Biochemical Pharmacology, Vol. 42, No. 3, pp. 679–683, 1991.
Printed in Great Britain.

0006-2952/91 $3.00 + 0.00

BEMORADAN—A NOVEL INHIBITOR OF THE ROLIPRAM-INSENSITIVE CYCLIC AMP PHOSPHODIESTERASE FROM CANINE HEART TISSUE

JOHN B. MOORE JR.,* DONALD W. COMBS and A. J. TOBIA
R. W. Johnson Pharmaceutical Research Institute, Raritan, NJ 08869-0602, U.S.A.

(*Received* 9 *October* 1990; *accepted* 1 *March* 1991)

Abstract—Canine cardiac muscle contains a type IV cyclic AMP (cAMP) phosphodiesterase (PDE) that is composed of two subtypes. One subtype is sensitive to rolipram inhibition (RSPDE), whereas the other is not inhibited significantly by rolipram (RIPDE). The RIPDE is inhibited by several cardiotonic agents operating by a PDE-inhibitory mechanism. Bemoradan [RWJ-22867; 7-(1,4,5,6-tetrahydro-4-methyl-6-oxo-3-pyridazinyl)-2*H*-1,4-benzoxazin-3(4*H*)-one], a novel, potent positive inotropic agent, demonstrated biphasic inhibition of the fraction III enzyme from canine cardiac muscle. Inhibition by rolipram of the RSPDE converted the IC_{50} curves of bemoradan, indolidan, pimobendan, and imazodan to sigmoidal, monophasic curves. Lineweaver–Burk analysis yielded competitive inhibition K_I values of 0.023, 0.09, 0.065 and 0.60 μM, respectively, for these compounds. The cardiotonic compounds, however, were not potent inhibitors of the Type I and Type II cAMP PDEs found in canine ventricular muscle. The order of potency for inhibiting the RIPDE cAMP PDE subtype was bemoradan > pimobendan > indolidan > imazodan. Bemoradan is, therefore, a potent inhibitor of the cardiac muscle cAMP PDE which could, in part, be responsible for its cardiotonic activity.

In an effort to investigate the biphasic response of bemoradan and to further clarify its enzyme inhibitory properties as compared to other cardiotonics, we utilized a system which classifies the isolated enzymes according to their properties rather than method of isolation [1]. Weishaar and his collaborators [2] correlated cardiotonic activity with the inhibition of one of the enzymes from cardiac muscle for several known positive inotropic agents. The enzyme used in those comparisons was denoted fraction III due to its elution profile from a DEAE cellulose column. This enzyme has a low K_m for the substrate cyclic AMP (cAMP) and is calmodulin independent. A number of more recent cardiotonic agents have also been shown to be selective fraction III enzyme inhibitors [3–6]. However, with at least one of the cardiotonics, indolidan, a clear biphasic inhibition curve was observed [4]. Our efforts with a novel cardiotonic agent, bemoradan [7(1,4,5,6-tetrahydro-4-methyl-6-oxo-3-pyridazinyl)-2*H*-1,4-benzoxazin-3(4*H*)-one], revealed that it is a very potent compound that demonstrates excellent cAMP phosphodiesterase (PDE) inhibition [7, 8]. Biphasic inhibition was also observed for bemoradan [8].

Very recent reports [9–11] have shown that the Type IV enzyme is composed of two subtypes—one that is sensitive to inhibition by cyclic GMP (cGMP) and one that is insensitive to cGMP inhibition. The cGMP-inhibitable subtype is also inhibited by several cardiotonic agents, while the cGMP-insensitive form can be inhibited by rolipram. These two subtypes will be designated rolipram-insensitive PDE (RIPDE) and rolipram-sensitive PDE (RSPDE), respectively. Recent work by several groups [12–15] has characterized these subtypes by tissue distribution and enzyme kinetics. Kithas *et al.* [16] have detailed the physical separation of two enzyme subtypes present in the type IV cAMP phosphodiesterase. By using a particulate fraction (associated with the sarcoplasmic reticulum vesicles), they could specifically study the enzyme inhibition by cardiotonic drugs.

In the present study, the inhibition by bemoradan of the different PDEs from canine heart tissue was compared to that of other known positive ionotropic agents. In addition, we used rolipram to selectively remove (by enzyme inhibition) one of the enzyme subtypes in the type IV fraction. We studied the kinetic effects of bemoradan on the remaining rolipram-insensitive enzyme and compared the different cardiotonic agents to bemoradan.

MATERIALS AND METHODS

Materials. Tissues (canine hearts) were obtained from mongrel dogs of either sex. DEAE-cellulose was purchased from Whatman Ltd. Snake venom (*Crotalus adamanteus*), cyclic AMP, Tris, theophylline and IBMX were obtained from the Sigma Chemical Co. [2,8-^{3}H]Cyclic AMP was obtained from New England Nuclear. Bio-Rad supplied the AG 1 × 8 anion exchange resin. The compounds used were obtained from the following sources: pimobendan, Boehringer-Ingelheim, U.S.A.; milrinone, Sterling Winthrop Research Institute; imazodan, Warner Lambert Co.; and rolipram, Berlex Laboratories. Indolidan and bemoradan were synthesized in our own Medicinal Chemistry Department. All other chemicals were reagent grade or of the highest purity commercially available.

* Correspondence: Dr. John B. Moore, Jr., Rm. B122, Ortho Pharmaceutical Corp., Route 202, Raritan, NJ 08869.

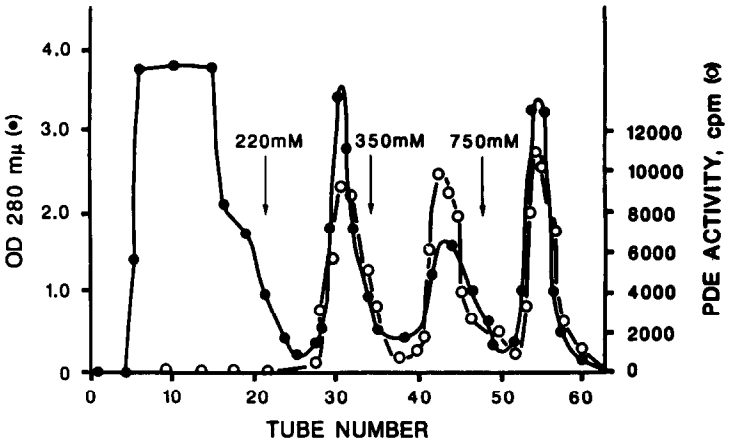

Fig. 1. DEAE-cellulose column elution profile of cyclic nucleotide phosphodiesterases from canine heart. A discontinuous elution from the column of the three fractions was performed as indicated. Fraction size was 8 mL. Aliquots (0.005 mL) were assayed as described under Materials and Methods. Key: (●) $O.D._{280\,m\mu}$, and (○) cyclic AMP hydrolysis.

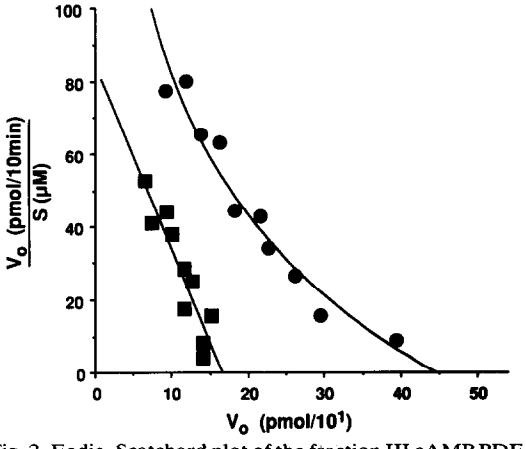

Fig. 2. Eadie–Scatchard plot of the fraction III cAMP PDE. The fraction III enzyme was assayed as described under Materials and Methods. Key: (●), no rolipram, and (■) 50 μM rolipram. Each experiment was run in duplicate.

Isolation of cAMP PDE. Isolation of the three isoenyzmes of cAMP PDE was based on the DEAE-cellulose anion exchange chromatographic procedure [3]. DEAE cellulose was prewashed with 0.5 M NaOH, neutralized, washed with 0.5 M HCl, and equilibrated with 70 mM sodium acetate (containing 5 mM β-mercaptoethanol and 30% ethylene glycol, pH 6.5).

The cAMP PDE homogenate was prepared by homogenizing 14–19 g of canine ventricle in 100 mL of distilled deionized water (4°) for 1 min, sonicating for 30 sec at 4°, and centrifuging at 40,000 *g* for 25 min. This clear, red supernatant was used as the source of the PDE isoenzymes.

Separation of the PDE isoenzymes was accomplished by loading the supernatant on the DEAE column (2.5 × 20 cm) and washing the column with two bed volumes of equilibration buffer at a flow rate of 50 mL/hr. Fractions I, II and III (Types I, II and IV) were eluted with the sodium acetate buffer containing 220, 350 and 750 mM acetate, respectively. Fractions containing the peak enzymatic activities were pooled and dialyzed against equilibration buffer before storage at −20°. No losses in enzymatic activity were observed over the course of these studies. The presence of the two PDE subtypes (cGMP inhibitable and cGMP insensitive) within the third elution peak was confirmed by inhibition with cGMP. cGMP (1 μM) inhibited 52% (±5) of the activity when cAMP (0.25 μM) was used as substrate.

Assay for cAMP PDE activity. PDE activity was measured as described earlier by Thompson and Appleman [17]. The assay buffer contained (unless otherwise noted) cAMP (0.25 μM containing 200,000 cpm of tritiated cAMP), enzyme, buffer (Tris–HCl, pH 7.4, 5 mM $MgCl_2$, and 5 mM β-mercaptoethanol), and compound to be evaluated. The PDE enzyme reaction was run for 20 min and terminated by heating (100°, 30 sec). Conversion of substrate to product was always less than 15%. Snake venom (0.10 mL, 1 mg/mL) was added for 30 min before the addition of 1 mL AG 1 × 8 resin to terminate the nucleotidase enzyme reaction. An aliquot of the supernatant (0.2 mL) was removed, placed into scintillation fluid, and counted in a scintillation counter. The assays were done at least in duplicate. The IC_{50} values were determined from inhibition curves (percent inhibition of enzyme activity versus concentration of drug).

RESULTS

A crude homogenate from canine ventricular muscle after sonication yielded three fractions of cAMP phosphodiesterase activity after elution from a DEAE-cellulose column (Fig. 1). In this procedure, the fraction III material can be completely resolved

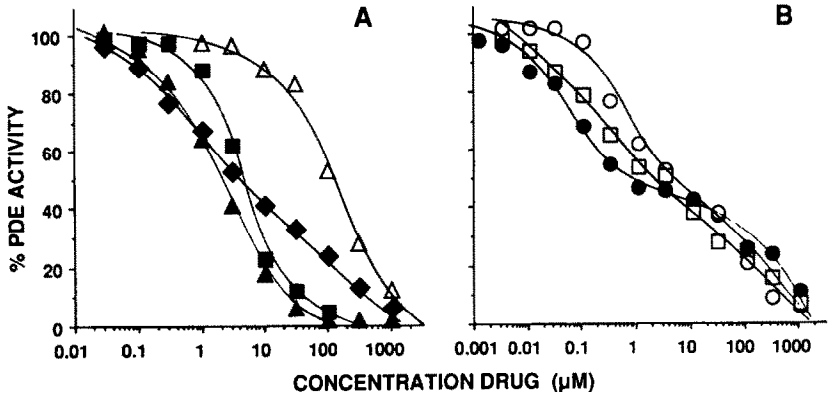

Fig. 3. Inhibition of fraction III cAMP PDE. Enzymatic activity was determined as described under Materials and Methods. Key: (A) (■) IBMX, (△) theophylline, (▲) milrinone, (◆) imazodan; (B) (●) bemoradan, (○) indolidan, and (□) pimobendan. Each point is the mean of duplicate assays. Control PDE values: (A) 3.2 pmol/min/μg protein, and (B) 3.5 pmol/min/μg protein.

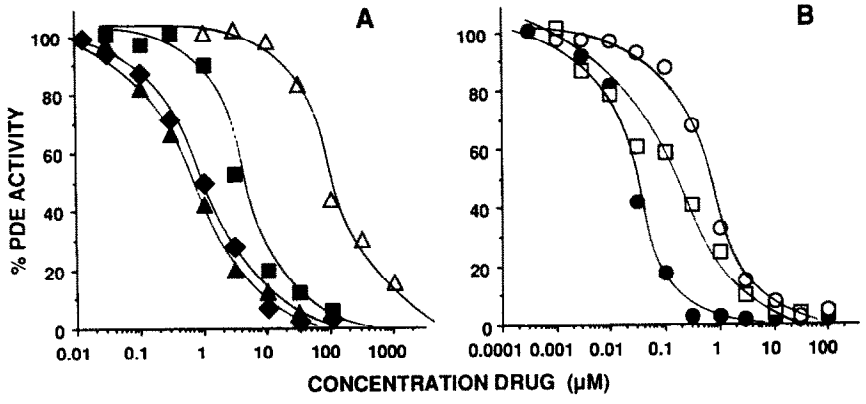

Fig. 4. Inhibition of fraction III cAMP PDE in the presence of 50 μM rolipram (RIPDE). Enzymatic activity was determined as described under Materials and Methods. Key: (A) (■) IBMX, (△) theophylline, (▲) milrinone, and (◆) imazodan; (B) (●) bemoradan, (○) indolidan, and (□) pimobendan. Each point is the mean of duplicate assays. Control PDE values: (A) 3.4 pmol/min/μg protein, and (B) 3.4 pmol/min/μg protein.

from the other two activities. The presence of the two subtypes within the fraction III peak was monitored by performing a kinetic analysis at various substrate concentrations (Fig. 2). This Eadie–Scatchard plot displayed a curved line suggesting that two enzymatic activities were present. The addition of rolipram (50 μM) inhibited one of the subtypes (RSPDE), leaving the single, active rolipram-insensitive enzyme (RIPDE) with an apparent K_m of 0.20 μM for cAMP.

The third enzyme fraction eluted from the DEAE column (750 mM sodium acetate) contained at least two subtypes of activity—a type sensitive to inhibition by rolipram and a type not inhibited by rolipram. The results obtained when this fraction was used to study the cardiotonic agents are shown in Fig. 3. For milrinone, theophylline and IBMX, sigmoidal inhibition curves yielded well-defined IC_{50} values. In contrast, the other four cardiotonics (indolidan, bemoradan, imazodan, and pimobendan) showed various degrees of biphasic inhibition. The addition of 50 μM rolipram did not alter the shape of the inhibition curve for milrinone, theophylline, or IBMX; however, there was a clear impact on the shape of the inhibition curve of the remaining four cardiotonics (Fig. 4). Rolipram inhibition caused the shapes of the inhibition curves to become monophasic so that the IC_{50} values could be determined easily.

To determine the relative potencies of these compounds for the cardiotonic-sensitive PDE (RIPDE) present in canine cardiac muscle, K_I values were determined. Figure 5 shows Lineweaver–Burk analyses for pimobendan, bemoradan, and indolidan. All demonstrated competitive inhibition of the RIPDE enzyme. Table 1 shows the results of these analyses for all the compounds. Bemoradan was the most potent compound tested with a K_I of 0.023 μM. Pimobendan also demonstrated potent inhibition of the cAMP PDE with a K_I of 0.065 μM.

The selectivity of the cardiotonic compounds

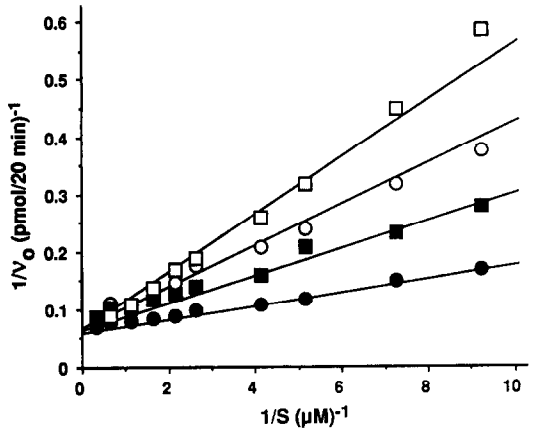

Fig. 5. Lineweaver–Burk analysis of fraction III cAMP PDE in the presence of 50 μM rolipram (RIPDE). Enzymatic activity was determined as described under Materials and Methods. Key: (●) control, (○) bemoradan (0.05 μM), (■) pimobendan (0.10 μM) and (□) indolidan (0.50 μM). Each point is the mean of at least duplicate assays.

Table 1. Inhibition constants (K_I) of inhibitors of the rolipram-insensitive cAMP PDE from canine heart tissue

Compound	K_I (μM)
Theophylline	39 ± 6
IBMX	2 ± 0.8
Milrinone	0.22 ± 0.01
Pimobendan	0.065 ± 0.023
Indolidan	0.09 ± 0.01
Imazodan	0.60 ± 0.08
Bemoradan	0.023 ± 0.001

The K_I values (± SEM) were determined from Lineweaver–Burk analyses. The enzymatic assays were performed as described under Materials and Methods. These data are the results of at least three experiments.

Table 2. Inhibition of canine cardiac muscle cAMP PDEs

Compound	IC_{50}* (μM)		
	Type I	Type II	RIPDE
Milrinone	187 ± 33	150 ± 10	0.47 ± 0.03
IBMX	10 ± 3	6 ± 1	3.8 ± 0.8
Theophylline	179 ± 5	155 ± 35	134 ± 19
Pimobendan	295 ± 39	242 ± 71	0.18 ± 0.08
Indolidan	358 ± 28	630 ± 73	0.43 ± 0.17
Imazodan	363 ± 66	412 ± 80	0.82 ± 0.18
Bemoradan	252 ± 48	505 ± 32	0.07 ± 0.02

* The IC_{50} is defined as the concentration of drug required to inhibit 50% of the enzymatic activity. Enzyme assays were done as described under Materials and Methods. Each value is the mean ± SEM of at least three experiments.

(milrinone, pimobendan, indolidan, imazodan, and bemoradan) for the RIPDE enzyme compared to the Type I and Type II enzymes can be seen in Table 2. Theophylline and IBMX had similar IC_{50} values for all three fractions, whereas the five cardiotonic agents had significantly lower IC_{50} values for the RIPDE as compared to the Type I and Type II enzymes.

DISCUSSION

The data presented in this report agree with the findings of other investigators that the fraction III cAMP PDE material is composed of two subtypes [10, 12]. One of these subtypes is not inhibitable by cGMP, but can be inhibited by rolipram (rolipram-sensitive PDE, RSPDE). The other enzyme subtype is inhibited by cGMP but not by rolipram (rolipram-insensitive PDE, RIPDE). A number of reports have characterized these subtypes as to tissue distribution and kinetic parameters [10, 12–14]. The inhibition of the RIPDE appears to be important for cardiotonic activity, while inhibition of the other subtype (RSPDE) may be important for antidepressant activity [15].

Recent work by Kithas *et al.* [16] showing that the particulate fraction of the type IV isoenzyme fraction contains the cardiotonic-sensitive enzyme (it is also inhibited by cGMP) is significant. The soluble enzyme is not inhibited by cGMP. In the present study, we utilized rolipram to selectively inhibit the enzyme that is not inhibited by cGMP. Approximately 50% of our type IV enzyme was the one inhibited by cGMP.

Bemoradan is a recently described potent inotropic drug [7], which markedly inhibits canine heart fraction III cAMP PDE [8]. However, the inhibition observed using fraction III with bemoradan is a non-sigmoidal function, i.e. the curve of percent inhibition versus bemoradan concentration is relatively flat in the middle. This observation suggests that bemoradan interacted with two enzymes present within the fraction III material. Similar results have been noted with indolidan [4]. In preliminary studies, a competitive interaction was observed at lower concentrations of bemoradan. In an effort to clarify the cAMP PDE inhibition by bemoradan, we utilized rolipram to inhibit one of the PDE subtypes known to be in the fraction III material. Rolipram converted the curvilinear Eadie–Scatchard plot without rolipram to a straight-line. This active enzyme species (RIPDE) had a high affinity for cAMP PDE with a K_m of 0.20 μM.

The presence of 50 μM rolipram in the incubation mixture altered the biphasic nature of inhibition observed with indolidan, pimobendan, imazodan, and bemoradan (Fig. 3 and 4). The inhibition with rolipram yielded sigmoidal curves with distinct IC_{50} values. Lineweaver–Burk analyses yielded IC_{50} values (Table 1) of the competitive inhibition. Bemoradan was the most potent cAMP PDE inhibitor with a K_I of 0.023 μM.

The K_I values reported here compare very favorably with those observed by others. Kauffman *et al.* [4] reported a K_I of 0.08 μM for indolidan in cardiac membranes. In that same study, milrinone and imazodan had calculated K_I values of 0.3 and 0.2 μM, respectively. Kariya and Dage [9] reported K_I values of 1.1 and 0.4 μM for IBMX and milrinone, respectively. Kithas *et al.* [16] demonstrated K_I

values of 0.2 and 1.5 μM, respectively, for milrinone and imazodan. Bemoradan with K_I of 0.023 μM is clearly the most potent *in vitro* phosphodiesterase inhibitor of these cardiotonics.

In addition, the cardiotonic agents showed their specificity by selectively inhibiting the high affinity cAMP PDEs. Fractions I and II (Type I and II) were not potently inhibited by the pyridazinones. Theophylline and IBMX showed no selectivity. Clearly, the pyridazinone compounds target the high affinity cAMP PDE. The order of *in vitro* RIPDE potency (K_I) for four of the cardiotonic agents was the same as that observed *in vivo* when measured against the EC_{50} for cardiac force increases: bemoradan > indolidan > milrinone > imazodan [7]. However, pimobendan, the second most potent RIPDE inhibitor, is the least potent compound *in vivo* [18, 19]. Whether this difference is due to additional mechanisms of action or metabolic changes of the parent pimobendan molecule must await further studies. Recently, reports by Solaro and others have suggested that both of these possibilities may account for the anomalous behavior observed for pimobendan [20, 21].

In conclusion, this report has demonstrated that bemoradan, a novel cardiotonic agent, is a potent (K_I ~0.02 μM) inhibitor of the cardiotonic-sensitive (RIPDE) cAMP PDE subtype present in canine cardiac tissue. Competitive inhibition of this enzyme was observed after inhibiting one of the subtypes with rolipram (RSPDE). Whether bemoradan has other mechanisms of action similar to those of pimobendan and indolidan remains to be shown.

REFERENCES

1. Strada SJ and Thompson WJ (Eds.), Nomenclature recommendations. In: *Advances in Cyclic Nucleotide and Protein Phosphorylation Research* Vol. 16, p. vi. Raven Press, New York, 1984.
2. Bristol JA, Sircar I, Moos WH, Evans DB and Weishaar RE, Cardiotonic agents. 1. 4,5-Dihydro-6-[4-(1*H*-imidazol-1-yl)phenyl]-3(2*H*)-pyridazinones: Novel positive inotropic agents for the treatment of congestive heart failure. *J Med Chem* **27**: 1099–1101, 1984.
3. Weishaar RE, Burrows SD, Kobylarz DC, Quade MM and Evans DB, Multiple molecular forms of cyclic nucleotide phosphodiesterase in cardiac and smooth muscle and in platelets. Isolation, characterization, and effects of various reference phosphodiesterase inhibitors and cardiotonic agents. *Biochem Pharmacol* **35**: 787–800, 1986.
4. Kauffman RF, Crowe VG, Utterback BG and Robertson DE, LY195115: A potent, selective inhibitor of cyclic nucleotide phosphodiesterase located in the sarcoplasmic reticulum. *Mol Pharmacol* **30**: 609–616, 1986.
5. Harrison SA, Chang ML and Beavo JA, Differential inhibition of cardiac cyclic nucleotide phosphodiesterase isozymes by cardiotonic drugs. *Circulation* **73**: III-109–III-116, 1986.
6. Silver PJ, Lepore RE, O'Connor B, Lemp BM, Hamel LT, Bentley RG and Harris AL, Inhibition of the low K_m cyclic AMP phosphodiesterase and activation of the cyclic AMP system in vascular smooth muscle by milrinone. *J Pharmacol Exp Ther* **247**: 34–42, 1988.
7. Combs DW, Rampulla MS, Bell SC, Klaubert DH, Tobia AJ, Falotico R, Haertlein B, Lakas-Weiss C and Moore JB, 6-Benzoxazinylpyridazin-3-ones: Potent, long-acting positive inotrope and peripheral vasodilator agents. *J Med Chem* **33**: 380–386, 1990.
8. Moore JB, Miller WC, Falotico R, Combs D and Tobia AJ, Inhibition of canine cardiac cAMP phosphodiesterase by bemoradan—A novel positive inotropic agent. *FASEB J* **3**: A1040, 1989.
9. Kariya T and Dage RC, Tissue distribution and selective inhibition of subtypes of high affinity cAMP phosphodiesterase. *Biochem Pharmacol* **37**: 3267–3270, 1988.
10. Komas N, Lugnier C, LeBec A, Serradeil-le Gal C, Barthelemy G and Stoclet JC, Differential sensitivity to cardiotonic drugs of cyclic AMP phosphodiesterases isolated from canine ventricular and sinoatrial-enriched tissues. *J Cardiovasc Pharmacol* **14**: 213–220, 1989.
11. Harris AL, Connell MJ, Ferguson EW, Wallace AM, Gordon RJ, Pagani ED and Silver PJ, Role of low K_M cyclic AMP phosphodiesterase inhibition in tracheal relaxation and bronchodilation in the guinea pig. *J Pharmacol Exp Ther* **251**: 199–206, 1989.
12. Torphy TJ and Cieslinski L, Characterization and selection inhibition of cyclic nucleotide phosphodiesterase isozymes in canine tracheal smooth muscle. *Mol Pharmacol* **37**: 206–214, 1990.
13. Fujimoto S and Matsuda T, Effects of pimobendan, a cardiotonic and vasodilating agent with phosphodiesterase inhibiting properties, on isolated arteries and veins of rats. *J Pharmacol Exp Ther* **252**: 1304–1311, 1990.
14. Ito M, Tamaka T, Saitoh M, Masuoka H, Nakano T and Hidaka H, Selective inhibition of cyclic AMP phosphodiesterase from various human tissues by milrinone, a potent cardiac bipyridine. *Biochem Pharmacol* **37**: 2041–2044, 1988.
15. Schultz J and Schmidt B, Rolipram, a stereospecific inhibitor of calmodulin-independent phosphodiesterase, causes β-adrenoceptor subsensitivity in rat cerebral cortex. *Naunyn Schmiedebergs Arch Pharmacol* **333**: 23–30, 1986.
16. Kithas PA, Artman M, Thompson WJ and Strada SJ, Subcellular distribution of high-affinity type IV cyclic AMP phosphodiesterase activity in rabbit ventricular myocardium: relations to the effects of cardiotonic drugs. *Circ Res* **62**: 782–789, 1988.
17. Thompson WJ and Appleman MM, Multiple cyclic nucleotide phosphodiesterase activities in rat brain. *Biochemistry* **10**: 311–316, 1971.
18. Berger C, Myer W, Scholz H and Starbatty J, Effects of the benzimidazole derivatives pimobendan and 2-(4-hydroxy-phenyl) - 5 - (5-methyl-3-oxo-4,5-dihydro - 2*H*-6-pyridazinyl) benzimidazole·HCl on phosphodiesterase activity and force of contraction in guinea pig hearts. *Arzneimittelforschung* **35**: 1668–1673, 1988.
19. Mul JCA, Cardiovascular effects of the positive inotropic agents pimobendan and sulmazole *in vivo*. *Arzneimittelforschung* **35**: 284–288, 1988.
20. Westfall MV, Wahler GM, Fujino K and Solaro R, Actions of the pimobendan metabolite UD-GG212 on electrophysiological and mechanical functions in myocardium. *FASEB J* **4**: 2716, 1990.
21. Fujino K, Sperelakis N and Solaro RJ, Differential effects of *k*- and *l*-pimobendan on cardiac myofilament calcium sensitivity. *J Pharmacol Exp Ther* **247**: 519–523, 1988.