J. Pharm. Pharmacol. 1993, 45: 530–534
Received July 30, 1992

Inhibitory Effect of NZ-105, a 1,4-Dihydropyridine Derivative, on Cyclic Nucloeotide Phosphodiesterase Activity

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Abstract—The effects of NZ-105, a 1,4-dihydropyridine calcium antagonist, on the intracellular cyclic nucleotide system were investigated in-vitro. In rabbit isolated aorta, both NZ-105 (1 and 10 μM) and nicardipine significantly and in a concentration-dependent manner increased intracellular cyclic AMP and cyclic GMP content. NZ-105 inhibited bovine cardiac phosphodiesterase activity (K_i 30 μM) by competitive antagonism. The concentration ranges for inhibition were consistent with the range of increases in cyclic nucleotides.

1,4-Dihydropyridine (DHP) derivatives have a regulatory effect on calcium influx through voltage-dependent calcium channels on the cell membrane. Several 1,4-DHP derivatives, such as nifedipine (Norman et al 1983; Minocherhomjee & Roufogalis 1984), nicardipine (Sakamoto et al 1978; Endoh et al 1980; Nishikori et al 1981) and nimodipine (Schachtele et al 1987), reportedly increase intracellular adenosine 3′,5′-cyclic monophosphate (cAMP) levels as a result of their inhibitory effect on cyclic nucleotide phosphodiesterase (PDE) activity.

NZ-105, (±)-2-[benzyl(phenyl)amino]ethyl 1,4-dihydro-2,6-dimethyl-5-(5,5-dimethyl-2-oxo-1,3,2-dioxaphosphorinan-2-yl)-4-(3-nitrophenyl)-3-pyridinecarboxylate hydrochloride ethanol, is a 1,4-DHP derivative, which we are developing as an antihypertensive agent (Masuda et al 1990; Sakai et al 1991a, b). We have previously shown that NZ-105 has a vasodilator action attributable to its calcium antagonistic effect in high-K^+ pre-contracted arteries of rabbit (Masuda et al 1991a). The results of earlier electrophysiological observations (Tamura et al 1991) and our previous biochemical studies involving $^{45}Ca^{2+}$-uptake experiments (Yamashita et al 1991) confirm that NZ-105 is a calcium antagonist. However, the effects of NZ-105 on intracellular cyclic nucleotide levels and PDE activity remain unclear. On the other hand, we have reported that the negative inotropic effect, but not chronotropic effect, of NZ-105 is much weaker than that of nicardipine (Masuda et al 1991b). If NZ-105 increases the cardiac cAMP level, its essential negative inotropic action may be offset.

In the present study, the inhibitory effect of NZ-105 on the activity of purified bovine heart cyclic nucleotide PDE and its effect on intracellular cyclic nucleotide levels in rabbit aortic strips were investigated in-vitro.

Materials and Methods

Assay of rat aorta cAMP and cGMP

Rabbits were killed, and their thoracic aortas were removed and cut into annular strips (about 5 mm long). The strips were equilibrated for 20 min at 37°C in Krebs-Henseleit solution through which a mixture of 95% O_2–5% CO_2 was bubbled. The solution was composed (mM) of NaCl 118·4, KCl 4·7, $MgSO_4$ 1·2, $CaCl_2$ 2·5, KH_2PO_4 1·2, $NaHCO_3$ 25, and glucose 11·7. After equilibrating the tissues, NZ-105 (1 and 10 μM), nicardipine (1 and 10 μM), 3-isobutyl-1-methylxanthine (IBMX) (100 μM and 1 mM), forskolin (1 and 10 μM) and sodium nitroprusside (10 and 100 μM) were added and incubated for 5 min. Following incubation, the tissues were blotted with filter paper (less than 3 s) and then frozen in liquid nitrogen.

The method of extracting cAMP and cGMP from the aorta was as follows: each frozen specimen was homogenized (2 min) in 1 mL of 6% trichloroacetic acid under ice-cold conditions using a Polytron. The homogenate was centrifuged at 1000 *g* at 4°C for 10 min, and the supernatant was collected. The pellet was re-homogenized under the same conditions, and the previous supernatant was added to this supernatant. The supernatant was shaken with triple the volume of water-saturated diethylether three times, and the aqueous layer was collected each time. The remaining diethylether was removed at 40°C.

The cAMP and cGMP extracted was succinylated at room temperature (21°C) with the same volume of a reaction mixture composed of succinic anhydride in 1,4-dioxane and triethylamine (9:1). After succinylation, the reaction was stopped with 0·3 M imidazole buffer (pH 6·5) under ice-cold conditions.

The cAMP and cGMP content of the assay samples was determined using a radioimmunoassay kit (Yamasa Shoyu Ltd, Choshi, Japan) which uses [^{125}I]succinyl cAMP tyrosine methylester as the standard and rabbit anti-cAMP serum and [^{125}I]succinyl cGMP tyrosine methylester as the standard and cGMP monoclonal antibody. The radioactivity of each was determined with an auto-gamma counter (Auto-Gamma 500C, Packard). The protein content of the final pellet in the extraction process was determined by the method described by Lowry et al (1951) using bovine serum albumin as the standard.

Assay procedures for bovine heart cAMP PDE activity

PDE activity was determined by the method described by Fukuda et al (1981) with slight modification. The standard

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reaction mixture consisted (mM) of: Tris-HCl buffer (pH 7·5) 30, NaCl 100, $MgCl_2$ 5, 2-mercaptoethanol 1 with [^{3}H]cAMP (1 or 10 μM) and bovine serum albumin (50 μg).

NZ-105, nicardipine or vehicle was added to the standard reaction mixture, which was maintained at 30°C, and the PDE enzyme preparation (8·36 μg) was then added to the standard reaction mixture, including the drugs. The reaction mixture was incubated for 10 min (or the various times shown in Results in the activity vs time experiment). After the reaction, the reaction tubes were immediately placed in boiling water to terminate the reaction. Basic and activated alumina 70–230 mesh, used for column chromatography, (Merck), and water were added to the resultant mixtures and stirred well (30 s). The mixtures were centrifuged at 10 000 *g* for 10 min, and the supernatant (including [^{3}H]cAMP) was collected and the radioactivity measured with 10 mL scintillator (Biofluor, New England Nuclear) with a liquid scintillation counter (Tri-Carb 460CD, Packard) at an efficiency of 45%.

Animals

Male Japanese White rabbits, 2·4–2·9 kg, were purchased from Tokyo Animal Laboratory Inc. (Tokyo, Japan). All rabbits were killed by venesection under deep anaesthesia induced by intravenous injection of an overdose of sodium pentobarbitone.

Enzyme preparations

Bovine heart phosphodiesterase 3′,5′-cyclic nucleotide (PDE) (0·092 units (mg protein)$^{-1}$) (Ho et al 1976) was used as the purified enzyme preparations. The enzyme preparations were purchased from Sigma Chemical Co. (St Louis, MO, USA). This PDE enzyme (P_I fraction) reportedly hydrolyses both cAMP and cGMP (Ho et al 1976).

Compounds

NZ-105 was synthesized at the Central Research Laboratories of Nissan Chemical Industries Ltd (Chiba, Japan). The other compounds used were nicardipine hydrochloride (Mediolast SpA, Italy), IBMX, forskolin, sodium nitroprusside and cAMP (Sigma Chemical Co., St Louis, MO, USA). cAMP was dissolved in 30 mM Tris-HCl buffer (pH 7·5), and the other compounds were dissolved in ethanol and diluted with water.

[5′,8-^{3}H]cAMP, ammonium salt (Amersham International plc) was used to measure PDE activity.

Statistical analysis

The inhibition style of each compound was determined by Lineweaver–Burk analysis. Statistically significant differences were calculated using a paired *t*-test.

Results

Effect of NZ-105 and reference compounds on rabbit aorta cAMP and cGMP

Both NZ-105 (1 and 10 μM) and nicardipine significantly and in a concentration-dependent manner increased both the cAMP and cGMP content of the rabbit aorta (Tables 1, 2). Forskolin, a direct activator of the adenylate cyclase system (Seamon et al 1981), significantly increased only the cAMP

Table 1. Effect of NZ-105 and reference compounds on the cAMP content of rabbit isolated aorta.

Compounds	cAMP (pmol (mg protein)$^{-1}$)	% of control
Control (vehicle)	5·26±0·47	
NZ-105		
1 μM	6·33±0·22*	125±23·4
10 μM	7·07±0·11**	140±27·3
Nicardipine		
1 μM	6·78±0·69**	129±6·7
10 μM	8·22±0·55**	162±14·8
IBMX		
100 μM	8·97±0·50***	174±7·5
1 mM	16·97±0·85***	342±38·3
Forskolin		
1 μM	7·83±0·34**	156±15·9
10 μM	9·91±0·63**	201±27·5

Data are means±s.e.m. of the results of seven experiments. *$P<0·05$, **$P<0·01$, ***$P<0·001$ compared with control.

Table 2. Effect of NZ-105 and reference compounds on the cGMP content of rabbit isolated aorta.

Compounds	cGMP (pmol (mg protein)$^{-1}$)	% of control
Control (vehicle)	0·50±0·08	
NZ-105		
1 μM	0·73±0·02*	173±23·9
10 μM	0·88±0·10**	201±33·5
Nicardipine		
1 μM	0·72±0·09	167±31·8
10 μM	0·96±0·12**	220±44·4
IBMX		
100 μM	1·69±0·21**	412±94·6
1 mM	5·02±0·52***	1233±254·5

Data are means±s.e.m. of the results of seven experiments. *$P<0·05$, **$P<0·01$, ***$P<0·001$ compared with control.

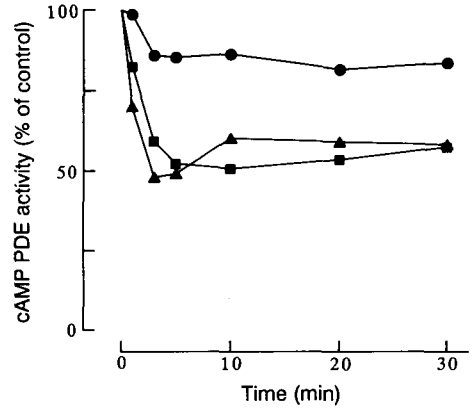

FIG. 1. Profiles of the effect of NZ-105 and reference compounds on bovine heart cyclic nucleotide PDE. PDE activities were determined using 8·36 μg of heart enzyme protein in the presence of 10 μM NZ-105 (●), 10 μM nicardipine (▲) and 100 μM IBMX (■). Each point represents the mean of triplicate determinations.

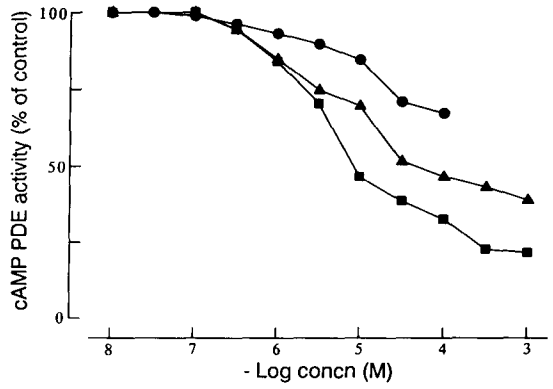

FIG. 2. Inhibitory effects of NZ-105 and reference compounds on bovine heart cyclic nucleotide PDE. PDE activities were determined using 8·36 μg heart enzyme protein in the presence of NZ-105 (●), nicardipine (▲) and IBMX (■). Each point represents the mean of triplicate determinations.

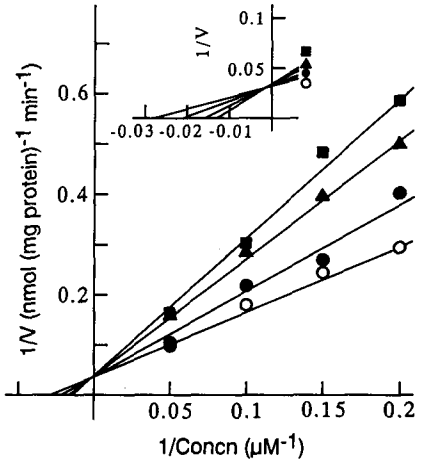

FIG. 3. Lineweaver–Burk plots (the inset is a magnification) of the inhibition of bovine heart cyclic nucleotide PDE. The PDE activities were determined using 8·36 μg of heart enzyme protein in the absence (○) and the presence of 10 μM NZ-105 (●), 10 μM nicardipine (▲) and 100 μM IBMX (■). Each point represents the mean of triplicate determinations.

levels of these preparations (Table 1). In other experiments, we confirmed that sodium nitroprusside, an activator of intracellular soluble guanylate cyclase, increases only cGMP (data not shown), as also reported in studies on rabbit aorta (Diamond & Chu 1983).

Effect of NZ-105 and reference compounds on cAMP PDE activity in purified bovine heart enzyme preparations

NZ-105 slightly inhibited bovine heart PDE activity (Figs 2, 3). The inhibitory effect of NZ-105 was less than that of nicardipine and IBMX. PDE inhibition by NZ-105 at higher concentrations could not be determined because of its low solubility in water. The onset of the inhibitory effect of these compounds was rapid (Fig. 1); maximum inhibition of PDE activity was reached within 3 min. The inhibition by each compound was shown at concentrations greater than 0·3 μM, and in a concentration dependent manner (Fig. 2). The IC50 of NZ-105 for PDE inhibition could not be calculated,

Table 3. Inhibition of bovine heart cyclic nucleotide PDE by NZ-105 and reference compounds.

Compounds	PDE inhibition (IC50, μM)
NZ-105	>100
Nicardipine	49·7
IBMX	8·4

Data are means of triplicate determinations. IC50 values were computed from the individual inhibition curves shown in Fig. 2.

Table 4. Apparent K_m values of bovine heart cyclic nucleotide PDE, and K_i values for NZ-105 and reference compounds.

Compounds	K_i (μM)	Apparent K_m (μM)
NZ-105	30·0	47·6
Nicardipine	13·3	62·5
IBMX	10·0	71·4

Data are means of triplicate determinations. Enzyme activities were determined in the presence of 8·36 μg of heart protein (K_m = 35·7 μM) in the range of 5–20 μM cAMP with and without 10 μM NZ-105 and nicardipine or 100 μM IBMX. Apparent K_i values were computed from the Lineweaver–Burk plots shown in Fig. 3. $K_i = K_m[I]/(\text{Apparent } K_m - K_m)$.

because NZ-105 failed to produce 50% inhibition of PDE activity (Table 3). The inhibitory style of NZ-105 revealed competitive inhibition on the basis of Lineweaver–Burk analysis (Fig. 3). As 1,4-DHP derivatives, NZ-105 and nicardipine, inhibited PDE activity by the same mechanism as the general PDE inhibitor, IBMX. Based on K_m values, NZ-105 was confirmed to inhibit PDE activity to a lesser extent than either nicardipine or IBMX (Table 4).

Discussion

The effects of NZ-105 on intracellular cAMP and cGMP levels were investigated using the rabbit aorta, continuing our previous studies (Masuda et al 1991a; Yamashita et al 1991). There is general agreement that these cyclic nucleotides are very important second messengers involved in intracellular calcium mobilization in various muscle contraction–relaxation pathways (Kobayashi et al 1985). At micromolar concentrations both NZ-105 and nicardipine significantly and concentration-dependently increased the content of these nucleotides. In this study, the minimal concentrations for significant increase could not be determined, because only two-step concentrations above 1 μM were tested. The minimal concentration of nicardipine causing significant cAMP increase is reportedly 0·1 μM (Nishikori et al 1981). The increasing ratios of nicardipine at the concentrations used in our study are consistent with other observations (Nishikori et al 1981), which suggested that the increase in intracellular cAMP (139% of the control at 1 μM) may be involved in smooth muscle relaxation. Hence, the present increase in response to NZ-105 (125 and 140% of the control at 1 and 10 μM, respectively) may also produce vasorelaxation in the concentrations used.

cAMP and cGMP are known to be biosynthesized by

different enzyme systems, i.e. adenylate cyclase (Sutherland & Robinson 1966) and guanylate cyclase (Ishikawa et al 1969), respectively, and that their hydrolysis is known to be caused by cyclic nucleotide PDEs, which are potential factors in the control of second messenger concentrations in target tissues (Drummond & Perrott-Yee 1961). The ratios of the increase in cyclic nucleotides in response to NZ-105 or nicardipine were approximately the same. Thus, NZ-105 presumably affects non-specific nucleotide PDE in vascular smooth muscle cells. Since basal release of cyclic nucleotides in vascular tissue is maintained by mechanisms such as basal neurotransmitter stimulation (cAMP) (Limbird 1981), basal release of endothelium-derived relaxing factor (Vanhoutte et al 1986) and atrial natriuretic peptide (cGMP) (Chinkers et al 1989), NZ-105 may potentiate these vasodilator mechanisms in the concentrations greater than 1 μM.

To evaluate its PDE inhibitory effect in further detail, cAMP PDE activity was investigated using enzyme purified from bovine heart (Ho et al 1976). Cyclic nucleotide PDEs have recently been classified according to their substrate preference and regulatory properties (Silver 1989). The effect of NZ-105 on individual PDE isozymes was not examined in the present study, since it has been reported that the PDE preparation used in this study was not selective with respect to nucleotide substrates (Ho et al 1976). Based on the present findings using the cardiac PDE enzyme preparation, NZ-105 at greater than micromolar concentrations inhibited cAMP PDE activity, but was less potent than nicardipine or IBMX. Such slight PDE inhibition by NZ-105 may permit enough accumulation of cyclic nucleotides as the same concentrations of nicardipine, since these compounds appear to affect cyclic nucleotide accumulation only by inhibiting their breakdown system, but not their synthetic system, and the difference between NZ-105 and nicardipine may be only in the rate of cyclic nucleotide accumulation. The results of the present study indicate that the increase in cyclic nucleotides in response to NZ-105 is attributable to PDE inhibition, since the concentration range for PDE inhibition was consistent with the above-mentioned ranges for increase in cyclic nucleotide.

Based on the double-reciprocal plots obtained from the PDE inhibition curves, the inhibition styles indicate competitive inhibition. This finding suggests that NZ-105, like nicardipine (Nishikori et al 1981), may act directly on the PDE enzyme protein. The PDE inhibitory effect of NZ-105 was manifested very rapidly, peaking within 3 min after mixing the compound and PDE. There were no significant differences between NZ-105 and the other compounds used with respect to onset of PDE inhibition. We previously reported that [^{3}H]NZ-105 specifically binds to DHP receptor proteins, and that the association rate is very slow; it took more than 3 h to achieve maximum binding (Yamashita et al 1991). Based on these findings, we may conclude that the binding mechanism for each functional protein (electrophysiological or chemical binding mechanism) or binding sites in each chemical structure differ completely.

We previously reported that the K_i value of NZ-105 for rabbit aortic DHP-receptor binding was 12·9 nM (Masuda et al 1991a), similar to the concentration for vasorelaxation (Masuda et al 1991a) and inhibition of $^{45}Ca^{2+}$ uptake (Yamashita et al 1991). The K_i value for PDE inhibition was about 2000 times that for DHP-receptor binding; hence, nanomolar concentration ranges sufficient for vasorelaxation may result mainly in DHP-receptor binding and a calcium antagonistic effect, with only a minimal PDE inhibitory effect. On the other hand, the negative inotropic effect of NZ-105 was very weak. NZ-105, even at concentration of 1 μM, had only a slight effect on contractile force (Masuda et al 1991b). The K_i value of NZ-105 for cardiac DHP-receptor binding was 140 nM (Masuda et al 1991b). In cardiac preparations, the PDE inhibitory effect of NZ-105 might be related to the inotropic action.

In conclusion, higher than micromolar concentrations of both NZ-105 and nicardipine significantly increased intracellular cAMP and cGMP levels in arterial tissue, and this may be attributable to inhibition of cyclic nucleotide PDE. These effects may contribute to vasorelaxation at greater than micromolar concentrations.

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