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ZARDAVERINE AS A SELECTIVE INHIBITOR OF PHOSPHODIESTERASE ISOZYMES

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Abstract—The pyridazinone derivative zardaverine has recently been introduced as a potent bronchodilator *in vivo* and *in vitro*. In addition, zardaverine exerts a positive inotropic action on heart muscle *in vitro*. The actions of zardaverine are thought to be mediated via inhibition of phosphodiesterase (PDE) activity. Recent data suggest that there are multiple forms of phosphodiesterases and at least five different isozyme families are now recognized. In the present study, the effects of zardaverine on the different PDE isozymes were investigated in several tissues. PDE isozymes were separated by chromatography on Q-sepharose. Zardaverine inhibited the cyclic GMP-inhibitable PDE III from human platelets and the rolipram-inhibitable PDE IV from canine trachea and human polymorphonuclear (PMN) cells with IC_{50}-values of 0.58, 0.79 and 0.17 μM, respectively. The pyridazinone derivative affected the calmodulin-stimulated PDE I, the cyclic GMP-stimulated PDE II and the cyclic GMP-specific PDE V only marginally at concentrations up to 100 μM. Zardaverine inhibits the ADP-induced aggregation of human platelets with an IC_{50} of 1.6 μM. This inhibition was synergistically increased by activators of adenylate cyclase such as PGE_1 and forskolin. In human PMN cells, zardaverine inhibited the zymosan-induced superoxide anion generation with an IC_{50} of 0.40 μM. Again, this effect was increased by activators of adenylate cyclase. These data clearly demonstrate that zardaverine is a selective inhibitor of PDE III and PDE IV isozymes.

Cyclic nucleotides mediate many different effects in mammalian cells. Cyclic nucleotide phosphodiesterases catalyse the hydrolysis of 3′,5′-cyclic nucleotides to the corresponding nucleoside 5′-monophosphates and thereby play important roles in the regulation of cyclic nucleotide concentrations. Principally, the development of phosphodiesterase inhibitors to increase intracellular cyclic nucleotide levels represents an important pharmacological approach to the treatment of several diseases. Thus, PDE inhibitors may have potential therapeutic relevance in a variety of areas, including heart failure, obstructive airways disease, thrombosis, hypertension and inflammation [1]. Although PDE inhibitors have been used therapeutically for many years, their utility has been limited by side effects and poor potency. Recently, multiple molecular forms of phosphodiesterases have been identified in a number of tissues, e.g. cardiac muscle, platelets, and tracheal smooth muscle [1, 2]. Recent data suggest that at least five different isozyme families exist and more than 20 distinct enzymes are now recognized [2]. These observations suggest that it will be possible to develop selective and possibly therapeutically useful PDE inhibitors that can be targeted for specific tissues.

Zardaverine [6-(4-difluoromethoxy -3- methoxyphenyl)-3(2*H*)pyridazinone] has recently been introduced as a potent bronchodilator and positive inotropic agent in several species [3–5]. Bronchodilation was demonstrated *in vitro* as well as *in vivo* in anaesthetized and conscious guinea-pigs [3, 4] and in allergic sheep (Abraham *et al.*, in preparation). In addition, zardaverine exerts a positive inotropic action on guinea-pig heart muscle *in vitro* [5]. The concentrations for the inotropic effects of zardaverine are about 10 times higher than those for bronchodilatation [3, 5]. The compound increases intracellular cyclic AMP concentrations via an inhibition of phosphodiesterase activity [3–5]. In the present study, we investigated the inhibitory effects of zardaverine on the five different PDE isozymes in different tissues.

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METHODS AND MATERIALS

Preparation of phosphodiesterase (PDE) isozymes. Separation of isozymes was carried out by a method similar to that described by Reeves *et al.* [6]. All subsequent procedures were carried out at 4°. Rat cardiac ventricle tissue (4 g) was minced with scissors in 10 volumes of buffer A containing 20 mM bis-Tris, 5 mM mercaptoethanol, 2 mM EDTA, 2 mM benzamidine and 50 mM sodium acetate, pH 6.5. For homogenization, 50 μM phenyl-methyl-sulfonyl-fluoride (PMSF), 10 μM pepstatin, 10 μM leupeptin, 0.1 mg/mL soybean trypsin inhibitor and 0.015% (v/v) genapol X-080 were added to buffer A. The suspension was homogenized for 3 × 15 sec in an ultra turrax, followed by 3 × 15 sec in an Potter-Elvejhem homogenizer at 1200 rpm. After centrifugation for 25 min at 25,000 *g*, the supernatant was filtered through a millipore filter (0.45 μm). The filtrate (about 40 mL) was applied to a column (17 × 1.6 cm) containing Q-sepharose "fast flow" pre-equilibrated with buffer A. The flow rate was 80 mL/hr throughout the chromatography. The column was washed with 90 mL buffer and was then

eluted with a linear gradient of 500 mL 0.05–1.0 M sodium acetate. Fractions (7.5 mL) were collected. Sodium acetate concentration in the eluted fractions was measured by osmometry. The fractions were tested for PDE activity, and the peaks containing the different isozymes were identified. Fractions preferentially containing one isozyme were pooled and 2 mL portions were frozen at −40°.

Canine tracheae were taken from euthanized dogs. Fat and adhering tissue were removed from four frozen tracheae and the remaining tissue was washed in buffer A. It was then sliced with scissors and was homogenized in 3 volumes of buffer A containing protease inhibitors (as above) for 3 × 60 sec in a Waring blender. The homogenate was centrifuged for 10 min at 1500 *g*, and the supernatant was again homogenized for 3 × 15 sec in an ultra turrax. After centrifugation at 25,000 *g* for 25 min, the resulting supernatant was filtrated, and chromatography was carried out as described above.

Calmodulin-activated PDE from bovine brain was obtained from Dr C. Gietzen (University Ulm, Germany). The procedure used for isolation was described by Gietzen *et al.* [7].

Assay of phosphodiesterase. PDE activity was determined in a standard reaction mixture containing 40 mM Tris (pH 8.0), 5 mM $MgCl_2$, 0.1 mg/mL bovine serum albumin (BSA) and 0.5 μM cyclic nucleotide/^{3}H-labelled cyclic nucleotide (about 50,000 cpm) in a total volume of 200 μL [8]. The reaction was initiated by addition of the enzyme solution (5–20 μg) and was carried out at 37° for 10 min. The reaction was stopped by addition of 50 μL 0.2 M HCl, followed by cooling on ice for 10 min. *Crotalus atrox* snake venom (50 μL; 1 mg/mL in 0.2 M Tris, pH 8.0) was added. After 10 min incubation at 37° the reaction was stopped on ice. A 0.2 mL aliquot of the assay volume was applied to small columns (Econo column, Biorad) containing 1 mL QAE-A25-sephadex, followed by 2 mL of 30 mM ammonium formate (pH 6.0). The effluent was collected directly in scintillation vials.

For cyclic GMP-selective PDE 0.5 μM cyclic GMP/[^{3}H]cyclic GMP was used as substrate.

For the assay of cyclic GMP-regulated PDE isozymes hydrolysing cyclic AMP (cyclic GMP-stimulated type II, cyclic GMP-inhibited type III), 1 μM cyclic GMP was added to the reaction mixture containing [^{3}H]cyclic AMP as substrate.

Calmodulin-dependent PDE was assayed in the presence of 10 nM calmodulin (M_r 16,700). Cyclic AMP/[^{3}H]cyclic AMP (0.5 μM) was used as a substrate for calmodulin-stimulated PDE from rat heart and canine trachea, while the corresponding PDE from bovine brain was assayed in the presence of cyclic GMP/[^{3}H]cyclic GMP (0.5 μM). Unstimulated activity (control value) was determined in the presence of 1 mM EGTA.

PDE inhibition studies. Inhibition studies with PDE isozymes from platelets were routinely performed using sonicates of platelet rich plasma (PRP) in the presence of either cyclic AMP or cyclic GMP as substrates. In parallel, isolated isozymes fractions were prepared chromatographically from homogenates of platelet pellets. Inhibition data for selected substances were identical using either homogenates or isolated fractions.

Preparation of platelet rich plasma (PRP). Human blood (20 mL) was diluted with 2 mL of 1.3% sodium citrate solution. PRP and platelet poor plasma (PPP) were obtained by centrifugation at 700 *g* for 10 min and 3500 *g* for 10 min, respectively. For PDE measurements in PRP homogenates, PRP was sonicated for 3 × 15 sec and was frozen in 2 mL portions.

Platelet aggregation. Aggregation of human platelets was measured in a four-channel aggregometer (PAP-4, Biodata Corporation). PRP was adjusted to 300,000 platelets/μL, and 193 μL were used per assay. Platelets were preincubated with the drugs for 2 min at 37°. Controls were measured in the presence of 0.5% DMSO. In order to increase sensitivity towards the drugs, 30 nM PGE_1 was added to the assay. Aggregation was initiated by addition of either 2 μM ADP (final concentration), 1.65 mM arachidonic acid, 1 μg/mL collagen, 1.5 μM epinephrine or 1 μM platelet activating factor (PAF), in a volume of 5 μL. The degree of aggregation was determined 4 min after addition of the aggregatory agent and was expressed as a percentage of the maximum.

Preparation of human polymorphonuclear (PMN) cells. Blood (50 mL) collected from healthy volunteers was first diluted with 5 mL 3.8% sodium citrate solution and then with PBS (1:2) containing 137 mM NaCl, 2.7 mM KCl, 8.1 mM NaH_2PO_4 and 1.5 mM KH_2PO_4 adjusted to 280 mOsM. Sedimentation of erythrocytes for 30 min in 1% dextran solution and subsequent centrifugation on Ficoll-paque (Pharmacia) were carried out as described previously [9]. The sediment containing polymorphonuclear (PMN) cells was washed once with PBS and resuspended in lysis buffer containing 155 mM NH_4Cl, 10 mM $KHCO_3$ and 0.1 mM EDTA at 4°. PMN cells were washed with PBS buffer and finally resuspended in buffer A containing 140 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$ and 10 mM Hepes, pH 7.4, at 10^7 cells/mL.

Measurement of superoxide release. This assay was performed according to Markert *et al.* [10]. The medium contained buffer A plus 1 mM $CaCl_2$, 5.6 mM glucose and 0.5 mg/mL bovine serum albumin. The production of superoxide was stimulated by 1 mg/mL opsonized zymosan and monitored by reduction of cytochrome *c*. Incubation was carried out at 37° for 50–60 min followed by cooling on ice and centrifugation. Absorption was read at 550 nm vs control in the presence of 30 μg/mL SOD. In order to increase the sensitivity to superoxide release of PDE inhibiting drugs, 5 μM forskolin was added to the assay, if not otherwise indicated.

Drug addition and statistics. All drugs were diluted in 50% DMSO/H_2O. They were added to the various assays at 100-fold higher concentrations in the appropriate volume. The final DMSO concentration of 0.5% did not influence PDE assays and reduced platelet aggregation and superoxide by about 5–15%. IC_{50} values were calculated from concentration inhibition curves by non-linear curve fitting using

the program GraphPad (GraphPad Software, San Diego, CA, U.S.A.).

Materials. ^{3}H-Labelled cyclic nucleotides were obtained from New England Nuclear (Dreieich, Germany). Q-sepharose "fast flow" and QAE sephadex were obtained from Pharmacia (Freiburg, Germany). 4-(3'-Cyclopentyloxy-4'-methoxyphenyl)-2-pyrrolidone (rolipram) was a gift from Schering A.G. (Berlin, Germany); 4-(butoxy-4-methoxybenzyl) - 2 - imidazolidinone (Ro 20 - 1724) from Hoffmann La Roche (Basel, Switzerland); 4,5-dihydro - 6 - (4 - (1*H* - imidazol - 1 - yl) - 2 - thienyl] - 5-methyl-3(2*H*)pyridazinone (motapizone) from Nattermann (Köln, Germany); 6 - (4 - 1*H* - imidazol - 1-yl)phenyl - 5 - methyl - 4,5 - dihydro - 3(2*H*) - pyridazinone (CI 930) from Warner Lambert (Parke Davies); 1,6-dihydro-2-methyl-6-oxo-(3,4-bipyridine)-5-carbonitril (milrinone) from Sterling Winthrop (Rensselaer, NY); 2-*O*-propoxyphenyl - 8-azapurine-6-one (zaprinast) from Rhone-Poulenc (Dagenham, U.K.); 6-(difluormethoxy-3-methoxyphenyl)-3-(2*H*)pyridazinone (zardaverine), 6-(3-isopropoxy - 4 - methoxyphenyl) - 3 - (2*H*)pyridazinone (B832-145) and 6-(3-methoxy-4-*n*-propoxyphenyl)-3(2*H*)pyridazinone (B832-07) were synthesized by Dr H. Amschler (Byk Gulden, Konstanz, Germany). All other chemicals and supplies were from standard commercial sources.

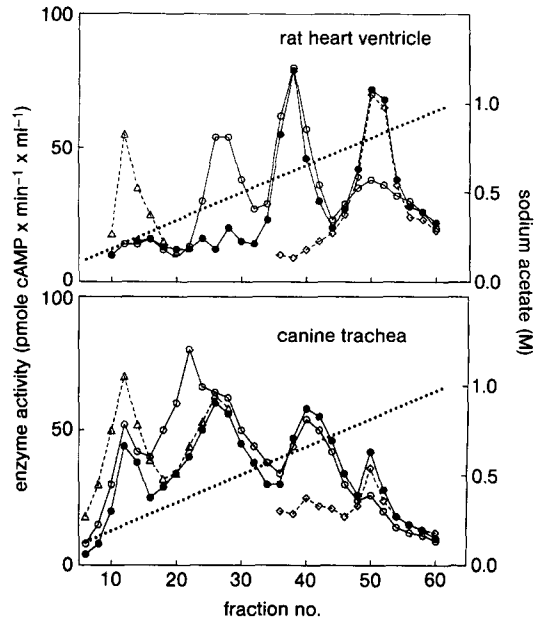

Fig. 1. Elution profile of phosphodiesterases isolated from rat heart ventricle and canine trachea. Supernatant of tissue homogenates was applied to Q-sepharose column and eluted by a sodium acetate gradient. Fractions (7.5 mL) were collected, and cyclic AMP hydrolysing activity was determined under various conditions: control (●); + 0.5 μM cyclic GMP (○); + Ca^{2+}/calmodulin (△); + 10 μM rolipram (◇).

RESULTS

Isolation and characterization of phosphodiesterase isozymes

Phosphodiesterase activities from rat cardiac ventricle and from supernatants of canine tracheal homogenates were separated by Q-sepharose chromatography. The profiles of PDE activities obtained from chromatography are shown in Fig. 1. In both tissues, four PDE activities could be distinguished, accomplished by measurements of [^{3}H]cyclic AMP cleavage in the presence of either 1 μM cyclic GMP, Ca^{2+}/calmodulin, or 10 μM rolipram, respectively. As shown in Fig. 1, the enzyme activity of the first peak obtained from chromatography of rat heart was stimulated several fold by the addition of Ca^{2+}/calmodulin. In the case of canine trachea, there was only a modest increase in peak I activity after addition of Ca^{2+}/calmodulin. In the present study, we did not try to separate any calmodulin-stimulated PDE subtype from the first peak. According to the nomenclature of phosphodiesterase isozymes as proposed by Beavo and Reifsnyder [2], the characteristics of the PDE activity of the first peak is similar to the PDE I_A/I_B type in other tissues.

The next PDE activity eluting from Q-sepharose columns possessed high K_m values for both cyclic AMP and cyclic GMP (Table 1). Cyclic AMP hydrolysis was increased approximately six-fold by the addition of 1 μM cyclic GMP. This cyclic GMP-stimulated enzyme, therefore, represents PDE II according to the classification outlined in [2]. Selective inhibitors of PDE II have not yet been identified.

The third peak of PDE activity from rat cardiac muscle eluting with 0.7–0.8 M sodium acetate hydrolysed cyclic AMP with a K_m of 2.5 μM and cyclic GMP with a K_m of > 100 μM (Table 1), and was selectively inhibited by rolipram (Fig. 1). K_m values of 2.0 μM (cyclic AMP) and of 7.4 μM (cyclic GMP) were obtained in canine trachea. This rolipram-inhibitable, cyclic AMP specific enzyme has been termed PDE IV [2].

The fourth peak of activity had a high affinity for cyclic AMP (K_m = 0.7 μM) and for cyclic GMP (K_m = 0.5 μM) (Table 1). In both tissue preparations, cyclic GMP inhibited cyclic AMP hydrolysis (Fig. 1). Rolipram had no effect on enzyme activity in the fourth peak. In contrast, compounds such as motapizone and milrinone selectively inhibited this PDE activity (Tables 1 and 2). The characteristics of these enzymes are, therefore, similar to those of PDE type III in other tissues [2, 6]. In agreement with the profile of PDE activities eluted from guinea-pig cardiac ventricle, PDE IV is eluted at lower ionic strength than PDE III in canine trachea and rat cardiac ventricle. This contrasts with the human cardiac ventricle PDE profile, in which PDE IV is eluted at higher ionic strength than PDE III [6].

Selective inhibition of phosphodiesterase isozymes

PDE isozymes differ in their kinetic and physical characteristics, substrate specificities, tissue distribution and subcellular localization [11, 12]. The function of these different enzymes and their involvement in regulation of signal transfer within

Table 1. Characteristics of phosphodiesterase isozymes

Tissue	Subtype*	Isozyme	K_M (μM) Cyclic AMP	K_M (μM) Cyclic GMP	Selective inhibitor
Bovine brain	I_B	Ca^{2+}/Cam-stimulated	110	7	Calmidazolium
Rat cardiac ventricle	II	Cyclic GMP-stimulated	14	ND	—
Rat cardiac ventricle	III	Cyclic GMP-inhibited	0.7	0.5	Motapizone
Human platelets	III	Cyclic GMP-inhibited	0.3	0.7	Motapizone
Rat cardiac ventricle	IV	Rolipram-inhibited	2.5	>100	Rolipram
Canine trachea	IV	Rolipram-inhibited	2.0	7.4	Rolipram
Human platelets	V	Cyclic GMP-specific	>20	3.4	Zaprinast

K_M values were calculated from saturation curves by nonlinear regression as well as by linearization procedures. Substrate concentrations in the range of $0.1 \times K_M$ to $20 \times K_M$ were used. Isozyme fractions and homogenates were prepared several times, and each preparation was characterized by K_M determination. The values are the means from 2 to 7 experiments.

Cam, calmodulin; ND, not determined; * according to Ref. 2.

cells is at present poorly understood. For some of the isozyme families inhibitors have been synthesized that possess a marked degree of isozyme selectivity [2, 12]. Some of these selective inhibitors are beginning to be used both experimentally and in the clinic. In order to characterize the inhibitory potency of zardaverine on selected PDE isozymes, PDE activities representing the five isozymes were separated chromatographically, as shown in Fig. 1. In platelets, however, homogenates were used as an enzyme source, since only one isozyme is predominantly active under the given conditions. The following isozymes were used (Table 1): PDE I_B from bovine brain, which is calmodulin-sensitive and displays selectivity for cyclic GMP [7]; cyclic GMP-stimulated PDE II obtained from rat cardiac ventricle; PDE III obtained from rat cardiac ventricle and human platelets (PDE III activity from human platelets is very similar to the enzymes separated from rat cardiac ventricle and canine trachea with respect to column elution and sensitivity towards different drugs, Fig. 1 [6, 13, 14]; in the case of PDE IV, the isozymes from rat cardiac ventricle and canine trachea were used; the cyclic GMP specific PDE V was obtained from human platelets [13].

Figure 2 shows the inhibition of PDE III activity from human platelets and of PDE IV from canine trachea in the presence of various concentrations of substrate and zardaverine. Transformation of the data according to Lineweaver–Burk and Dixon, respectively, suggests competitive inhibition of both PDE III and IV by zardaverine. From these experiments, K_i values of 0.45 μM (PDE III) and 0.16 μM (PDE IV) were calculated, demonstrating a slightly higher affinity of zardaverine for PDE IV compared to PDE III.

In order to determine the potency of zardaverine and other agents as inhibitors of the different PDE isozymes, concentration–response curves were conducted to define IC_{50} values. The cyclic AMP concentration of 0.5 μM was kept constant in all assays. The IC_{50} values are listed in Table 2. In contrast to PDE III and IV, activities of PDE I, II and V were affected only at zardaverine concentrations above 100 μM. Thus, zardaverine exhibits an at least 100-fold selectivity for PDE III and IV over PDE I, II and V. A similar selectivity is found for another new pyridazinone derivative, B832-07 [15], but at variance, this compound is about 30-fold more potent as inhibitor of PDE III than as inhibitor of PDE IV. However, B832-145, a third compound of this class, exhibits remarkable PDE IV selectivity as compared to zardaverine.

The classical PDE inhibitor 3-isobutyl-1-methylxanthine (IBMX) had IC_{50} values in the 10 μM range and was, therefore, approximately 10–40-fold more potent than theophylline (1,3-dimethylxanthine). Enprofylline (3-propylxanthine) has been shown to be an effective bronchodilator, being about five times more potent than theophylline [16]. In contrast to the latter compound, enprofylline is a weak adenosine receptor antagonist [16, 17]. The PDE inhibitory potency of enprofylline is similar to that of theophylline (Table 2). None of these xanthines showed any selectivity for PDE isozymes.

Motapizone, milrinone and CI 930 are new cardiotonic agents which increase the force of contraction [18–20]. Milrinone and CI 930 have been shown to selectively inhibit cardiac PDE III [18]. An excellent agreement was found between PDE III inhibition *in vitro* and cardiotonic activities in anesthetized dogs [21]. In agreement with these results, all three compounds are potent inhibitors of PDE III activities from rat cardiac ventricle and human platelets (Table 2). Of all compounds tested, motapizone was the most potent agent with an IC_{50} of 30 nM for inhibition of platelet PDE III. The potency of motapizone for inhibition of PDE I, II and IV was up to 10,000-fold lower than that for inhibition of PDE III. Similarly, milrinone and CI 930 exerted inhibitory effects on PDI III isozymes which were several orders of magnitude greater than their inhibitory effects on the other PDE isozymes.

Rolipram and RO 20-1724 have been implicated as selective inhibitors of PDE IV activity [1, 2]. The IC_{50} values of rolipram for inhibition of PDE III activities from cardiac ventricle and platelets were about 70–200-fold higher than those for inhibition of PDE IV from cardiac ventricle and trachea. Ro 20-1724 was nearly as potent as rolipram as inhibitor of PDE IV activities. Ro 20-1724 showed a marked difference in its inhibitory potency for PDE III

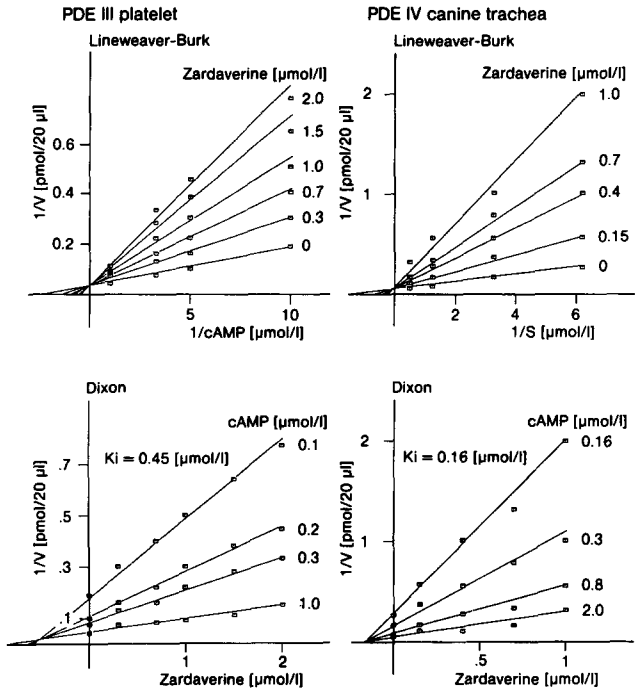

Fig. 2. Cyclic AMP hydrolysis by PDE III of human platelets and by PDE IV of canine trachea in the presence of zardaverine. Cyclic AMP hydrolysing activity was determined in pooled fractions of PDE III from platelets and PDE IV from canine trachea. Activities in the presence of various concentrations of zardaverine and cyclic AMP are plotted according to Lineweaver–Burk and Dixon. V_{max} values of 700 and 200 pmol/min/mg were calculated for the isozymes from platelets and trachea. A representative experiment is presented. Similar values were obtained in different enzyme preparations.

Table 2. Inhibition of phosphodiesterase isozymes by various compounds

	$-\log IC_{50} \pm SD$						
	PDE I_B	PDE II	PDE III		PDE IV		PDE V
	Bovine brain	Rat cardiac ventricle	Rat cardiac ventricle	Human platelets	Rat cardiac ventricle	Canine trachea	Human platelets
Zardaverine	3.00 ± 0.07	3.35 ± 0.35	6.21 ± 0.19	6.24 ± 0.09	5.76 ± 0.12	6.10 ± 0.08	3.86 ± 0.18
B832-07	3.48 ± 0.18	3.62 ± 0.08	6.01 ± 0.36	6.22 ± 0.14	4.79 ± 0.26	4.88 ± 0.27	3.72 ± 0.06
B832-145	3.17 ± 0.08	3.70 ± 0.13	5.52 ± 0.24	5.56 ± 0.15	6.27 ± 0.21	6.33 ± 0.29	3.33 ± 0.24
IBMX	5.05 ± 0.26	5.20 ± 0.21	4.98 ± 0.12	5.03 ± 0.14	5.12 ± 0.25	5.04 ± 0.10	5.01 ± 0.06
Theophylline	3.55 ± 0.36	3.57 ± 0.06	3.41 ± 0.30	3.79 ± 0.11	4.01 ± 0.35	3.81 ± 0.06	3.20 ± 0.27
Enprofylline	3.33 ± 0.10	3.81 ± 0.06	3.97 ± 0.32	4.62 ± 0.22	3.66 ± 0.30	4.02 ± 0.09	3.24 ± 0.16
Motapizone	3.40 ± 0.36	3.21 ± 0.25	6.12 ± 0.18	7.47 ± 0.17	3.86 ± 0.26	4.27 ± 0.21	4.10 ± 0.28
Milrinone	3.57 ± 0.32	3.58 ± 0.07	6.14 ± 0.23	6.05 ± 0.18	4.91 ± 0.27	4.74 ± 0.11	3.84 ± 0.13
CI 930	3.38 ± 0.29	3.24 ± 0.25	6.20 ± 0.32	6.43 ± 0.11	3.55 ± 0.27	4.19 ± 0.37	4.08 ± 0.18
Rolipram	3.09 ± 0.40	3.90 ± 0.40	3.80 ± 0.12	3.97 ± 0.21	6.10 ± 0.18	5.82 ± 0.31	3.20 ± 0.14
Ro 20-1724	3.35 ± 0.45	3.20 ± 0.21	5.99 ± 0.16	3.88 ± 0.26	5.77 ± 0.07	5.33 ± 0.34	3.33 ± 0.10
Zaprinast	3.95 ± 0.33	3.81 ± 0.13	ND	4.13 ± 0.31	ND	4.00 ± 0.26	5.82 ± 0.34
Calmidazolium	6.81 ± 0.10	5.42 ± 0.27	ND	ND	ND	ND	3.57 ± 0.33

IC_{50} values were calculated from inhibition curves using inhibitor concentrations from 0.01 × IC_{50} to 10 × IC_{50}. Assays were performed in duplicate, and IC_{50} values were calculated by nonlinear regression. Values represent the means of 5 to 8 independent experiments and are derived from at least two different preparations.

ND, not determined.

Table 3. Potency of zardavarine as inhibitor of aggregation of human platelets

Aggregatory agent	$-\log_{10} IC_{50} \pm SD$
ADP (3 μM)	4.81 ± 0.18
ADP (3 μM) + PGE_1 (0.03 μM)	5.80 ± 0.22
PAF (1 μM)	5.24 ± 0.08
Collagen (1 μM/mL)	4.79 ± 0.22
Arachidonic acid (1.65 mM)	5.82 ± 0.23

Values are the means of 5 independent experiments done in duplicate.

activities. The IC_{50} value for inhibition of PDE III from rat cardiac ventricle was about 100-fold lower than that for inhibition of PDE III from human platelets and, therefore, in the same order of magnitude as the IC_{50} values for inhibition of PDE IV activities. Zaprinast exerted a potent inhibitory effect on cyclic GMP specific PDE V (IC_{50} = 1.5 μM), while having modest inhibitory effects on cyclic AMP hydrolysis by other PDE isozymes. Calmidazolium potently antagonized the Ca^{2+}/calmodulin dependent PDE I_B with an IC_{50} of 0.15 μM.

Selective inhibition of aggregation of platelets and PMN cells by PDE inhibitors

The pharmacological effects of the various PDE inhibitors were evaluated in functional studies using human platelets and human polymorphonuclear granulocytes (PMN). These studies were designed to detect synergistic interactions between PDE inhibitors and activators of adenylate cyclase. The aggregation of human platelets was used as a model. The pyridazinone derivative zardaverine inhibited aggregation of platelets induced by a variety of different agonists such as ADP, PAF, collagen and arachidonic acid. Under the assay conditions used, IC_{50} values for zardaverine inhibition of platelet aggregation were in the range of 1.5 to 16.2 μM (Table 3). The anti-aggregatory potency of zardaverine was markedly increased in the presence of activators of adenylate cyclase. A 10-fold decrease of IC_{50} after addition of 30 nM PGE_1 is demonstrated in Table 3. Furthermore, as shown in Fig. 3, both forskolin and PGE_1 induced a parallel left-shift of the concentration–response curve of zardaverine without a change of slope and maximal effect. These results are consistent with a synergistic interaction between zardaverine and activators of adenylate cyclase.

The anti-aggregatory potencies of the various PDE inhibitors were determined in the presence of 30 nM PGE_1. The IC_{50} values of the compounds are listed in Table 4. Compared to zardaverine, B832-07 was approximately three-fold more potent and B832-145 approximately two-fold less potent as inhibitors of platelet aggregation. The xanthines IBMX, theophylline and enprofylline were only weak inhibitors of platelet aggregation with IC_{50} values of 17.8, 316 and 50 μM, respectively. Motapizone (IC_{50} = 4.7 nM) was the most potent compound, followed by CI 930 (IC_{50} = 182 nM), B832-07 (IC_{50} = 646 nM) and milrinone (IC_{50} = 1.05 μM). Inhibitors

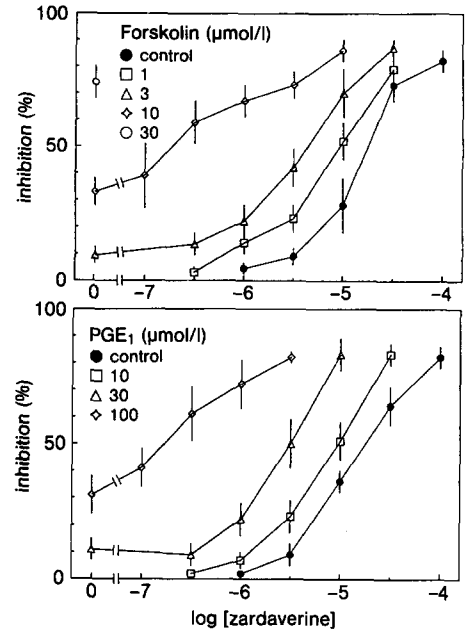

Fig. 3. Inhibition of ADP-induced platelet aggregation by zardaverine in the presence of forskolin and PGE_1. Platelets were preincubated for 2 min with the given substance and forskolin or PGE_1 concentrations. Aggregation was quantified 4 min after addition of 2 μM ADP as per cent of control in the absence of any substance. Values are means ± SD of 5 independent experiments done in duplicate.

Table 4. Inhibitory effects of phosphodiesterase inhibitors on aggregation of human platelets and on O_2^--release of human polymorphonuclear cells

Compound	Aggregation $-\log_{10} IC_{50} \pm SD$	O_2^--release
Zardaverine	5.71 ± 0.58	6.23 ± 0.30
B832-07	6.19 ± 0.56	4.88 ± 0.32
B832-145	5.47 ± 0.34	8.40 ± 0.41
IBMX	4.75 ± 0.21	4.72 ± 0.43
Theophylline	3.50 ± 0.24	3.87 ± 0.27
Enprofylline	4.31 ± 0.16	3.78 ± 0.46
Motapizone	8.33 ± 0.60	4.07 ± 0.24
Milrinone	5.98 ± 0.18	3.52 ± 0.34
CI 930	6.74 ± 0.18	4.05 ± 0.49
Rolipram	3.50 ± 0.26	6.35 ± 0.29
Ro 20-1724	3.45 ± 0.17	6.36 ± 0.35

Aggregation was measured in the presence of 2 μM ADP and 30 nM PGE_1. Zymosan-induced O_2^--release was measured in the presence of 5 μM forskolin. Values represent the means of 5 to 8 independent experiments.

with selectivity for PDE IV, such as rolipram and Ro 20-1724, showed low potencies as inhibitors of aggregation even in the presence of 30 nM PGE_1 with IC_{50} values higher than 300 μM.

In the case of PMN cells, superoxide anion (O_2^-) release was used as a marker of cell activation.

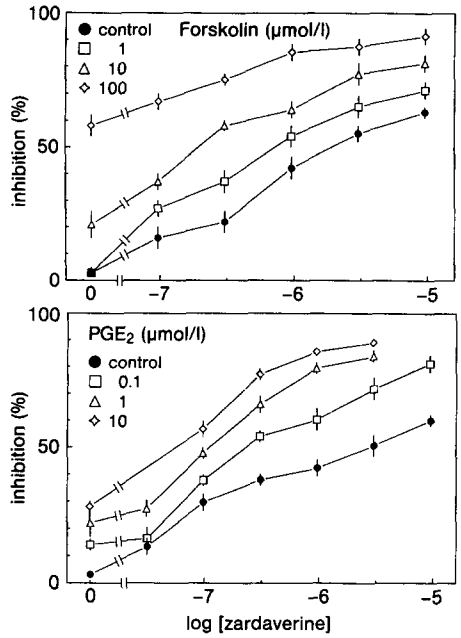

Fig. 4. Inhibition of zymosan-induced superoxide anion release from human PMN cells by zardaverine in the presence of forskolin and PGE_2. In the presence of the given concentration of zardaverine and forskolin or PGE_2, 10^6 cells/mL were incubated for 1 hr. O.D. at 550 nm was determined after cooling and centrifugation in the assay supernatant vs SOD containing controls. Values are means ± SD from 5 independent experiments done in duplicate.

Superoxide anion generation stimulated by opsonized zymosan was time- and dose-dependent (figure not shown). None of the agents used in this study had any significant effect on baseline release of O_2^-. In the absence of any activator of adenylate cyclase, zardaverine inhibited zymosan-induced O_2^--release with an IC_{50} of 0.4 μM. Both forskolin and PGE_2 synergistically increased the inhibitory potency of zardaverine in a concentration-dependent manner (Fig. 4). However, the combined effects of the adenylate cyclase activator and zardaverine do not significantly differ from the additive mode. Furthermore, the inhibition curves of zardaverine are shallow with indirect Hill-coefficients of approximately 0.3, indicating a complex type of interaction between zardaverine and these cells.

With both platelets and PMN cells, there was no significant difference in the release of LDH in the presence or absence of any PDE inhibitor, indicating that none of the compounds were cytotoxic (data not shown).

The IC_{50} values of the various PDE inhibitors for inhibition of zymosan-induced O_2^--release from PMN cells are listed in Table 4. As was the case with inhibition of platelet aggregation, the xanthines IBMX, theophylline and enprofylline were rather weak inhibitors of O_2^--release with IC_{50} values of 19, 135 and 162 μM, respectively. The IC_{50} of motapizone in PMN cells was about 18,000-fold higher than that for inhibition of platelet aggregation. Milrinone was about 300-fold, CI 930 about 500-fold, and B832-07 about 20-fold less potent as inhibitors of O_2^--release than as inhibitors of platelet aggregation. In contrast, rolipram (IC_{50} = 0.45 μM) and Ro 20-1724 (IC_{50} = 0.44 μM) were about 700-fold more potent in PMN cells than in platelets.

In order to correlate inhibition of PDE isozymes with the functional experiments on the cellular level, the IC_{50} values of the various compounds for inhibition of PDE III from platelets and of PDV IV from trachea were compared with the IC_{50} values of the same compounds as inhibitors of platelet aggregation and of O_2^--release from PMN cells. Figure 5 reveals a high correlation between inhibition of aggregation and PDE III inhibition and, on the other hand, between inhibition of O_2^--release and PDE IV inhibition.

DISCUSSION

Diversity of PDE isozymes and selectivity of inhibitors

In the present study, several distinct isozymes were used to evaluate the selectivity of zardaverine for PDE isozymes. The PDE fractions were isolated by anion exchange chromatography, which has been established in many laboratories [6, 14, 22, 23]. The use of Q-sepharose, particularly, proved to be of advantage in separating PDE III and IV over any other ion exchange material [24]. In agreement with the studies of Torphy and Cieslinski [22] and Silver *et al.* [14] both PDE III and IV were found to be present in homogenates of tracheal tissue. These two isozymes, the cyclic GMP-inhibited PDE III and the cyclic AMP-specific PDE IV, have been the primary focus of research in the last few years [2, 20].

With respect to inhibition of PDE III and PDE IV, the results presented in this study clearly indicate that three groups of PDE III/IV inhibitors can be differentiated: (a) compounds such as motapizone, milrinone and CI 930 with selectivity for PDE III; (b) PDE IV selective inhibitors such as rolipram and Ro 20-1724; (c) a third group of compounds combine both PDE III and PDE IV inhibitor activity, e.g. zardaverine. The benzonaphthyridine derivative AH 21–132 also exhibits selectivity as an inhibitor of the isozymes types III and IV [25]. As shown in the present study, zardaverine inhibits PDE III and PDE IV activities with IC_{50} values ranging from 0.56 to 1.7 μM and shows, therefore, an 80–1700-fold selectivity for these isozymes compared to PDE I, II and V. Based on the K_i values as calculated in Fig. 2, zardaverine appears to show a slight preference for inhibition of the PDE IV subtype over that of the PDE III subtype. Whether PDE III or PDE IV isozymes present in different human tissues will be inhibited with similar potency or will differentiate into further subtypes remains to be clarified.

The existence of both PDE III and PDE IV was demonstrated in homogenates of the tracheal tissue of dogs, in agreement with previously published studies [22, 23]. However, the broad peak of PDE IV from canine trachea homogenates as shown in Fig. 1 suggests that the mucosal tissue contains PDE IV preferentially, since in eluates from isolated smooth muscle tissue the peaks of PDE III and IV

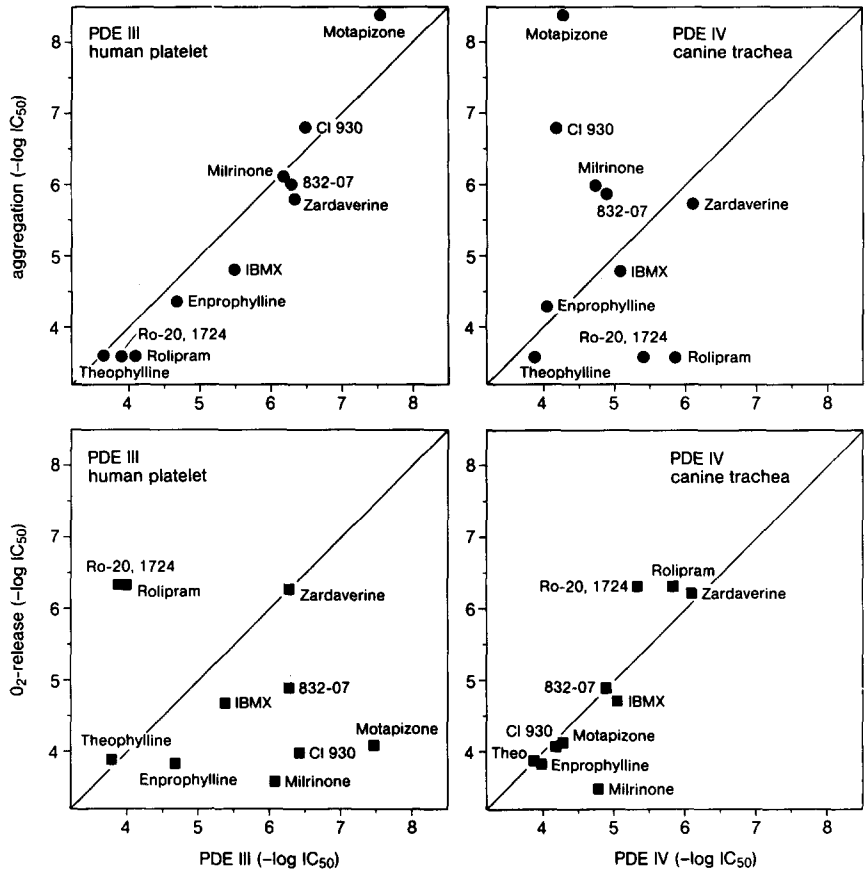

Fig. 5. Correlation of cellular function vs PDE inhibition. Inhibitory potencies of PDE inhibitors on platelets (above) and PMN cells (bottom) were plotted vs their inhibitory potencies on PDE III from human platelets (left) and PDE IV from canine trachea (right). Values were taken from Tables 2 and 4.

were of similar size. These biochemical data on distribution and pharmacological characteristics of isozymes in smooth muscle correlate with recent results from functional studies. With isolated smooth muscle of tracheal or vascular origin, it was shown that a number of PDE inhibitors with selectivity for either PDE III or PDE IV were comparably capable of inducing relaxation or preventing agonist-induced constriction (Eltze and Schudt, in preparation). In view of the presence of both isozymes in human tracheal smooth muscle [26], the inhibition of both PDE III and IV isozymes by zardaverine could correlate to a bronchodilatory effect of the compound in the clinic.

Merely demonstrating the presence of a PDE isozyme in a tissue homogenate does not confirm that it is important for the regulation of cyclic AMP content in intact cells. Accordingly, studies were conducted with intact human platelets and human PMN cells to determine the functional role of the individual PDEs. These studies were designed to detect synergistic interactions between PDE inhibitors and activators of adenylate cyclase. Zardaverine inhibited platelet aggregation regardless of the aggregatory agent with IC_{50} values ranging from 1.5 to 16 μM. On the other hand, the pyridazinone derivative inhibited zymosan-induced superoxide anion release from PMN cells with an IC_{50} of 0.6 μM. Zardaverine is, therefore, approximately three times more potent in PMN cells than in platelets. This result corresponds with the three-fold lower K_i-value of zardaverine for inhibition of PDE III compared to PDE IV isozymes in Fig. 2. This excellent correlation should be emphasized, since data in intact human cells are compared with those from enzyme preparations in human and canine tissue.

Cellular distribution and function of PDE isozymes

In both platelets and PMN cells, combining any activator of adenylate cyclase with zardaverine resulted in a marked left shift of the concentration–response curve of the PDE inhibitor. Obviously, in platelets zardaverine and the activators of adenylate cyclase act synergistically to increase cyclic AMP content, whereas in PMN cells a more complex interaction might be present. Thus, as a consequence of the synergistic interaction between a PDE inhibitor and an endogenous activator of adenylate cyclase such as epinephrine or PGE_2, plasma concentrations

of PDE inhibitors that are considerably lower than IC_{50} values for PDE inhibition may produce increases in cyclic AMP content in target cells, thereby inhibiting or preventing cell activation.

Based on the inhibition of platelet aggregation and of superoxide anion release from PMN cells by PDE inhibitors, the functional role of the individual PDEs can also be determined. Inhibitors with selectivity for PDE III were potent in platelets, whereas those with selectivity for PDE IV were potent inhibitors of O_2^--release in PMN cells. It can be concluded, therefore, that with respect to the particular response, PDE III is important for regulating cyclic AMP content in human platelets, whereas PDE IV is the predominant isozyme in PMN cells.

Anti-inflammatory potential of selective PDE inhibitors

Zardaverine does not have an inhibitory effect only on PMN cells — as shown in the present study — but also influences the function of other inflammatory cells. As recently shown, zardaverine and rolipram inhibited zymosan-induced O_2^--production in guinea-pig eosinophils [27]. A significant inhibition was observed at 0.1 μM zardaverine. In contrast, selective PDE III inhibitors did not affect cell activation at concentrations up to 100 μM [27]. Therefore, inhibition of O_2^--release from eosinophils seems to be mediated by PDE IV. Additionally, a synergism between zardaverine and salbutamol was observed in human eosinophils [27]. These data suggest that zardaverine also possesses the prerequisites to inhibit activation of proinflammatory cells *in vivo* and could possibly be regarded as an anti-inflammatory drug. With respect to the treatment of inflammatory diseases such as asthma zardaverine could represent a new class of anti-obstructive drugs that, by virtue of inhibition of PDE III and IV isozymes, possess bronchodilator and anti-inflammatory activities.

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