Uptake of CD-349 and Nitrendipine into Bovine Intrapulmonary Artery and Vein

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The uptake of CD-349 into the bovine intrapulmonary artery (BPA) and vein (BPV) was examined and characteristics of this newly synthesized 1,4-dihydropyridine Ca^{2+} antagonist were compared with those of nitrendipine. One hundred minutes after initiation of incubation of the vessels, the uptake of [^3H]CD-349 and [^3H]nitrendipine reached a plateau. The concentrations of CD-349 inside the muscle cell of the BPA and BPV were 11.1 and 6.8 times higher than that of the incubation medium, respectively, and concentrations of nitrendipine in the BPA and BPV were 8.6 and 6.1 times higher, respectively. The degree of uptake was not influenced by the lowering the incubation temperature nor by adding 2,4-dinitrophenol. The partition coefficient of CD-349 measured in the corn oil/Ringer and octanol/Ringer systems was greater than the partition coefficient of nitrendipine, independent of the concentration tested. These results suggest that CD-349 permeates smooth muscle membrane by passive diffusion and accumulates inside the muscle cells, much in the same manner as seen with nitrendipine. The lipid solubility of DC-349 presumably accounts for these events.

Keywords — CD-349; nitrendipine; calcium ion antagonist; 1,4-dihydropyridine; uptake; bovine intrapulmonary artery; bovine intrapulmonary vein; vascular smooth muscle

Introduction

Ca^{2+} antagonists are structurally and pharmacologically diverse and are classified into four groups: (1) 1,4-dihydropyridines, (2) phenylalkylamine, (3) benzothiazepines and (4) diphenylalkylamines.¹⁾ Despite differences in potency and specificity, the main function of all these drugs seems to be the blocking of Ca^{2+} influx across excitatory membranes, such as smooth and cardiac muscles, and neurons. These Ca^{2+} antagonists permeate plasma membranes of cardiac and smooth muscle cells and accumulate in the cell interior.²⁾ In addition to functions of a calcium channel blockade, there is evidence which suggests that drugs which contain 1,4-dihydropyridines exert their pharmacological effect *via* intracellular mechanisms, including: (1) the binding of Ca^{2+} antagonists to calmodulin,³⁾ which inhibits subsequent calmodulin-activated enzymes, such as cyclic nucleotide phosphodiesterase,⁴⁾ and myosin light chain kinase⁵⁾, enzymes closely linked to muscle contraction, (2) the inhibition of the Ca^{2+} extrusion pump ($\text{Ca}^{2+} + \text{Mg}^{2+}$ -ATPase) in the plasmalemma,⁶⁾ (3) the stimulation of $\text{Ca}^{2+} + \text{Mg}^{2+}$ -ATPase in the sarcoplasmic reticulum⁷⁾

and (4) the inhibition of Ca^{2+} release from intracellular stores.⁸⁾

CD-349 (Fig. 1) is a newly synthesized 1,4-dihydropyridine derivative, which has Ca^{2+} -antagonistic tendencies: CD-349 selectively binds to the calcium channel with an affinity similar to that of other 1,4-dihydropyridines.⁹⁾ CD-349 also completely inhibits the high K^{+} -induced canine basilar arterial contraction and decreases blood pressure in the anesthetized dog.¹⁰⁾ In addition, CD-349 might have an intracellular mechanism on muscle relaxation since it inhibits Ca^{2+} -dependent cyclic nucleotide phosphodiesterase,^{4c,d)} and elevates cyclic guanosine monophosphate (GMP) levels in the bovine intrapulmonary artery and vein.^{4e)}

Fig. 1. Chemical Structure of CD-349 (2-Nitratopropyl 3-nitratopropyl 2,6-dimethyl-4-(3-nitrophenyl)-1,4-dihydropyridine-3,5-dicarboxylate)

In the present study, we investigated the degree to which CD-349 accumulates in blood vessels, the objective being to determine the possible involvement of intracellular mechanisms in the vasodilative action.

Materials and Methods

Materials — Bovine lungs obtained from a local slaughterhouse were immersed in an ice-cold Ringer solution containing (in mM): NaCl (145), KCl (4), MgCl₂ (1), CaCl₂ (2), Tris/HCl [pH 7.5] (20), and glucose (10).

CD-349 and nitrendipine were synthesized in the Research Center of Taisho Pharmaceutical Co., Ltd. [³H]CD-349 (28 Ci/mmol) was synthesized at Amersham International. [³H]nitrendipine (87 Ci/mmol) was purchased from New England Nuclear. Corn oil used was purchased from Ajinomoto.

Uptake of Drugs into the Bovine Vessels — Bovine intrapulmonary vessels dissected from the lung parenchyma were placed in ice-cold Ringer solution saturated with 95% O₂ and 5% CO₂. The vessels were cut into small strips (40–60 mg wet weight), after removing the connective tissue. The uptake of the drug into the vessels was measured as described by Pang and Sperelakis.^{2a)} Incubation was carried out at 37 °C or the indicated temperature, with protection from light, in 2.0 ml of Ringer solution containing from 1 × 10⁻⁹ to 1 × 10⁻⁶ M of either CD-349 or nitrendipine and a tracer amount of tritiated drugs (0.1 μCi/ml). At definite time intervals, the blood vessels were removed from the incubation medium and blotted dry on the filter paper. To wash out any of the drug which were taken up into the interstitial fluid spaces, the vessels were soaked for 10 min in ice-cold Ringer solution. After re-blotting and weighing, the vessels were dissolved overnight with 1.0 ml of NCS[®] tissue solubilizer (Amersham) at 50 °C. Following the addition of 50 μl of acetic acid, radioactivity in the solubilized vessels was measured in a liquid scintillation counter (Tri Carb Model 4430, Packard) using 10 ml of Aquasol-2 scintillator (New England Nuclear).

The tissue/medium (T/M) ratio was calculated by dividing the concentration of drugs taken up

into the blood vessels at a steady state by the concentration of drugs in the original medium (pmol per 100 mg tissue wet weight/pmol per 100 μl of medium). $[\text{Drug}]_{\text{i(intracellular)}}/[\text{Drug}]_{\text{o(outside)}}$ was calculated from the T/M ratio after the incorporation of correction factors for: (1) the water content being 80% of the total wet weight and (2) the interstitial or extracellular spaces being about 30% of the muscle.^{2a)}

Determination of Partition Coefficient — The partition coefficient of the drugs was measured using 2 systems, namely the corn oil/Ringer and octanol/Ringer solution systems, as described by Pang and Sperelakis.^{2b)} One ml of Ringer solution was vigorously mixed for 5 min with either 1.0 ml of corn oil or octanol, using a Vortex mixer. The corn oil and octanol contained from 1 × 10⁻⁸ to 1 × 10⁻⁶ M of the drug and a tracer amount of tritiated drug (0.1 μCi/ml). Thereafter, the samples were centrifuged at 3000 rpm for 5 min. A 0.5 ml aliquot of the upper phase were transferred to scintillation vials, and the remaining upper phase was aspirated off and discarded. Then 0.5 ml of aliquot from the lower phase was transferred into another scintillation vial. Radioactivity was measured, as described above. All procedures were carried out at room temperature and under subdued light. The partition coefficient was defined as the ratio of the amount of radioactivity in the upper phase (organic solution) to the amount of the radioactivity in the lower phase (Ringer solution).

Data Analysis — Drug uptake data in Fig. 2 were fitted by the non-linear Marquardt-Levenberg curve fitting procedure of the RS/1 program (BBN Research Systems, Cambridge, MA), based on a hyperbolic relationship, running on a VAX-8600/VMS system.

Results

The time course for the uptake of CD-349 and nitrendipine into the bovine intrapulmonary arteries and veins is shown in Fig. 2. The uptake of CD-349 and nitrendipine increased linearly with incubation time for the first 2 to 5 min and reached a plateau in both the artery and vein at 100 min and 60 min, respectively, after start of

Fig. 2. Time Course of Uptake of CD-349 and Nitrendipine into the Bovine Intrapulmonary Artery and Vein

Uptake of CD-349 (○, ●) and nitrendipine (△, ▲) was measured at 37 °C in 2.0 ml of Ringer solution containing 1×10^{-7} M of non-labeled drugs and 0.1 μ Ci/ml of tritiated drugs. Uptakes of these drugs into artery and vein were indicated by open and closed remarks, respectively. Half time ($T_{1/2}$) of maximal uptake was obtained graphically and is indicated by arrows. Data are expressed as the mean value obtained from 2 separate experiments each done in 3 tissues.

the incubation. At a concentration of 1×10^{-7} M, the maximal uptake of CD-349 was several times larger than that of nitrendipine. The half time of the maximal uptake ($T_{1/2}$) of CD-349 into the arteries and veins was 20 min and 23 min, respectively. $T_{1/2}$ of nitrendipine was 9 min in both the artery and vein. As shown in Table I, the initial rate and maximal uptake of these drugs increased linearly with the concentrations of the drugs and a state of saturation was not reached (from 1×10^{-9} to 1×10^{-6} M). After the water content and interstitial or extracellular fluid spaces were taken into consideration, the intracellular concentration of CD-349 was 9.02- to 13.11-fold (mean value: 11.1) and 5.23- to 8.02-fold (6.8) higher than outside of the bovine intrapulmonary arteries and veins, respectively (Table I). The intracellular concentration of nitrendipine was 6.52- to 11.18-fold (8.6) and 5.80- to 6.46-fold (6.1) higher than outside, respectively. These results suggest that CD-349 has a relatively higher permeability into arteries than into veins (Fig. 1 and Table I).

TABLE I. Characteristics of the Uptake of CD-349 and Nitrendipine into the Bovine Intrapulmonary Artery and Vein

		[Drug] _o (M)	Drug uptake (at 37 °C)			
			Initial rate ^{a,e)}	Maximal uptake ^{b,e)}	T/M ^{c)}	[Drug] _i ^{d)}
			(pmol/100 mg tissue/min)	(pmol/100 mg tissue)	ratio	[Drug] _o
CD-349	Artery	1 × 10 ⁻⁹	0.045 ± 0.012	0.629 ± 0.093	6.29	11.23
		1 × 10 ⁻⁸	0.427 ± 0.051	6.232 ± 0.876	6.23	11.12
		1 × 10 ⁻⁷	3.961 ± 0.545	73.42 ± 9.19	7.34	13.11
		1 × 10 ⁻⁶	33.45 ± 7.52	504.6 ± 150.4	5.05	9.02
	Vein	1 × 10 ⁻⁹	0.078 ± 0.046	0.390 ± 0.040	3.90	6.96
		1 × 10 ⁻⁸	0.295 ± 0.037	4.493 ± 0.563	4.49	8.02
		1 × 10 ⁻⁷	3.982 ± 1.077	29.31 ± 3.82	2.93	5.23
		1 × 10 ⁻⁶	29.78 ± 2.88	392.6 ± 50.1	3.93	7.02
Nitrendipine	Artery	1 × 10 ⁻⁹	0.031 ± 0.007	0.365 ± 0.038	3.65	6.52
		1 × 10 ⁻⁸	0.383 ± 0.096	6.262 ± 0.578	6.26	11.18
		1 × 10 ⁻⁷	3.134 ± 0.618	48.06 ± 4.78	4.81	8.59
		1 × 10 ⁻⁶	34.75 ± 9.17	442.9 ± 37.1	4.43	7.91
	Vein	1 × 10 ⁻⁹	0.028 ± 0.008	0.325 ± 0.026	3.25	5.80
		1 × 10 ⁻⁸	0.247 ± 0.053	3.617 ± 0.724	3.62	6.46
		1 × 10 ⁻⁷	2.726 ± 0.810	32.69 ± 2.82	3.27	5.84
		1 × 10 ⁻⁶	27.59 ± 7.89	351.1 ± 61.9	3.51	6.27

a) Initial rates were calculated from values obtained at 2 or 5 min. b) Maximal uptake was calculated from values obtained at 100 or 120 min. c) T/M ratio (tissue/medium ratio) was expressed as pmol per 100 mg tissue/pmol per 100 μ l medium. d) $[\text{Drug}]_i/[\text{Drug}]_o$ was calculated from the T/M ratio after correction as described in Materials and Methods. e) Values are expressed as the mean $\pm$ S.E.M. of 7–8 tissues from 2 separate experiments.

TABLE II. Effect of Incubation Temperature and 2,4-Dinitrophenol (DNP) on the Uptake of CD-349 and Nitrendipine

	Temp. (°C)	Maximal uptake (pmol/100 mg tissue) ^{a)}	
		Artery	Vein
CD-349	7	26.79 ± 3.76	19.26 ± 2.51
(1 × 10 ⁻⁷ M)	17	28.55 ± 3.53	21.36 ± 2.71
	27	43.06 ± 7.39	25.40 ± 2.85
	37	53.54 ± 7.05	34.39 ± 6.05
+ 50 μM DNP	37	55.82 ± 4.60	59.63 ± 10.0
Nitrendipine	7	26.08 ± 2.93	17.03 ± 1.95
(1 × 10 ⁻⁷ M)	17	28.62 ± 2.41	18.43 ± 1.72
	27	42.24 ± 3.87	31.14 ± 3.74
	37	44.93 ± 4.70	33.97 ± 2.84
+ 50 μM DNP	37	55.07 ± 7.84	37.79 ± 3.27

a) Values are expressed as the mean ± S.E.M. of 11 tissues from 3 separate experiments, except in the experiment with 50 μM DNP, in which values of 4 tissues from 1 experiment are indicated.

TABLE III. Partition Coefficients of CD-349 and Nitrendipine

	Conc. (M)	Partition coefficients ^{a)}	
		Corn oil/Ringer	Octanol/Ringer
CD-349	1 × 10 ⁻⁸	3.80 ± 0.09	9.91 ± 0.16
	1 × 10 ⁻⁷	4.25 ± 0.19	10.29 ± 0.15
	1 × 10 ⁻⁶	3.58 ± 0.06	13.01 ± 0.22
Nitrendipine	1 × 10 ⁻⁸	2.59 ± 0.05	8.59 ± 0.13
	1 × 10 ⁻⁷	3.04 ± 0.09	8.02 ± 0.09
	1 × 10 ⁻⁶	3.49 ± 0.18	8.27 ± 0.20

a) Values are expressed as the mean ± S.E.M. from 6 experiments.

The effects of temperature and the presence of 2,4-dinitrophenol (uncoupler of oxidative phosphorylation) on the accumulation of CD-349 and nitrendipine are shown in Table II. The maximal uptake of CD-349 and nitrendipine was not affected by the lowering the incubation temperature from 37 °C to 27 °C or by the addition of 50 μM of 2,4-dinitrophenol. However, when the incubation temperature was lowered from 37 °C to 17 °C or 7 °C, a marked decrease in the accumulation of CD-349 and nitrendipine occurred.

The partition coefficients of CD-349, as measured in the corn oil/Ringer and octanol/Ringer systems, were larger than those of nitrendipine at any concentrations tested (1 × 10⁻⁸ to 1 × 10⁻⁶ M) (Table III).

Discussion

We obtained evidence that the concentration of accumulated CD-349 in bovine intrapulmonary arterial and venous smooth muscles cells was approximately 10 times higher than outside. The differences in concentrations may be due to the accumulation and distribution of CD-349 in the inside of vascular smooth muscle since the concentrations were determined after the wash out of the drug taken up into interstitial and extracellular fluid spaces. The binding reaction of nitrendipine to plasma membranes was slower than CD-349 in the rat myocardium membrane fraction, yet the maximal binding sites ($B_{\max}$) of [³H]CD-349 and [³H]nitrendipine were much the same.⁹⁾ On the other hand, $T_{1/2}$ of CD-349 was about 2-fold greater than that of nitrendi-

pine, and a larger amount of CD-349 was taken up, as compared with nitrendipine. The uptake of these drugs required a longer incubation time before reaching a plateau, as compared with the binding reactions. These results suggest that the uptake of CD-349 and nitrendipine is clearly different from their binding to the Ca^{2+} channel in the plasma membrane and that these drugs apparently accumulate within the smooth muscle.

The uptake of CD-349 might not be due to active transport of the Ca^{2+} antagonist into the muscle cells, since (1) a saturation of the accumulated CD-349 was not observed at the concentration range of $1 \times 10^{-9} - 1 \times 10^{-6}$ M, (2) there was no decrease in the uptake after lowering the incubation temperature from 37 °C to 27 °C, and (3) the uptake was not affected by adding 2,4-dinitrophenol (50 μM). However, by lowering the incubation temperature from 37 °C to 17 °C or 7 °C, a marked decrease in the maximal uptake did occur. This decrease can probably be explained by the occurrence of a phase transition or a change of fluidity of plasma membrane of arterial and venous muscle cells at a temperature near 20 °C. The accumulation of these drugs in the blood vessels might be explained by passive diffusion through a concentration gradient. However, a simple passive diffusion would not account for the comparatively higher internal concentration of CD-349 and nitrendipine. One possible explanation of this phenomenon is the difference in solubility of CD-349 and nitrendipine to outer and inner phospholipids component of the plasma membrane. The phospholipid component of inner and outer plasma membrane is quite different¹¹⁾ and the partition coefficient is changed by oil used.^{2e)} If the partition coefficient of these drugs to outer phospholipids is higher than that to inner phospholipids, these drugs might be transported interiorly, against the concentration gradient. The longer half time of efflux, compared to that of uptake, as demonstrated by Pang and Sperelakis,¹²⁾ support this possibility. CD-349 and nitrendipine both have highly hydrophobic properties. The present experiment revealed evidence of a high lipid solubility and that the partition coefficient was changed by oil. It may be due to the solubilization of these drugs in the

lipid bilayer matrixes of cell membranes that they have such a high capacity to permeate the cell membranes and to accumulate within the vascular smooth muscle cells. The amount of CD-349 and nitrendipine taken up into the arteries was higher than that in the veins, presumably because the ratio of different kinds of polar lipids in the membrane determines the fluidity and permeability of membrane.¹³⁾ Another possible mechanism of accumulation of CD-349 and nitrendipine is the binding of these drugs to internal vesicles. Nitrendipine binds to the isolated sarcoplasmic reticulum^{2b, 14)} and mitochondrial membranes.¹⁵⁾

It has been reported that CD-349 inhibits Ca^{2+} -dependent cyclic nucleotide phosphodiesterase activity with an IC_{50} value of about 10^{-6} M.^{4c,d)} CD-349, at $10^{-6} - 10^{-5}$ M levels, accumulates cyclic GMP generated from GTP in the homogenate of bovine intrapulmonary arteries and veins, by inhibiting Ca^{2+} -dependent cyclic GMP phosphodiesterase activity. In particular, guanylate cyclase activity is stimulated.^{4e)} Both cyclic adenosine monophosphate (AMP) and cyclic GMP dilate vascular smooth muscle.¹⁶⁾ Also, cyclic nucleotide phosphodiesterase inhibitors which inhibit cyclic AMP or GMP metabolism, are clinically useful for treating hypertension.¹⁷⁾ This suggests that a CD-349 ($> 10^{-6}$ M) may stimulate the vasodilative action through an intracellular mechanism, such as a cyclic nucleotide elevation, by accumulating in the blood vessels.

The clinically induced plasma concentration of Ca^{2+} antagonists was 10^{-7} M.¹⁸⁾ Preliminary experiments demonstrated that the peak plasma concentration of CD-349 reached 10^{-8} to 10^{-7} M in case of 0.2 mg/kg of single oral administration to the dog. On the other hand, the Ca^{2+} antagonist, felodipine, accumulated 200- to 500-fold in vascular smooth muscle¹⁹⁾ and 480-fold in rabbit heart muscle,²⁰⁾ as compared to the peripheral concentrations. $[\text{Nitrendipine}]_i/[\text{Nitrendipine}]_o$ ratios obtained by simple incubation of smooth muscle strips in Ringer solution were 8.6 and 6.1 in the artery and the vein, respectively, and were analogous with those obtained by Pang and Sperelakis.¹¹⁾ However, when smooth muscle cells of a guinea-pig are

electrically stimulated, the nitrendipine concentration inside the cells was 120-fold higher than the outside.^{2c)} These data suggest that the concentration of CD-349 in vascular smooth muscle cells is likely to reach to 10^{-6} to 10^{-5} M levels of concentration, under physiological conditions, *in vivo*.

In considering these data, it is suggested that the accumulation of CD-349 in the vascular smooth muscle may relate to the intracellular mechanism in its vasodilating action.

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