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INHIBITION OF CALCIUM INFLUX IN RABBIT AORTA BY NICARDIPINE HYDROCHLORIDE (YC-93)

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Abstract—The Ca^{2+} antagonistic effects of a potent vasodilator, nicardipine hydrochloride [2-(*N*-benzyl-*N*-methylamino)ethyl methyl 2,6-dimethyl-4-(*m*-nitrophenyl)-1,4-dihydropyridine-3,5-dicarboxylate hydrochloride], were investigated. Both nicardipine and verapamil, neither of which exhibited a significant effect on cellular $^{45}Ca^{2+}$ uptake by the rabbit aorta in normal buffer containing 2.68 mM KCl as measured by the "lanthanum method", inhibited the enhancement of $^{45}Ca^{2+}$ uptake induced by equimolar replacement of 120 mM NaCl by KCl. Nicardipine with an IC_{50} (the concentration required for 50 per cent inhibition) of 10^{-9} M was sixty-eight times more potent than verapamil in the inhibition of KCl-induced $^{45}Ca^{2+}$ uptake. Nicardipine also repressed KCl-induced contraction of the aorta, and its IC_{50} was about 2×10^{-9} M, eighty-five times smaller than that of verapamil. These comparative studies on the relation between cellular $^{45}Ca^{2+}$ uptake and KCl-induced contraction of the rabbit aorta suggest that nicardipine causes relaxation of smooth muscle mainly by interfering with Ca^{2+} influx.

Nicardipine hydrochloride [2-(*N*-benzyl-*N*-methylamino)ethyl methyl 2,6-dimethyl-4-(*m*-nitrophenyl)-1,4-dihydropyridine-3,5-dicarboxylate hydrochloride] is a potent cerebral and coronary vasodilator and appears to act directly on vascular smooth muscle cells without involving known specific receptors such as α-adrenergic, β-adrenergic, cholinergic, and adenosine receptors [1]. The vasodilator effect of nicardipine is highly specific to KCl-induced rather than norepinephrine-induced contraction. These findings, along with the result that the inhibition of KCl-induced contraction is diminished by raising extracellular Ca^{2+} concentrations [2], have suggested that the inhibition of Ca^{2+} influx is responsible for relaxation of arteries by nicardipine.

The present report demonstrates the extremely potent inhibitory effect of nicardipine on $^{45}Ca^{2+}$ uptake by the rabbit aorta, using the "lanthanum method" [3], as well as on KCl-induced contraction of the smooth muscle.

MATERIALS AND METHODS

Drugs. Verapamil hydrochloride, $^{45}CaCl_2$ (about 20 mCi/mg Ca), $LaCl_3 \cdot 7H_2O$, and Soluene 350 were purchased from the Eisai Co. Ltd. (Tokyo, Japan), the New England Nuclear Corp. (Boston, MA, U.S.A.), Koso Chemicals Co. Ltd. (Tokyo, Japan), and the Packard Instrument Co., Inc. (Downers Grove, IL, U.S.A.) respectively. Nicardipine hydrochloride was prepared by Iwanami *et al.* [4].

Determination of $^{45}Ca^{2+}$ *uptake.* Cellular $^{45}Ca^{2+}$ uptake was determined by the lanthanum method described by van Breemen *et al.* [3, 5]. The thoracic aorta of a Japanese white rabbit (weighing about 2.5 kg) was removed and placed in warm oxygenated buffer A containing 140 mM NaCl, 2.68 mM KCl, 1.25 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM glucose, and 5 mM HEPES–Tris buffer, pH 7.4.† The aorta was cleaned of fat and connective tissue, and cut transversely into rings weighing about 25 mg. Following equilibration in buffer A at 37° for 60 min, the aortic rings were preincubated with nicardipine or verapamil for 10 min. $^{45}Ca^{2+}$ uptake was then initiated by transferring the tissue rings to fresh buffer A or B (the isotonic solution containing similar components to those in buffer A except for the presence of 20 mM NaCl and 123 mM KCl), including 1.25 mM $^{45}CaCl_2$ (about 0.2 μCi/ml), and continued for 30 min under various conditions. The uptake was terminated by addition of 15 mM $LaCl_3$, and the aortic rings were exposed to $LaCl_3$ for 5 min to block completely $^{45}Ca^{2+}$ exchange between the inside and outside of the cells. The aortic rings were then transferred to buffer A in which $CaCl_2$ was replaced by 15 mM $LaCl_3$, and further incubated at 37° for 60 min to expel the extracellularly bound $^{45}Ca^{2+}$. Through the experiments up to this stage of incubation, all mixtures including the tissue rings were continuously oxygenated by 100% O_2. The rings were then blotted, weighed, and solubilized with Soluene 350 at 50° for 3 hr. The radioactivity of $^{45}Ca^{2+}$ was determined by a Packard liquid scintillation counter.

Determination of isometric contraction. Helical strips (3.0 cm × 0.2 cm) were prepared from a rabbit thoracic aorta according to the method of Furchgott and Bhadrakom [6]. The strips were incubated at 37°

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† HEPES = 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid.

in 30 ml of buffer A, and the organ baths were constantly aerated with 100% O_2. The isometric contraction with loading tension of 1.5 g was recorded by a strain gauge transducer (SB-1T, Nihon Koden) connected to an oscillograph (TO2N2, Fujisoku). While the strips were preincubated for 120 min, the medium was replaced every 30 min, with a concomitant adjustment of the loading tension. The strips were then contracted in modified buffer B containing 63 mM KCl. When the contraction fully developed, nicardipine or verapamil was added to the bath progressively. Under these conditions, 63 mM KCl caused a stable contraction. The results were expressed as percent inhibition of the initial contraction.

In another experiment, the strips were equilibrated for 20 min in Ringer solution containing 154 mM NaCl, 5.4 mM KCl, 1.5 mM $CaCl_2$, 6 mM $NaHCO_3$, and 11 mM glucose with constant aeration with a 95% O_2 and 5% CO_2 gas mixture. A stepwise isometric contraction with loading tension of 2 g was then induced by the cumulative addition of KCl or norepinephrine from the threshold concentration to the one capable of inducing maximal contraction without osmolarity adjustment. Ninety minutes after washing the strips with Ringer solution, the cumulative effects of KCl or norepinephrine on tissue contractility were studied in the presence of vasodilators. The drugs were added to the medium at 30 min prior to the addition of KCl or norepinephrine. Dose–response curves for KCl-induced contractions using buffer A were similar to those in the Ringer solution, but the equilibration time required for constant contractility in buffer A was longer than that in the Ringer solution. Thus, mainly for this reason, the Ringer solution was used in the present pharmacological experiments.

Table 1. Effects of drugs on La^{3+}-inhibitable $^{45}Ca^{2+}$ uptake by rabbit aorta*

Addition (10^{-5} M)	Relative $^{45}Ca^{2+}$ uptake	
	2.68 mM KCl	123 mM KCl
None	100.0 ± 5.3 (24)	180.3 ± 18.7 (23)
Nicardipine	102.7 ± 11.0 (6)	90.4 ± 7.9† (6)
Verapamil	123.8 ± 23.5 (6)	88.4 ± 6.2† (6)
Norepinephrine	109.1 ± 10.8 (6)	ND‡

* $^{45}Ca^{2+}$ uptake was defined as La^{3+} (15 mM)-inhibitable $^{45}Ca^{2+}$ uptake. The results were expressed as per cent of the $^{45}Ca^{2+}$ uptake (mean, 111.1 ± 17.6 nmoles/g wet tissue per 30 min) at 2.68 mM KCl. The La^{3+}-uninhibitable $^{45}Ca^{2+}$ uptake in the presence of 15 mM $LaCl_3$ at 2.68 mM KCl or at 123 mM KCl was 71.9 ± 10.2 (N = 4) nmoles/g tissue or 87.5 ± 4.7 (N = 20) nmoles/g wet tissue respectively. Each value is the mean ± S.E. Figures in parentheses are the number of experiments.

† Significantly different from the control (results in the absence of drugs) ($P < 0.05$).

‡ Not determined.

RESULTS

Effect on $^{45}Ca^{2+}$ uptake. $^{45}Ca^{2+}$ uptake by the rabbit aorta, in normal (buffer A) and 123 mM KCl-containing (buffer B) buffers, augmented with time and leveled off at 30 min with about 100 and 140 nmoles/g

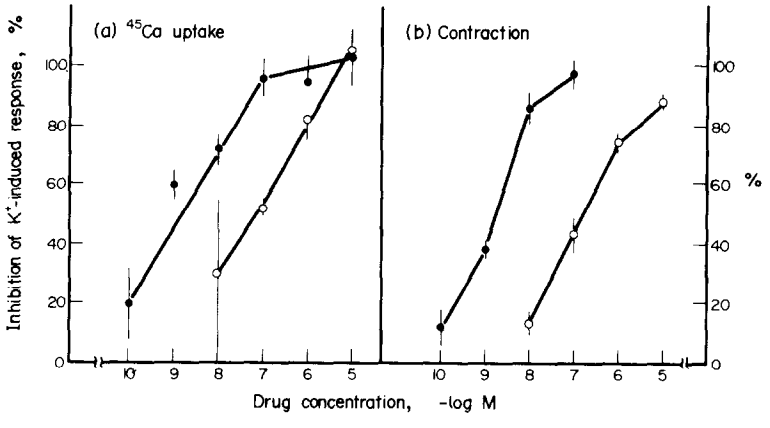

Fig. 1. Effects of nicardipine (●——●) and verapamil (○——○) on KCl-induced $^{45}Ca^{2+}$ uptake (A) and contraction (B) of the rabbit aorta. Panel A: The percent inhibition of KCl-induced $^{45}Ca^{2+}$ uptake by the individual aorta was calculated as follows:

$$\frac{\left[\begin{array}{c}^{45}Ca^{2+}\text{ uptake in 123 mM KCl-}\\ \text{containing buffer (buffer B)}\end{array}\right] - \left[\begin{array}{c}^{45}Ca^{2+}\text{ uptake in buffer B}\\ \text{with vasodilators}\end{array}\right]}{\left[\begin{array}{c}^{45}Ca^{2+}\text{ uptake in}\\ \text{buffer B}\end{array}\right] - \left[\begin{array}{c}^{45}Ca^{2+}\text{ uptake in normal}\\ \text{buffer (buffer A)}\end{array}\right]} \times 100$$

Since the inhibition of $^{45}Ca^{2+}$ uptake was calculated according to this formula, it was possible to obtain more than 100 per cent inhibition. Each point is the mean ± S.E. of three to six experiments. The mean of 123 mM KCl-induced increase in $^{45}Ca^{2+}$ uptake was 89.2 ± 20.8 nmoles/g wet tissue per 30 min. Panel B: After the aortic preparations were fully contracted by isotonic 63 mM KCl, the drugs were added in a cumulative manner. Each point is the mean ± S.E. of six experiments.

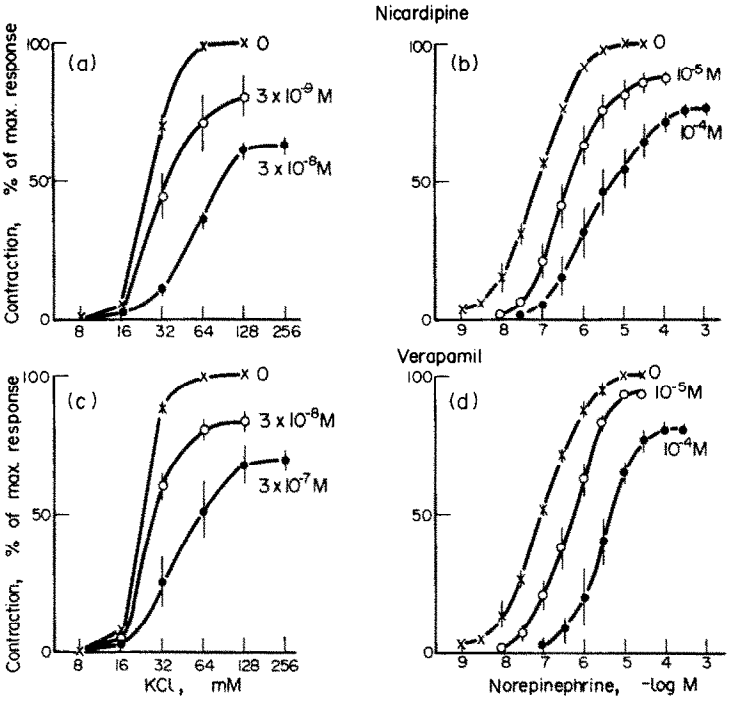

Fig. 2. Effects of nicardipine (A and B) and verapamil (C and D) on contraction of rabbit aorta induced by various concentrations of KCl (A and C) or norepinephrine (B and D). Each point is the mean ± S.E. of four experiments.

wet tissue, respectively, much like the results described by van Breemen *et al.* [3]. The addition of 15 mM $LaCl_3$ markedly depressed $^{45}Ca^{2+}$ uptake in both buffer A and B with almost identical time courses having plateau levels of 43 nmoles/g wet tissue at 30 min.

Norepinephrine at 10 μM did not significantly affect $^{45}Ca^{2+}$ uptake in buffer A, as shown in Table 1. The increase in $^{45}Ca^{2+}$ uptake in response to depolarization of cell membranes by KCl was entirely abolished by either nicardipine or verapamil at 10 μM, whereas neither vasodilator significantly altered $^{45}Ca^{2+}$ uptake in the presence of 2.68 mM KCl.

Figure 1A shows the inhibitory effects of various concentrations of nicardipine and verapamil on the 123 mM KCl-induced increase in $^{45}Ca^{2+}$ uptake. Nicardipine and verapamil both inhibited KCl-induced $^{45}Ca^{2+}$ uptake in a dose-dependent fashion with IC_{50} values (the concentration required for 50 per cent inhibition) of about 1.0×10^{-9} M and 6.8×10^{-8} M respectively.

Effects on contractions. In view of the physiological significance of the inhibitory effects of drugs on cellular $^{45}Ca^{2+}$ uptake, some pharmacological experiments were performed to see whether the vasodilators depressed contraction of the rabbit aorta induced by KCl or norepinephrine. As shown in Fig. 1B, nicardipine and verapamil inhibited the contraction induced by isotonic 63 mM KCl, with IC_{50} values of about 1.9×10^{-9} M and 1.7×10^{-7} M, respectively, indicating that nicardipine was eighty-five times more potent than verapamil.

Figure 2 shows the effects of nicardipine and verapamil on the contraction of rabbit aorta induced by various concentrations of KCl or norepinephrine. The threshold concentration of KCl to induce contraction of the aorta was 8 mM, and the maximal contraction occurred at 128 mM. In the time control, contractility of the aorta was not impaired significantly during the entire period of the experiments. The addition of nicardipine or verapamil caused the dose–response curve to shift to the right concomitantly with a decrease in the maximal contractile responses. The IC_{50} values at hypertonic 64 mM KCl were approximately 1.4×10^{-8} M for nicardipine and 3.3×10^{-7} M for verapamil, indicating that nicardipine was apparently about twenty-four times more potent than verapamil. When norepinephrine was used as a contracting agent, nicardipine and verapamil also shifted the dose–response curves to the right with a reduction in maximal contractile responses. It was found that 1.6×10^{-4} M nicardipine and 3.3×10^{-4} M verapamil were needed to inhibit by 50 per cent the maximal contractions elicited by 10 μM norepinephrine, indicating no significant difference in inhibition between the two vasodilators.

DISCUSSION

Van Breemen *et al.* [3] and Kreye *et al.* [7] have demonstrated by the lanthanum method that verapamil, D-600 (a methoxy derivative of verapamil), and nitroprusside mainly inhibit increased $^{45}Ca^{2+}$ influx in response to depolarization of cell membranes by KCl in the rabbit aorta. We have shown in the present experiments with the rabbit aorta that a new vasodilator, nicardipine, also mainly inhibits increased $^{45}Ca^{2+}$ uptake, as determined by the lanthanum method, in response to an increase in a KCl concentration, without affecting $^{45}Ca^{2+}$ uptake in the normal buffer containing 2.68 mM KCl. The inhibi-

tory effect of nicardipine on Ca^{2+} influx is consistent with our recent observation that nicardipine depresses a Ca^{2+} spike in *Taenia coli* of the guinea pig (unpublished data by Asano *et al.*). Thorens and Haeusler [8] have reported that the IC_{50} of verapamil for $^{45}Ca^{2+}$ influx in the primary pulmonary arteries is 3×10^{-7} M, which is similar to the IC_{50} in the present study. It is noteworthy that, in terms of the IC_{50} values, nicardipine appears to be by far the most potent inhibitor of $^{45}Ca^{2+}$ influx among the known vasodilators—for instance, sixty-eight times more potent than verapamil. Interestingly, the IC_{50} of nicardipine for the inhibition of KCl-induced contractions is very similar to that of KCl-induced $^{45}Ca^{2+}$ uptake, indicating a close relationship between the antagonistic actions of nicardipine on $^{45}Ca^{2+}$ uptake and on KCl-induced contraction of the artery.

Far greater concentrations of nicardipine and verapamil are required to inhibit norepinephrine-induced, than KCl-induced, contractions. Norepinephrine at a concentration of 10 μM, which induces at least partial depolarization but maximum contraction of rabbit aorta, does not significantly augment $^{45}Ca^{2+}$ uptake over the control value in the normal buffer, in agreement with van Breemen *et al.* [3] and Karaki and Weiss [9]. Thus, the result supports the hypothesis that Ca^{2+} required for norepinephrine-induced contraction is released from the intracellular storage compartments, but not from the outside of cells, to enter the cytosol during the contraction phase.

In conclusion, nicardipine may act on two independent mechanisms for altering intracellular Ca^{2+}: (1) that low concentrations of nicardipine inhibit KCl-induced contractions by blocking calcium influx, and (2) that the high concentrations inhibit norepinephrine-induced contractions by potentiating intracellular calcium sequestration.

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