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INHIBITION OF CYCLIC AMP PHOSPHODIESTERASE BY 2,6-DIMETHYL-4-(3-NITROPHENYL)-1,4-DIHYDROPYRIDINE-3,5-DICARBOXYLIC ACID 3-[2-(*N*-BENZYL-*N*-METHYLAMINO)] ETHYL ESTER 5-METHYL ESTER HYDROCHLORIDE (YC-93), A POTENT VASODILATOR

NOBUO SAKAMOTO, MICHIO TERAI, TOICHI TAKENAKA and HIROO MAENO*

Department of Pharmacology and Biochemistry, Central Research Laboratories, Yamanouchi Pharmaceutical Co., Ltd., Itabashi-ku, Tokyo, Japan

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Abstract—A new, potent vasodilator (YC-93), 2,6-dimethyl-4-(3-nitrophenyl)-1,4-dihydropyridine-3,5-dicarboxylic acid 3-[2-(*N*-benzyl-*N*-methyl amino)] ethyl ester 5-methyl ester hydrochloride, competitively inhibited cyclic adenosine 3′,5′-monophosphate (cyclic AMP) phosphodiesterase in the 105,000 *g* supernatant solutions from canine basilar, carotid, coronary and femoral arteries. The inhibition constant (K_i) of YC-93 for these enzyme preparations was in the range of 2.0–4.3 μM at substrate concentrations near the low K_m (about 1 μM for each enzyme preparation), and was 4.0–12 μM at substrate concentrations near the high K_m (50–70 μM). The potency of YC-93 for inhibition of coronary phosphodiesterase at 1 μM cyclic AMP and 50 μM cyclic AMP was much greater than that of papaverine and 3-isobutyl-1-methyl xanthine (IBMX). Commercially available cyclic AMP phosphodiesterase purified from beef heart was also strongly inhibited by YC-93 in a competitive manner and its K_i value was 2.0 μM in the wide range of substrate concentrations tested. Studies on the structure-activity relationship using low K_m phosphodiesterase from canine coronary artery and high K_m phosphodiesterase from beef heart, demonstrated that 3,5-diethoxycarbonyl-1,4-dihydro-2,4,6-collidine, the simplest 1,4-dihydropyridine derivative (tested in the present studies) resulted in slightly less inhibition than papaverine, and the inhibitory potency of the former compound was greatly increased mainly by two structural modifications. Firstly, addition of a nitrophenyl group at position 4 of the 1,4-dihydropyridine ring, secondly, the replacement of ethylester at position 3 of the 1,4-dihydropyridine ring by *N*-benzyl-*N*-methylaminoethyl ester. A few dihydropyridine derivatives together with YC-93 were the most potent inhibitors of cyclic AMP phosphodiesterase among the compounds tested. The finding that the level of cyclic AMP in canine arterial strips was increased by 64 per cent ($P < 0.01$) even after 1 min exposure to 1 μM YC-93 supports the possibility of at least a partial involvement of phosphodiesterase inhibition in vasodilation by the drug.

INTRODUCTION

It was recently demonstrated by Takenaka *et al.* [1] that a 1,4-dihydropyridine derivative, 2,6-dimethyl-4-(3-nitrophenyl)-1,4-dihydropyridine-3,5-dicarboxylic acid 3-[2-(*N*-benzyl-*N*-methylamino)] ethyl ester 5-methyl ester hydrochloride (YC-93)† was a hundred times more potent than similarly acting drugs such as papaverine, isoxsupurine, or cinnarizine in causing cerebral and coronary vasodilation. Neither β-adrenergic, cholinergic, nor histaminergic antagonists exhibited any significant effect on vasodilation caused by administration of YC-93 to dogs under anesthesia. In addition, YC-93 failed to potentiate the coronary vasodilator effect of adenosine. Aminophylline, an antagonist to the vasodilating action of adenosine, did not affect the increase in coronary blood flow by YC-93, ruling out a possible role of adenosine as a mediator in vasodilation by YC-93. These pharmacological characteristics strongly suggest a direct action of YC-93 on smooth muscle of blood vessels without acting through specific receptors [1].

In view of the mechanism of smooth muscle relaxation, the drugs to vasodilate may be classified into two major groups. One, the inhibitors of cyclic AMP phosphodiesterase such as papaverine, increase the intracellular level of cyclic AMP, thereby possibly triggering vasodilation through biochemical steps yet to be clarified [2, 3]. The other group, which is much less active in the inhibition of cyclic AMP phosphodiesterase, has been assumed to produce vasodilation primarily by reducing the influx of calcium ion across cell membranes. An example of this group is verapamil [4].

The inhibition of cyclic AMP phosphodiesterase by YC-93 and its structural analogues was investigated in an attempt to elucidate the mechanism of vasodilation of these compounds. These analogues

* To whom correspondence should be sent: Hiroo Maeno, Ph.D. Department of Pharmacology and Biochemistry, Central Research Laboratories, Yamanouchi Pharmaceutical Co., Ltd., No. 1-8, Azusawa-1-chome, Itabashi-ku, Tokyo 174, Japan.

† The abbreviations used are: YC-93, 2,6-dimethyl 4-(3-nitrophenyl)-1,4-dihydropyridine-3,5-dicarboxylic acid 3-[2-(*N*-benzyl-*N*-methylamino)]-ethyl ester 5-methyl ester hydrochloride; IBMX, 3-isobutyl-1-methylxanthine; DDC, 3,5-diethoxycarbonyl-1,4-dihydro-2,4,6-collidine; cyclic AMP, cyclic adenosine 3′,5′-monophosphate.

were found to be a structurally new type of extremely potent cyclic AMP phosphodiesterase inhibitors. It was also demonstrated that YC-93 increased the intracellular level of cyclic AMP in canine coronary artery. These results suggest that at least a partial involvement of phosphodiesterase inhibition in vasodilation by YC-93.

MATERIALS AND METHODS

Materials. Beef heart phosphodiesterase (specific activity, 0.15 unit/mg protein) and cyclic AMP were purchased from Boehringer Mannheim Yamanouchi. 8-[^{3}H]-Cyclic AMP (specific radioactivity, 27.5 Ci/m-mole), *Naja haja* venom and IBMX were from Radiochemical Centre, Amersham, Sigma and Aldrich, respectively. DDC, theophylline and papaverine hydrochloride were from Tokyo Kasei. YC-93 and its analogues were supplied by Dr. M. Iwanami in our Laboratories.

Enzyme preparation. Mongrel dogs weighing 8–25 Kg, anesthetized with sodium pentobarbital (25 mg/kg i.v.), were sacrificed by bleeding from the common carotid artery. Basilar, carotid, coronary and femoral arteries were dissected and suspended in cold 0.04 M Tris-HCl buffer, pH 7.5, containing 0.25 M sucrose, 5 mM 2-mercaptoethanol and 5 mm $MgCl_2$. All subsequent preparation processes were performed at 4°. Each dissected artery was minced with scissors and homogenized in 9 volumes of the Tris-HCl buffer by a glass homogenizer with a motor-driven glass pestle. The homogenates were centrifuged at 105,000 *g* for 1 hr and the supernatant fractions were used as the source of cyclic AMP phosphodiesterase.

Epididymal fat cells were prepared by the method of Rodbell [5]. Canine cerebral cortex, heart, liver and epididymal fat cells were also homogenized as described for the preparation of arterial homogenates, and the 105,000 *g* supernatants were used in the measurement of phosphodiesterase activity.

Enzyme assay. Cyclic AMP phosphodiesterase activity was determined by measuring the appearance of [^{3}H]-5′-AMP resulting from the enzymatic hydrolysis of [^{3}H]-cyclic AMP as described by Terai *et al.* [6]. The standard incubation mixture contained in a total volume of 0.5 ml 30 mM Tris-HCl, pH 7.5, 5 mM $MgCl_2$, 1 mM 2-mercaptoethanol, 100 mM NaCl, 1 μM or 50 μM [^{3}H]-cyclic AMP, 50 μg bovine serum albumin and the appropriate amount of enzyme protein to give less than 20 per cent hydrolysis of the added substrate. The incubation was initiated by addition of enzyme, carried out at 37° for 10 min and stopped by placing the incubation tube in a boiling water bath for 2 min. After cooling, 10 μl of the aqueous solution of 50 μg *Naja haja* venom was added as the source of 5′-nucleotidase and then the incubation mixture was maintained at 37° for 30 min. Following the addition of 0.5 ml of 0.5 mM adenosine as a carrier, the reaction mixture was applied to a Dowex-1 (Cl-form) column (0.7 cm × 2.0 cm). Adenosine was eluted with 8 ml of 0.1 M Tris-HCl buffer, pH 7.5, and the radioactivity was determined in 10 ml of Bray's scintillator in a Packard liquid scintillation spectrometer (Model 3385) [7]. Under these experimental conditions, neither YC-93 nor its analogues affected 5′-nucleotidase and the recovery of adenosine from the Dowex-1 column was more than 95 per cent. In addition, the assay method of Pöch [8], in which the amount of unhydrolyzed cyclic AMP remaining was determined after removal of the reaction product (5′-AMP) by precipitation with $ZnSO_4$-$Ba(OH)_2$, gave similar results for the inhibition of beef heart cyclic AMP phosphodiesterase (data not shown).

Measurement of cyclic AMP level in the coronary artery. Descending and circumflex branches of the left coronary artery (outer diameter, 1.5–2.0 mm) were sliced with a razor blade into circular pieces of approximately 2 cm in length. Each piece was then cut lengthways into halves. One of the strips was used to measured the level of cyclic AMP in the presence of YC-93 and the other half was for the control experiment in the absence of the drug. The tissue fragment was placed in a 10 ml organ bath filled with modified Ringer solution consisting of 145.4 mM NaCl, 5.4 mM KCl, 2.2 mM $CaCl_2$, 14.9 mM $NaHCO_3$ and 10 mM glucose. The organ bath was aerated with a gas mixture of 95% O_2 and 5% CO_2 at 37°. The tissue was preincubated for 120 min with occasional changes of the medium at 30 min intervals. The incubation was initiated by addition of YC-93 and terminated by freezing the tissue fragments in liquid nitrogen after 1 or 5 min exposure to the drug. The frozen tissue was then weighed, put into a glass tube containing 2 ml of 6% (w/v) cold trichloroacetic acid and homogenized in a Polytron (PT 10–35 Kinematica GmbH) for 2 min in ice. After centrifugation of the homogenate at 1800 *g* for 20 min, the supernatant was washed seven times with 10 ml of water-saturated ethylether. An aliquot of the washed extract was dried under reduced pressure and then used to determine the amount of cyclic AMP according to Brown *et al.* [9]. Protein was measured by the method of Lowry *et al.* [10] using bovine serum albumin as a standard.

RESULTS

Inhibition by YC-93 of cyclic AMP phosphodiesterases from canine arteries and beef heart. YC-93 was tested for its ability to inhibit cyclic AMP phosphodiesterases from canine basilar, carotid, coronary and femoral arteries as well as the purified enzyme from beef heart. Each enzyme preparation from the four arteries exhibited two apparent K_m values as determined by a double reciprocal plot. The lower K_m values of the canine arterial enzymes were estimated to be about 1 μM, while the higher K_m values ranged from 50 to 70 μM (Table 1). The inhibition by YC-93 was competitive with respect to cyclic AMP with all four arterial enzyme preparations, as represented by that from the coronary artery in Fig. 1. The K_i values of these enzyme preparations for YC-93 were only slightly different, ranging from 2.0 to 4.3 μM at substrate concentrations near the low K_m. In the presence of high concentrations of cyclic AMP the K_i's were between 4.0 and 12 μM (Table 1). These results indicate that the kinetic parameters of the inhibition of the four arterial phosphodiesterases by YC-93 were quite similar. Figure 2 illustrates kinetic studies on

Table 1. Apparent K_m for cyclic AMP and K_i for YC-93 of canine arterial phosphodiesterases*

Artery	K_m (μM)	K_i (μM)
Basilar	1.1	4.2
	50.0	10.0
Carotid	1.3	2.7
	68.0	12.0
Coronary	1.0	2.0
	50.0	4.0
Femoral	1.0	4.3
	50.0	10.0

* Each 105,000 g supernatant of the four arteries was used for the assay of cyclic AMP phosphodiesterase activity. The enzyme activity was measured under the standard conditions in the presence of 20 μg protein in the range of 0.5–5.0 μM cyclic AMP or in the presence of 100 μg protein in the range of 25–500 μM cyclic AMP, with and without 10 μM YC-93. Apparent K_i values were computed according to the Lineweaver–Burk plot.

beef heart phosphodiesterase which possessed only a single K_m of 45 μM under the standard assay conditions. The inhibition type of YC-93 was competitive and the K_i was found to be 2 μM, indicating that beef heart phosphodiesterase is slightly more susceptible to YC-93 than the arterial enzymes.

Effects of YC-93 and some known inhibitors on phosphodiesterases from canine coronary artery and beef heart. The concentrations (I_{50}) of YC-93, papaverine, theophylline, and IBMX necessary to inhibit by 50 per cent the hydrolysis of cyclic AMP by low K_m phosphodiesterase from the canine coronary artery and high K_m phosphodiesterase from beef heart were determined graphically by plotting the enzyme activity against the logarithm of five different concentrations of the drugs. YC-93, although structurally unrelated to such known inhibitors as illustrated in Table 2, was the most potent among the inhibitors and inhibited almost completely the phosphodiesterase activity of both enzyme preparations at the concentration of 80 μM.

Structure-activity relationship of 1,4-dihydropyridine derivatives. The I_{50} values of a series of 1,4-dihydropyridine derivatives were determined in comparison with YC-93 under the standard assay conditions using coronary arterial low K_m phosphodiesterase (Table 3). Removal of a nitro group from the phenyl ring at position 4 of the dihydropyridine ring reduced considerably the inhibitory effect of YC-93 on coronary arterial phosphodiesterase as seen in the comparison among the four compounds (YC-93, compound 1, 2 and 3). Although the effect of the position of the nitro group was not as great, it was evident that YC-93 with a *m*-nitro group exhibited the highest inhibitory effect. Since the substitution of a methyl group (compound 4) or a hydrogen (compound 5) for the benzyl group (YC-93) in the side chain at position 3 of the 1,4-dihydropyridine ring resulted in a marked reduction in the inhibitory potency, the benzyl group seemed to be important for the inhibitory effect of YC-93. It was also demonstrated that replacement of the benzyl group (YC-93) by a phenyl group (compound 6) at position 3 showed a slight increase in the inhibitory potency.

Regarding the effects of modifying R_3 in the side chain at position 5 of the dihydropyridine ring, only

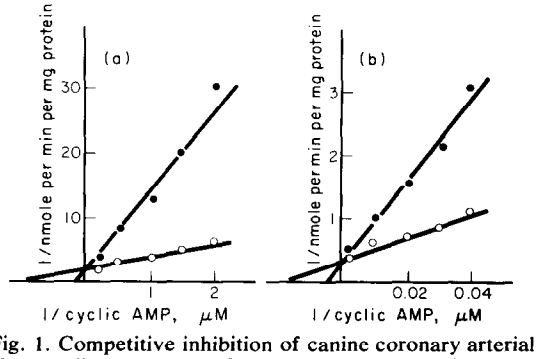


Fig. 1. Competitive inhibition of canine coronary arterial phosphodiesterase by YC-93. (a) The phosphodiesterase activity was determined with 20 μg of enzyme protein under the standard assay conditions in the presence (—●—) and absence (—○—) of 10 μM YC-93. (b) The phosphodiesterase activity was determined with 100 μg of enzyme protein under the standard assay conditions in the presence (—●—) and absence (—○—) of 10 μM YC-93.

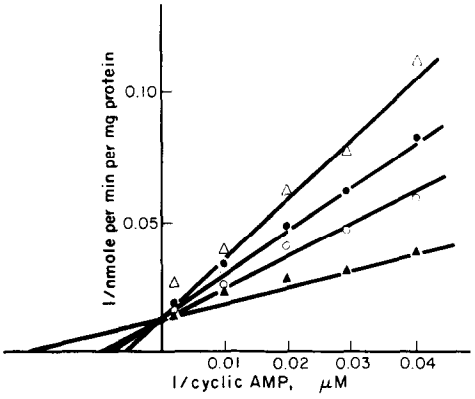


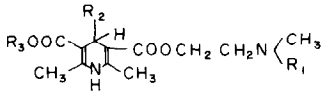
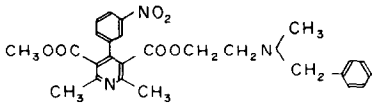
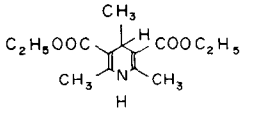
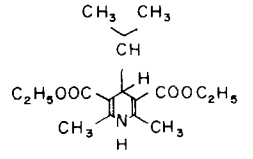
Fig. 2. Competitive inhibition of beef heart phosphodiesterase by YC-93. The phosphodiesterase activity was determined with 5 μg enzyme protein under the standard assay conditions in the presence of 2 μM (—○—), 4 μM (—●—), 6 μM (—△—) YC-93 and in its absence (—▲—).

Table 2. Inhibition of phosphodiesterase from canine coronary artery and beef heart by YC-93 and some known inhibitors*

Compound	Phosphodiesterase inhibition I_{50} (μM) Coronary artery (low K_m enzyme)	Beef heart (high K_m enzyme)
YC-93	5.0 ± 0.5	4.5 ± 0.3
Papaverine	15.0 ± 4.0	19.0 ± 1.0
Theophylline	310.0 ± 50.0	190 ± 20.0
IBMX	34.0 ± 5.0	8.0 ± 0.4

* The phosphodiesterase activity was determined under the standard assay conditions in the presence of 1 μM cyclic AMP and 20 μg coronary arterial enzyme protein, or in the presence of 50 μM cyclic AMP and 5 μg beef heart enzyme protein. The I_{50} values were obtained from the dose response curves with five different concentrations of the inhibitors, and the mean values ± S.E. from three experiments are presented.

Table 3. Structure-activity relationship in inhibition of phosphodiesterase by 1,4-dihydropyridine derivatives*

Compound	Structural formula			Phosphodiesterase inhibition $I_{50}(\mu M)$	
				Coronary artery (low K_m enzyme)	Beef heart (high K_m enzyme)
					
	R_1	R_2	R_3		
YC-93†	CH_2-Ph	m-NO_2-Ph	CH_3	5.0 ± 0.5	4.5 ± 0.3
1†	CH_2-Ph	o-NO_2-Ph	CH_3	15.0 ± 2.5	4.9 ± 0.7
2†	CH_2-Ph	p-NO_2-Ph	CH_3	14.0 ± 1.5	6.3 ± 0.8
3†	CH_2-Ph	Ph	CH_3	37.0 ± 5.0	11.6 ± 1.9
4†	CH_3	m-NO_2-Ph	CH_3	50.0 ± 5.0	16.4 ± 1.9
5†	H	m-NO_2-Ph	CH_3	98.0 ± 4.0	34.8 ± 4.3
6	Ph	m-NO_2-Ph	CH_3	2.7 ± 0.6	3.3 ± 0.3
7†	CH_2-Ph	m-NO_2-Ph	C_2H_5	5.0 ± 0.5	1.2 ± 0.1
8†	CH_2-Ph-OCH_3	m-NO_2-Ph	C_2H_5	2.0 ± 0.3	0.87 ± 0.02
9†	CH_2-Ph-Cl	m-NO_2-Ph	C_2H_5	2.7 ± 0.6	0.83 ± 0.05
10†	CH_3	m-NO_2-Ph	C_2H_5	49.5 ± 15.5	5.0 ± 0.9
11	CH_3	i-C_3H_7	C_2H_5	390.0 ± 25.0	233.0 ± 40.8
12†	CH_2-Ph	m-NO_2-Ph	i-C_3H_7	2.0 ± 0.6	1.5 ± 0.2
13				100.0 ± 17.8	43.7 ± 9.5
14 (DDC)				33.0 ± 4.0	29.3 ± 6.3
15				87.3 ± 3.3	57.3 ± 6.7

* The phosphodiesterase activity was determined as described in Table 2.

† All compounds were hydrochloride salt and dissolved in water. The other compounds without a dagger symbol were dissolved in absolute ethanol and then an aliquot of 10 μl was directly added to the reaction mixture. The final concentration of ethanol in the reaction mixture was 2 per cent, which had no effect on the enzyme activity.

three compounds (YC-93, compound 7 and 12) were comparable in the present experiments. No significant difference was detected in the inhibitory potency between methyl (YC-93) and ethyl groups (compound 7) at R_3, while the isopropyl ester (compound 12) exhibited 2.5-fold higher potency than the former two. Introduction of a methoxy (compound 8) or a chlor group (compound 9) onto para position of the phenyl ring at R_1 of compound 7 increased the inhibition of phosphodiesterase slightly more than 2-fold. The substitution of a methyl (compound 10) for the benzyl group (compound 7) at R_1 reduced greatly the inhibitory potency as already seen between YC-93 and compound 4, which has a methyl group at R_3. These results indicate clearly that the benzyl group at R_1 is important for the inhibition of

Table 4. Inhibition of low K_m phosphodiesterase from several canine tissues by YC-93, compound 8, papaverine and IBMX*

Tissue	Phosphodiesterase inhibition I_{50} (μM) YC-93	Compound 8	Papaverine	IBMX
Coronary artery	5.0 ± 0.5	2.0 ± 0.3	15.0 ± 4.0	34.0 ± 5.0
Cerebral cortex	3.0 ± 0.1	1.2 ± 0.2	15.0 ± 0.4	31.8 ± 2.7
Heart	18.4 ± 0.9	12.5 ± 0.8	21.5 ± 0.5	23.5 ± 1.5
Liver	13.7 ± 0.2	12.2 ± 1.5	10.6 ± 0.3	25.3 ± 2.6
Fat cells	17.0 ± 0.5	12.3 ± 0.2	12.1 ± 1.2	30.0 ± 1.7

* Enzyme activity was determined under the standard assay conditions in the presence of 1 μM cyclic AMP. The I_{50} values were obtained from dose response curves with three different concentrations of inhibitors, and the mean values ± S.E. from the three experiments were presented in the Table.

phosphodiesterase. It appears that the *m*-nitrophenyl group at position 4 of the dihydropyridine ring is important for the inhibition of phosphodiesterase, since the replacement of the *m*-nitrophenyl group of compound 10 by an isopropyl group (compound 11) markedly decreased the inhibitory potency.

It is noteworthy that one (compound 13) of the biological oxidation metabolites of YC-93 [11], which has a pyridine ring instead of 1,4-dihydropyridine, also competitively inhibited the phosphodiesterase though the potency was only one twentieth as great as that of the mother compound. These data demonstrate that the 1,4-dihydropyridine ring of YC-93 was an important structural requirement for the inhibitory potency. Compound 14 (DDC), structurally simplest among 1,4-dihydropyridine derivatives tested, was much less inhibitory than YC-93 as presented in Table 3. Replacement of the methyl group of compound 14 by an isopropyl group (compound 15) at position 4 of the dihydropyridine ring reduced significantly the inhibitory potency.

Similar experiments on the structure-activity relationship were also performed with the high K_m enzyme of the Boehringer beef heart preparation (Table 3). The order of the inhibitory potency of 1,4-dihydropyridine derivatives was on the whole very similar to that with the low K_m enzyme from canine coronary artery. Most potent were compound 8 and compound 9, both of which have a substitution (methoxy and chlor group, respectively) at the para position of the phenyl group in R_1. The biological metabolite of YC-93, compound 13, exhibited far less inhibitory potency than YC-93, as in the case of coronary artery enzyme.

Inhibition of low K_m phosphodiesterase from several canine tissues by YC-93, compound 8, papaverine and IBMX. The inhibitory effects of YC-93, compound 8, papaverine and IBMX on the phosphodiesterase activity of high-speed supernatants from coronary artery, cerebral cortex, heart, liver and fat cells were compared under the standard assay conditions in the presence of 1 μM cyclic AMP (Table 4). It appeared that the compounds with the 1,4-dihydropyridine ring tended to inhibit more selectively phosphodiesterases of coronary artery and cerebral cortex rather than those of the other tissues. With cerebral cortex as an enzyme source, compound 8 (I_{50} = 1.2 μM) was approximately 13-fold more potent than papaverine (I_{50} = 15 μM) when the I_{50} values were compared. However, with liver phosphodiesterase, YC-93, compound 8 and papaverine exhibited relatively similar inhibitory potencies.

Effect of YC-93 on cyclic AMP level of canine coronary artery. In order to see whether the intracellular level of cyclic AMP actually increased in tissues as the result of phosphodiesterase inhibition, the strips of canine coronary artery were incubated with 1 μM or 0.1 μM YC-93. As shown in Table 5, the cyclic AMP level was already increased significantly by 64 per cent ($P < 0.01$) after 1 min exposure of the tissue to 1 μM YC-93, and continued to increase with time. In the presence of 0.1 μM YC-93, 5 min incubation did not alter significantly the amount of cyclic AMP in the tissue.

DISCUSSION

The present experiments demonstrate that YC-93, a new potent vasodilator structurally characterized by the 1,4-dihydropyridine ring, inhibits very strongly the activity of cyclic AMP phosphodiesterase

Table 5. Effect of YC-93 on cyclic AMP level of canine coronary artery*

Concentration of YC-93 (μM)	Incubation time, min	Amount of cyclic AMP (pmoles/mg protein) −YC-93	+YC-93	Percent of control in cyclic AMP level
0.1	1	22.8 ± 3.8	23.6 ± 4.3	98.3 ± 7.6
	5	28.3 ± 5.7	38.6 ± 6.4	138.5 ± 13.1
1	1	18.9 ± 4.1	31.9 ± 9.2	163.6 ± 15.0†
	5	20.2 ± 3.3	37.0 ± 5.5	189.6 ± 20.8†

* The experimental conditions were described in Methods. The mean values ± S.E. from five experiments are presented.

† Significantly different from the control level of cyclic AMP by a paired *t*-test ($P < 0.01$).

from canine artery and beef heart in a competitive manner despite the lack of structural similarity to cyclic AMP. However, apparent structural similarity between cyclic AMP and drugs in general may not necessarily be a determinant of inhibition type. For instance, it has been shown that the type of inhibition of phosphodiesterase by papaverine, SQ-20,009, theophylline or cyclic GMP depends upon the enzyme source used [12–16]. In addition, other important factors contributing to the kinetic nature of the inhibition may be the relative purity of the enzyme, the presence of activator or calcium and multiple forms of the enzyme [14]. Thus, the possibility cannot be ruled out that the kinetic nature of the inhibition by YC-93 is altered under other experimental conditions.

YC-93 evidently can be classified into a structurally new family of phosphodiesterase inhibitors. The structural importance of the 1,4-dihydropyridine ring in the inhibition of phosphodiesterase could be demonstrated by the finding that the biological oxidation of the 1,4-dihydropyridine ring of YC-93 to a corresponding pyridine derivative (compound 13, Table 3) reduced the original inhibitory activity to one twentieth. It is of interest to note that this oxidation product still preserved the competitive type of inhibition.

YC-93 appeared to inhibit specifically only cyclic AMP phosphodiesterase among the enzymes involving cyclic AMP for their activities. The drug at 80 μM was maximally effective in the inhibition of phosphodiesterase and showed no significant effect on adenylate cyclase, endogenous cyclic AMP-dependent protein kinase and endogenous cyclic AMP-dependent protein phosphatase from the microsomes of rat uterus or on the basal activity of adenylate cyclase of canine caudate nucleus [17]. In addition, Ca-dependent ATPase of uterine microsomes as well as myosine ATPase from uterus, skeletal muscle or cardiac muscle were not affected by YC-93 [17]. Accordingly, YC-93 may be a good reagent to protect cyclic AMP from enzymatic hydrolysis and allow for the accurate assessment of its formation during incubation.

It is not surprising that such a low concentration of YC-93 as 0.5 K_i caused such a large, rapid increase in the concentration of intracellular cyclic AMP of canine arterial strips without stimulating adenylate cyclase as shown in Table 5. Assuming no change in the enzymatic formation of intracellular cyclic AMP in the presence of a competitive inhibitor of phosphodiesterase, the steady state concentration of intracellular cyclic AMP would be $(1+I/K_i)$ fold higher than that in the absence of the inhibitor, according to the equation: $V = V_{max}/[1+(K_m/S)\ (1+I/K_i)]$ (V; velocity in the presence of the inhibitor, V_{max}; maximum velocity, K_m; Michaelis constant of the substrate (S), S; cyclic AMP concentration, I; inhibitor concentration). In the present experiments the content of cyclic AMP is 1 nmole/g tissue (equivalent to the low K_m of phosphodiesterase) and the specific activity of cyclic AMP phosphodiesterase at 1 μM is 12.5 nmoles/min/g tissue. When YC-93 is added at 1 μM (equal to a half of K_i) which may cause 20 per cent inhibition of low K_m phosphodiesterase activity, one may expect within a min a new steady state of cyclic AMP content which is one and a half times higher than that in the absence of YC-93. Moreover, it must be taken into account that the drug accumulates to about 2-fold concentration (equivalent to the K_i) in a min in the arterial preparation in our preliminary experiment. Thus, a 64 per cent increase in a min in intracellular cyclic AMP by addition of 1 μM YC-93 is not abnormally high but in the range of theoretical prediction.

Since much evidence has accumulated indicating that cyclic AMP is a mediator for the relaxation of smooth muscle of various tissues [3, 18, 19], it seems likely that the increase in intracellular cyclic AMP may be responsible at least partly for the relaxation of arterial smooth muscle induced by YC-93 Recently, Nishikori *et al.* [20] demonstrated that the relaxation of uterine smooth muscle by dibutyryl cyclic AMP might occur through the increase in microsomal calcium uptake following the stimulation of cyclic AMP-dependent phosphorylation of a microsomal protein with 49,000 daltons. Furthermore, they demonstrated that YC-93 caused an increase in intracellular cyclic AMP in the uterus as well as an increase in microsomal Ca-uptake [17]. In the coronary artery, similar experiments are now under way.

REFERENCES

1. T. Takenaka, S. Usuda, T. Nomura, H. Maeno and T. Sado, *ArzneimittelForsch.* **26**, 2172 (1976).
2. W. R. Kukovetz and G. Pöch, *Naunyn-Schmiedebergs Arch. exp. Path. Pharmak.* **267**, 189 (1970).
3. G. Pöch and W. R. Kukovetz, in *Advances in Cyclic Nucleotide Research* (Eds P. Greengard and G. A. Robinson), Vol. 1, p. 195. Raven Press, NY (1972).
4. G. Haeusler, R. Eigenmann and W. P. Burkard, *Naunyn-Schmiedebergs Arch. exp. Path. Pharmack.* **274**, R48 (1972).
5. M. Rodbell, *J. biol. Chem.* **239**, 375 (1964).
6. M. Terai, C. Furihata, T. Matsushima and T. Sugimura, *Archs Biochem. Biophys.* **176**, 621 (1976).
7. G. A. Bray, *Analyt. Biochem.* **1**, 279 (1960).
8. G. Pöch, *Naunyn-Schmiedebergs Arch. exp. Path. Pharmak.* **268**, 272 (1971).
9. B. L. Brown, J. D. M. Albano, R. P. Ekins and A. M. Sgherzi, *Biochem. J.* **121**, 561 (1971).
10. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. biol. Chem.* **193**, 265 (1951).
11. S. Higuchi, H. Sasaki, Y. Shiobara and T. Sado, *Xenobiotica*, **7**, 469 (1977).
12. G. Pöch and W. R. Kukovetz, *Life Sci.* **10**, Part 1, 133 (1971).
13. M. Chasin, D. N. Harris, M. B. Phillips and S. M. Hess, *Biochem. Pharmac.* **21**, 2443 (1972).
14. R. W. Butcher and E. W. Sutherland, *J. biol. Chem.* **237**, 1244 (1962).
15. D. G. Therriault and V. G. Winters, *Life Sci.* **9**, Part II, 1053 (1970).
16. T. R. Russell, W. L. Terasaki and M. M. Appleman. *J. biol. Chem.* **248**, 1334 (1973).
17. K. Nishikori, T. Takenaka and H. Maeno, *Seikagaku*, **48**, 367 (1976).
18. R. Andersson, L. Lundholm, E. Mohme-Lundholm and K. Nilsson, in *Advances in Cyclic Nucleotides Research* (Eds P. Greengard and G. A. Robison), Vol. 1, p. 213. Raven Press, NY (1972).
19. H. P. Bär, in *Advances in Cyclic Nucleotide Research* (Eds P. Greengard and G. A. Robison), Vol. 4, p. 195. Raven Press, NY (1974).
20. K. Nishikori, T. Takenaka and H. Maeno, *Molec. Pharmac.* **13**, 671 (1977).