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Selective Inhibitory Effect of New Phosphodiesterase Inhibitors on PDE Isozymes In Guinea pig Cardiac Muscle

Sun Kyeong Lee, Kwang Il Kwon* and Ok Pyo Zee
Korea Research Institute of Chemical Technology, Taejeon 302-343, and
**College of Pharmacy, Chung Nam National University, Taejeon 302-764, Korea*
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Abstract□Selective inhibition of seven new PDE inhibitors on cyclic nucleotide PDE isozymes was investigated. Three PDE isozymes (PDE I, II and III) of guinea pig left ventricular muscles were used. All tested agents inhibited cyclic AMP hydrolysis by PDE III in a concentration-dependent manner. Some agents represented more potent and selective inhibitory effect on PDE III than that of imazodan.

Keywords□Inotropic agents, congestive heart failure, selective PDE inhibitor.

For many years digitalis glycosides have been used as principal agents in the treatment of cardiac dysfunction.[1] These agents increase the inotropic activity of the failing heart, resulting in increased oxygen utilization. More recently, application of systemic vasodilators to reduce ventricular afterload and improve cardiac performance has led to their use in the treatment of heart failure.[2] Experimental and clinical data currently emphasize the advantages of combining ventricular overload reduction and positive inotropic stimulation to provide the most efficient pharmacologic enhancement of cardiac pump function.[3]

Cardiotonic agents of the bipyridine, pyridazinone, benzimidazole and imidazole chemical classes have combining positive inotropism with vasodilating properties.[4-6] Investigations into the mechanism of action on these agents have shown that the cardiotonic activity of these agents is normally attributed to their selective inhibitory effect on the low K_m, cyclic AMP specific form of phosphodiesterase (PDE III), which has been identified in ventricular muscle.[7-11]

The relationship between cyclic AMP and myocardial contractility has been well documented,[12-14] and agents that increase the cyclic AMP, e.g., β-receptor stimulants, as well as agents that inhibit the degradation of cyclic AMP such as phosphodiesterase inhibitors, all increase myocardial contractility.[15] Multiple molecular forms of phosphodiesterase have been identified in ventricular muscle[16-18] and it has been demonstrated that the many of novel cardiotonic agents, most notably amrinone, enoximone and imazodan, are selective inhibitors of PDE III, a low K_m, cyclic AMP specific form of the enzyme.[19,20] Supportive evidence that inhibition of PDE III represents the cardiotonic mechanism of action for these agents is provided by the observation that these agents (1) produce selective concentration dependent increase in tissue levels of cyclic AMP but not cyclic GMP (2) potentiate the positive inotropic response to isoproterenol, and (3) restore contractility to isolated potassium-depolarized cardiac muscle.[21]

The purpose of the present study was to determine the inhibitory effect as well as the selectivity on PDE III of the new synthesized agents to develop a newer cardiotonic agent for the treatment of congestive heart failure. In this study, the multiple molecular forms of phosphodiesterase were isolated from guinea pig cardiac muscle. K_m and V_{max} values were determined for each molecular form, as well as substrate specificity (cyclic AMP *vs* cyclic GMP). In addition, we examined the effect of variety of seven new synthesized agents, which have pyridazinone structure (KR30045, KR30075 and KR30081) or tetrazole structure (KR30091, KR30117, KR30120 and KR30121) and two standard PDE inhibitors on three PDE isozymes in guinea pig cardiac muscle to find the selective PDE III inhibitors.

EXPERIMENTAL METHODS

Isolation of phosphodiesterases from cardiac muscle

Male guinea pigs were sacrified by cervical dis-

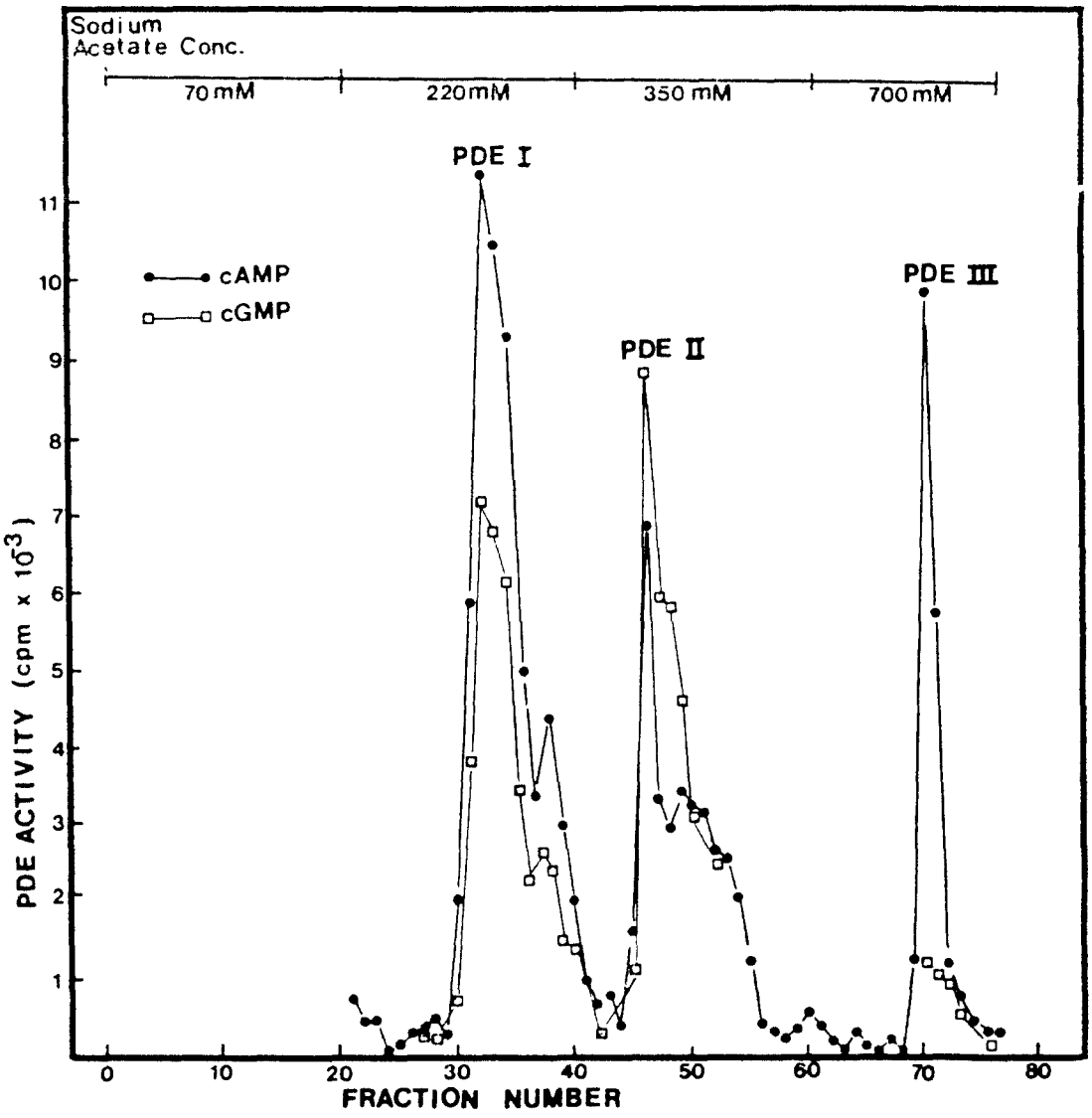

Fig. 1. Typical DEAE-cellulose chromatographic profile of guinea pig ventricular cyclic nucleotide PDEs. Aliquots (40 u*l*) of eluates were assayed for enzyme activity using 1 uM cyclic AMP and 1 uM cyclic GMP as the substrate. Fractions 32-34, 46-48, 70-71 were pooled, respectively, for peaks I, II and III. The major peaks were labeled PDE I, II and III according to the order of elution from the column.

location, after which the heart was rapidly excised and placed in ice-cold saline (5 hearts were used for isolation). The left ventricle and septum were dissected and minced. The mince were immediately homogenized at 4 °C in 10 volume buffer (10 mM Tris/HCl, 1mM DTT, 2mM EDTA, 2mM $MgCl_2$, pH 7.5) using a Brinkmann Polytron PT-10 (30 sec, setting 5) and then sonicated (30 sec/m*l* homogenate). The sonicated homogenates were centrifuged at 30,000g for 20 min at 4 °C. The resulting supernatant fraction was filtered through glass wool and applied to a DEAE cellulose column (1.5 × 40 cm) equilibrated with freshly prepared 70 mM sodium acetate/5 mM 2-mercaptoethanol (pH 6.5). Three major forms of guinea pig ventricular cyclic nucleotide PDE were isolated by DEAE cellulose anion exchange chromatography using the stepwise elution procedure described by Thompson

et al.[22] The loaded column was washed with two column volumes of buffer (70 mM sodium acetate buffer, pH 6.5, containing 5 mM 2-mercaptoethanol) followed by succesive elutions with each 100 m*l* of 220, 350 and 700 mM sodium acetate buffers, pH 6.5, containing 5 mM 2-mercaptoethanol at a flow rate of 0.5 m*l*/min.[5] The each 5 m*l* fractions were collected and assayed for cyclic AMP and cyclic GMP phosphodiesterase activity. Appropriate fractions pooled and dialyzed overnight against 70 mM sodium acetate/5 mM 2-mercaptoethanol. Protein concentration was determined by the method of Lowry *et al.*[23] with bovine serum albumin as standard.

Assay for phosphodiesterase inhibition

Substances under examination were preincubated for 5 min with the phosphodiesterase in Tris HCl buffer (50 mM Tris HCl, 5 mM $MgCl_2$, pH 7.5), the reaction was initiated at 30 °C by adding [^{3}H]cyclic AMP (1 uM) or [^{3}H]cyclic GMP (1 uM).[24] The phosphodiesterase was dosed so that a maximum of 15% of the substrate was hydrolyzed during the reaction. The reaction product, 5′-[^{3}H]-AMP or 5′-[^{3}H]GMP, was split to [^{3}H]adenosine by a 5′-nucleotidase in the venom of *Crotalus atrox*.[25] [^{3}H]Adenosine or [^{3}H]guanosine was separated from [^{3}H]cyclic AMP or [^{3}H]cyclic GMP, which was not hydrolyzed by phosphodiesterase, by batch procedure at the anion exchanger Bio-Rad AG1-X2[26] and was determined in a scintillation counter. All drugs were dissolved in DMSO and the final concentration of DMSO in the reaction medium was 2.5%.[4] This concentration of DMSO inhibited enzyme activity by approximately 10%. The IC_{50} values of the various agents examined were determined from concentration-response curves, in which concentrations ranged from 10^{-8} to 10^{-3} M (semi-log increments).

Materials

Imazodan and new PDE inhibitors (KR30045, KR30075, KR30081, KR30091, KR30117, KR30120 and KR30121) were synthesized by Medicinal Chemistry Department, Korea Research Institute of Chemical Technology (KRICT). All reagents used were of the highest obtainable commercial pure. DEAE-cellulose Cellex D and the anion exchanger AG1-X2 were from Bio Rad, USA; [8-^{3}H]-labeled cyclic AMP and [8-^{3}H]-labeled cyclic GMP were from Amersham Buchler; Venom (*Crotalus atrox*) was purchased from Sigma.

Tabable I. Kinetic parameters of isolated guinea pig ventricular PDE peaks I, II and III

	PDE I		PDE II		PDE III
	cAMP	cGMP	cAMP	cGMP	cAMP
K_m (uM)	0.8	0.8	34	27	1.0
V_{max} (pmoles/min/ ug protein)	3.5	3.5	91	71	2.3

For Lineweaver-Burk analysis of cyclic AMP and cyclic GMP hydrolysis by PDEs, the concentrations of both substrates used ranged from 0.1 to 100 uM.
The K_m and V_{max} values for cGMP of PDE III did not be determined because PDE III hardly hydrolyzes cGMP.[4]

RESULTS AND DISCUSSION

Isolation of multiful forms of phosphodiesterase

For this study, the cyclic AMP specific phosphodiesterase (PDE III) was isolated from guinea pig left ventricular muscle. Fig. 1 is a representative chromatograph[5,22] demonstrating the isolation of different PDEs. As can be seen, three forms of PDEs were identified, which were labelled PDE I, PDE II and PDE III based on their order of elution from DEAE cellulose. PDE I hydrolyzed cyclic AMP and cyclic GMP with equal affinify (K_m = 0.8 uM for both substrates). PDE II also hydrolyzed cyclic AMP and cyclic GMP with similar affinity (K_m = 30 uM for both substrates), but PDE III preferentially hydrolyzed cyclic AMP.

The kinetics of the hydrolysis of cyclic AMP and cyclic GMP by guinea pig ventricular PDE I, II and III were investigated by Lineweaver-Burk analysis and are summarized in Table I.

Effects of drugs on guinea pig ventricular PDE I, II and III

To evaluate the potency and the selectivity of the new PDE inhibitors, effects of these agents on PDE I, II and III were examined by using cyclic AMP (1 uM) or cyclic GMP (1 uM) as a substrate. Two standard PDE inhibitors, theophylline and imazodan, were tested for comparison. All tested drugs inhibited PDE III activity in a concentration dependent manner. Their IC_{50} values for the inhibition of PDE III are listed in Table II.

Potencies of the new agents on PDE III varied widely: The pyridazinone chemical classes of KR30045, KR30045 and KR30081 showed higher potency than imazodan (Fig. 2). The other agents

Table II. Effects of selective and nonselective PDE inhibitors on phosphodiesterases in guinea pig left ventricular muscle

Agent	IC_{50} (uM) PDE I cAMP	PDE I cGMP	PDE II cGMP	PDE III cAMP
Imazodan	1000	1000	414	4.5
KR30045	1000	1000	1000	2.2
KR30075	1000	1000	1000	1.9
KR30081	1000	1000	1000	3.7
KR30091	364	450	312	11.2
KR30117	56	58	35.8	14.9
KR30120	30	44	38	17.8
KR30121	407	530	194	29.5
Theophylline	352	411	355	324

The IC_{50} values were determined from concentration-responce curves, in which concentrations ranged from 10^{-8}-10^{-3}M. Each experiment assayed in triplicate. Substrate (cyclic AMP or cyclic GMP) concentration used was 1 uM.

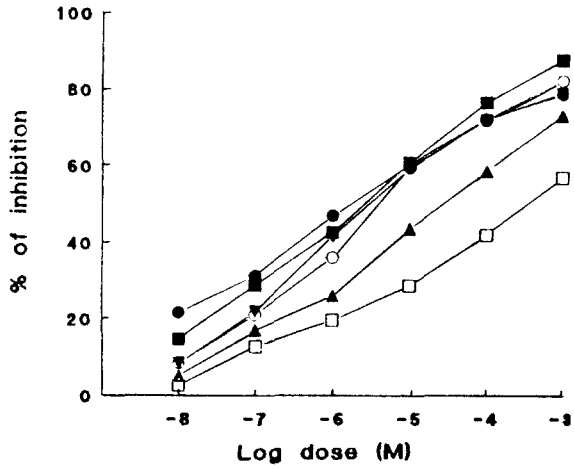

Fig. 2. Inhibitory effects of PDE inhibitors on the activity of the cyclic AMP specific phosphodiesterase isolated from guinea pig left ventricular muscle. (-○-; Imazodan, -■-; KR30045, -●-; KR30075, -▼-; KR30081, -▲-; KR30121, -□-; Theophylline) Enzymes were isolated as described in the "Experimental methods". Each symbol represents the mean of triplicate determinations of percent inhibition on PDE III hydrolytic activity. Substrate concentration for these experiments was 1.0 uM cAMP.

were less potent than imazodan. PDE I and PDE II activities, as measured by cyclic AMP or cyclic GMP hydrolysis, were inhibited significantly by theophylline, KR30091, KR30117, KR30120 and KR30121 but not KR30045, KR30075 and KR30081 (Table III) in a marked contrast to PDE III. The inhibition of PDE I and PDE II required higher concentrations of KR30091, KR30117, KR30120 and KR30121 than that of PDE III.

Table III. Effect of relatively selective PDE inhibitors on PDE I and PDE II in guinea pig left ventricular muscle

Agent	Conc. (uM)	Inhibition % cAMP PDE I	cGMP PDE I	cGMP PDE II
Imazodan	10	13.9*	6.7	5.4
	100	28.7	24.8	33.3
	1000	42.7	41.5	60.5
KR30045	10	17.7	8.7	12.0
	100	27.2	9.4	14.8
	1000	-2.3	-10.0	27.3
KR30075	10	7.2	3.6	7.2
	100	2.9	4.9	12.8
	1000	-2.0	-1.5	27.3
KR30081	10	4.9	8.6	12.6
	100	-0.4	-2.4	19.1
	1000	-5.0	-4.3	30.4

*Each value represents the mean of triplicate.
Substrate (cyclic AMP or cyclic GMP) concentration used was 1 uM.

In summary, some new PDE inhibitors, KR-30045, KR30075 and KR30081, respresented more selective and potent PDE III inhibitory effects than that of imazodan. These pyridazinone chemicals are expected to be positive inotropic agent, further studies on cardiotonic activities and cardiotoxic effect of these agents are being performed.

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