rosa P, Sala C, Clementi F, Sher E. N-type Ca2+ channels are present in secretory granules and are transiently translocated to the plasma membrane during regulated exocytosis. J Biol Chem. 1996;271:30096–30104. 10. Greene LA, Tischler AS. PC12 pheochromocytoma cultures in neurobiological research. Adv Cell Neurobiol. 1982;3:373–414. 11. Takahashi M, Tsukui H, Hamada H. Neuronal differentiation of Ca2+-channel antagonist by nerve growth factor. Brain Res. 1985;341:381–384. 12. Usowicz M, Porzig M, Becker C, Reuter H. Differential expression by nerve growth factor of three types of Ca2+-channels present in rat pheochromocytoma cell line. J Physiol (Lond). 1990;426:95–116. 13. Chen X, Westfall TC. Modulation of intracellular calcium transients and norepinephrine release by cilnidipine. J Pharmacol Exp Ther. 1995;277:489–497. 14. Takano K, Yamashita N, Fujii S, Kameyama K, Hosono M, Hayashi Y, Kitamura K. Effect of cilnidipine, a novel dihydropyridine Ca(2+)-channel antagonist, on N-type Ca2+ channel in rat dorsal root ganglion neurons. J Pharmacol Exp Ther. 1997;280:1184–1191. 15. Uneyama H, Takahara A, Dohmoto H, Yoshimoto R, Inoue K, Akaake N. Blockade of N-type Ca2+ current by cilnidipine (FRC-8653) in acutely dissociated rat sympathetic neurons. Br J Pharmacol. 1997;122:37–42. 16. Friedman DJ, Dackles SP. Influence of age on control of norepinephrine release: Ca2+ channels and dopamine D2 receptors. Eur J Pharmacol. 1994;252:1–9. 17. Kimura T, Takeuchi A, Satoh S. Inhibition by omega-conotoxin GVIA of adrenalin catecholamine release in response to endogenous and exogenous acetylcholine. Eur J Pharmacol. 1994;264:169–175. 18. Uneyama et al May 1998 1199

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