Biochemical Pharmacology, Vol. 33, No. 21, pp. 3339–3344, 1984.
Printed in Great Britain.

0006-2952/84 $3.00 + 0.00

PURIFICATION OF CYCLIC ADENOSINE MONOPHOSPHATE PHOSPHODIESTERASE FROM HUMAN PLATELETS USING NEW-INHIBITOR SEPHAROSE CHROMATOGRAPHY

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(*Received* 12 *December* 1983; *accepted* 16 *April* 1984)

Abstract—Cilostamide derivatives are potent inhibitors of human platelet aggregation and selectively inhibit human platelet cyclic adenosine monophosphate (cyclic AMP) phosphodiesterase. *N*-Cyclohexyl-*N*-(2-hydroxybutyl)-5-[6-1,2,3,4-tetrahydro-2-oxoquinolyloxy)]-butyramide (OPC-13135) is one of these derivatives, and the concentration of OPC-13135 producing 50% inhibition of human platelet aggregation induced by 2 μg/ml collagen was 5 μM. On the other hand, the concentrations of OPC-13135 producing 50% inhibition of human platelet cyclic AMP phosphodiesterase and cyclic guanosine monophosphate (cyclic GMP) phosphodiesterase were 0.073 and 21.8 μM, respectively. We purified over 480-fold the soluble low K_m form of cyclic AMP phosphodiesterase from human platelets, using OPC-13135 Sehparose column as a final step in the purification procedure. The purified protein has a molecular weight of 175,000, determined by gel filtration and is an acidic protein, as determined by isoelectric focussing (pI = 4.9). Kinetic measurements indicated that the enzyme protein had a K_m value for the substrate cyclic AMP and cyclic GMP of 0.34 and 0.11 μM respectively, and a V_{max} value of 85.3 and 19.8 nmole/min/mg protein, respectively. K_i value of the OPC-13135 for the enzyme was 0.015 μM and was of competitive fashion against cyclic AMP.

Since inhibition by cyclic AMP of platelet aggregation *in vitro* was reported in 1965 [1], changes in the endogenous cyclic AMP levels with platelet activity have been demonstrated [2, 3]. It was subsequently shown that various pharmacological agents known to affect platelet function influence cyclic nucleotide metabolism of platelets [4]. Cyclic nucleotide phosphodiesterase (EC3) [1, 4, 5] catalyzes the hydrolysis of cyclic AMP and cyclic GMP, the only known catabolic mechanism for these important regulatory nucleotides. Kinetic and physical criteria provided support as to the existence of multiple molecular forms of this enzyme system in human platelets and also in a wide variety of tissues [6]. We reported that human blood platelets contained at least three kinetically distinct forms of 3′,5′-cyclic nucleotide phosphodiesterase which could be clearly separated by DEAE-cellulose column chromatography [7]. More recently, we found that *N*-cyclohexyl-*N*-methyl-4 -(1,2-dihydro-2 -oxo-6 -quinoloxy) butyramide (OPC-3689, cilostamide) inhibited selectively platelet cyclic AMP phosphodiesterase, separated from cyclic GMP phosphodiesterase by DEAE-cellulose column. This novel compound was found to inhibit human platelet aggregation induced by ADP, collagen and arachidonic acid (8). We now report that cilostamide derivatives (OPC-13013, OPC-13135, OPC-3911, OPC-13032 and OPC-13120) suppress human platelet aggregation induced by collagen and selectively inhibit a high affinity cyclic AMP phosphodiesterase from human platelets. We purified the low K_m cyclic AMP phosphodiesterase from human platelets and the final step was affinity chromatography using OPC-13135 coupled to Sepharose 6B. OPC-13135 Sepharose proved to be a useful tool for purifying cyclic AMP phosphodiesterase from human platelets.

MATERIALS AND METHODS

Materials. Cyclic [8-^{3}H]AMP (specific activity 26 Ci/mole) and cyclic [8-^{3}H]GMP (specific activity 16 Ci/nmole) were purchased from Amersham International Ltd. (U.K.). Unlabeled cyclic nucleotide (cyclic AMP and cyclic GMP), soybean trypsin inhibitor and snake venom were purchased from Sigma Chemical Company (St. Louis, MO). DEAE-cellulose (DE-52) was purchased from Whatman Biochemicals Ltd. (Clifton, NJ). Blue Sepharose 6B, Sephacryl S-300, epoxy-activated Sepharose 6B and the pH 3–10 Pharmalyte were from Pharmacia Fine Chemicals (Sweden). OPC-13013, OPC-13135,

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Abbreviations: W-7, *N*-(6-aminohexyl)-5-chloro-1-naphthalene-sulfonamide; EGTA, ethylene glycol bis (β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; PMSF, phenylmethyl-sulfonylfluoride; OPC-13013, 6-[4-(1-cyclohexyl-5-tetrazolyl) butoxyl]-1,2,3,4-tetrahydro-2-oxoquinoline; OPC-13135, q*N*-cyclohexyl-*N*-(2-hydroxybutyl) -5-[6-(1,2,3,4-tetrahydro-2-oxoquinolyloxy)] butyramide; OPC-3911, / *N*-cyclohexyl-*N*-(2-hydroxyethyl)-4-[6-(1,2-dihydro-2-oxoquinolyloxy)] butyramide; OPC-13032, 6-[3-(4-phenylpiperazinylcarbonyl) propoxy]-2-quinolone; OPC-13120, *N*-cyclohexyl-*N*-(4-hydroxybutyl)-4-[6-(1,2,3,4-tetrahydro-2-oxoquinolyloxy)] butyramide.

This work was supported in part by a Grant-In-Aid for Scientific Research from the Ministry of Education, Science and Culture, Japan (1983–1984).

OPC-3911, OPC-13032 and OPC-13120 were synthesized in the Laboratory of Medical Chemistry, Otsuka Pharmaceutical Co., Ltd. (Tokushima, Japan). The chemical structures are shown in Fig. 1. Calmodulin (CaM) was isolated from bovine brain by Ca^{2+}-dependent affinity chromatography on W-7 Sepharose column, as previously described [9].

Platelet aggregation studies. Blood samples were collected from healthy volunteers by venipuncture, into acid–citrate dextrose (volume preparation 1 : 6). Platelet-rich plasma (PRP) was prepared by centrifugation at 120 *g* for 15 min. Platelet-poor plasma (PPP) was obtained by further centrifugation at 1500 *g* for 10 min. Platelet aggregation was studied by the turbidimetric method of Born [10], using a Rikadenki 4 channel Aggregometer (RAM, 41, Rikadenki Kogyo Co., Ltd., Japan) modified to provide continuous stirring and maintenance of constant temperature (37°). The standard platelet reaction mixture consisted of 270 μl of PRP, 30 μl of a drug solution in saline, and the aggregating agent. All concentrations given refer to the final ones. PRP was kept tightly capped at all times, and experiments were completed within 2–3 hr of blood collection. Each experiment in which inhibition was obtained was followed by a control experiment which clearly demonstrated that the platelets remained responsive to the stimulus in the absence of the inhibitor.

Assay procedures. Cyclic nucleotide phosphodiesterase activity was measured, as previously described [11]. Unless otherwise noted, the enzymatic reaction was in a total volume of 0.5 ml containing buffer (50 mM Tris–HCl, pH 8.0 and 5 mM $MgCl_2$), substrate (0.4 μM cyclic [^{3}H]adenosine monophosphate, 100,000 cpm or 0.4 μM cyclic [^{3}H] guanosine monophosphate, 100,000 cpm), and enzyme preparation. The reaction was run at 30° for 15 min before termination by boiling for 60 sec. 5′-[^{3}H]AMP or GMP formed by the phosphodiesterase is converted to [^{3}H]adenosine or [^{3}H]guanosine by the action of nucleotidase and the product isolated by cation exchange resin was counted in a liquid scintillation counter. Adenosine or guanosine was confirmed by thin-layer chromatography to be the only breakdown product from cyclic AMP or cyclic GMP. Over 95% of adensone or guanosine is recovered by this method. About 10% of the added cyclic nucleotide is generally hydrolyzed during the incubation. The product formation was linear up to 30 min, using this assay.

Others. Protein concentrations were determined according to Lowry *et al.* [12] with bovine serum albumin as a standard. CaM was assayed for its ability to activate a fixed amount of CaM-deficient phosphodiesterase, under standard conditions [13]. The coupling of OPC-13135 to epoxy-activated Sepharose 6B was carried out, as previously described [9].

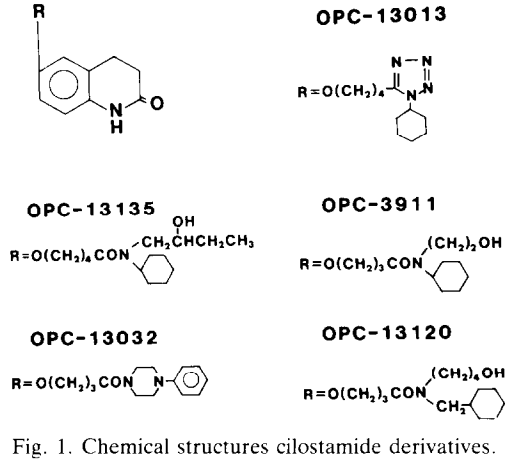

Fig. 1. Chemical structures cilostamide derivatives.

RESULTS

Effects of cilostamide derivatives on human platelet aggregation in vitro. Collagen, when added to the human PRP, aggregated the platelets, in a dose-dependent fashion. All the cilostamide derivatives, at low concentrations, prevented the platelet aggregation induced by 2 μg/ml of collagen, the minimum dose required to produce maximum aggregation of human platelets under the experimental conditions used. The addition of cilostamide derivatives resulted in a marked inhibition of the aggregation and the extent of inhibition was concentration dependent. A typical result seen with the cilostamide derivatives (OPC-3911) is shown in Fig. 2. The concentrations of cilostamide derivatives producing 50% inhibition of human platelet aggregation by collagen are summarized in Table 1. Among these derivatives, OPC-3911 had the most potent effect on collagen-induced aggregation.

Inhibition of human platelet cyclic nucleotide phosphodiesterase by cilostamide derivatives. The effects of cilostamide derivatives on human platelet cyclic nucleotide phosphodiesterase were examined using DEAE-resolved phosphodiesterase as the enzyme preparation. Human platelets contain at least three distinct cyclic nucleotide phosphodi-

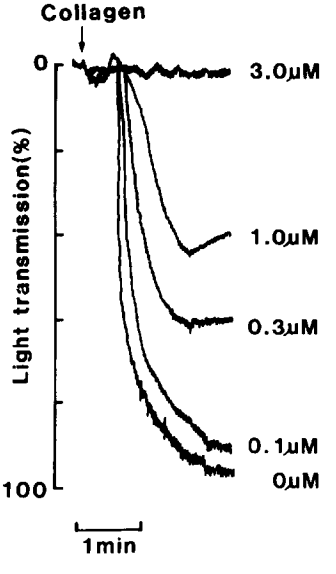

Fig. 2. Effect of OPC-3911 on human platelet aggregation induced by collagen (2 μg/ml). The extent of aggregation was expressed as the change in light transmission (vertical scale).

Table 1. Effect of cilostamide derivatives on human platelet aggregation*

Agents	IC_{50} (μM)†
OPC-13013	12 ± 2.3
OPC 13135	5 ± 0.09
OPC-3911	0.7 ± 0.01
OPC-13032	7.2 ± 0.56
OPC-13120	1.8 ± 0.38

* Collagen (2 μg/ml) was used as an inducer and platelets were preincubated for 1 min, with or without cilostamide and at concentrations ranging from 1×10^{-7} to 1×10^{-5} M.

† The IC_{50} values are mean ± S.E. of six experiments with six different populations.

esterases which can be separated by DEAE-cellulose column chromatography [7]. These enzymes are specific cyclic GMP (FI), a cyclic nucleotide (FII) and a specific cyclic AMP phosphodiesterase (FIII). All of the cilostamide tested inhibited these forms of the phosphodiesterase to different degrees. Table 2 summarizes the effect of these compounds on FI and FIII. The results indicated that cilostamide derivatives selectively inhibited FIII. OPC-3911, OPC-13032 and OPC-13135 were 300 times more potent inhibitors of FIII than of FI. In addition, these compounds showed no significant effects on Ca^{2+}-calmodulin activated phosphodiesterase from rat brain below 1 mM.

Purification of cyclic AMP phosphodiesterase. DEAE-cellulose resolved phosphodiesterase was prepared, as previously reported [7]. FIII fractions (82.7 mg protein) of DEAE-cellulose chromatography were pooled and dialyzed overnight against 50 mM Tris–acetate buffer (pH 6.0) containing 1 mM ethylene glycol bis (β-aminoethyl ether)-*N*,*N*,*N*′,*N*-tetraacetic acid (EGTA), 3.75 mM 2-mercaptoethanol, 75 mg/l of phenylmethylsulfonylfluoride (PMSF). The dialyzed material was applied to a Blue Sepharose column equilibrated with the same buffer. The column was washed extensively with the equilibrating buffer until the u.v. absorbance at 280 nm returned to the base-line level and eluted with the same buffer containing 0.2 mM cyclic AMP and 1 M NaCl. As shown in Fig. 3(A), a peak of cyclic AMP phosphodiesterase activity appeared. Protein could not be monitored because of the absorbance of the eluting nucleotide. This peak fraction was concentrated to a volume of 5.5 ml and applied to a column (2.2 × 80 cm) of Sephacryl S-300 (Fig. 3B). A single peak of cyclic AMP phosphodiesterase activity was eluted from the column and was pooled and dialyzed against 50 mM Tris–acetate buffer (pH 6.0) containing 1 mM EGTA, 3.75 mM 2-mercaptoethanol, 75 mg/l of PMSF.

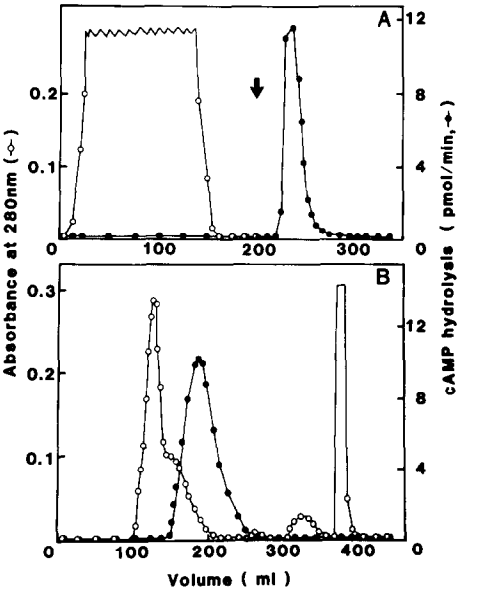

Fig. 3. Typical elution profile of a Blue Sepharose column chromatography (A). The change to the buffer containing additionally 0.2 mM cyclic AMP and 1 M NaCl was indicated by the arrow. Gel filtration of cyclic AMP phosphodiesterase (B). 5.5 ml of the concentrated peak fraction was applied to a column (2.2 × 80 cm) of Sephacryl S-300 equilibrated and eluted in a solution of 50 mM Tris–acetate buffer (pH 6.0), 0.5 M NaCl, 1 mM EGTA, 3.75 mM 2-mercaptoethanol and 75 mg/l of PMSF. Fractions were assayed for cyclic AMP phosphodiesterase activity (—●—) as outlined under Materials and Methods.

The final step of purification was affinity chroma-

Table 2. Effect of cilostamide derivatives on human platelet cyclic nucleotide phosphodiesterase

Agents	IC_{50} (μM)* FI(cyclic GMP-PDE)	FIII(cyclic AMP-PDE)
OPC-13013	7.8 ± 0.4	0.188 ± 0.066
OPC-13135	21.8 ± 0.75	0.073 ± 0.003
OPC-3911	16.1 ± 2.0	0.017 ± 0.002
OPC-13032	24.8 ± 5.2	0.046 ± 0.009
OPC-13120	14.9 ± 1.9	0.130 ± 0.059

*The IC_{50} value is defined as the concentration of drug required to produce 50% inhibition of enzyme activity. Values are mean ± S.E. of four experiments with four different populations. Each experiment was run in triplicate.

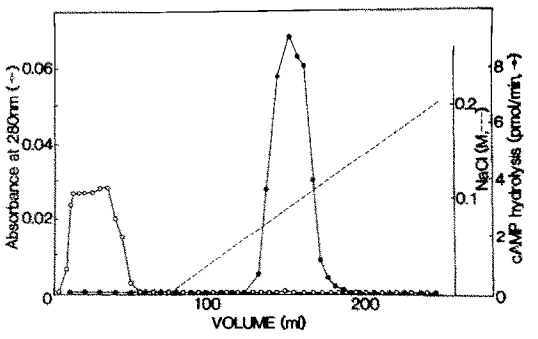

Fig. 4. OPC-13135 affinity chromatography of cyclic AMP phosphodiesterase. Approximately 60 ml (0.08 mg/ml) of the pooled peak following Sephacryl S-300 chromatography was applied to a column of OPC-13135 Sepharose 6B equilibrated with 50 mM Tris–acetate buffer (pH 6.0), 1 mM EGTA, 3.75 mM 2-mercaptoethanol and 75 ml/l of PMSF. The enzyme was eluted with a linear NaCl gradient (0–0.2 M NaCl) made up in the equilibration buffer.

tography on a column of OPC-13135 coupled to Sepharose 6B (Fig. 4). Following dialysis, the pooled Sephacryl S-300 peak was chromatographed on this column. The cyclic AMP phosphodiesterase activity was eluted when a linear gradient from 0 to 0.2 M NaCl was applied.

The enzyme from OPC-13135 Sepharose step was free from contaminations of other cyclic nucleotide phosphodiesterases, adenylate cyclase, and quanylate cyclase, and cyclic nucleotide dependent protein kinases (data not shown).

Table 3 summarises the recovery and activity of the phosphodiesterase, at various steps. The overall recovery was approximately 3%. The specific activity of the final product using 0.4 μM cyclic AMP was 55 nmole/min/mg protein at 30°.

Properties of the purified enzyme. Properties of human platelet cyclic AMP phosphodiesterase are shown in Table 4. The purified enzyme had the ability to hydrolyze both cyclic AMP and cyclic GMP. When Lineweaver–Burk plots were used to analyse the initial data related to velocity, a K_m of 0.34 and 0.11 μM, and a V_{max} value of 85.3 and 19.8 nmole/min/mg protein were obtained, respectively. When the data were re-plotted, the Hill coefficient of 0.98 was apparent. The molecular weight of the enzyme determined by a Sephacryl S-300 gel filtration column chromatography was 175,000 daltons. The pH optimum of the purified enzyme for the hydrolysis of the two substrates at 0.4 μM was approximately pH 8.0. Neither Ca^{2+} nor CaM purified from bovine brain stimulated the activity in the presence or absence of calcium. Incubation with trypsin at various concentrations revealed significant losses of enzymatic activity. Trypsin did not enhance the enzymatic activity (data not shown).

Magnesium was the most efficient metal cation and a maximal effect was observed at concentrations over 5 mM. Manganese had an inhibitory effect at concentrations above 1 mM and a stimulatory effect at concentrations of less than 1 mM. Co^{2+} activated the enzyme slightly. Zn^{2+}, Cu^{2+}, Sr^{2+}, Ca^{2+}, Ni^{2+}, Cd^{2+} and Ba^{2+} showed no ability as a cofactor for the enzymatic reaction (data not shown). Inhibition of this purified cyclic AMP phosphodiesterase by OPC-13135 was then analyzed by Dixon and double reciprocal plots (Fig. 5). The resulting K_i value of OPC-13135 for the phosphodiesterase was 0.015 μM. OPC-13135 inhibited the enzyme activity competitively against its substrate, cyclic AMP.

DISCUSSION

These cilostamide derivatives inhibited human platelet aggregation and showed a selective inhibition toward cyclic AMP phosphodiesterase. Although nanomolar concentrations of these compounds inhibited cyclic AMP phosphodiesterase, the concentrations producing inhibition of aggregation were 100-fold higher. This evidence would be the result of a penetration of these compounds through the platelet membrane. Among these derivatives, OPC-13135, OPC-3911 and OPC-13032 are not only potent inhibitors of human platelet aggregation, but also highly and selectively inhibited cyclic AMP phosphodiesterase which OPC-13135 has a residue in the compound molecule couples readily Sepharose. OPC-13032 has no residues in its molecule to couple to Sepharose. Therefore, OPC-13135 was selected as a ligand of the affinity chromatography. Mohindru *et al.* [14] reported that succinylated trimethylpapaverolin- and 5-acetic acid derivative of 1-methyl-3-isobutylxanthine were coupled to diaminodiprophylamine-substituted-agarose, but these inhibitor-substituted columns showed a low recovery and a low selectivity. More recently, Prigent *et al.* [15] described affinity chromatography on phenylbutenolide immobilized Sepharose, with good yields. However, several phosphodiesterase forms were absorbed on this column. On the other hand, it should be noted that only the high-affinity cyclic

Table 3. Purification of human platelet cyclic AMP phosphodiesterase

	Volume (ml)	Protein concentration (mg/ml)(total mg)	Total activity (nmole/min)(%)	Specific activity (nmole/min/mg)	Activity ratio hydrolysis (cyclic AMP/GMP)
Homogenate	65	32.4 2106	242.2 100	0.115 (1.0)	—
100,000 *g* Sup	50	17.0 850	215.1 89	0.253 2.2	—
DEAE-Cellulose	176	0.47 82.7	144.6 60	1.748 (15.2)	3.11
Blue Sepharose	5.5	3.48 19.1	92.5 38	4.830 (42.0)	—
Sephacryl S-300	59	0.077 4.54	47.15 19	10.38 (90.3)	3.03
OPC-13135 Affinity	40	0.0035 0.14	7.75 3.2	55.41 (482)	3.22

Table 4. Properties of high affinity cyclic AMP phosphodiesterase from human platelets

Property	Value
Molecular weight	175,000
Isoelectric point	4.9
pH optimum	8.0
Kinetic constants	
K_m for cyclic AMP (μM)	0.34
K_m for cyclic GMP (μM)	0.11
K_i for cyclic GMP (μM)	0.15
V_{max} for cyclic AMP (nmole/min/mg)	85.3
V_{max} for cyclic GMP (nmole/min/mg)	19.8
Hill coefficient	0.98

AMP phosphodiesterase was absorbed on OPC-13135-Sepharose column but not other forms of phosphodiesterase from human platelets. After the human platelet cytosolic fraction had been to this column and the column washed to eliminate the contaminating protein with loading buffer, only the low K_m cyclic AMP phosphodiesterase activity was eluted with a salt gradient. These observations support the idea that inhibition of platelet aggregation by OPC-13135 may be due to suppression of cyclic AMP phosphodiesterase activity. Analysis of the purified cyclic AMP phosphodiesterase by electrophoresis, under non-denaturing conditions, showed that over 92% of the phosphodiesterase activity co-migrated with the protein. However, contamination was evident on the electrophoresis in the presence of sodium dodecyl sulfate. Attempts at further purification of this enzyme resulted in a great loss of activity. Therefore, subunit compositions of the enzyme remain nuclear. Human platelet cyclic AMP phosphodiesterase has a molecular weight of 175,000 daltons (Table 4), this weight being higher than that of dog kidney [5], human lung [16] and rat liver plasma membranes [17]. However, enzyme resembles others with regard to isoelectric point, pH optimum, divalent cation requirements and K_m value for cyclic AMP. However, cyclic GMP also acts as a good substrate for the enzyme (Table 4). Cyclic AMP and cyclic GMP competitively inhibit the hydrolysis in each, with inhibition constants (apparent K_i values) similar to their respective K_m values of the human platelet enzyme. This suggests that the purified enzyme has a common catalytic site and with a similar preference for cyclic AMP and cyclic GMP as substrate [7]. Whitson *et al.* reported that cyclic AMP phosphodiesterase from the rat liver particulate fraction hydrolyses cyclic AMP and cyclic GMP, with a high affinity, however, cyclic AMP is hydrolysed at a much higher rate [18]. It is of interest, from the physiological standpoint, that these enzymes hydrolyse both cyclic AMP and cyclic GMP and that each of these nucleotides acts as a competitive inhibitor of the hydrolysis of the other. OPC-13135 Sepharose proved to be a useful tool to purify human platelet cyclic AMP phosphodiesterase. This purified enzyme will enhance understanding of tissue differences in this enzyme system.

Acknowledgements—We thank M. Ohara of Kyushu University for critical reading of the manuscript.

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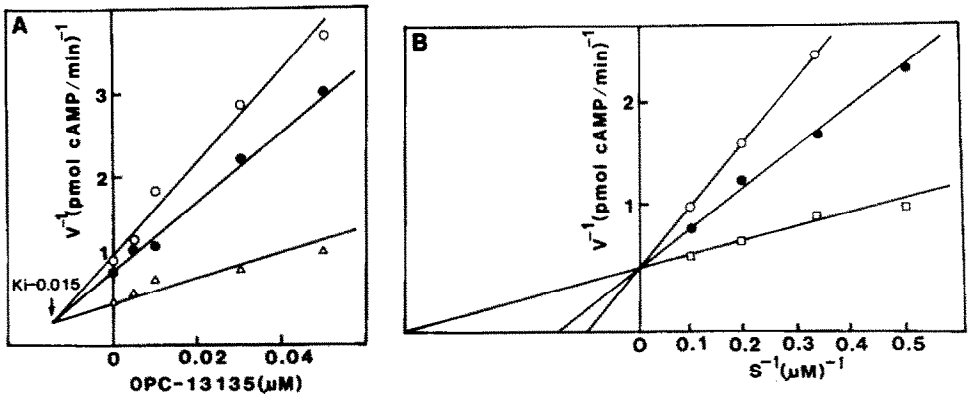

Fig. 5. Kinetic analysis of inhibition of cyclic AMP phosphodiesterase by OPC-13135, (A) Dixon plots of inhibition of cAMP hydrolysis. Substrate concentrations were 0.2 μM (○), 0.4 μM (●) and 10 μM (△) cyclic AMP. (B) double reciprocal plots of inhibition of cyclic AMP phosphodiesterase in the presence or absence of OPC-13135. OPC-13135 concentration were 0 μM (□), 0.01 μM (●), 0.03 μM (○), respectively.

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